

## The effects of 6-month hydrogen-rich water intake on molecular and phenotypic biomarkers of aging in older adults aged 70 years and over: A randomized controlled pilot trial

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### ABSTRACT

In this randomized controlled pilot trial, we investigated the effects of a 6-month intake of hydrogen-rich water (HRW) on several molecular and phenotypic biomarkers of aging in older adults aged 70 years and over. Forty older adults (20 women) were randomly allocated in a parallel-group design to receive 0.5 L per day of HRW (15 ppm of hydrogen) or control drink (0 ppm of hydrogen) during a 6-month intervention period. The biomarkers assessed at baseline and 6-month follow up were molecular markers in the blood (DNA and chromosomes, nutrient sensing, protein, and lipid metabolism, oxidative stress and mitochondria, cell senescence, inflammation), brain metabolism, cognitive functioning, physical function and body composition, resting blood pressure, facial skin features, sleep outcomes, and health-related quality of life. The mean age, weight, and height of study participants were 76.0 ± 5.6 years, 78.2 ± 16.1 kg, height 167.5 ± 11.5 cm, respectively. A significant treatment vs. time interaction was found for telomere length ( $P = 0.049$ ), with the length increased after HRW intervention (from 0.99 ± 0.15 at baseline to 1.02 ± 0.26 at follow up) and decreased after drinking control water (from 0.92 ± 0.27 to 0.79 ± 0.15). A marker of DNA methylation (Tet methylcytosine dioxygenase 2, TET2) expression at 6-month follow-up increased in both groups, yet the degree of elevation was significantly higher in HRW (from 0.81 ± 0.52 at baseline to 1.62 ± 0.66 at follow up) comparing to the control water (from 1.13 ± 0.82 to 1.76 ± 0.87) ( $P = 0.040$ ). A strong trend for treatment vs. time interaction was found for a degree of DNA methylation ( $P = 0.166$ ), with the methylation increased in the HRW group (from 120.6 ± 39.8 ng at baseline to 126.6 ± 33.8 ng at follow up) and decreased after taking control water (from 133.6 ± 52.9 ng to 121.2 ± 38.4 ng). HRW was superior to control water to increase brain choline and NAA levels in the left frontal grey matter, brain creatine at the right parietal white matter, and brain NAA at the right parietal mesial grey matter ( $P < 0.05$ ). No significant differences were found between interventions for other outcomes ( $P > 0.05$ ), except for a significantly improved chair stand performance after HRW intervention compared to the control water ( $P = 0.01$ ). Owing to pleiotropic mechanisms of hydrogen action, this simple biomedical gas could be recognized as a possible anti-aging agent that tackles several hallmarks of aging, including loss of function and telomere length shortening. The study was registered at [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT04430803).

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## 1. Introduction

The increase in the number of older adults across developed countries is unprecedented. In 2016, approximately 50 million U.S. adults were aged 65 years or older, representing 15% of the population. That number is expected to reach 71 million by 2030 and 98 million by 2060 when older adults will make up nearly 25% of the population (U.S. National Center for Chronic Disease Prevention Health Promotion, 2021). Age is the leading risk factor for many prevalent non-communicable diseases, including cardiovascular and metabolic conditions, neurodegenerative disorders, and cancer (Niccoli and Partridge, 2012). Thus, improving prevention and management of age-related disorders remains of utmost importance for health caregivers in helping the older adults remain healthy as long as possible while tackling the ever-increasing population with effective and safe approaches. Diet appears to be a practical, flexible, and affordable approach that could help older people live longer and healthier lives (Calder et al., 2018), yet the effects of specific dietary components or nutritional formulations in the aging population have yet to be clarified. While the beneficial effects of dietary food components for cognitive function in older adults are well recognized (Ozawa et al., 2021), much less information is available how various nutritional compounds affect other biomarkers of aging. As getting old is accompanied by telomere attrition and DNA damage, any nutraceutical that is able to directly influence telomeres metabolism, slow their deterioration, and diminish aging might extend the life and health span (Vidacek et al., 2017). Among others, hydrogen-rich water (HRW) recently emerged as a novel drinkable dietary product that might favorably affect various aging-related features in interventional trials. For instance, HRW reduces inflammatory responses and prevents programmed cell death (Sim et al., 2020), improves nutrients metabolism (Kajiyama et al., 2008; Song et al., 2013), enhances psychophysiological outcomes (Mizuno et al., 2018), and represses wrinkles formation (Kato et al., 2012). Although promising, the above studies typically recruited mid-age or pre-elderly individuals, administered HRW for a relatively short interval (e.g., seven days to 4 weeks), used open-label or non-randomized research designs, or employed a limited set of aging biomarkers (Xia et al., 2017), leaving many open questions concerning the effects of HRW in older adults. In this randomized controlled pilot trial, we investigated the effects of 6-month HRW intake on several molecular and phenotypic biomarkers of aging in older adults aged 70 years and over. We hypothesized that drinking HRW would improve quantitative indicators of aging, including telomere length and DNA methylation, brain metabolism, cognitive and physical functioning, and skin viability.

## 2. Methods

### 2.1. Participants

Forty older adults signed an informed consent to participate in this randomized placebo-controlled parallel-group interventional trial voluntarily. The inclusion criteria were: age 70 years and over (with at least 20% of participants aged over 80 years), body mass index normal or overweight range, no current acute disorders or major chronic diseases (e.g., cancer, neurodegenerative disorders, stroke, psychiatric diseases), able to read and understand written consent form, and willing to take blood tests and other measurements, and consume assigned intervention. Exclusion criteria included a previous history of dietary supplement use during the four weeks before the study commences. All participants were subjected to a pre-participation general health screening to identify potentially *eligible participants*. The minimal sample size ( $n = 34$ ) was calculated using power analysis (G\*Power 3.1), with the effects size set at 0.25 (small effect), alpha error probability 0.05, power 0.80 for two groups, and two measurements of study outcomes. The primary outcome was the change in telomere length at baseline and 6-month post-administration. The study design was approved by the local IRB at the University of Novi Sad (# 46-06-01/2020-1), with the

study systematized following the Declaration of Helsinki (7th Revision) and the International Conference of Harmonization Efficacy Guidelines. The study was registered at [ClinicalTrials.gov](https://clinicaltrials.gov), a registry of clinical trials run by the U.S. National Library of Medicine at the National Institutes of Health (NCT04430803).

### 2.2. Experimental intervention

All participants were randomly allocated in a parallel-group design to receive HRW (15 ppm of hydrogen) or control drink (0 ppm of hydrogen) during a 6-month intervention period. HRW was produced by dissolving a tablet of Rejuvenation (HRW Natural Health Products Inc., Drink HRW™, New Westminster, Canada) into a cup of tap water (250 mL). The participants were asked to take HRW or control drink two times a day in the morning and the evening on an empty stomach. The following reaction produced hydrogen in HRW:  $Mg + H_2O \rightarrow H_2 + Mg(OH)_2$ , with the concentration of hydrogen measured by gas chromatography, as previously described (Zanini et al., 2020). Both drinks were similar in appearance, texture, and sensory characteristics and normalized for total magnesium amount. The adherence to supplementation regimen over the 6-month period was checked by the tablet counts, with participants connected by phone every 4 weeks to look over for any major changes in participants' status. In addition, the participants were asked to refrain from using any other dietary supplements and maintain their usual lifestyle (including diet and physical activity) during the study, as monitored by physical activity questionnaires and 3-day food records. The biomarkers assessed at baseline and 6-month follow up were molecular markers in the blood (DNA and chromosomes, nutrient sensing, protein, and lipid metabolism, oxidative stress and mitochondria, cell senescence, inflammation), brain metabolism, cognitive functioning, physical function, and body composition, resting blood pressure, facial skin features, sleep outcomes, and health-related quality of life.

### 2.3. Study design

The laboratory assessments were carried out between 09:00 and 13:00 after an overnight fast. First, the venous blood was drawn from a peripheral antecubital vein and centrifuged immediately at 3000g, with serum separated, refrigerated at  $-80\text{ }^\circ\text{C}$  and analyzed at the end of the study. Telomere length was determined with the modified Cawthon qPCR method. In short, genomic DNA was extracted using a commercial DNA kit (Flexi GENE DNA kit, Qiagen). Quantity of extracted DNA and contamination with organic solvents and proteins was checked spectrophotometrically at 260, 230, and 280 nm, respectively (SPECTROstar Nano, BMG Labtech, GMBH, Allmendgrün 8, 77799 Ortenberg, Germany). All samples had good quality ( $A_{260}/280 \approx 1.8$ ,  $A_{260}/230 > 2.0$ ) and were suitable for subsequent qPCR analysis. Albumin was used as a single copy reference gene. Primers used in PCR reaction were: Telomere-forward primer 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3', telomere-reverse primer 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACCA-3', albumin-forward primer 5'-CGGCGCGGGCGGGCGGGCTGGGCGGAAATGCTGCACAGAATCC T TG-3', albumin-reverse primer 5'-GCCCGGCCCGCCGCGCCCGTCCGCGGAAAAGCATGGTCGCCTGTT-3'. The final reaction mixtures for the telomere length and the single-copy reference gene (albumin) contained: qPCR Master Mix (5× HOT FIREPol® EvaGreen® qPCR SuperMix, Solis Biodyne, Tartu, Estonia), forward primer, reverse primer, and DNA template. The thermal cycling profile for the telomere length had the following steps: i)  $95\text{ }^\circ\text{C}$  for 12 min, ii) 4 cycles of  $95\text{ }^\circ\text{C}$  for 15 s,  $49\text{ }^\circ\text{C}$  for 20 s, iii) 40 cycles of  $95\text{ }^\circ\text{C}$  for 15 s,  $62\text{ }^\circ\text{C}$  for 10 s,  $72\text{ }^\circ\text{C}$  for 35 s. The PCR profile for albumin amplification contained steps: i)  $95\text{ }^\circ\text{C}$  for 12 min, ii) 40 cycles of  $95\text{ }^\circ\text{C}$  for 15 s,  $62\text{ }^\circ\text{C}$  for 10 s,  $87\text{ }^\circ\text{C}$  for 15 s. Telomere length was calculated as a T/S ratio suggested by Cawthon (2002). PCR measurements for telomere length were performed on Real-time PCR 7500 (Applied Biosystems, USA). Total RNA was isolated

from peripheral blood mononuclear cells (PBMC) by liquid-phase extraction with TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) applying modified protocol (Vujovic et al., 2013). RNA purity and integrity were assessed by spectrophotometric and 1% agarose gel electrophoresis methods, respectively. Reverse transcription was done using a high-capacity complementary deoxyribonucleic acid reverse transcription kit (Applied Biosystems, Foster City, CA, USA) using manufacturer recommendations for thermal protocols. TET2-TERT gene expression levels were measured by quantitative PCR 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using specific primers (F: 5'-TGA CAC CTC ACC TCA CCC AC-3'; R: 5'-CAC TGT CTT CCG CAA GTT CAC-3') and 5× HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia). mRNA levels were normalized to GAPDH (F: 5'-TGCACCACCAACTGCTTAGC-3'; R: 5'-GGCATG-GACTGTGGTCATGAG-3') as a housekeeping gene. For global DNA methylation analysis, peripheral blood samples were collected in EDTA tubes and kept at −20 °C. Genomic DNA was extracted by a modified salting-out method of Lahiri and Schnabel (1993). DNA quantity and integrity were confirmed using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan) and electrophoresis on a 1% (w/v) agarose gel. Global DNA methylation analyses were performed using the Imprint Methylated DNA Quantification Kit MDQ1 (Sigma Aldrich) based on the ELISA principle, according to manual instructions. The input DNA for the assay was 200 ng. The percent methylation of the samples was calculated relative to the methylated control DNA.

Malondialdehyde (MDA) levels in serum were measured according to the method described by Satoh (1978), which is based on the principle that MDA, the specific product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a pink-colored complex with maximum absorption at 532 nm. The results were calculated using the standard curve prepared with ranges of MDA standards' concentrations of 0.1–25 μmol/L and expressed as nmol MDA per mL of serum. The antioxidant capacity of serum was measured by FRAP assay (Ferric Reducing Antioxidant Power assay) according to the method described by Benzie and Strain (1996). The method is based on reducing a colorless ferric-tripyridyltriazine complex (Fe<sup>3+</sup>-TPTZ) into an intense blue ferrous-tripyridyltriazine complex (Fe<sup>2+</sup>-TPTZ) in interaction with a potential antioxidant at low pH, with an absorption maximum at 593 nm. Ascorbic acid was used as standard, and results were expressed as equivalent of vitamin C per mL of serum. The NAD<sup>+</sup> and NADH serum concentration and their ratio were measured using the NAD<sup>+</sup>/NADH assay kit (AB65348, Abcam, Cambridge, UK) following manufacturer instructions. After separation from the whole blood, serum samples were immediately snap frozen in liquid nitrogen and stored at −80 °C. Before performing the assay, samples were thawed on ice and deproteinized by filtering through a 10 kD Spin Column (AB93349, Abcam, Cambridge, UK) by centrifuging at 16,000g for 30 min at 4 °C. Lipid profiles, fasting blood glucose, insulin, serum magnesium, total testosterone, advanced glycation end products, high-sensitive C-reactive protein, and interleukin 1 beta (IL-1β) were measured with ELISA spectrophotometric systems (Cobas Pro, Cobas c 501, Cobas c 6000, Roche Diagnostics, Basel, Switzerland). At the same time, erythrocyte sedimentation rate was assessed via the Westergreen method. Insulin-like growth hormone (IGF-I) and human growth hormone were evaluated using commercial kits on an automated analyzer (ChemWell 2910, AWARENESS Technology Inc., Palm City, FL).

After the biochemical sampling was finished, anthropometrical variables were obtained. Height was measured using a stadiometer (Seca 217, Hamburg, Germany); weight and body fat were measured by a bioelectrical impedance analyzer (Omron BF 511, Kyoto, Japan). Body mass index was calculated as weight in kilograms divided by height in meters squared. The subjects were measured in underwear after voiding, while the same trained technician did the anthropometric assessment intending to minimize the testing error. Resting blood pressure and heart rate were measured after 5 min of seated rest with an automated system (OMRON Hem 907XL IntelliSense, Kyoto, Japan).

Proton magnetic resonance spectroscopy was performed on a 1.5 T Avanto scanner (Siemens, Erlangen, Germany) using matrix head coil in circularly polarized mode, with metabolite spectra in the specific brain regions (thalamus, frontal, precentral, paracentral, and parietal white and grey matter) processed as previously described (Ostojic et al., 2016). In short, non-water-suppressed two-dimensional chemical shift imaging (CSI) and single-voxel spectroscopy (SVS) data were obtained to provide an internal water reference for the absolute quantification of brain choline, *N*-acetyl aspartate (NAA), and creatine. Metabolites were calculated using water-suppressed CSI, and SVS data sets were acquired with point-resolved spectroscopy with repetition time/echo time of 1500/135 ms. Mono-exponential spin-lattice and spin-spin relaxation were assumed, and standard values of T1 and T2 relaxation times of water and total creatine measured at 1.5 T were used for relaxation corrections.

A senior fitness test (SFT) was conducted to assess six various domains of functional fitness in older adults, as previously described (Rikli and Jones, 2013). The individual SFT items (e.g., chair stand, arm curl, chair sit-and-reach, back scratch, 8-foot up-and-go, step in place) involve everyday activities to measure aerobic fitness, strength, and flexibility of older adults. Sleep performance was evaluated via the Sleep Quality Scale test, an efficient measure suitable for evaluating sleep quality in various patient and research populations (Shahid et al., 2011). This 28-item tool evaluates six domains of sleep quality, including daytime symptoms, restoration after sleep, problems initiating and maintaining sleep, difficulty waking, and sleep satisfaction. The quality of life was assessed with the Short Form Health Survey (SF-36), a 36-item patient-reported survey of patient's health (Ware, 2000). SF-36 evaluates eight health concepts: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions, with items are scored so that a high score defines a more favorable health state. The cognitive function of participants was assessed using the Mini-Mental State Exam (MMSE) and Alzheimer's disease assessment scale cognitive subscale (ADAS-Cog) (Lin et al., 2013). MMSE is a 30-point questionnaire used extensively in clinical and research settings to measure cognitive impairment in the older adults. ADAS-Cog is a cognitive testing instrument that consists of 11 tasks measuring the disturbances of memory, language, praxis, attention, and other cognitive abilities in the assessment of dementia. In addition, investigators rated facial photographs of participants (front and right and left sides, to provide a full view of the face) to evaluate facial skin features using Medicis Midface Volume Scale (Lorenec et al., 2012). Finally, the participants were asked to report any adverse effects (e.g., gut disturbances, headache) of either intervention during the study through an open-ended questionnaire. Early termination criteria included subjective severe side effects of the intervention and/or significant health status changes due to other reasons.

#### 2.4. Statistical analyses

Initially, data were tested with the Shapiro–Wilk test for the normality of distribution and Bartlett's test for the homogeneity of the variances. When homogenous variances were verified for normally distributed data, summary measures were compared by two-way ANOVA with repeated measures, with a posthoc test employed to identify the differences between individual interventions. When non-homogenous variances were identified, data were compared using the Kruskal–Wallis test. The significance level was set at  $P \leq 0.05$ . Effect sizes in two-way ANOVA and Kruskal–Wallis test with replication after 6 months of administration were assessed by Cohen statistics, with  $d \geq 0.8$  indicated large effect. The missing data were removed from any analyses. The data were analyzed using the statistical package SPSS version 24.0 for Mac (IBM SPSS Statistics, Chicago, IL).

### 3. Results

Forty older adults (mean age  $76.0 \pm 5.6$  years, weight  $78.2 \pm 16.1$  kg, height  $167.5 \pm 11.5$  cm; 20 women) were enrolled in this pilot trial; the average duration of education was  $13.0 \pm 4.5$  years. A total of thirty-four participants ( $n = 34$ ) completed the follow-up measures, with six subjects dropped out from the study (four from the HRW group and two participants from the control group) due to the reasons not related to the study *per se*. The volunteers reported no major side effects that precluded their participation in the study; the adherence to the intervention was 96.2% for the HRW group and 97.8% for the control group, as calculated by the tablet counts.

A significant treatment vs. time interaction was found for telomere length ( $P = 0.049$ ), with the length increased after HRW intervention (from  $0.99 \pm 0.15$  at baseline to  $1.02 \pm 0.26$  at follow up; percent change 4.1%) and decreased after drinking control water (from  $0.92 \pm 0.27$  to  $0.79 \pm 0.15$ ; percent change 11.1%) (Fig. 1). TET2 expression at 6-month follow-up increased in both groups, yet the degree of elevation was significantly higher in HRW (from  $0.81 \pm 0.52$  at baseline to  $1.62 \pm 0.66$  at follow up) comparing to the control water (from  $1.13 \pm 0.82$  to  $1.76 \pm 0.87$ ) ( $P = 0.040$ ). A strong trend for treatment vs. time interaction was found for a degree of DNA methylation ( $P = 0.166$ ;  $d = 4.6$ ), with the methylation increased in the HRW group (from  $120.6 \pm 39.8$  ng at baseline to  $126.6 \pm 33.8$  ng at follow up) and decreased after taking control water (from  $133.6 \pm 52.9$  ng to  $121.2 \pm 38.4$  ng).

Nutrient sensing and metabolic biomarkers of protein and lipid metabolism remained essentially unchanged during the study ( $P > 0.05$ ) (Table 1). Still, human growth hormone and serum IGF-1 levels (also advanced glycation end-products) tended to rise after HRW intervention and diminish in the control group. As opposed to the control water, HRW tended to reduce LDL blood cholesterol levels, while serum triglycerides showed a tendency for a reduction in both groups at post-administration. No significant differences were found between groups for biomarkers of glucose metabolism ( $P > 0.05$ ); changes in serum testosterone shown a tendency for a decrease in the HRW group and an increase in the control group ( $P = 0.22$ ;  $d = 2.0$ ).

Oxidative status and inflammation biomarkers were depicted in Table 2. Although no significant differences were found across groups ( $P > 0.05$ ), serum malondialdehyde levels and ferric reducing ability of plasma strongly tended to decrease after HRW intervention (for 48.7% and 8.4%, respectively) as opposed to a mild rise noted for both biomarkers in the control group; effect sizes for both outcomes were  $> 0.8$ . No differences were found between interventions for inflammation biomarkers ( $P > 0.05$ ); HRW tended to be superior to control water to increase serum magnesium levels ( $P = 0.09$ ;  $d = 8.6$ ).

No significant differences were found between interventions for body composition, physical fitness outcomes, and resting blood pressure (Table 3), except for a significantly improved chair stand performance after HRW intervention compared to the control water ( $P = 0.01$ ). A trend was found for improved arm curl ( $P = 0.13$ ) and 8-foot up-and-go performance ( $P = 0.25$ ) in HRW group vs. placebo; effect sizes for both

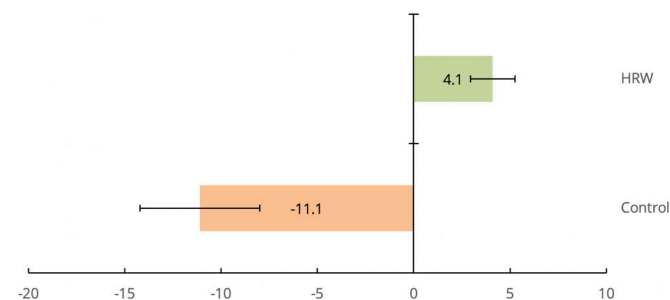


Fig. 1. Percent change in telomere length at 6-month follow-up. HRW = hydrogen-rich water.

Table 1

Biochemical indices of metabolism during the study. Values are mean  $\pm$  SD.

Variable	Hydrogen-rich water		Control water		P
	Baseline	Follow-up	Baseline	Follow-up	
<b>Nutrient sensing</b>					
Insulin-like growth factor 1 (nmol/L)	98.3 $\pm$ 32.4	100.6 $\pm$ 39.6	117.0 $\pm$ 36.3	101.1 $\pm$ 26.5	0.34
Human growth hormone (IU/L)	1.11 $\pm$ 0.79	0.94 $\pm$ 1.31	2.14 $\pm$ 2.05	1.25 $\pm$ 1.31	0.29
NAD <sup>+</sup> /NADH ratio	9.7 $\pm$ 10.4	8.2 $\pm$ 5.6	1.7 $\pm$ 1.1	4.8 $\pm$ 6.6	0.72
<b>Protein metabolism</b>					
Advanced glycation end-products (IU/mL)	280 $\pm$ 19	286 $\pm$ 29	274 $\pm$ 35	269 $\pm$ 34	0.23
<b>Lipid metabolism</b>					
Total cholesterol (mmol/L)	6.0 $\pm$ 1.4	5.6 $\pm$ 0.9	5.4 $\pm$ 1.2	5.3 $\pm$ 1.0	0.43
LDL-cholesterol (mmol/L)	3.7 $\pm$ 1.2	3.3 $\pm$ 0.8	3.1 $\pm$ 1.0	3.2 $\pm$ 0.9	0.37
HDL-cholesterol (mmol/L)	1.5 $\pm$ 0.5	1.6 $\pm$ 0.5	1.6 $\pm$ 0.5	1.6 $\pm$ 0.4	0.88
Triglycerides (mmol/L)	1.7 $\pm$ 0.7	1.5 $\pm$ 0.5	1.6 $\pm$ 0.7	1.2 $\pm$ 0.5	0.19
<b>Glucose metabolism</b>					
Fasting glucose (mmol/L)	5.7 $\pm$ 0.8	5.9 $\pm$ 1.1	5.7 $\pm$ 1.1	5.7 $\pm$ 1.2	0.89
Insulin (IU/mL)	12.6 $\pm$ 5.5	13.6 $\pm$ 8.8	11.5 $\pm$ 5.5	9.7 $\pm$ 3.9	0.87
Testosterone (ng/mL)	3.1 $\pm$ 3.1	2.8 $\pm$ 3.2	2.5 $\pm$ 2.9	2.8 $\pm$ 3.1	0.22

Table 2

Biomarkers of oxidative stress and inflammation. Values are mean  $\pm$  SD.

Variable	Hydrogen-rich water		Control water		P
	Pre	Post	Pre	Post	
<b>Oxidative status</b>					
Malondialdehyde ( $\mu$ mol/L)	1.54 $\pm$ 0.92	0.79 $\pm$ 0.57	1.63 $\pm$ 0.93	1.68 $\pm$ 1.37	0.17
Ferric reducing ability ( $\mu$ mol/L)	120.4 $\pm$ 20.2	110.3 $\pm$ 17.0	105.8 $\pm$ 22.6	106.2 $\pm$ 22.6	0.10
<b>Inflammation</b>					
High-sensitive C-reactive protein (mg/L)	3.2 $\pm$ 3.1	3.3 $\pm$ 2.2	3.0 $\pm$ 3.7	2.2 $\pm$ 1.8	0.82
Erythrocyte sedimentation rate (mm/h)	32 $\pm$ 21	29 $\pm$ 19	22 $\pm$ 18	19 $\pm$ 15	0.57
Interleukin 1 beta (IU/mg)	5.0 $\pm$ 1.0	5.5 $\pm$ 1.2	7.5 $\pm$ 7.1	11.0 $\pm$ 17.0	0.90
Magnesium (mmol/L)	0.80 $\pm$ 0.06	0.89 $\pm$ 0.09	0.83 $\pm$ 0.09	0.86 $\pm$ 0.08	0.09

outcomes were  $> 0.8$ .

Two-way ANOVA revealed no significant treatment vs. time interaction for most sleep quality outcomes (Table 4). Nevertheless, the participants who received HRW demonstrated a trend towards favorable sleep quality outcomes for daytime dysfunction ( $P = 0.15$ ;  $d = 9.0$ ) and difficulties in getting asleep ( $P = 0.11$ ;  $d = 4.5$ ). A significant interaction was found for the pain domain of the quality of life profile ( $P = 0.05$ ), with HRW increased favorable health scores for pain as opposed to the placebo. No significant treatment vs. time interaction for cognition biomarkers was found during the trial ( $P > 0.05$ ). Finally, no significant treatment vs. time interaction was found for facial features ( $P = 0.98$ ), with midface volume remained unaffected by HRW intervention ( $3.25 \pm 0.4$  point at baseline to  $3.3 \pm 0.4$  point at follow up;  $P = 0.50$ ), and decreased insignificantly after drinking control water (from  $3.24 \pm 0.56$  points to  $3.18 \pm 0.53$  point;  $P = 0.30$ ).

A significant treatment vs. time interaction was found for brain

**Table 3**  
Body composition and physical fitness during the study. Values are mean  $\pm$  SD.

Variable	Hydrogen-rich water		Control water		P
	Pre	Post	Pre	Post	
<b>Body composition</b>					
Body mass index (kg/m <sup>2</sup> )	28.6 $\pm$ 3.9	29.0 $\pm$ 4.3	28.4 $\pm$ 4.6	28.8 $\pm$ 4.5	0.83
Fat mass (kg)	33.3 $\pm$ 10.1	34.1 $\pm$ 10.7	32.5 $\pm$ 11.2	33.2 $\pm$ 11.0	0.91
Muscle mass (kg)	26.4 $\pm$ 4.1	27.7 $\pm$ 4.9	28.0 $\pm$ 6.6	28.2 $\pm$ 5.8	0.52
<b>Senior fitness test</b>					
Chair stand (score)	10.6 $\pm$ 2.4	11.6 $\pm$ 2.6	12.8 $\pm$ 2.1	12.5 $\pm$ 1.8	0.01
Arm curl (score)	12.8 $\pm$ 3.2	13.6 $\pm$ 3.1	15.3 $\pm$ 3.5	15.3 $\pm$ 3.0	0.13
Chair sit-and-reach (cm)	2.3 $\pm$ 10.1	1.4 $\pm$ 8.9	2.5 $\pm$ 14.1	0.9 $\pm$ 11.6	0.95
Back scratch (cm)	-15.5 $\pm$ 14.1	-17.3 $\pm$ 10.2	-8.3 $\pm$ 14.5	-10.7 $\pm$ 12.0	0.46
8-Foot up-and-go (s)	5.7 $\pm$ 1.3	5.6 $\pm$ 1.6	4.9 $\pm$ 0.5	5.1 $\pm$ 1.3	0.25
Step in place (score)	87 $\pm$ 24	89 $\pm$ 20	109 $\pm$ 21	107 $\pm$ 24	0.44
Handgrip strength (kg)	70.0 $\pm$ 23.2	69.2 $\pm$ 23.3	72.2 $\pm$ 25.2	68.7 $\pm$ 22.5	0.53
Heart rate (bpm)	69 $\pm$ 11	73 $\pm$ 12	71 $\pm$ 12	72 $\pm$ 10	0.47
<b>Resting blood pressure</b>					
Systolic (mmHg)	142 $\pm$ 13	143 $\pm$ 16	134 $\pm$ 20	136 $\pm$ 17	0.82
Diastolic (mmHg)	80 $\pm$ 8	81 $\pm$ 12	78 $\pm$ 10	77 $\pm$ 8	0.91

metabolites levels (choline, creatine, and NAA) at several brain locations ( $P < 0.05$ ) (Fig. 2), with HRW was superior to control water to increase brain choline and NAA levels in the left frontal grey matter, brain creatine at the right parietal white matter, and brain NAA at the right parietal mesial grey matter. In addition, control water was superior to HRW to increase brain creatine concentrations at the left parietal mesial grey matter ( $P < 0.05$ )

#### 4. Discussion

This is the first study that comprehensively evaluated the effectiveness and safety of medium-term intake of hydrogen-rich water on molecular and phenotypic biomarkers of aging in a cohort of men and women aged 70 years and older. We found that drinking HRW for six months was harmless, and favorably affected several aging-related features, including telomere length, lower body strength, general pain, and brain metabolism indices, and tended to improve DNA methylation, antioxidant status, sleep quality, and upper body strength in this population. Owing to the pleiotropic mechanisms of hydrogen action, this simple biomedical gas could be thus recognized as a possible anti-aging agent that tackles several hallmarks of aging, including loss of function and telomere length shortening. More studies are highly warranted to corroborate these promising findings and advance hydrogen use in gerontology.

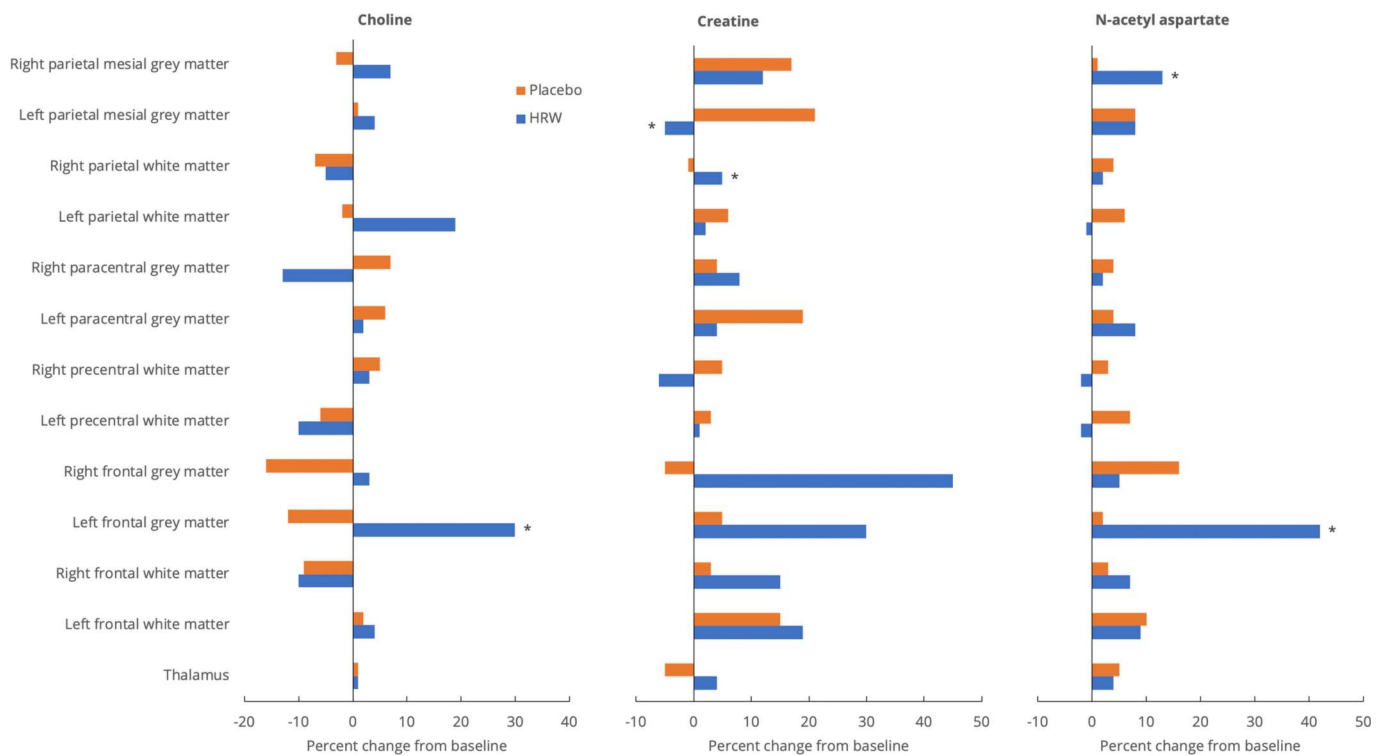
Either administered as an element of inhalational gas mixture or through water enriched with gaseous H<sub>2</sub>, molecular hydrogen has started to be evaluated in geriatric medicine during the past decade or so. Arguably the first human trial that investigated the effects of hydrogen in preventing and treating diseases and disabilities in the older population was published in 2008. The authors from the Kyoto Prefectural University of Medicine evaluated the effects of hydrogen-rich water in men and women (mean age 59 years) with either mild type 2 diabetes mellitus or impaired glucose tolerance (Kajiyama et al., 2008). The authors reported that the intake of HRW (900 mL per day for eight weeks) has beneficial effects on lipid and glucose metabolism in older adults, suggesting that HRW may prevent or delay the development and progression of diabetes and insulin resistance in this sensitive

**Table 4**  
Sleep quality, quality of life, and cognition during the study. Values are mean  $\pm$  SD.

Variable	Hydrogen-rich water		Control water		P
	Pre	Post	Pre	Post	
<b>Sleep quality</b>					
Day time dysfunction (score)	14.9 $\pm$ 3.7	15.7 $\pm$ 5.6	15.3 $\pm$ 5.5	17.9 $\pm$ 7.7	0.15
Restoration after sleep (score) <sup>a</sup>	6.7 $\pm$ 2.2	6.4 $\pm$ 2.5	5.8 $\pm$ 2.3	5.7 $\pm$ 3.2	0.74
Difficulties in getting asleep (score)	6.3 $\pm$ 2.4	7.1 $\pm$ 3.2	6.3 $\pm$ 2.7	6.2 $\pm$ 3.2	0.11
Difficulties in getting up (score)	4.6 $\pm$ 1.4	4.7 $\pm$ 1.6	3.9 $\pm$ 1.4	4.2 $\pm$ 2.1	0.69
Satisfaction with sleep (score) <sup>a</sup>	6.2 $\pm$ 1.9	5.5 $\pm$ 2.8	6.2 $\pm$ 1.6	5.2 $\pm$ 2.7	0.61
Difficulties in maintaining sleep (score)	4.7 $\pm$ 2.0	4.7 $\pm$ 2.0	5.1 $\pm$ 1.8	5.3 $\pm$ 2.1	0.54
Sleep quality scale (score)	43.3 $\pm$ 9.3	44.1 $\pm$ 13.3	42.5 $\pm$ 11.9	44.6 $\pm$ 16.6	0.69
<b>Quality of life</b>					
Physical functioning (score)	81 $\pm$ 20	79 $\pm$ 18	84 $\pm$ 16	81 $\pm$ 15	0.79
Role limitation due to physical health (score)	70 $\pm$ 34	60 $\pm$ 41	71 $\pm$ 35	69 $\pm$ 33	0.21
Role limitation due to emotional problems (score)	67 $\pm$ 42	60 $\pm$ 40	78 $\pm$ 34	78 $\pm$ 26	0.69
Energy and fatigue (score)	74 $\pm$ 15	68 $\pm$ 26	74 $\pm$ 16	65 $\pm$ 13	0.61
Emotional well-being (score)	82 $\pm$ 15	80 $\pm$ 18	82 $\pm$ 12	79 $\pm$ 11	0.87
Social functioning (score)	73 $\pm$ 31	77 $\pm$ 28	77 $\pm$ 30	68 $\pm$ 22	0.33
Pain (score)	75 $\pm$ 23	79 $\pm$ 25	78 $\pm$ 23	65 $\pm$ 27	0.05
General health (score)	66 $\pm$ 15	67 $\pm$ 21	58 $\pm$ 20	70 $\pm$ 17	0.08
Physical health (score)	73 $\pm$ 16	71 $\pm$ 23	73 $\pm$ 17	71 $\pm$ 18	0.49
Mental health (score)	74 $\pm$ 22	71 $\pm$ 24	78 $\pm$ 15	73 $\pm$ 14	0.66
<b>Cognition</b>					
ADAS-Cog					
Memory (no. of words)	7.9 $\pm$ 2.7	6.8 $\pm$ 2.7	6.3 $\pm$ 2.0	5.0 $\pm$ 1.6	0.64
Trail making test A (sec)	59.0 $\pm$ 20.1	61.8 $\pm$ 21.8	48.0 $\pm$ 14.7	46.3 $\pm$ 10.5	0.48
Numbers backward-forward (sum)	9.1 $\pm$ 2.5	8.9 $\pm$ 3.3	11.6 $\pm$ 3.8	12.3 $\pm$ 3.5	0.12
Symbol digital modality test (no. of symbols)	24.4 $\pm$ 8.7	25.3 $\pm$ 10.6	31.4 $\pm$ 7.6	32.0 $\pm$ 8.1	0.86
Symbol digital modality test (no. of errors)	0.4 $\pm$ 0.9	1.1 $\pm$ 2.7	0.8 $\pm$ 1.3	0.9 $\pm$ 1.4	0.73
Total score (sum)	10.7 $\pm$ 4.4	9.3 $\pm$ 3.9	8.1 $\pm$ 2.5	7.0 $\pm$ 3.2	0.97
Mini-Mental State Examination (point)	27 $\pm$ 3	27 $\pm$ 3	28 $\pm$ 2	28 $\pm$ 3	0.81

<sup>a</sup> Lower levels mean better sleep quality.

population. Favorable lipid-lowering effects of HRW have been corroborated in the subsequent study with pre-elderly patients (mean age 55.8  $\pm$  10.6 years) with potential metabolic syndrome (Song et al., 2013), where drinking 0.9–1.1 L of HRW per day for ten weeks decreases total serum cholesterol and LDL-cholesterol levels and improve HDL-cholesterol functions. Drinking 1 L of HRW per day for 28 days also reduced liver fat accumulation in pre-elderly overweight patients (mean age 56.2  $\pm$  10.0 years) suffering from mild-to-moderate non-alcoholic fatty liver disease (Korovljev et al., 2019). Another exciting study demonstrates that 3-month HRW administration repressed wrinkle formation in healthy men and women aged up to 65 (Kato et al., 2012), perhaps by providing protection against oxidative stress. HRW reduces inflammatory responses and prevents apoptosis of peripheral blood cells in healthy adults aged up to 59 years who consumed 1.5 L of HRW per day for four weeks (Sim et al., 2020). Interestingly, biological antioxidant potential in this trial increased greater in the subpopulation of participants who are advanced in years. Hydrogen administration in older men and women was found to reduce inflammation associated



**Fig. 2.** Percent change in brain metabolites at 6-month follow-up. HRW = hydrogen-rich water. Asterisk (\*) indicates statistical significance at  $P < 0.05$  for treatment vs. time interaction.

with psoriasis (Ishibashi et al., 2015), improve ventricular remodeling after myocardial infarction (Katsumata et al., 2017), ameliorate clinical outcomes of acute cerebral infarction (Ono et al., 2017), positively affect cardiometabolic risk factors (Korovljev et al., 2018), restore immunological activity in colorectal cancer (Akagi and Baba, 2019), improve quality-of-life and controls cancer progression (Chen et al., 2019), ameliorate airway inflammation in asthma and chronic obstructive pulmonary disease (Wang et al., 2020), and upgrade cognitive function (Korovljev et al., 2020). Our findings back up the results from the previous trials about the advantageous effects of hydrogen in the older adults while extending the line of evidence to a more comprehensive profile of hydrogen impact in geriatric biomedicine.

To our knowledge, this is the first interventional study that evaluated the effects of hydrogen on specific DNA components related to aging. We found that drinking HRW for six months extended mean telomere length by ~4%, while placebo intervention induced a progressive shortening of telomeres by ~11% during the trial; a large variability in telomere length reduction in the placebo group may be caused by a small sample size. Still, since longer telomeres are associated with decreased incidence of diseases and better survival (Shammas, 2011), HRW could be recognized as a dietary intervention that can lead to delayed onset of age-associated diseases and increased lifespan. This was accompanied by the upregulation of TET2 expression and a trend for amplified DNA methylation after HRW intervention, suggesting an additional protective impact of molecular hydrogen on DNA-chromosomal viability. HRW may instigate DNA-associated outcomes in the older adults due to various mechanisms that involve altering the expression of genes and signaling pathways (e.g., nuclear factor-kappa B) that transactivate telomerase (Sobue et al., 2015) or through prevention against systemic DNA-oxidative injuries (Asada et al., 2020). Along with telomere lengthening, we reported here favorable effects of HRW on physical fitness components and a tendency for better sleep quality. Telomere length appears to be a significant biomarker of normal aging with respect to crucial cognitive and physical abilities (Harris et al., 2012), suggesting a possible connection between drinking HRW and healthy

aging from subcellular to systematic context. However, only a handful of human studies evaluated the effects of HRW in older adults so far, with most omitted to analyze detailed molecular and phenotypic biomarkers of aging (for a review, see Ge et al., 2017 and Ishibashi, 2019). More studies are thus highly warranted before HRW can be endorsed as an anti-aging and telomere-lengthening intervention.

We found that HRW tended to reduce lipid peroxidation as reported by a drop in serum MDA levels at 6-month follow-up, with lowering lipid peroxidation that may rescue cellular membranes from oxidative degeneration. While HRW is known to effectively correct elevated MDA levels and restore antioxidant defense (Paulis et al., 2018), no clear explanation is available why HRW largely failed to affect biomarkers of inflammation and metabolism in our trial. One possible reason for the differing results may have to do with the fact that we recruited here a cohort of apparently healthy male and female seniors distinguished by a low-level inflammation and minor metabolic disturbances. In contrast, previous studies recruited older adults with inflammatory diseases, including cardiometabolic conditions and cancer. Still, HRW tended to modulate serum levels of IGF-1, a hormonal proxy of nutrient sensing, thus suggesting a possible role of hydrogen in regulating metabolic pathways; our findings corroborate previous *in vitro* research where hydrogen was found to promote the expression of IGF-1 (Fang et al., 2018). Interestingly, nutrient-sensing modifying therapeutics can improve cognitive outcomes, and growth hormone analogs are the most promising therapeutic to ameliorate cognition (Kioussis et al., 2021). A possible HRW-driven adjustment in IGF-1 and growth hormone output might be therefore seen as an alternative channel to advance cognition in the older adults. Tracking down other biomarkers of nutrient sensing (e.g., mechanistic target of rapamycin, 5'-adenosine monophosphate-activated protein kinase, sirtuins) after HRW intervention might be needed to clarify the potential of hydrogen to interfere with nutrient signaling and metabolism. Interestingly, HRW significantly impacted brain metabolism, with frontal grey matter particularly susceptible to hydrogen intervention. This largely corroborates our recent findings (Ostojic et al., unpublished data) that HRW can improve brain viability

and target brain regions relevant for executive functions, attention, and problem solving.

The major strengths of this study were the use of randomized-controlled design for administering HRW and placebo, a relatively long duration of the intervention, and a broad list of biomarkers and patient-reported outcomes used. The limitations include the recruitment of apparently healthy participants that prevented any analysis about the effects of HRW in older adults with prevalent aging-specific disorders; the relatively small sample size that restricted any gender-related comparison and analyses; the lack of employing weight-based dosing that allows equivalent exposure to HRW for each individual; and the inability to follow the participants for a more extended period of time after the end of the treatment to check for residual effects.

In conclusion, drinking hydrogen rich-water positively affected several molecular and phenotypic biomarkers of aging when administered for six months in apparently healthy men and women aged 70 years and over. An HRW-instigated telomere lengthening accompanied by improved functioning and towards improved sleep quality and biochemical milieu justify further exploration of hydrogen as a possible anti-aging agent. Despite the promising results reported here, building the case for molecular hydrogen in geriatric medicine requires more well-sampled multicentric trials that evaluate its long-term effects using a mechanistic approach.

### Ethical review

The study was approved by the local IRB at the University of Novi Sad (# 46-06-01/2020-1), with the study systematized following the Declaration of Helsinki and International Conference of Harmonization Efficacy Guidelines E6.

### Informed consent

All participants signed an informed consent to voluntarily participate in this trial.

### Data availability statement

The data that support the findings of this study have already been included in the manuscript. Raw data are available from the corresponding author upon reasonable request.

### CRedit authorship contribution statement

**Dragana Zanini:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Nikola Todorovic:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Darinka Korovljev:** Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing - original draft; Writing - review & editing. **Valdemar Stajer:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review & editing. **Jelena Ostojic:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Jelena Purac:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Danijela Kojic:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Elvira Vukasinovic:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Srdjana Djordjievski:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Miron Sopic:** Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review & editing. **Azra Guzonjic:** Data curation; Formal analysis; Investigation;

Methodology; Writing - original draft; Writing - review & editing. **Ana Ninic:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Sanja Erceg:** Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Sergej M. Ostojic:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualization; Roles/Writing - original draft; Writing - review & editing.

### Declaration of competing interest

The authors declare no conflicts of interest.

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