Nicotinamide adenine dinucleotide metabolism and arterial stiffness after long-term nicotinamide mononucleotide supplementation: a randomized, double-blind, placebo-controlled trial

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Abstract

Many animal studies have shown that oral administration of the nicotinamide adenine dinucleotide (NAD^+) precursor nicotinamide mononucleotide (NMN) prevents the reduction of NAD^+ levels in organs and tissues, helping alleviate aging-related diseases. However, there are very few clinical reports of NMN supplementation in humans. Thus, this study aimed to investigate the influence of a 12-week NMN oral supplementation on biochemical and metabolic health parameters. A 12-week randomized, double-blind, placebo-controlled, parallel-group clinical trial was conducted. A total of 36 healthy middle-aged participants received one capsule of either 125 mg NMN or placebo twice a day. Among the NAD^+ metabolites, the levels of nicotinamide in the blood were significantly higher in the NMN intake group than in the placebo group. Pulse wave velocity values indicating arterial stiffness tended to decrease in the NMN intake group. However, no significant difference was found between the two groups. Long-term NMN supplementation at 250 mg/day was well tolerated and did not cause adverse events. NMN safely and effectively elevated NAD^+ metabolism in healthy middle-aged adults. Additionally, NMN supplementation showed potential in alleviating arterial stiffness.

Introduction

The elderly population is rapidly growing globally due to declining fertility and increasing longevity. Aging is a major risk factor for various diseases, particularly cardiovascular disease (CVD)[1]. The prevalence of these diseases increases dramatically with age. Therefore, from the viewpoint of aging of the heart or blood vessels, research on anti-aging medicines is considered useful for the treatment and prevention of CVD.

Several studies on anti-aging interventions have shown that dietary restriction (DR) is one of the most effective strategies for delaying aging and prolonging lifespan[2]. DR has also been reported to alleviate various conditions associated with CVDs, such as hypertension and atherosclerosis[2, 3]. Although the mechanism underlying the anti-aging effects of DR is not fully understood, inhibition of insulin-like growth factor-1/insulin signaling, activation of adenosine monophosphate-activated protein kinase (AMPK) and sirtuins (SIRTs), and inhibition of the mammalian target of rapamycin (mTOR) have been proposed as plausible mechanisms[2]. However, suitable DR without resulting in malnutrition is considered impractical owing to the difficulty in practicing continuously over a long period. Therefore, research on DR mimetics has drawn increasing attention. DR mimetics do not require restrictions and are potential alternatives to prevent or treat CVD[4]. To date, the following compounds have been reported as major DR mimetics: aspirin and metformin as activators of AMPK, resveratrol and nicotinamide adenine dinucleotide (NAD^+) precursors as activators of SIRTs, and rapamycin as an inhibitor of mTOR[4].

There are two main pathways for NAD^+ biosynthesis in mammals: the de novo pathway from tryptophan and the salvage pathway consisting of precursors, such as nicotinamide (NAM), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR)[5]. Systemic NAD^+ levels and nicotinamide

phosphoribosyltransferase (NAMPT) activity, which serves the rate-limiting enzymatic step that converts NAM to NMN in the salvage pathway, have been found to decrease with age in various tissues[5]. Because NMN is a downstream product of the NAMPT reaction, NMN supplementation is assumed to increase NAD^+ biosynthesis in a way that avoids the need for NAMPT. Thus, NMN has been shown to have beneficial effects, including SIRT activation, as an NAD^+ booster in various animal models of age-related diseases[5]. In terms of CVD, Natalie et al. showed that oral NMN supplementation enhanced arterial SIRT1 expression and activity and restored some age-associated endothelial impairments, such as endothelium-dependent dilation and elastic artery stiffness, in mice[6]. Using an ex vivo model, Mateuszuk et al. showed that NMN improved angiotensin II-induced impairment of vasodilation of aortic rings[7]. They also revealed that NMN is incorporated into endothelial tissue via extracellular conversion of NMN to NR by ecto-5'-nucleotidase. Using SIRT1-knockout mice, Das et al. showed that NMN promotes angiogenesis by inhibiting Notch signaling, which is negatively regulated by SIRT1[8].

Compared to preclinical trials involving cell and animal studies, clinical trials on NMN supplementation in humans have only begun in the last few years[9]. Notably, these studies did not report adverse events. Some clinical reports have shown that NMN supplementation increases NAD^+ metabolites, indicating an increase in NAD^+ metabolism[10, 11]. NMN supplementation has been reported to increase muscle sensitivity in prediabetic women[11] and improve lower limb function and mental fatigue in older adults[12]. However, the effects of NMN supplementation on blood vessels have not been verified. In this study, we conducted a 12-week randomized, double-blind, placebo-controlled, parallel-group clinical trial in 36 middle-aged men and women who appeared healthy to investigate the effects of 12-week NMN supplementation on biochemical tests and metabolic health parameters, including indicators of blood vessel condition.

Results

Baseline characteristics and completion of clinical study

Figure 1 illustrates the study flowchart, which includes the processes of participant enrolment, allocation, follow-up, and analysis. Thirty-six healthy men and women aged 40–59 years agreed to participate in the study. Participants were randomly assigned to either the NMN intake (250 mg/day) or placebo group. The baseline characteristics of participants are shown in Table 1. At the eligibility assessment stage, no participants were excluded, including those who did not meet the selection criteria, declined participation, or were excluded for other reasons. No significant differences were found between the groups in any baseline parameters (Table 1). Following the 12-week intervention, one participant in each group was untraceable and was considered a dropout. All participants who completed the study showed no significant changes in parameters such as hematology, clinical chemistry, and hormones after the 12-week intervention (Table 2). These results indicate that long-term NMN supplementation at a dose of 250 mg/day is safe and well-tolerated in healthy middle-aged adults.

Table 1
Baseline characteristics of study participants

	Placebo (n = 18)	NMN (n = 18)	P-value
Sex (Male/Female)	6/12	8/10	0.733
Age (years)	47.9 ± 5.5	48.1 ± 5.4	0.951
Height (cm)	164.1 ± 6.2	167.7 ± 10.1	0.216
Weight (kg)	58.7 ± 9.1	62.9 ± 18.6	0.405
BMI (kg/M^2)	21.7 ± 2.3	21.9 ± 4.3	0.829
Systolic Blood Pressure (mmHg)	124.2 ± 14.5	119.8 ± 17.5	0.419
Diastolic Blood Pressure (mmHg)	76.2 ± 12.0	71.7 ± 16.6	0.316

Values are presented as mean ± standard deviation. Each parameter was measured prior to the initiation of the intervention. To analyze any significant differences between groups, data on sex were assessed by the chi-square test, and other parameters such as age, weight, BMI, and blood pressure were tested using Welch's t-test. Statistical significance was set at $p < 0.05$. BMI, body mass index; NMN, nicotinamide mononucleotide.

Table 2
Body composition and metabolic parameters of the placebo and NMN intake groups before and after the intervention period

	Placebo (n = 17)			NMN (n = 17)			P-value
	Baseline	12 weeks	% Change from baseline	Baseline	12 weeks	% Change from baseline	
Weight (kg)	57.8 ± 8.4	57.6 ± 7.8	-0.4	61.7 ± 18.5	61.9 ± 18.6	0.4	0.324
BMI (kg/M^2)	21.5 ± 2.3	21.5 ± 2.2	0.0	21.7 ± 4.2	21.9 ± 4.3	0.8	0.273
Blood glucose level (mg/dL)	90.1 ± 5.9	89.9 ± 6.5	-0.2	87.0 ± 4.5	89.0 ± 5.6	2.3	0.324
Blood count							
WBC (counts/ μL)	6015.3 ± 1876.9	5999.4 ± 1732.2	-0.3	5937.6 ± 1552.5	5853.5 ± 1871.5	-1.4	0.838
RBC (counts×10^4// μL)	454.3 ± 42.7	442.1 ± 36.8	-2.7	452.5 ± 41.9	445.8 ± 41.7	-1.5	0.376
Hemoglobin (g/dL)	13.6 ± 1.0	13.4 ± 0.9	-2.0	13.6 ± 1.8	13.3 ± 1.8	-2.0	0.971
Hematocrit (%)	42.7 ± 2.9	41.6 ± 2.9	-2.7	42.6 ± 4.1	41.7 ± 4.1	-2.1	0.686
Blood pressure							
Systolic (mmHg)	124.3 ± 15.0	127.1 ± 13.0	2.2	118.0 ± 16.2	119.2 ± 18.2	1.0	0.414
Diastolic (mmHg)	76.0 ± 12.4	79.4 ± 10.3	4.4	69.8 ± 15.0	70.8 ± 15.5	1.5	0.196
Liver function							
AST (U/L)	25.3 ± 7.9	23.1 ± 6.1	-8.6	22.8 ± 7.5	20.8 ± 5.4	-9.0	0.472
ALT (U/L)	24.9 ± 12.2	22.4 ± 9.8	-10.4	20.6 ± 10.9	17.4 ± 8.3	-16.0	0.193

Values are presented as mean ± standard deviation. Primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied for significance between the two groups using the baseline as a covariate. Statistical significance was set at $p < 0.05$. NMN, nicotinamide mononucleotide; BMI, body mass index; WBC, white blood cell; RBC, red blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DHEA-S, dehydroepiandrosterone sulfate.

	Placebo (n = 17)			NMN (n = 17)			P-value
	Baseline	12 weeks	% Change from baseline	Baseline	12 weeks	% Change from baseline	
γ-GTP (U/L)	32.4 ± 19.2	29.6 ± 18.7	-8.5	37.1 ± 46.2	33.0 ± 37.3	-11.1	0.956
Lipids							
HDL-cholesterol (mg/dL)	73.5 ± 16.5	74.2 ± 16.9	1.0	72.2 ± 15.5	73.5 ± 16.2	1.7	0.885
LDL-cholesterol (mg/dL))	122.7 ± 54.1	121.3 ± 47.6	-1.2	119.9 ± 25.9	120.3 ± 24.9	0.3	0.863
Triglyceride (mg/dL)	86.4 ± 54.7	83.1 ± 46.6	-3.8	69.1 ± 35.9	70.6 ± 42.1	2.1	0.804
Hormones							
Testosterone (ng/dL)	143.1 ± 193.7	139.7 ± 187.8	-2.4	212.2 ± 249.1	208.2 ± 257.6	-1.9	0.933
Progesterone (ng/mL)	2.1 ± 4.4	3.0 ± 4.8	43.6	2.4 ± 6.2	1.9 ± 4.4	-22.1	0.860
Estradiol (pg/mL)	75.2 ± 87.2	101.7 ± 123.8	35.1	81.0 ± 121.1	78.3 ± 82.4	-3.3	0.361
DHEA-S (ng/mL)	1610.1 ± 566.1	1864.2 ± 751.2	15.8	1875.8 ± 1087.3	1923.1 ± 972.4	2.5	0.166
Serum cortisol (μg/dL)	8.4 ± 2.6	7.9 ± 2.5	-6.6	8.1 ± 3.1	7.2 ± 1.9	-11.0	0.422

Values are presented as mean ± standard deviation. Primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied for significance between the two groups using the baseline as a covariate. Statistical significance was set at $p < 0.05$. NMN, nicotinamide mononucleotide; BMI, body mass index; WBC, white blood cell; RBC, red blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DHEA-S, dehydroepiandrosterone sulfate.

Measurement of NAD^+ and related metabolites in the blood

To investigate whether the 12-week administration of NMN alters NAD^+ metabolism, the serum concentrations of NAD^+ and related metabolites, such as NAM and NMN, were measured using isotope dilution liquid chromatography with tandem mass spectrometry analyses. As shown in Table 3, NAM in the placebo group decreased from 14.9 ± 3.8 to 10.9 ± 4.8 ng/mL, while that in the NMN group increased from 10.4 ± 3.5 to 16.5 ± 6.3 ng/mL. Blood NAM levels in the NMN intake group were significantly

increased after the intervention compared to the placebo group ($p = 0.037$). NAD^+-consuming enzymes such as SIRTs and poly ADP-ribose polymerase hydrolyze NAD^+ to produce NAM and ADP-ribosyl products[5]. On the other hand, NAD^+ and NMN in the serum were detectable in both groups, but the values were below the lower limit of quantification. Therefore, it was difficult to compare these two parameters between the groups. These results indicate that NMN supplementation effectively enhanced NAD^+ metabolism in middle-aged adults.

Table 3

NAD^+ metabolites of the placebo and NMN intake groups before and after the intervention period

	Placebo (n = 17)			NMN (n = 17)			P-value
	Baseline	12 weeks	% Change from baseline	Baseline	12 weeks	% Change from baseline	
NAM (ng/mL)	14.9 ± 3.8	10.9 ± 4.8	-26.8	10.4 ± 3.5	16.5 ± 6.3	57.6	0.037*
NMN (ng/mL)	< 2	< 2	N.A.	< 2	< 2	N.A.	N.A.
NAD (ng/mL)	< 5	< 5	N.A.	< 5	< 5	N.A.	N.A.

Values are presented as mean ± standard deviation. Primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied for significance between the two groups using the baseline as a covariate. Statistical significance was set at $p < 0.05$. NAM, nicotinamide; NMN, nicotinamide mononucleotide; NAD, nicotinamide adenine dinucleotide.

Measurement of indicators related to blood vessel condition

We evaluated the effects of NMN supplementation on the conditions of blood vessels. In addition to biochemical and hematological tests, we measured the ankle-brachial index (ABI) and brachial-ankle pulse wave velocity (baPWV) to evaluate blood flow and arterial stiffness, respectively. There were no characteristic clinical findings in the blood pressure, number of blood cells, and ABI values in both groups (Tables 2 and 4). The average baPWV values in the NMN intake group tended to decrease by 25.1 ± 14.5 cm/s (Fig. 2). However, no significant difference was observed in the average baPWV values between the two groups ($p = 0.097$). Since hypertension, obesity, and hyperglycemia are considered cardiovascular risk factors, we performed subgroup analyses of NMN and placebo groups in participants with above-average blood pressure, body mass index (BMI), or blood glucose level. In the analyses of participants with higher-than-mean systolic or diastolic blood pressure, there was no significant change in baPWV values between the groups during the test period (Figs. 3A and B). In contrast, in participants with above-average BMI or blood glucose levels, baPWV values in the NMN intake group were significantly decreased after the test

period compared to the placebo group (Figs. 3C and D). These results suggest that NMN supplementation potentially improves vascular health in middle-aged adults, especially in those with high BMI or blood glucose levels.

Table 4
Parameters for assessing the vascular function of the placebo and NMN intake groups before and after the intervention period

	Placebo (n = 17)			NMN (n = 17)			P-value
	Baseline	12 weeks	% Change from baseline	Baseline	12 weeks	% Change from baseline	
ABI							
Right	1.11 ± 0.04	1.12 ± 0.06	1.3	1.10 ± 0.07	1.11 ± 0.07	0.6	0.623
Left	1.10 ± 0.06	1.13 ± 0.06	2.2	1.10 ± 0.07	1.11 ± 0.08	0.6	0.359
Average	1.11 ± 0.04	1.12 ± 0.06	1.8	1.10 ± 0.06	1.11 ± 0.06	0.6	0.367
BaPWV (cm/s)							
Right	1332.7 ± 227.1	1339.6 ± 217.1	0.5	1211.0 ± 153.8	1176.4 ± 161.7	-2.9	0.070
Left	1343.6 ± 216.5	1349.4 ± 211.7	0.4	1205.1 ± 153.6	1189.5 ± 131.1	-1.3	0.147
Average	1338.1 ± 219.9	1344.5 ± 212.9	0.5	1208.0 ± 152.2	1182.9 ± 145.3	-2.1	0.097

Values are presented as mean ± standard deviation. Primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied for significance between the two groups using the baseline as a covariate. Statistical significance was set at $p < 0.05$. ABI, ankle-brachial index; baPWV, brachial-ankle pulse wave velocity; NMN, nicotinamide mononucleotide.

Measurements of other health parameters

We assessed the effects of NMN administration on three health care parameters: urinary 8-hydroxydeoxyguanosine (8-OHdG), SIRT1 mRNA expression in the blood, and advanced glycation end products (AGEs) in the skin. However, no significant differences were observed between the two groups (Table 5).

Table 5
Health parameters of the placebo and the NMN intake groups before and after the intervention period

	Placebo (n = 17)			NMN (n = 17)			P-value
	Baseline	12 weeks	% Change from baseline	Baseline	12 weeks	% Change from baseline	
SIRT1 mRNA level	3.6 ± 1.0	4.9 ± 1.4	38.5	3.6 ± 1.6	4.3 ± 2.0	18.8	0.211
AGEs (a.u.)	0.6 ± 0.1	0.5 ± 0.1	-3.9	0.5 ± 0.1	0.5 ± 0.1	7.6	0.283
8-OHdG (ng/mg creatinine)	7.1 ± 2.4	7.3 ± 2.6	2.3	7.9 ± 2.6	8.0 ± 3.3	0.7	0.735

Values are presented as mean ± standard deviation. Primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied for significance between the two groups using the baseline as a covariate. Statistical significance was set at $p < 0.05$. Abbreviations: SIRT1, sirtuin 1; AGEs, advanced glycation end products; 8-OHdG, 8-hydroxydeoxyguanosine; NMN, nicotinamide mononucleotide.

Discussion

In this study, NMN supplementation was shown to be safe and beneficial for the activation of NAD metabolism in apparently healthy middle-aged individuals. We also found that 12-week supplementation with 250 mg NMN tended to reduce baPWV values. A previous meta-analysis of longitudinal cohort studies investigating baPWV showed that the risk of cardiovascular events associated with high baPWV is almost three times that associated with low baPWV[13]. Thus, NMN supplementation may alleviate vascular stiffness and reduce the risk of CVD events. To date, there has been one clinical trial report on the effect of another NAD precursor NR on PWV, demonstrating that a 6-week supplementation with 1000 mg/day of NR tended to reduce carotid-femoral PWV in healthy middle-aged and older people[14]. Our results show that NMN supplementation can affect PWV values, along with NR supplementation. NMN supplementation significantly increased the blood levels of the NAD metabolite NAM, suggesting that the ingested NMN was utilized in the blood. PWV has been reported to correlate well with age, and the estimated age of blood vessels can be calculated from a participant's baPWV value[15]. The change in the estimated age of blood vessels in the placebo and NMN intake groups was calculated using the equation reported by Tomiyama and was 0.5 ± 6.1 and − 2.0 ± 4.3, respectively. However, the mechanism by which NMN supplementation improves baPWV remains unclear. A previous animal study showed that oral NMN ingestion activates arterial SIRT1 and normalizes age-related changes in the composition of constituent proteins such as collagen and elastin within the vessel wall[6]. To further elucidate the mechanism, we also assessed several health parameters, including urinary 8-OHdG, SIRT1 expression, and AGEs in the skin, but no significant changes were found.

In a subgroup analysis limited to participants with higher-than-mean BMI, baPWV levels in the NMN intake group were significantly reduced after the intervention compared to the placebo group. Since this study was aimed at healthy individuals, their BMI levels were in the normal range; however, obesity tendencies may make the effect of NMN supplementation on vascular stiffness more pronounced. In cross-sectional studies, PWV is often reported to be negatively correlated with obesity-related parameters[16, 17]. However, some cohort studies have reported a positive association between obesity and PWV[18, 19]. Wildman et al. showed that weight loss reduces PWV in clinical trials including obese patients[18]. In contrast, in this study, no significant change in body weight was observed in the NMN intake group with higher-than-mean BMI during the intervention. Therefore, it appears that the decrease in baPWV after NMN supplementation was not due to weight loss. In recent years, it has been clarified that adipocytokines secreted by adipocytes are closely related to CVD[20], and clinical studies have also reported a significant association between decreased serum adiponectin and increased PWV[21]. *In vivo* studies have reported that deficiency of the NAMPT gene leads to reduced adiponectin production, while oral NMN intake normalizes it[22]. Considering that oral intake of NMN in aged mice has been reported to improve PWV[6], restoration of adiponectin production may be a reasonable mechanism by which NMN improves PWV. In contrast, in a study on NMN supplementation in prediabetic women, Yoshino et al. reported that a 10-week NMN supplementation (250 mg/day) did not cause any significant change in the plasma levels of high-molecular-weight adiponectin[11]. As there are still very few reports of clinical trials in humans compared to animal studies, it is necessary to further increase the number of participants in NMN clinical trials in the future. Measuring PWV and adiponectin levels together may be a good strategy to elucidate the mechanisms underlying NMN supplementation.

We also conducted a subgroup analysis limited to participants with higher-than-mean blood glucose levels and showed that NMN supplementation significantly reduced baPWV levels after 12 weeks. Interestingly, there was no change in blood glucose levels in participants with higher-than-mean blood glucose levels after 12-week NMN supplementation, but diastolic blood pressure significantly decreased (Supplementary Table S1). Takase et al. have reported that baPWV was positively correlated with blood pressure[23]. Thus, the ameliorating effect of NMN supplementation on baPWV might reduce diastolic blood pressure in middle-aged participants. Hypertension is a known risk factor for CVD. It has been clarified that a relatively mild increase in blood pressure increases cardiovascular risk even when the blood pressure is within a range that is considered healthy[24]. Thus, from the viewpoint of CVD prevention, it is important to control blood pressure at any stage, even if it falls within the healthy range. Vidal-Petiot et al. showed that a diastolic blood pressure of 80–89 mmHg increased CVD risk compared with 70–79 mmHg, while a systolic blood pressure of 130–139 mmHg did not, and concluded that it is more significant for patients with coronary artery disease to prioritize diastolic blood pressure < 80 mmHg as a blood pressure-lowering target than systolic blood pressure < 130 mmHg[25]. Therefore, as observed in the subgroup analysis, the ameliorating effect of NMN supplementation on diastolic blood pressure may help reduce CVD risk, even in people who seem to be healthy.

In conclusion, in this study, we showed that long-term NMN supplementation in healthy middle-aged individuals is safe, beneficial for activating NAD metabolism, and beneficial for reducing baPWV. For future studies, more evident effects of NMN on human health may be observed by further increasing the number of participants and dose of NMN. In addition, in the subgroup analysis of participants with high BMI or high blood glucose levels, baPWV values were significantly lower in the NMN intake group than in the placebo group. Thus, it may be possible to see a more evident effect of NMN on vascular stiffness by conducting tests on not only healthy but also obese or hyperglycemic participants. This study is the first to show that NMN supplementation could help reduce CVD risk and may be useful for advancing larger-scale studies in the future.

Materials And Methods

Study design

Thirty-six healthy male and female participants were enrolled in this randomized, double-blind, placebo-controlled, parallel study. Written informed consent was obtained from all participants involved in the study. Participants were randomly assigned to two groups by Orthomedico Inc. (Tokyo, Japan). Participants visited the clinic in Tokyo, Japan, for laboratory tests and safety assessments at baseline prior to the initiation of the intervention. All results were reviewed by a physician. Participants were provided with either NMN (125 mg/capsule) or placebo capsules in batches for 12 weeks, and one capsule was ingested twice a day after meals for 12 weeks. After the 12-week intervention period, participants returned to the clinic for laboratory tests and safety assessment. The study was completed when the last participant visited the clinic on 08/12/2021. The researchers involved in collecting and analyzing the results were not informed of the treatment conditions. All the procedures in this study were approved by the ethics committee of our institution on 04/08/2021 (No. 202101). This study was conducted in accordance with the ethical principles based on the Declaration of Helsinki and its subsequent amendments and was registered with the identifier UMIN000045205 on 20/08/2021 in the clinical trial registration system of the University Hospital Medical Information Network and met the criteria of the International Committee of Medical Journal Editors.

Participants

Eligible participants were men and non-pregnant or non-breastfeeding women between 40 and 65 years of age who seemed healthy at the time of provision of consent to participate in the study. All the participants were recruited between 22/06/2021 and 07/07/2021. The key exclusion criteria were as follows: 1) history of serious hepatic, renal, cardiac, pulmonary, or gastrointestinal (including gastrectomy) disease; diabetes, food allergies, or other serious comorbidities; 2) use of drugs that may affect the test values in this study; and 3) participation in other clinical trials. Participants continued to take dietary supplements or medicines that they had taken prior to the study and avoided starting any new dietary supplements during the study.

Randomization and blinding

An allocation controller at Orthomedico Inc. randomly assigned participants to two supplementation groups (NMN or placebo) by block random allocation. The allocation ratio of each group was 1:1. An allocation table was created based on the algorithm of block random allocation. The allocation table contained the types of groups (NMN or placebo) and the identification code of each individual and was kept concealed using an emergency key by the allocation controller until the blinding process was completed. DHC Corporation provided the NMN and placebo capsules to the allocation controller. After ensuring that the capsules were indistinguishable, the allocation controller assigned an identification code to all the capsules based on the allocation table. After the screening test, the capsules with the identification code were given to participants by a blinded investigator. Information on allocation was not revealed until the participants for analysis were determined at a clinical meeting after test completion.

Characteristics of participants

BMI (kg/m^2) was calculated as weight divided by height squared. Systolic and diastolic blood pressure were measured using a Heart Station S MPV-550 (A&D, Tokyo, Japan). Blood and urine samples were collected at the clinic, and subsequent tests investigating blood glucose levels, liver function, lipids, and hormones were performed by outsourcing their analyses to BML (Tokyo, Japan). ABI and baPWV were measured using a waveform analyzer (BP-203RPE III, Omron, Kyoto, Japan).

Quantification of serum NAD^+, NMN, and NAM

The serum concentrations of NAM, NMN, and NAD^+ were calculated by outsourcing their analyses to LSI Medience (Tokyo, Japan). Briefly, the serum was transferred to a test tube and mixed with methanol (1/6, v/v). After being centrifuged at 1,000 × g for 15 min, the supernatant was dried in nitrogen gas at 40°C. The residue was dissolved in a 5 mM ammonium acetate solution and analyzed using a liquid chromatography-tandem mass spectrometer (6470 B Triple Quadrupole system, Agilent, CA, US) equipped with a reverse-phase Aqcuity HSS T3 column (2.0 × 100 mm, 1.8 μm, Waters, MA, US). Quantitative analyses were conducted using Mass Hunter software (Agilent). The peak area was corrected using an internal standard, and the concentration of each component was determined using a calibration curve.

Real-time PCR analysis for SIRT1 mRNA expression

SIRT1 mRNA expression in the blood was evaluated by outsourcing the analysis to the Research Center for Immunological Analysis (Okayama, Japan). Briefly, whole blood was collected in a PAXgene® RNA blood collection tube (Qiagen, Mississauga, Canada). Total RNA was isolated from the blood using a PAXgene® Blood RNA Kit (Qiagen) and stored in RNase-free water at − 80°C. First-strand cDNA was reverse-transcribed with total RNA using a T100® Thermal Cycler (Bio-Rad, CA, USA). The mRNA expression level of the target genes was measured using a CFX384® Touch Real-Time PCR Detection System (Bio-Rad). *β-Actin* was used as a housekeeping gene for normalization. The comparative C_T method was used to calculate the relative levels of SIRT1 mRNA.

Measurement of urine 8-OHdG

Urine 8-OHdG content was measured by outsourcing the analysis to the Nikken Seil (Shizuoka, Japan). Briefly, urine 8-OHdG was quantified using an enzyme-linked immunosorbent assay kit for 8-OHdG (Nikken Seil), according to the manufacturer's instructions. Measured urinary 8-OHdG levels were normalized to urinary creatinine concentrations and are shown as the urinary 8-OHdG (ng/mL)/creatinine (mg/mL) ratio.

Measurement of AGEs

AGEs were measured on the middle finger using an AGE sensor RQ-1201J (Sharp Life Science, Kobe, Japan), according to the manufacturer's instructions. Excitation light from the light source through the lens illuminates the skin surface of the fingertip inserted into the sensor. The emission spectrum reflected from the skin is detected as the accumulation of AGEs in the skin. The value of AGEs obtained with this device has been reported to correlate with the blood levels of methylglyoxal-derived hydroimidazolone-1, a major AGE in proteins[26].

Statistical analyses

Statistical analyses of the baseline characteristics of participants were performed in an intention-to-treat population. For analyzing significant differences between groups, data on sex were assessed by the chi-square test, and other parameters, such as age, height, weight, BMI, and blood pressure, were tested using Welch's t-test.

The primary efficacy variables were statistically analyzed using a full-analysis-set population. Analysis of covariance was applied to the significance between groups using the baseline as a covariate.

Statistical analyses were performed using IBM SPSS Statistics version 23 software (IBM, NY, USA). Statistical significance was set at $p < 0.05$.

Declarations

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Author contributions

Conceptualization: KTN

Validation: TK, KTN

Investigation: TK, SU, NN

Methodology: TK, SU, NN, KTN

Writing - Original Draft: KTN

Writing - Review & Editing: TK, SU, NN, TN, NK, MK

Visualization: TK

Supervision: KTN

Project administration: MK

Data Availability

The datasets generated during and/or analyzed during the current study are available on request.

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Competing interests

The authors declare no competing interests.

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Figures

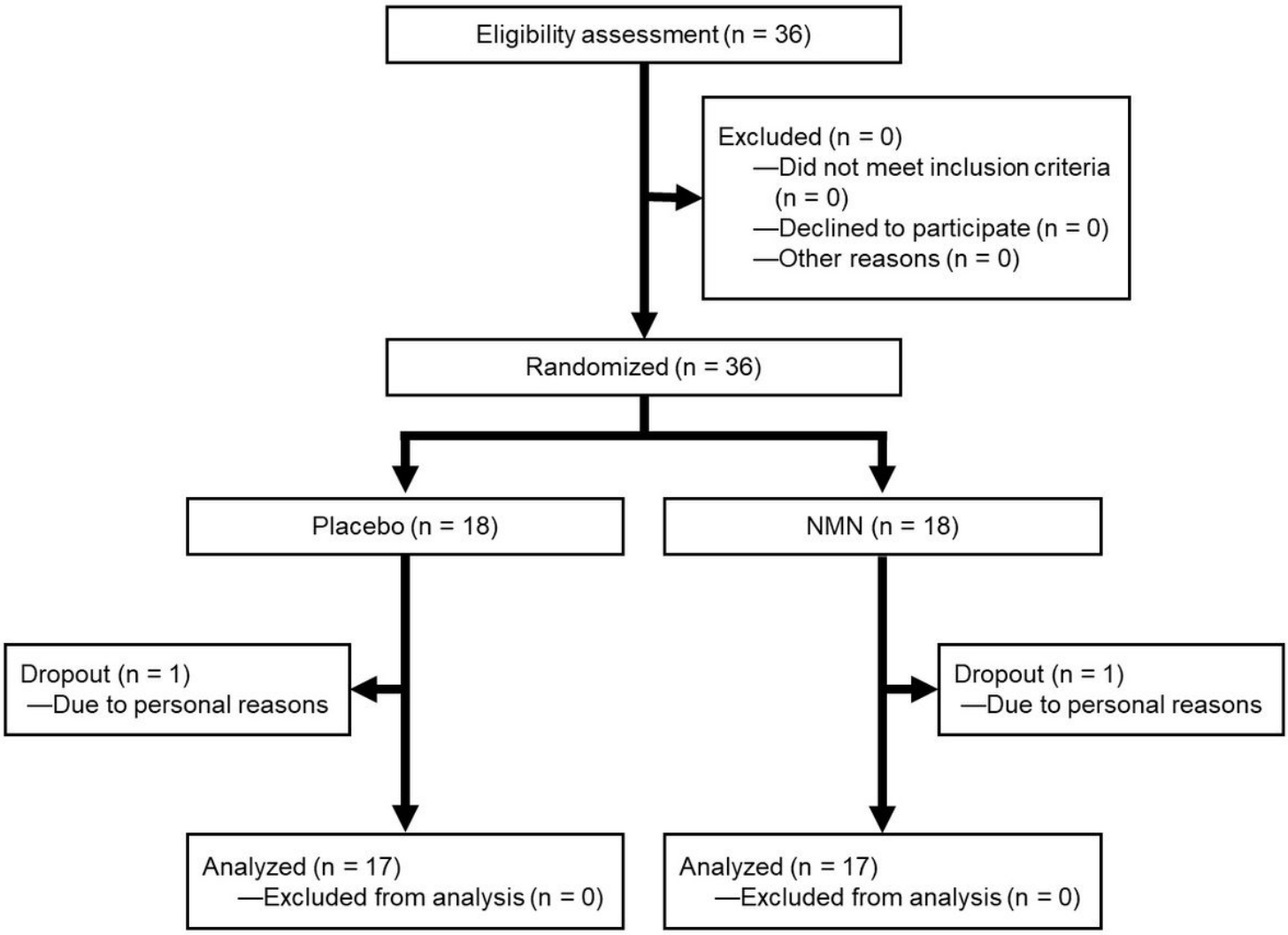

Figure 1

Clinical trial flowchart

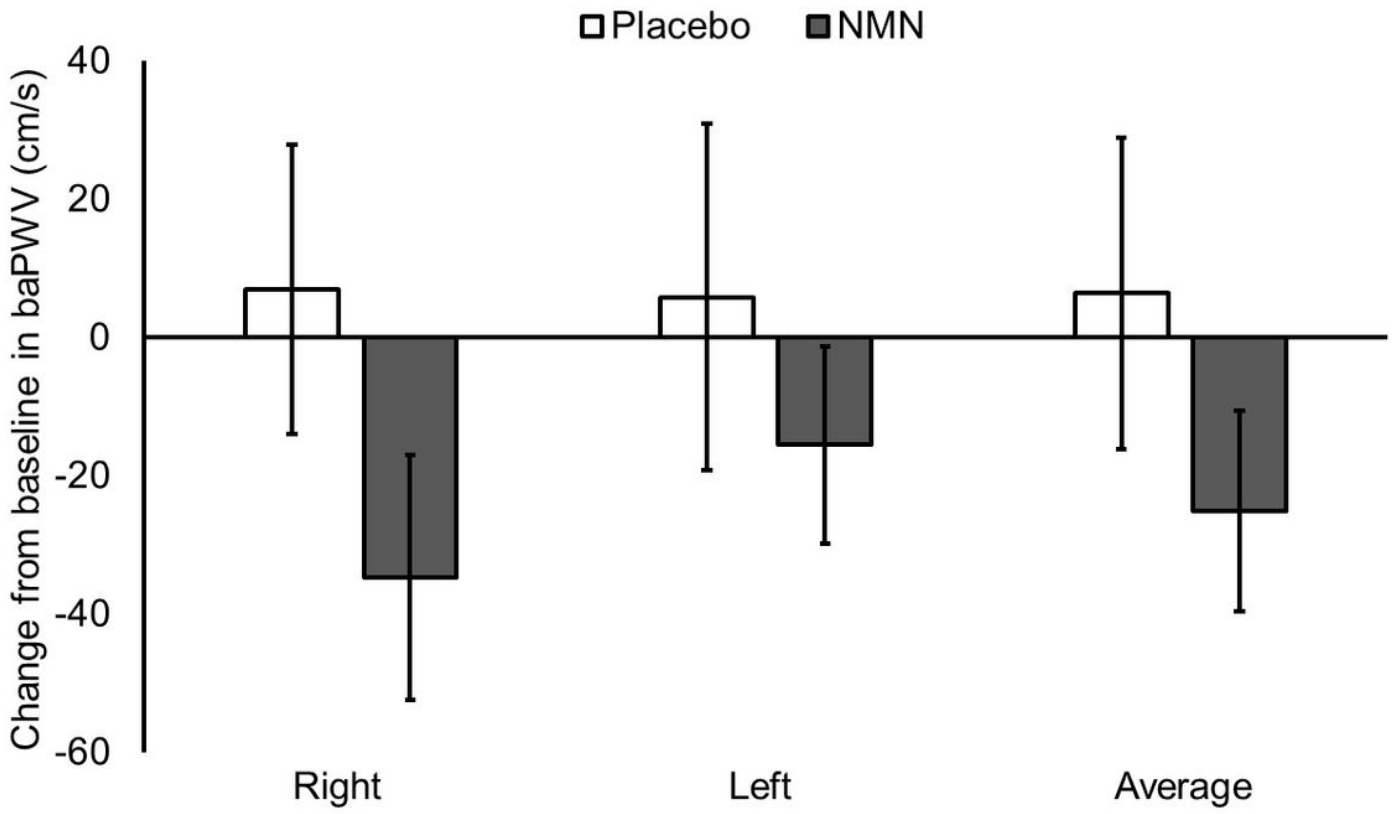

Figure 2

Change from baseline in brachial-ankle pulse wave velocity (baPWV) after nicotinamide mononucleotide (NMN) or placebo supplementation

Values are expressed as mean ± standard error.

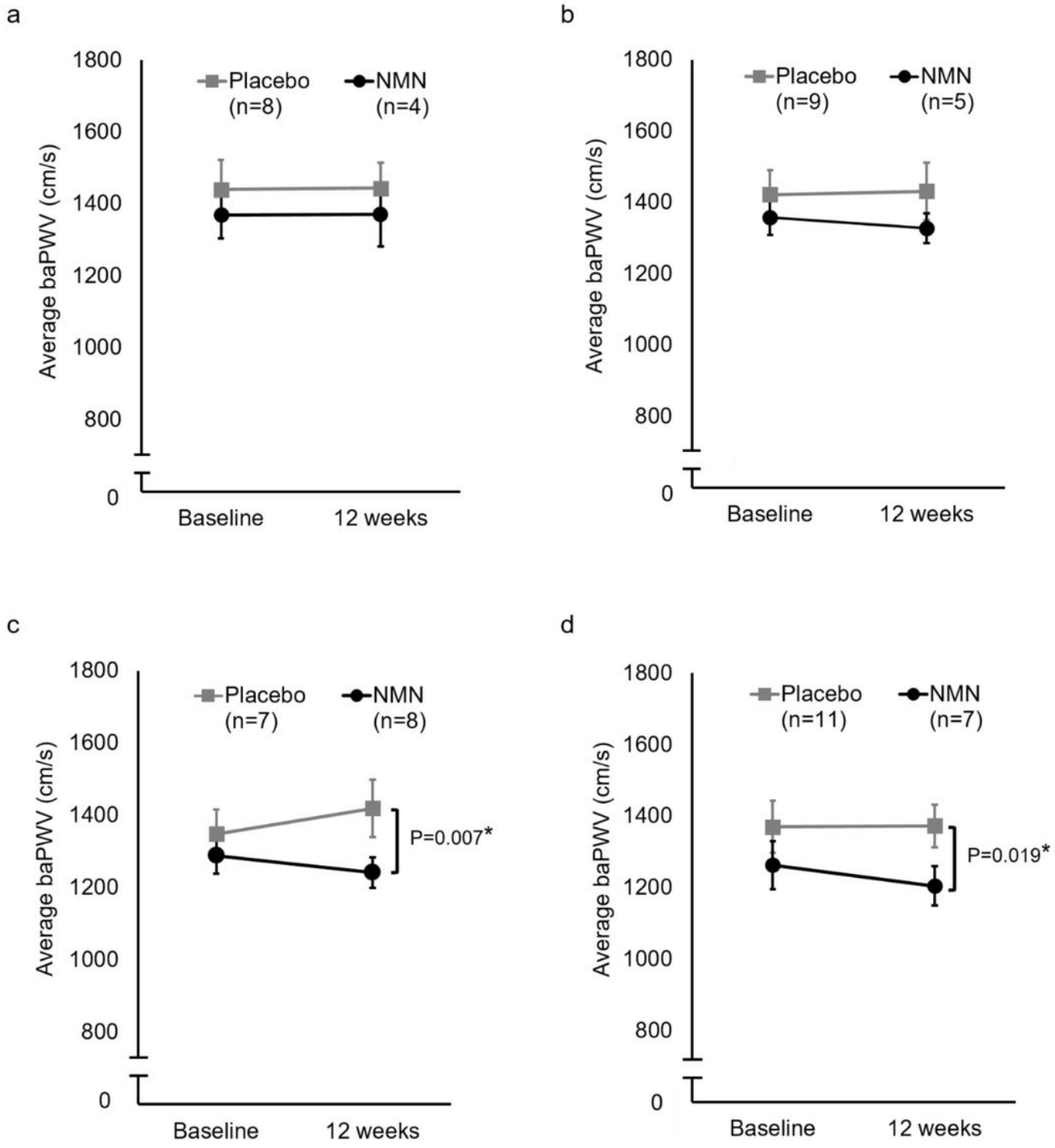

Figure 3

Subgroup analyses of NMN and placebo groups for baPWV

(**a**) Participants with above-average systolic blood pressure, (**b**) participants with above-average diastolic blood pressure, (**c**) participants with above-average BMI, and (**d**) participants with above-average blood glucose levels. Values are expressed as mean ± standard error.

Supplementary Files

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- SupplementaryTableS1.docx