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Abstract

Preclinical studies have revealed that the elevation of nicotinamide adenine dinucleotide (NAD+) levels on administration of an NAD+ precursor, nicotinamide mononucleotide (NMN), can mitigate aging-related disorders; however, human data are sparse. Therefore, we aimed to investigate whether the chronic oral supplementation of NMN can elevate blood NAD+ levels and alter physiological dysfunctions, including muscle weakness, in healthy elderly participants. We administered 250 mg NMN per day to aged men for 6 or 12 weeks (n=21 for 6 weeks, n=10 for 12 weeks) in a placebo-controlled, randomized, double blind, parallel-group trial. Chronic supplementation with NMN was well tolerated and did not cause any significant deleterious effect. Metabolomic analysis of whole blood demonstrated that the oral supplementation of NMN significantly increased the concentrations of NAD+ and NAD+ metabolites. Moreover, NMN significantly improved muscle strength and performance, which were evaluated using the 30-second chair stand test, walking speed, and grip strength, and it showed no significant effect on body composition. Thus, our evidence indicates that chronic oral NMN supplementation can be an efficient NAD+ booster for preventing aging-related muscle dysfunctions in humans.

Aging is a risk factor for diabetes, cardiovascular diseases, cancer, and neurological diseases such as Alzheimer's disease, and the suppression of physiological decline in aging is an important approach to prevent aging-related diseases[1]. Calorie restriction is known to have life-extending and health-promoting effects in many species, including mammals[2], and it regulates several molecular mechanisms that cause health-promoting effects. Among them, nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase sirtuins, discovered in yeast and proven to extend the lifespan in mice, mediate the health-promoting effects of calorie restriction[2].

Aging- and age-related diseases have been shown to be closely related to decreased NAD+ levels and sirtuin activity[3]. In animal studies, administration of intermediate NAD+ metabolites, nicotinamide mononucleotide (NMN) or nicotinamide riboside (NR), increases NAD+ concentrations and activates sirtuins to improve health and extend the lifespan[3-5]. For example, NR administration ameliorates age-related decline in the self-renewal of intestinal stem cells[6]. Thus, the potential of intermediate NAD+ metabolites to improve tissue rejuvenation in humans has led to many clinical trials with NR and NMN.

NR, a vitamin B3 analog, is a major vitamin component found in milk (~1 mg/L) [7]. NMN is found in foods such as edamame, broccoli, and meat (~1 mg/100 g

food)[8]. However, due to their extremely low amounts in foods, it is difficult to obtain a sufficient amount of these components from food. Therefore, purified and concentrated NR and NMN have been used in clinical trials.

The results of NR clinical trials have already been reported. NR (100–2000 mg/day) has been administered to healthy or obese participants for a maximum of 12 weeks[9-19]. Most NR clinical trials have reported the safety of NR administration[9-18] and elevated NAD+ or NAD+-related metabolites in the blood or tissues[9-17]. The most recent report showed that NR increases the fat-free body mass in obese participants, although no effect was observed on insulin sensitivity, mitochondrial function, and hepatic and intramyocellular lipid accumulation[17].

Recently, for the first time, the safety of single-day NMN oral administration has been reported in humans[20]. However, the evidence of human interventions with NMN is limited and the safety and efficacy of long-term NMN administration have not been determined.

Therefore, to elucidate the safety and efficacy of NMN administration, we conducted a placebo-controlled, randomized, double-blind, parallel-group study with the administration of 250 mg of NMN to healthy men aged 65 years or above for 12 weeks. We demonstrated that the oral supplementation of 250 mg/day NMN in healthy old men for 12 weeks was safe, well tolerated, and significantly increased

NAD+-and NAD+- related metabolites in the whole blood. Furthermore, NMN administration significantly improved muscle performance in healthy elderly men. Thus, the chronic oral administration of NMN can be a therapeutic strategy for aging-related disorders in humans such as sarcopenia.

Results

Participant enrollment and baseline characteristics

Sixty-five elderly men aged 65 years and above were screened for the study, which was conducted between July 2019 and November 2019 and registered on UMIN-CTR under the identifier UMIN000036321. Eight participants were excluded due to a specific medical history or abnormal laboratory data. Three participants were enrolled in other clinical trials after obtainment of consent. The other two participants were excluded because they requested to withdraw immediately after providing consent. The 42 enrolled subjects were randomized between the two treatment groups (placebo group and 250 mg NMN/day group) (Figure 1).

The supplements (placebo or NMN) were supplied to each group of participants at 0- and 6-week visits. However, after completion of the study, it came to light that at the 6-week visit, 11 participants each in the NMN and placebo groups received the other supplement due to the supplier's mistake. According to the decision of the Ethics Committee of the University of Tokyo Hospital, we decided to exclude the acquired data from the 22 participants at the 12-week visit (Figure 1).

The main physical and metabolic features of the NMN (n=21) and placebo groups (n=21) are summarized in Table 1. Key parameters were comparable between the two groups at baseline. Excluding the 22 participants, the physical characteristics of all participants in the NMN and placebo groups at baseline are shown in Table S1.

Supplementation of 250 mg/day NMN for 12 weeks is well tolerated

Adherence to the study treatment was excellent, with all participants consuming greater than 90% of all NMN and placebo supplements administered. NMN (250 mg/day) was well tolerated, and no serious adverse event occurred. Clinical laboratory values were obtained from blood samples collected at baseline and at the 12-week visit. No significant difference was observed between the NMN and placebo groups for hematology and blood chemistry, including liver enzymes and renal function markers (Tables S2 and S3). Importantly, all clinical laboratory values remained within the normal range in the NMN group. These results support that the supplementation of 250 mg/day NMN for 12 weeks is well tolerated in healthy old men.

Chronic oral administration of NMN increases NAD+ and related metabolites in the whole blood

Whole blood was collected at baseline and at the 12-week visit from participants for subsequent analysis of NAD+ and related metabolites using liquid chromatography-mass spectrometry (LC-MS/MS). As shown in Figure 2, oral NMN supplementation effectively elevated the levels of NMN and NAD+ as compared to the placebo. We also observed an increase in NR, which may indicate the possible conversion of NMN to NR by CD73[21]. Notably, NMN also significantly elevated nicotinic acid mononucleotide (NAMN) and nicotinic acid riboside (NAR) levels, which are intermediates of the NAD+ *de novo* synthesis pathway. Collectively, these findings indicate that the chronic oral supplementation of NMN effectively stimulates NAD+ metabolism in healthy elderly men.

Chronic oral administration of NMN improves motor functions

To examine the effect of oral administration of NMN on skeletal muscle mass in healthy old men, skeletal mass index (SMI) and segmental lean (lean trunk, arms, and legs) were measured using bioimpedance analysis (BIA), and the mean values

in the NMN and placebo groups at baseline and the 6-and 12-week visits were evaluated using mixed model analysis or mixed-effect model for repeated measures (MMRM) [22]. The means of each group at both the visits were compared using the Mann-Whitney U and t-tests for non-normal distribution. Furthermore, the difference between pre- and post-placebo and pre- and post-NMN supplementation (∆Placebo and ∆NMN, respectively) at the 6-and 12-week visits were analyzed using ANCOVA. No significant difference was observed in the skeletal muscle mass in any of these analyses (Table 2).

On the other hand, to examine muscle strength and performance, gait speed, counts in 30-second chair stand test, and grip strength were assessed and analyzed using the same statistical method. (Table 3). Mixed model analysis or MMRM showed a significant improvement in gait speed (p=0.033) and left grip test (p=0.019) after NMN administration. We also observed a significant difference in gait speed between the mean values of each group at the 6-and 12-week visits (p=0.023 and p=0.002, respectively). Furthermore, a significant difference was observed in the 30-second chair stand test between ∆Placebo and ∆NMN groups at the 6-week visit (p=0.031) (Table 3).

These findings indicate that the chronic oral supplementation of NMN improved muscle strength and performance in healthy old men, although NMN did

not affect skeletal muscle mass.

Liver and visceral fat mass are not affected on NMN supplementation

Next, we investigated the effect of NR on fat mass distribution because animal studies suggest a positive role of NMN on insulin sensitivity and hepatic steatosis[4, 5] (Figure 3). Chronic NMN supplementation did not affect the visceral fat area (Figure 3A) and CT values of the liver and spleen (L/S ratio) in the CT scan (Figure 3B), in accordance with the measurement of fat mass using BIA (Figure 3B). Likewise, NMN administration did not affect the homeostatic model assessment of insulin resistance (HOMA-IR), an indicator of hepatic insulin sensitivity in blood analysis (Figure 3C). Adiponectin and interleukin (IL) 6, which are also related to insulin sensitivity, were not affected by NMN administration (Table S4). These data indicate that insulin sensitivity and fat mass were not affected by NMN supplementation in our study. Consistently, no significant difference or trend was observed in triglycerides, LDL cholesterol, HDL cholesterol, HbA1c, FBG, HOMA-β, calculated AUC of glucose, insulin, and C-peptide in 75 g of the sample using oral glucose tolerance test OGTT after NMN supplementation (Figure 3C and TableS4).

Effect of NMN on other aging-related phenotypes

To gain exploratory insight into the potential benefits of NMN supplementation on other domains of physiological functions in healthy old men, we assessed a wide variety of outcomes indicative of sensory, vascular, and cognitive functions. Right audibility improved with a significant difference ($p=0.0268$, mixed model analysis) (Table S5). On the other hand, no difference was observed in the indicators of vascular functions, such as assessed blood pressure and Flow Mediated Dilation (Table S6). Finally, no effect was observed of the intervention on overall cognitive function, as assessed by mini-mental state examination-Japanese (MMSE-J) and the Japanese version of the Montreal Cognitive Assessment (MOCA-J) (Table S5).

Discussion

In this study, we reported that the chronic oral supplementation of 250 mg NMN per day is safe and a well-tolerated and effective strategy for boosting NAD+ metabolism in healthy elderly men. Additionally, our exploratory analyses of the effects of NMN supplementation on physiological functions suggest the ability of NMN to improve muscle strength, which is an important clinical indicator of aging.

When this study was designed, the results of the NMN clinical trial were not available. Some studies have reported the effects of oral or intraperitoneal NMN (100–500 mg/kg/day) administration in mice[4, 5]. Particularly, long-term administration of NMN at doses of 100 or 300 mg/kg/day for one year showed no significant side effect; however, insulin sensitivity and eye functions deteriorate with aging[8]. If this dosage is converted to the absorption area of the small intestine, 100 mg/kg/day NMN in mice is considered equivalent to 8 mg/kg/day intake in humans[23]. On the other hand, some clinical studies have been conducted on humans in Japan and the US. The US study (ClinicalTrials.gov Identifier: NCT03151239) was performed at a dose of 250 mg/day for 8 weeks, and the Japanese study (UMIN ID UMIN000025739) was performed at a dose of 100 or 200 mg/day for 24 weeks. Additionally, several human clinical trials have been conducted on NR, another

precursor of NAD+, in which NR has been administered at doses of 100-2000 mg/day for up to 12 weeks with no serious side effects[9-19]. Finally, the NMN dose in this study was set in consideration of the dose in previously reported NR (100–2000 mg/day) and ongoing NMN clinical trials (100, 200, 250 mg/day).

In the first NMN study, a single oral administration of 500 mg NMN did not show any specific deleterious side effects in healthy men[20]. Likewise, the 12-week chronic administration of 250 mg NMN also showed no significant side effect. NR has been demonstrated to be well-tolerated in all published clinical studies[9-19] whereas niacin analogs, including nicotinic acid (NA), nicotinamide (NAM), and NAD+ precursors, are known to induce nausea and flushing, leading to a difficulty in using high doses of niacin to increase NAD+[24,25]. Niacin has also been reported to induce hepatotoxicity, hyperglycemia, and hyperuricemia[24, 25]; however, we did not find any abnormality in clinical laboratory values, including those of liver or muscle enzymes in our study. Overall, NMN was well tolerated up to a chronic dose of 250 mg.

A previous study reported that NR administration significantly increases tissue NAD+ and plasma or whole blood NAD+ levels in healthy participants[9-12, 15, 16]. On the other hand, no previous NMN study directly detected NAD+ increase in the blood whereas the major final metabolites of NMN, such as methylnicotinamide, N-methyl-

2-pyridone-5-carboxamide, and N-methyl-2-pyridone-5-carboxamide, have been analyzed[20]. Thus, this is the first study to report that NMN administration significantly increased NAD+ and NAD+ metabolites in the whole blood. One unexpected finding was a remarkable elevation in NAMN and NAR levels, which was not an *en route* for the conversion of NMN to NAD+. Previous reports have shown that as the rate of NAD+ synthesis increases, the deamination of NAD+ to nicotinic acid adenine dinucleotide (NAAD) occurs in competition with NAD+ turnover to nicotinamide, suggesting that NAAD can serve as a sensitive biomarker for increased NAD+ metabolism[9]. Alternatively, an increase in NAD+ can result in NMN deamination, giving rise to NAMN. Another explanation is the deamination of NMN by gut microbiota. Oral NAM or NR can be deamidated into NA, NAR, NAAD, and NAMN by gut microbiota in the small intestine and colon[26]. These deamidated NAD+ metabolites circulate to the tissues, contributing to NAD+ synthesis[26].

Skeletal muscle mass and strength decrease with aging because of muscle atrophy, leading to a reduced quality of life[27]. The application of NMN *in vivo* ameliorates muscle decline in rodent models[4, 5]. NMN has also been reported to improve mitochondrial functions in the skeletal muscles of rodents[4, 5, 28]. In agreement with the evidence reported in rodents, we found that our chronic NMN supplementation improved muscle strength and performance in old men, which was

evaluated using the 30-second chair stand test, walking speed, and grip strength. On the other hand, contrary to the animal study, in which 300 mg/kg/day NMN tended to have an increased lean mass as compared to that of the controls[8], NMN did not affect skeletal muscle mass in our study. Recently, several NR human studies have reported that skeletal muscle mitochondrial functions do not increase following NR supplementation[16, 17, 19]. Therefore, our findings suggest that the chronic supplementation of NMN may support overall muscle health, but further studies are warranted to elucidate the mechanisms behind this observed increase in mobility.

NMN supplementation has also been suggested to improve insulin sensitivity and metabolic health in rodent models[4, 5]. A previous NR human study reported a decrease in haptic lipid content in obese men, although it was not significant[13]. In this study, no effect of NMN was observed on hepatic lipid accumulation and insulin sensitivity. This may be attributed to the normal metabolic status of our study population.

In this study, we also performed a preliminary evaluation of auditory capacity using an audiometer before and after intervention with NMN. In mice, SIRT3, an NAD+-dependent protein deacetylase localized in the mitochondria, has been reported to be involved in the regulation of hearing ability during aging[29]. NR supplementation in rodents has also been reported to improve noise-induced and

age-related hearing loss via SIRT3 activation[29-31]. In our study, NMN supplementation partially improved the auditory capacity of elderly people. However, little is known about the underlying mechanisms by which NAD+ precursors may improve hearing in humans. Based on preclinical studies[29-32], NMN can similarly affect hearing in humans through mechanisms involving SIRT3 activation and the increased ratio of reduced to oxidized glutathione in the mitochondria. However, future mechanistic studies are needed to test this hypothesis. Such studies will be technically challenging in humans, and it will be important to dissociate the effects of SIRT3 activation from the possible pleotropic effects of elevating NAD+ metabolites.

While this study offers novel insights into NMN as a nutritional supplement and potential therapeutic entity, there are some limitations. First, the enrolled 42 participants were randomized between the two treatment groups that were adjusted for age, body mass index (BMI), and SMI. However, 22 participants dropped out and the adjustment between the two groups was broken, which may compromise some results in this study. Second, we included only healthy elderly men in this study. Elderly participants were included in the study because NAD+ levels decrease with age in rodents and humans[33] and NAD+ supplementation can be more effective in elderly people. Moreover, men were chosen as participants, considering the possibility that the data from older women are affected by the rapid decrease in

estrogen or progesterone levels associated with menopause. We still speculate that NMN administration may be effective in different populations, such as middle-aged adults or elderly women, because the apparent difference in the response to NR, another NAD+ precursor, due to age or sex has not been reported in human clinical studies[9-19]. However, it remains to be determined whether NMN supplementation is effective in populations that are different in gender, age, or baseline physiological functions, which is critical in determining the therapeutic potential of oral NMN supplementation. Thus, further clinical studies should be conducted in specific populations in this regard.

Conclusion

We report that supplementation of 250 mg/d NMN for 12 weeks in healthy old men was safe, well tolerated, and significantly increased NAD+ and NAD+ metabolites in whole blood. Additionally, NMN induced improvements in muscle strength and performance. Thus, chronic oral administration of NMN could be an effective strategy for the prevention of age-related muscle disorders such as sarcopenia.

Materials and Methods

Ethical approval, informed consent, and study location

The study was conducted in accordance with the Declaration of Helsinki guidelines, and it was approved by the Graduate School of Medicine and Faculty of Medicine, The University of Tokyo Research Ethics Committee (2018013P). The study was registered at UMIN-CTR (UMIN000036321) before the patients were recruited. The participants received oral and written information before obtaining written consent. The study was conducted at the Clinical Research Support Center Phase 1 Unit at the University of Tokyo Hospital.

Study design, randomization, and intervention

The study was designed as a placebo-controlled, randomized, double blind, parallel-group trial. Participants were examined at baseline and the 6-week and 12-week visits. After completion of the baseline investigations, participants were randomized to a 12-week supplementation with NMN or placebo with daily administration by a third party, C&C QUALITATIVE RESEARCH INSTITUTE INC

(Tokyo, Japan), with no significant difference in age, BMI, or SMI between the two groups (Table 1). The allocation of NMN or placebo group was also managed by the C&C QUALITATIVE RESEARCH INSTITUTE INC until the end of the study. The participants received oral supplementation of 250 mg of NMN (Mitsubishi Corporation Life Sciences Limited, Tokyo, Japan) once daily or placebo for 12 weeks. The participants and data collectors were blinded to the treatment. Once all participants completed the study, the randomization code was released.

The primary objective of this study was to evaluate the potential benefits of NMN in increasing blood NAD+ concentration and affecting the body composition of elderly participants after the 12-week treatment. The secondary objective of the study was to evaluate aging-related parameters such as muscle strength and performance, bone density, vision, and hearing ability.

Study participants

Sixty-five healthy Japanese male volunteers were recruited in the study. The inclusion criteria were as follows: male, over 65 years of age, BMI (in kg/m^2) 22-28, nonsmokers, and having no active diseases. Participants with a history of treatment for malignancy, heart failure, or myocardial infarction; with a prescription medication

and supplement that may affect clinical research; or who had exercised for at least one hour daily for at least six months continuously were excluded. Participants underwent a physical examination by a physician, including routine clinical biochemistry, to evaluate their eligibility for the study.

During the intervention, participants were instructed not to change their lifestyle and to abstain from Vitamin B3-related dietary supplements. Finally, 20 participants completed the study whereas 22 dropped out because of a mistake in the distribution of NMN or placebo at the 6-week visit (Figure 1).

Evaluation of safety, tolerability, and adherence

Participants were instructed to record any adverse event in a diary, and they were asked during the visit if they had experienced any difficulty or problem since the last visit. Participants were also requested to immediately report any serious adverse event during the study to the investigators. Adverse events were monitored via a blood test and by observing the participants during safety checkups at the 6- and 12-week visits. Adherence was checked using the pill count.

Laboratory measurements

Blood was collected from the forearm of each participant at baseline and the 12-week visit. Hematological tests, including white blood cell count, red blood cell count, hemoglobin, hematocrit level, platelet count, mean red blood cell pigment content, mean red blood cell volume, and mean red blood cell pigment concentration, were measured.

An OGTT was performed using 75 g of glucose. Blood glucose, insulin, and C-peptide levels were measured at 0, 30, 60, and 120 min after oral glucose loading. The areas under the curves (AUC) for glucose, insulin, and C-peptide were calculated using the trapezoidal formula. Insulin resistance, determined using HOMA-IR, was calculated using the following equation: fasting glucose (mg/dL) × fasting insulin (μU/mL)/405. HOMA-β was calculated using the following formula: 360 × fasting insulin (μU/mL) / (fasting glucose [mg/dL] - 63).

Biochemical tests, including triglyceride, total cholesterol, LDL-cholesterol, HDL-cholesterol, glucose, HbA1c, insulin, blood C-peptide, AST, ALT, γ-GTP, CK, total protein, albumin, uric acid, uric acid nitrogen, creatinine, sodium, potassium, high-sensitivity C-reactive protein, adiponectin, and IL-6 levels, were measured.

For adiponectin and IL-6, blood samples were left to stand for 30 min,

centrifuged at 25 °C and 3500 rpm for 5 min, and stored at -30 °C. Blood samples for adiponectin and IL-6 were sent to SRL (SRL, Inc. Tokyo, Japan) for testing. Other blood tests were performed at the University of Tokyo Hospital.

Extraction of NAD+ and LC-MS analysis

At baseline and the 12-week visit, blood samples were collected in heparinized tubes, frozen at -80 °C, and analyzed at University of Toyama. Metabolite extraction and NAD+ metabolomics were performed as previously described[34]. Briefly, metabolites were extracted by mixing 50 μL of blood and 450 μL of MeOH, and this was followed by vortexing for 10 s. An equal volume of chloroform was added to the solution. The mixture was centrifuged at 13,000 × g at 4 °C for 10 min. The separated upper aqueous phase was transferred into a new tube, and the same procedure was repeated. The aqueous phase was dried and reconstituted in LC/MS-grade water. Metabolites were analyzed using an Agilent 6460 Triple Quad mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, United States) coupled with an Agilent 1290 HPLC system (Agilent Technologies Inc., Santa Clara, CA, United States). Analytes were separated on an Atlantis T3 column (2.1 × 150 mm, particle size 3 μm, Waters) using mobile phase A (5 mM ammonium formate)

and mobile phase B (methanol) with a flow rate of 150 μL/min and column temperature of 40 °C. The programmed mobile phase gradient was as follows: 0-10 min, 0%-70% B; 10-15 min, 70% B; 15-20 min, 0% B. Data were analyzed using MassHunter Quantitative Analysis software (Agilent). A standard curve was obtained from various concentrations of the standard compounds and used for quantification.

Body composition

A direct segmental multifrequency bioelectrical impedance analyzer (InBody S10®; InBody Japan Inc., Tokyo, Japan) was used to measure body composition. We recorded whole-body skeletal muscle mass, segmental lean (right arm, left arm, trunk, right leg, and right leg), fat mass, and percentage of body fat at baseline and 6- and 12-week weeks. SMI was calculated by dividing the whole-body skeletal muscle mass by height squared (kg/m^2).

Computed tomography

Abdominal CT was performed to assess liver and visceral fat (Aquilion PRIME/TSX-303A/BI, Aquilion Precison/TSX-304A/2A, Aquilion ONE/TSX-101A Vision Edition) at baseline and 12-week visit.

The ratio of the CT values of the liver and spleen (L/S ratio) was evaluated to assess liver fat using the images on a Centricity RA1000 workstation (GE Healthcare, Chicago, Illinois, United States). Three circular or ovoid regions of interest (ROIs) (diameter, ~15 mm) in the liver were placed on the left lobe and ventral and dorsal parts of the right lobe at the level of the umbilical portion of portal vein. In contrast, two ROIs in the spleen were placed on the ventral and dorsal parts of the spleen at the level of its maximum diameter. The apparent main vasculature, bile duct, and calcification were avoided when ROIs were placed in each image set. The CT values and standard deviations (i.e., image noise) were recorded after placing ROIs on each image. CT (L/S) was calculated as the ratio of the mean CT values of three ROIs in the liver (CT[L]) to two ROIs in the spleen (CT[S]).

Visceral fat was assessed using Fat Scan (East Japan Institute of Technology Co., Ltd. Ibaraki, Japan). The visceral fat area was measured at the slice at the umbilicus (Figure 3)[35].

Assessment of exercise capacity and physical function

To evaluate physical functions, the participants were tested for gait speed, grip strength, and 30-s chair-stand test at baseline and the 6- and 12-week visits.

Gait speed was measured twice for a 10 m walking time, as described previously[36], and the average of two measurements was used as the outcome data.

Grip strength was measured as previously described[37] using a Smedley-type digital hand dynamometer (Grip D®; Matsuyoshi & Co., Ltd., Tokyo, Japan). Measurements were repeated twice for each hand. The highest hand grip strength value was used for calculations.

30-s chair-stand test was performed as previously described[38,39]. The number of times a participant stood up from the chair in 30 seconds was recorded.

Hearing tests

The hearing ability of both ears was measured using an audiometer (Audiometer AA-79, RION Co., Ltd.) at baseline and the 12-week visit. In the hearing test, only air conduction was measured, and the pure tone hearing level averages of 500, 1000 × 2, and 2000 Hz were evaluated.

Cognitive function test

MMSE-J and MOCA-J were performed to assess the cognitive performance of participants at the start and after the 12-week intervention[40,41].

Flow Mediated Dilation

After 10 min of rest, a blood flow-dependent vasodilatation response test was performed using a vascular ultrasound system (UNEX EF 18VG, UNEX Corporation) at baseline and 6- and 12-week weeks. When the cuff was used to stop and release the blood flow from the forearm, the amount of blood vessel dilation was measured as the percentage of vessel diameter dilatation (lrb%).

Statistical analysis

Statistical analysis was performed using Easy R for Microsoft Windows[42] using the data at baseline (N=21) and 6- (N=21) and 12-week (N=10) visits in the NMN or placebo group. Outcome data are reported as mean ± standard deviation. For comparisons between the NMN and placebo groups, each outcome data followed the Shapiro-Wilk test as a normality test. Data that followed a normal distribution were analyzed using an unpaired t-test. Changes in NMN and placebo groups from baseline to Week 6 or Week 12 were compared using ANCOVA to adjust for baseline. Data that did not follow a normal distribution were compared using the Mann-Whitney U test.

Treatment comparison was performed using mixed model analysis, in which intercept and visit were included as random effects, and group, visit, and group-by-visit interaction were included as fixed effects. As some endpoints could not be calculated using mixed model analysis, all endpoints followed MMRM, in which the fixed effects were group, visit, group-by-visit interaction. The values of the outcomes at the baseline visit and covariance structure between visits was estimated without restriction. The p-values denote group-by-visit interaction.

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Author contributions

M.I., M.M., Y.F., T.S., and T.S. conceived the trial; M.I., M.M., T.K., and T.Y. designed the trial; M.I, M.M., and Y.N. conducted the clinical trial; K.Y. and T.N. undertook targeted metabolomics and quantitation of NAD+ related metabolites; M.S., R.S., and N.K. provided technical support regarding the measurement and analysis of the body composition; J.S. and K.I. provided technical support regarding the CT techniques and aided in CT data analysis; Y.N. and K.K. conducted statistical analyses. M.I., M.M., Y.N., and T.Y. interpreted the data. M.I., M.M., and Y.N. took the leading roles in writing the manuscript and creating the figures. All authors read and approved of the final manuscript.

Competing interests statement

Yuichiro Fukamizu, Toshiya Sato, and Takanobu Sakurai are employees of Mitsubishi Corporation Life Sciences Limited. The other authors declare no conflicts of interest. This study was funded by Mitsubishi Corporation Life Sciences Limited, and the firm provided NMN.

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Figure legends

Figure 1

Clinical trial diagrams

Clinical trial flow chart illustrating the procedures for the selection of study participants and data analyses. A total of 65 potential participants were screened, and 42 eligible participants were selected and randomized in a 1:1 ratio into the two groups. Clinical examinations were performed at the 0-, 6-, and 12-week visits. Twenty-two participants deviated from the protocol after the 6-week visit, and 20 participants completed the 12-week study.

Figure 2

Chronic oral NMN administration increases whole blood NAD+ and related metabolite levels.

(A-G) Changes in whole blood NAD+ and NAD+ metabolite levels; NMN (A), NAD+ (B), NR (C), NAMN (D), NAR (E), NA (F), and NAM (G) after 12 weeks of placebo

(n=10) or NMN (n=10) supplementation.

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using Mann-Whitney *U* test.

*P<0.05; **P<0.01; ***P<0.001

Figure 3

NMN supplementation does not affect metabolic parameters.

(A) A representative single CT slice of the navel level of an NMN or placebo group participant at the 0- or 12-visit to calculate the area of visceral fat; the red region is visceral fat and the blue region is subcutaneous fat. (B) The effect of NMN on CT L/S ratio, visceral fat area calculated from CT slices, and fat mass (lrb%) measured using the BIA method. (C) The effect of NMN on HbA1c, FBG, HOMA-β, HOMA-IR, glucose AUC, insulin AUC, and C-peptide AUC. The AUC was calculated from the result of 75 g OGTT.

a. Treatment was compared using a mixed model analysis. The p-value denotes the interaction.

b. Treatment was compared using MMRM. The p-value denotes interaction.

c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).

d. Inter-group comparisons were made using the Mann-Whitney *U* test (no adjustment for baseline).

e. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

*P<0.05; **P<0.01; ***P<0.001

Table 1. Clinical characteristics of 42 study participants prior to NMN supplementation.

	Placebo Mean ± SD (21)	NMN Mean ± SD (21)	Between group p-value
Age (year)	71.8 ± 6.1	71.1 ± 3.9	0.960[b]
BMI (kg/m^2)	24.5 ± 1.4	24.1 ± 1.4	0.283[b]
Fat Mass (%)	26.7 ± 3.9	25.7 ± 3.8	0.424[a]
SMI (kg/m^2)	7.62 ± 0.42	7.64 ± 0.29	0.867[a]
Gait Speed (m/sec)	1.36 ± 0.16	1.45 ± 0.17	0.106[a]
A 30-s Chair-Stand Test (Counts/30sec)	14.0 ± 4.2	13.9 ± 3.9	0.909[a]
Right hand grip strength (kg)	37.7 ± 6.1	39.1 ± 4.7	0.686[b]
Left hand grip strength (kg)	34.6 ± 4.9	35.1 ± 4.5	0.970[b]
HbA1c (%)	5.82 ± 0.29	5.90 ± 0.53	0.761[b]
FBG (mg/dL)	95.7 ± 10.2	101.0 ± 11.6	0.332[b]
HOMA-IR	1.30 ± 0.83	1.66 ± 1.57	0.406[b]
CT L/S ratio	1.16 ± 0.12	1.14 ± 0.13	0.460[a]
Visceral adipose tissue (cm^2)	123.0 ± 32.0	124.4 ± 38.7	0.842[b]

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using Mann-Whitney *U* test.

Table 2. Effect of NMN on muscle mass.

	Placebo Mean ± SD (n)	NMN Mean ± SD (n)	Between group p-value		Placebo Mean ± SD (n)	NMN Mean ± SD (n)	Between group p-value
SMI (kg/m²) p=0.979[a], p=0.874[b]				**Segmental Lean/Trunk (kg) p=0.094[a], p=0.605[b]**			
Baseline	7.62 ± 0.42 (21)	7.64 ± 0.29 (21)	0.867[c]	Baseline	21.2 ± 1.97 (21)	22.2 ± 1.51 (21)	0.076[c]
Week 6	7.69 ± 0. 40 (21)	7.64 ± 0.32 (21)	0.703[c]	Week 6	21.2 ± 1.94 (21)	21.9 ± 1.47 (21)	0.113[d]
Week 12	7.64 ± 0.52 (10)	7.52 ± 0.43 (10)	0.582[c]	Week 12	21.1 ± 2.25 (10)	21.9 ± 1.89 (10)	0.412[c]
Change from Baseline to Week 6	0.06 ± 0.15 (21)	0.0 ± 0.13 (21)	0.167[e]	Change from Baseline to Week 6	-0.1 ± 0.53 (21)	-0.3 ± 0.41 (21)	0.328[e]
Change from Baseline to Week 12	-0.15 ± 0.22 (10)	-0.13 ± 0.21 (10)	0.805[e]	Change from Baseline to Week 12	-0.2 ± 0.37 (10)	-0.3 ± 0.51 (10)	0.706[e]
Segmental Lean/Right Arm (kg) p=0.374[b]				**Segmental Lean/Right Leg (kg) p=0.268[a], p=0.702[b]**			
Baseline	2.54 ± 0.32 (21)	2.68 ± 0.24 (21)	0.120[c]	Baseline	7.92 ± 0.81 (21)	8.16 ± 0.58 (21)	0.274[c]
Week 6	2.53 ± 0.30 (21)	2.62 ± 0.23 (21)	0.345[d]	Week 6	8.05 ± 0.81 (21)	8.27 ± 0.57 (21)	0.332[c]
Week 12	2.49 ± 0.35 (10)	2.60 ± 0.28 (10)	0.443[c]	Week 12	7.91 ± 0.94 (10)	8.15 ± 0.70 (10)	1.000[d]
Change from Baseline to Week 6	-0.01 ± 0.10 (21)	-0.05 ± 0.08 (21)	0.283[e]	Change from Baseline to Week 6	0.14 ± 0.23 (21)	0.11 ± 0.19 (21)	0.804[e]
Change from Baseline to Week 12	-0.05 ± 0.08 (10)	-0.07 ± 0.09 (10)	0.667[e]	Change from Baseline to Week 12	-0.19 ± 0.36 (10)	-0.05 ± 0.31 (10)	0.367[e]
Segmental Lean/Left Arm (kg) p=0.761[b]				**Segmental Lean/Left Leg (kg) p=0.251[a], p=0.891[b]**			
Baseline	2.51 ± 0.33 (21)	2.67 ± 0.25 (21)	0.081[c].	Baseline	7.78 ± 0.82 (21)	8.02 ± 0.50 (21)	0.264[c]
Week 6	2.49 ± 0.32 (21)	2.61 ± 0.24 (21)	0.183[c]	Week 6	7.84 ± 0.77 (21)	8.07 ± 0.53 (21)	0.256[c]
Week 12	2.50 ± 0.39 (10)	2.60 ± 0.33 (10)	0.538[c]	Week 12	7.89 ± 1.03 (10)	7.92 ± 0.60 (10)	0.923[c]
Change from Baseline to Week 6	-0.01 ± 0.11 (21)	0.06 ± 0.09 (21)	0.351[e]	Change from Baseline to Week 6	0.06 ± 0.23 (21)	0.06 ± 0.17 (21)	0.818[e]
Change from Baseline to Week 12	-0.04 ± 0.06 (10)	-0.06 ± 0.11 (10)	0.695[e]	Change from Baseline to Week 12	-0.13 ± 0.33 (10)	-0.13 ± 0.24 (10)	0.979[e]

a. Treatment was compared using a mixed model analysis. The p-value denotes

interaction.

b. Treatment was compared using MMRM. The p-value denotes interaction.

c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).

d. Inter-group comparisons were made using the Mann-Whitney *U* test (no adjustment for baseline).

e. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

Table 3. NMN improves muscle strength and performance.

	Placebo Mean ± SD (n)	NMN Mean ± SD (n)	between group p-value		Placebo Mean ± SD (n)	NMN Mean ± SD (n)	between group p-value
Gait Speed (m/sec) p=0.033[a]*, p=0.015[b]*				**Right Grip Strength (kg) p=0.242[a], p=0.194[b]**			
Baseline	1.36 ± 0.16 (21)	1.45 ± 0.17 (21)	0.106[c]	Baseline	37.7 ± 6.07 (21)	39.1 ± 4.67 (21)	0.686[d]
Week 6	1.34 ± 0.18 (21)	1.47 ± 0.16 (21)	0.023[c]*	Week 6	39.2 ± 5.33 (21)	40.6 ± 4.01 (21)	0.348[c]
Week 12	1.30 ± 0.22 (10)	1.60 ± 0.13 (10)	0.002[c]**	Week 12	37.3 ± 5.91 (10)	41.9 ± 5.59 (10)	0.090[c]
Change from Baseline to Week 6	-0.02 ± 0.13 (21)	0.02 ± 0.09 (21)	0.111[e]	Change from Baseline to Week 6	1.6 ± 3.38 (21)	1.5 ± 4.01 (21)	0.669[e]
Change from Baseline to Week 12	-0.01 ± 0.10 (10)	0.09 ± 0.13 (10)	0.066[c]	Change from Baseline to Week 12	1.3 ± 3.16 (10)	1.8 ± 4.24 (10)	0.479[e]
A 30-s Chair-Stand Test (Counts/30sec) p=0.509[a], p=0.309[b]				**Left Grip Strength (kg) p=0.019[b]***			
Baseline	14.0 ± 4.19 (21)	13.9 ± 3.85 (21)	0.909[c]	Baseline	34.6 ± 4.88 (21)	35.1 ± 4.48 (21)	0.970[d]
Week 6	13.6 ± 4.44 (21)	15.1 ± 4.22 (21)	0.261[c]	Week 6	34.7 ± 4.98 (21)	36.6 ± 5.38 (21)	0.207[d]
Week 12	14.0 ± 5.23 (10)	16.3 ± 3.59 (10)	0.267[c]	Week 12	34.1 ± 4.63 (10)	37.4 ± 5.80 (10)	0.177[c]
Change from Baseline to Week 6	-0.5 ± 2.64 (21)	1.2 ± 2.11 (21)	0.031[e]*	Change from Baseline to Week 6	0.095 ± 3.42 (21)	1.5 ± 4.25 (21)	0.204[e]
Change from Baseline to Week 12	0.5 ± 3.66 (10)	1.5 ± 1.65 (10)	0.311[e]	Change from Baseline to Week 12	-0.80 ± 2.70 (10)	1.3 ± 2.71 (10)	0.081[e]

a. Treatment was compared using a mixed model analysis. The p-value denotes interaction.

b. Treatment was compared using MMRM. The p-value denotes interaction.

c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).

d. Inter-group comparisons were made using the Mann–Whitney *U* test (no adjustment for baseline).

e. Inter-group comparisons were made using ANCOVA for adjusting for baseline.

*P<0.05; **P<0.01; ***P<0.001

Supplementary Table 1. Clinical characteristics of 20 study participants completing the 12-week study prior to NMN supplementation, Related to Table 1.

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using Mann–Whitney *U* test.

*P<0.05

Supplementary Table 2. The effect of NMN on clinical laboratory data

(hematology and CRP).

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using the Mann–Whitney *U* test.

c. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

*P<0.05

Supplementary Table 3. The effect of NMN on clinical laboratory data (hematology and CRP) (blood chemistry).

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using Mann–Whitney *U* test.

c. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

*P<0.05

Supplementary Table 4. NMN supplementation does not affect metabolic parameters. Related to Figure 3.

a. Inter-group comparisons were made using an unpaired t-test.

b. Inter-group comparisons were made using the Mann–Whitney *U* test.

c. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

*P<0.05

Supplementary Table 5. The effect of NMN on auditory and cognitive functions.

a. Treatment was compared using a mixed model analysis. The p-value denotes interaction.

b. Treatment was compared using MMRM. The p-value denotes interaction.

c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).

d. Inter-group comparisons were made using the Mann–Whitney *U* test (no adjustment for baseline).

e. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

*P<0.05

Supplementary Table 6. The effect of NMN on vascular function.

a. Treatment was compared using a mixed model analysis. The p-value denotes

interaction.

b. Treatment was compared using MMRM. p-value denotes interaction.

c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).

d. Inter-group comparisons were made using the Mann–Whitney *U* test (no adjustment for baseline).

e. Inter-group comparisons were made using ANCOVA for adjusting the baseline.

Figure 1

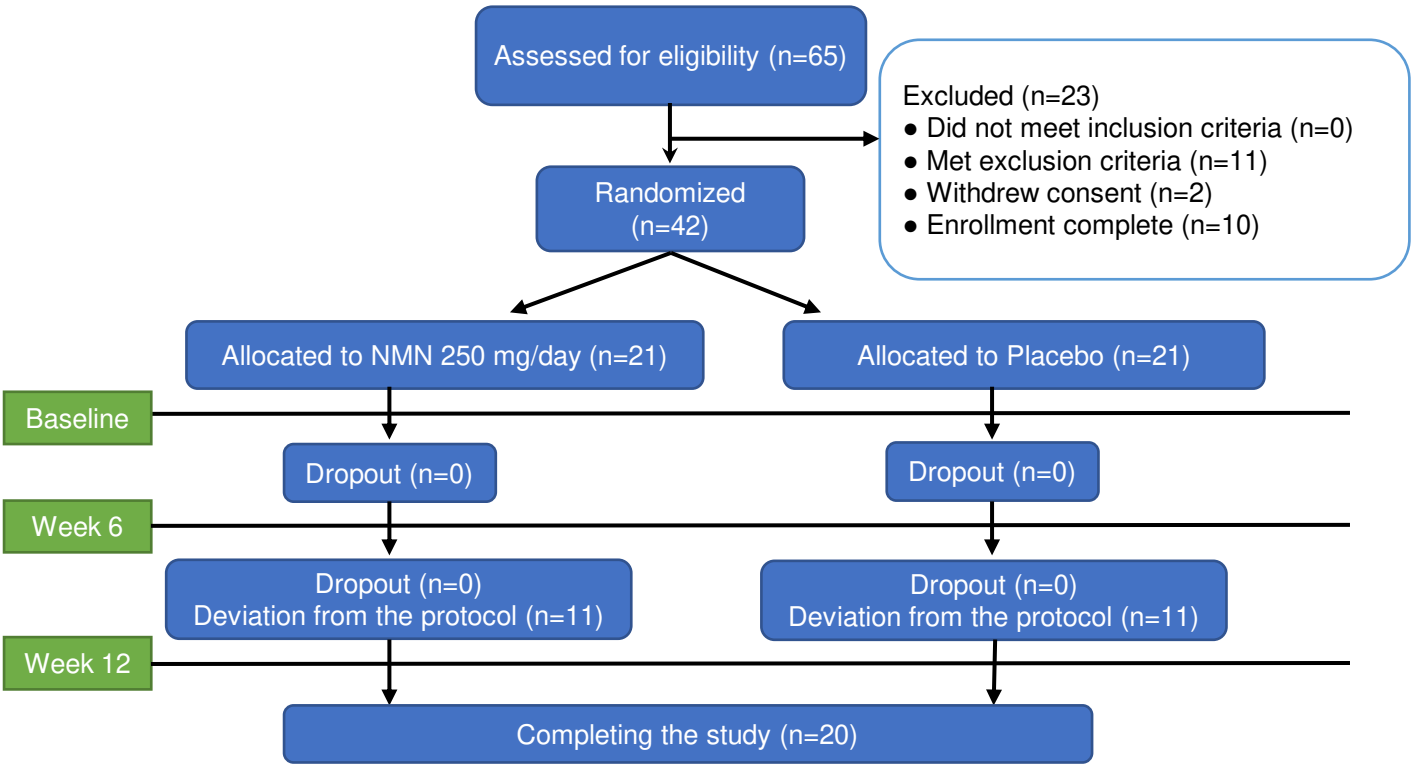


Figure 1 Clinical trial diagrams
Clinical trial flow chart illustrating the procedures for the selection of study participants and data analyses. A total of 65 potential participants were screened, and 42 eligible participants were selected and randomized in a 1:1 ratio into the two groups. Clinical examinations were performed at the 0-, 6-, and 12-week visits. Twenty-two participants deviated from the protocol after the 6-week visit, and 20 participants completed the 12-week study.

Figure 2

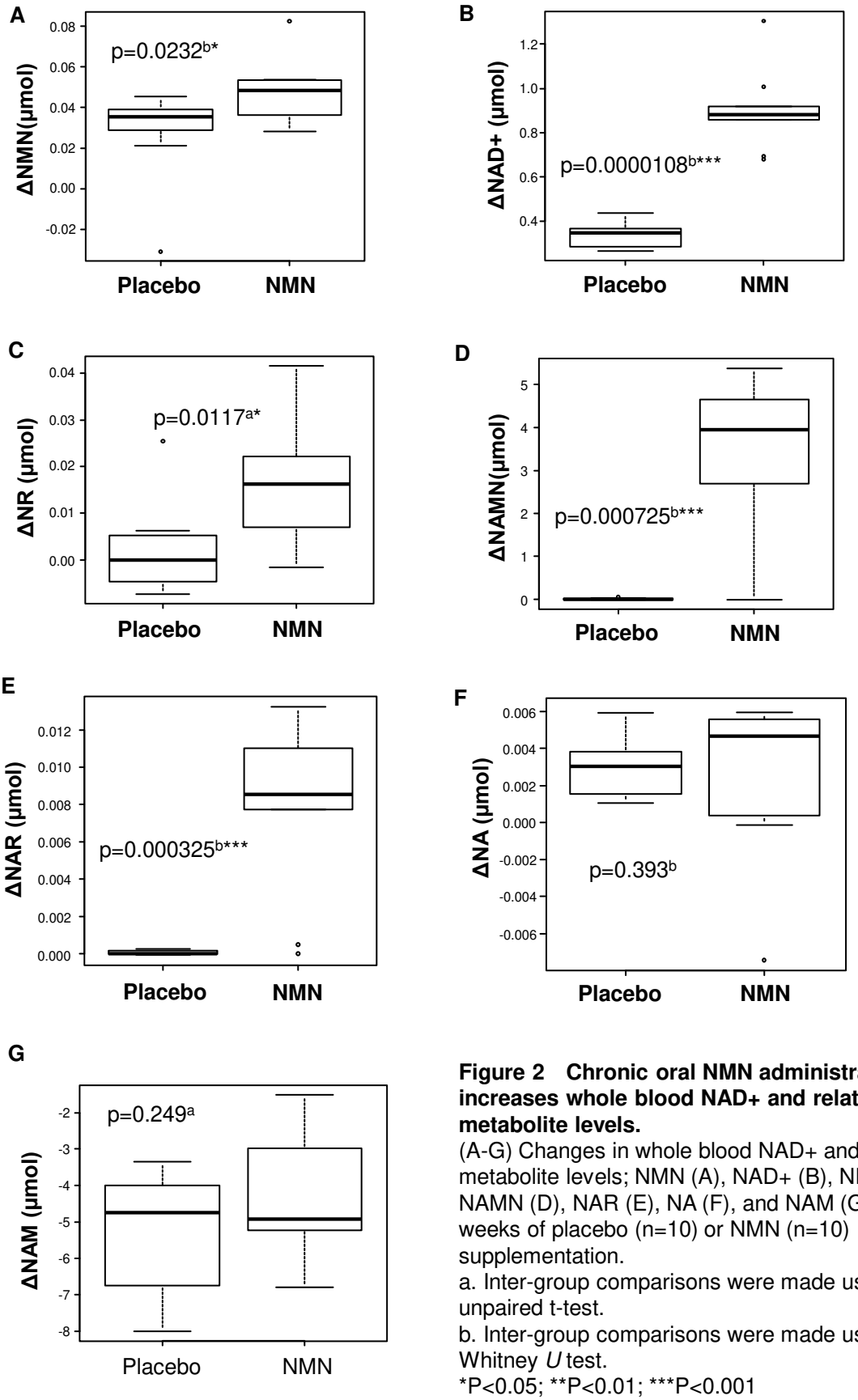


Figure 2 Chronic oral NMN administration increases whole blood NAD+ and related metabolite levels.

(A-G) Changes in whole blood NAD+ and NAD+ metabolite levels; NMN (A), NAD+ (B), NR (C), NAMN (D), NAR (E), NA (F), and NAM (G) after 12 weeks of placebo (n=10) or NMN (n=10) supplementation.

a. Inter-group comparisons were made using an unpaired t-test.

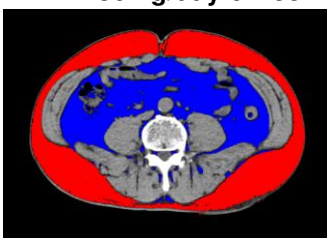
b. Inter-group comparisons were made using Mann-Whitney *U* test.

*P<0.05; **P<0.01; ***P<0.001

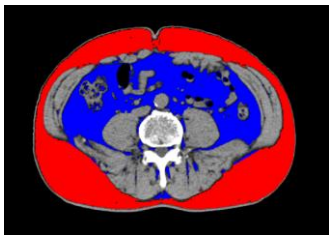
Figure 3

A

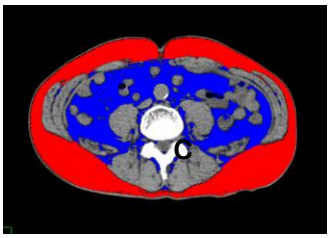
NMN 250mg/day 0 week



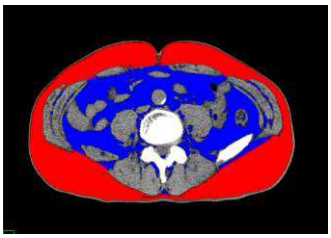
NMN 250mg/day 12 weeks



Placebo 0 week



Placebo 12 weeks



B

	Placebo Mean ± SD (n)	NMN Mean ± SD (n)	Between groups p-value
CT L/S ratio			
Baseline	1.16 ± 0.12 (21)	1.13 ± 0.13 (21)	0.430[c]
Week 12	1.18 ± 0.08 (10)	1.17 ± 0.15 (10)	0.631[d]
Change from Baseline to Week 12	0.03 ± 0.05 (10)	0.02 ± 0.04 (10)	0.651[c]
Visceral fat by CT (m^2)			
Baseline	123.0 ± 32.0 (21)	124.4 ± 38.7 (21)	0.842[d]
Week 12	126.3 ± 36.7 (10)	120.1 ± 46.4 (10)	0.744[c]
Change from Baseline to Week 12	0.0 ± 11.6 (10)	-9.2 ± 19.7 (10)	0.234[e]
Fat Mass by InBody (%) p= 0.442[a], p=0.700[b]			
Baseline	26.7 ± 3.9 (21)	25.7 ± 3.8 (21)	0.424[c]
Week 6	26.7 ± 4.1 (21)	26.0 ± 3.5 (21)	0.543[c]
Week 12	26.5 ± 4.8 (10)	24.9 ± 2.5 (10)	0.342[c]
Change from Baseline to Week 6	0.0 ± 1.8 (21)	0.3 ± 1.2 (21)	0.750[e]
Change from Baseline to Week 12	-0.2 ± 1.3 (10)	-0.2 ± 2.4 (10)	0.991[c]

C

	Placebo Mean ± SD (n)	NMN Mean ± SD (n)	Between group p-value		Placebo Mean ± SD (n)	NMN Mean ± SD (n)	Between group p-value
HbA1c (%)				**FBG (mg/dL)**			
Baseline	5.82 ± 0.29 (21)	5.90 ± 0.53 (21)	0.761[d]	Baseline	95.7 ± 10.2 (21)	101.0 ± 11.6 (21)	0.332[d]
Week 12	5.69 ± 0.30 (10)	5.88 ± 0.56 (10)	0.647[d]	Week 12	97.2 ± 8.95 (10)	101.4 ± 11.1 (10)	0.365[c]
Change from Baseline to Week 12	-0.05 ± 0.09 (10)	0.03 ± 0.13 (10)	0.0861[e]	Change from Baseline to Week 12	2.8 ± 7.5 (10)	2.4 ± 7.3 (10)	0.921[e]
HOMA-β				**HOMA-IR**			
Baseline	46.9 ± 17.7 (21)	45.7 ± 16.9 (21)	0.821[d]	Baseline	1.32 ± 0.83 (21)	1.67 ± 1.57 (21)	0.481[d]
Week 12	45.1 ± 16.8 (10)	43.1 ± 16.1 (10)	0.791[c]	Week 12	1.11 ± 0.32 (10)	1.26 ± 0.49 (10)	0.448[c]
Change from Baseline to Week 12	-0.6 ± 15.7 (10)	1.3 ± 16.0 (10)	0.992[e]	Change from Baseline to Week 12	0.03 ± 0.43 (10)	-0.09 ± 0.55 (10)	0.812[e]
Glucose AUC				**Insulin AUC**			
Baseline	19362.1 ± 2467.4 (21)	20804.3 ± 5426.4 (21)	0.633[d]	Baseline	4889.3 ± 1941.3 (21)	4761.4 ± 1875.8 (21)	0.829[d]
Week 12	18706.5 ± 2279.3 (10)	19299.0 ± 4790.6 (10)	0.728[c]	Week 12	5508.0 ± 2639.8 (10)	4389.0 ± 1582.7 (10)	0.326[d]
Change from Baseline to Week 12	687.0 ± 1989.7 (10)	-859.5 ± 2092.9 (10)	0.201[e]	Change from Baseline to Week 12	1057.5 ± 2215.9 (10)	-94.5 ± 845.4 (10)	0.142[e]
C-peptide AUC							
Baseline	704.9 ± 140.1 (21)	694.7 ± 180.0 (21)	0.840[c]				
Week 12	772.2 ± 170.2 (10)	694.2 ± 153.5 (10)	0.296[c]				
Change from Baseline to Week 12	117.6 ± 199.9 (10)	41.9 ± 88.2 (10)	0.244[e]				

Figure 3 NMN supplementation does not affect metabolic parameters.
(A) A representative single CT slice of the navel level of an NMN or placebo group participant at the 0- or 12-visit to calculate the area of visceral fat; the red region is visceral fat and the blue region is subcutaneous fat. (B) The effect of NMN on CT L/S ratio, visceral fat area calculated from CT slices, and fat mass (lrb%) measured using the BIA method. (C) The effect of NMN on HbA1c, FBG, HOMA-β, HOMA-IR, glucose AUC, insulin AUC, and C-peptide AUC. The AUC was calculated from the result of 75 g OGTT.
a. Treatment was compared using a mixed model analysis. The p-value denotes the interaction.
b. Treatment was compared using MMRM. The p-value denotes interaction.
c. Inter-group comparisons were made using an unpaired t-test (no adjustment for baseline).
d. Inter-group comparisons were made using the Mann-Whitney *U* test (no adjustment for baseline).
e. Inter-group comparisons were made using ANCOVA for adjusting the baseline.
*P<0.05; **P<0.01; ***P<0.001

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure3.pdf
- ResearchProtcolEnglishversion16208848682.pdf