N-DOSE AD: A randomized, double blind, dose optimization trial of nicotinamide riboside in Alzheimer's disease.

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SIGNATURE PAGE

Title **N-DOSE AD: A randomized, double blind, dose optimization trial of nicotinamide riboside in Alzheimer's disease.**

Protocol ID no: 428878

I hereby declare that I will conduct the study in compliance with the Protocol, ICH GCP and the applicable regulatory requirements:

To be signed by sponsor's representative, Coordinating Investigator, Principal Investigator, statistician etc. This section and table above should be completed as appropriate for the type of study conducted and the parties involved.

Name	Title	Role	Signature	Date
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PROTOCOL SYNOPSIS

N-DOSE AD: A randomized, double blind, dose optimization trial of nicotinamide riboside in Alzheimer's disease.

Sponsor	Haukeland University Hospital
Phase and study type	Phase II, Dose-Optimization study
Investigational Product (IP) (including active comparator and placebo):	Nicotinamide Riboside Placebo
Centers:	**Haukeland University Hospital** **Haraldsplass Deaconess Hospital**
Study Period:	Estimated date of first patient enrolled: 20.10.2022 Anticipated recruitment period: 20.10.2022 – 20.04.2025 Estimated date of last patient completed: 30.06.2025.
Treatment Duration:	12 weeks

Objectives and endpoints

Objective		**Endpoint**
Primary	To compare the effect of orally administered nicotinamide riboside (NR), escalated to 1500 mg twice per day (3000 mg/day) in the dose-escalation group (DE-group) - versus stable dosing of 500 mg twice per day (1000 mg/day) in the dose-stable group (DS-group) on cerebral NAD-levels, at week 12.	Change in cerebral NAD/ATP-α ratio measured by 31 Phosphorus magnetic resonance spectroscopy (31P-MRS) in the posterior brain (encompassing the occipital, parietooccipital and posterior parts of the temporal cortex).
Secondary	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in cerebral NAD levels from baseline to weeks 4, 8 and 12.	Change in cerebral NAD/ATP-α ratio measured by 31 Phosphorus magnetic resonance spectroscopy (31P-MRS) in the posterior brain (encompassing the occipital, parietooccipital and posterior parts of the

		temporal cortex).
	To compare the effectiveness of orally administered nicotinamide riboside (NR) 1500 mg twice per day versus 500 mg twice per day in augmenting the NAD-metabolome in the central nervous system (CNS) at week 12.	Change in the cerebrospinal fluid (CSF) levels of NAD or other metabolites of the NAD metabolome*, measured by LC-MS.
Exploratory	**Neuroimaging**	
	To compare the effect of orally administered NR in the DE-group versus DS-group on the NR related metabolic pattern (NRRP) expression at week 12.	Change in NRRP expression, measured by FDG-PET.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in NRRP expression from baseline to weeks 4, 8 and 12.	Change in NRRP expression, measured by FDG-PET.
	To compare the effect of orally administered NR DE-group versus DS-group on the AD-related pattern (ADRP) expression at week 12.	Change in ADRP expression, measured by FDG-PET.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in ADRP expression from baseline to weeks 4, 8 and 12.	Change in ADRP expression, measured by FDG-PET.
	Metabolism & molecular markers	
	To compare the effect of orally administered NR in the DE-group versus DS-group on the NAD metabolome* in the blood, urine and central nervous system (CNS) at week 12.	Change in levels of NAD metabolites in blood, urine and CSF, measured by HPLC-MS and/or the NADMed method.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in the NAD metabolome* in blood and urine from baseline to weeks 4, 8 and 12.	Change in levels of NAD metabolites in blood and urine, measured by HPLC-MS and/or the NADMed method.
	To compare the effect of orally administered NR in the DE-group versus DS-group on serum and CSF inflammatory markers at week 12.	Change in inflammatory cytokines in serum and CSF, measured by ELISA.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in serum inflammatory markers from baseline to weeks 4, 8 and 12.	Change in inflammatory cytokines in serum, measured by ELISA.
	Clinical – cognitive & non motor symptom severity, quality of life	
	To compare the effect of orally administered NR in the DE-group versus DS-group on clinical severity of AD symptoms at week 12.	Change in the total ADAS-Cog 13 score, CDR sum of boxes, MoCA score, TMT.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in clinical severity of PD symptoms from baseline to weeks 4, 8 and 12.	Change in the total ADAS-Cog 13 score, CDR sum of boxes, MoCA score, TMT.
	To compare the effect of orally administered	Change in IADL and PADL scores.

	NR in the DE-group versus DS-group on ADL severity in AD at week 12.	
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in ADL scores in AD from baseline to weeks 4, 8 and 12.	Change in IADL and PADL scores..
	To compare the effect of orally administered NR in the DE-group versus DS-group on depressive symptoms in AD at week 12.	Change in MADRS score.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and depressive symptoms in AD from baseline to weeks 4, 8 and 12.	Change in MADRS score.
	To compare the effect of orally administered NR in the DE-group versus DS-group on neuropsychiatric symptoms in AD at week 12.	Change in the NPI-Q score.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in neuropsychiatric symptoms in AD from baseline to weeks 4, 8 and 12.	Change in the NPI-Q score.
	Hypothesis-generating or resource-dependent endpoints (may be reported in follow-up or secondary publications).	
	To compare the effect of orally administered NR in the DE-group versus DS-group on gene expression at week 12.	Change in gene expression, measured by RNA sequencing (RNAseq).
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in gene expression from baseline to weeks 4, 8 and 12.	Change in gene expression, measured by RNA sequencing (RNAseq).
	To compare the effect of orally administered NR in the DE-group versus DS-group on protein expression at week 12.	Change in protein levels, measured by LC-MS.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in protein expression from baseline to weeks 4, 8 and 12.	Change in protein levels, measured by LC-MS.
	To compare the effect of orally administered NR in the DE-group versus DS-group on serum and CSF inflammatory markers at week 12.	Change in inflammatory cytokines in serum and CSF, measured by ELISA.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in serum inflammatory markers from baseline to weeks 4, 8 and 12.	Change in inflammatory cytokines in serum, measured by ELISA.
	To compare the effect of orally administered NR in the DE-group versus DS-group on histone acetylation in AD at week 12.	Change in histone panacetylation, measured by immunoblotting.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in histone acetylation in AD from baseline to weeks 4, 8	Change in histone panacetylation, measured by immunoblotting.

	and 12.	
	To compare the effect of orally administered NR in the DE-group versus DS-group on H3K27 and H4K16 histone acetylation in AD at week 12.	Changes in levels of H3K27 and H4K16 acetylation, measured by immunoblotting.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in on H3K27 and H4K16 histone acetylation in AD from baseline to weeks 4, 8 and 12.	Changes in levels of H3K27 and H4K16 acetylation, measured by immunoblotting.
	To compare the effect of orally administered NR in the DE-group versus DS-group on the genomic distribution of H3K27 and H4K16 histone acetylation in AD at week 12.	Change in the genomic distribution of H3K27 and H4K16 acetylation, measured by chromatin immunoprecipitation sequencing (ChIPseq).
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in the genomic distribution of H3K27 and H4K16 histone acetylation in AD from baseline to weeks 4, 8 and 12.	Change in the genomic distribution of H3K27 and H4K16 acetylation, measured by chromatin immunoprecipitation sequencing (ChIPseq).
	To compare the effect of orally administered NR in the DE-group versus DS-group on folate and one-carbon metabolism in AD at week 12.	Change in folate and one-carbon metabolites in blood and CSF, measured by HPLC-MS.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in folate and one-carbon metabolism in AD from baseline to weeks 4, 8 and 12.	Change in folate and one-carbon metabolites in blood, measured by HPLC-MS.
	To compare the effect of orally administered NR in the DE-group versus DS-group on methyl donors in AD at week 12.	Change in methyl-donors (e.g., SAM), measured by HPLC-MS, in the blood and/or CSF.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in methyl-donors in AD from baseline to weeks 4, 8 and 12.	Change in methyl-donors (e.g., SAM), measured by HPLC-MS, in the blood.
	To compare the effect of orally administered NR in the DE-group versus DS-group on DNA methylation at week 12.	Change in level and genomic distribution of DNA methylation, measured by Illumina Infinium MethylationEpic kit.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in methyl-donors in AD from baseline to weeks 4, 8 and 12.	Change in level and genomic distribution of DNA methylation, measured by Illumina Infinium MethylationEpic kit.
	To compare the effect of orally administered NR in the DE-group versus DS-group on synthesis of neurotransmitters in AD at week 12.	Change in neurotransmitters in CSF, measured by HPLC-MS.
	Determine whether NR-therapy affects the gut microbiome in a dose-responsive manner at week 12.	Change in gut microbiome composition, measured by metagenomics in fecal samples.
	To compare the effect of orally administered NR in the DE-group versus DS-group on the gut metabolome at week 12.	Change in fecal metabolomics, measured by LC-MS in fecal samples.

Safety	To determine the safety and tolerability of NR at a dose of 1000 mg, 2000 mg, and 3000 mg per day in AD.	Number and severity of adverse events from baseline to week 12 across treatment groups and NR dose levels.

***The NAD metabolome is comprised of:** Nicotinamide adenine dinucleotide oxidized (NAD^+), Nicotinamide adenine dinucleotide reduced (NADH), NAD^+/NADH ratio, total NAD (sum of NAD^+ and NADH), Nicotinamide adenine dinucleotide phosphate oxidized ($NADP^+$), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), $NADP^+$/NADPH ratio, total NADP (sum of $NADP^+$ and NADPH, 1-methyl nicotinamide (Me-Nam), nicotinic acid-adenine dinucleotide (NAAD), N1-methyl-2-pyridone-5-carboxamide (Me-2-PY), Nicotinamide (Nam), Nicotinamide N-oxide (Nam N-oxide), ADP-ribose (ADPR), Nicotinic acid riboside (NAR), Nicotinamide riboside (NR), Nicotinamide mononucleotide (NMN), Nicotinic acid (NA).

Study Design:	Multi-center, double-blinded, randomized, placebo controlled, dose-optimization
Inclusion Criteria:	● Diagnosis of probable AD according to the core clinical criteria updated in the NIA and Alzheimer's Association guidelines. ● Biomarker evidence consistent with AD neuropathologic change, defined by CSF markers. ● Diagnosed with AD within two years from enrollment. ● CDR 0.5-1 (inclusive) at enrolment. ● Age 50 to 85 years (inclusive) at the time of enrollment. ● A study partner able to study data and assist the participant in the study drug administration, i.e. contact ≥ 3 times weekly. ● Capacity to provide written informed consent for study participation defined as Montreal Cognitive Assessment (MoCA) score ≥ 16 or Mini Mental State Evaluation (MMSE) score ≥ 20. MMSE or MoCA must have been performed within 6 months prior to baseline. If there is any doubt regarding the participants capacity to give informed consent we will ask for an independent evaluation by a consultant clinician who is not associated with the N-DOSE AD study. ● Cholinesterase inhibitors and memantine can be used if stable for 8 weeks prior to baseline visit. ● Able to undergo lumbar puncture ● Able to undergo MRI

Exclusion Criteria	● Diagnosis of dementia other than probable AD. ● Comorbidity that precludes study participation or data interpretation. ● Any psychiatric disorder that would interfere with compliance in the study. ● Any severe somatic illness that would interfere with compliance and participation in the study. ● Use of high dose vitamin B3 supplementation within 30 days of enrollment. ● Metabolic, neoplastic, or other physically or mentally debilitating disorder at baseline visit. ● Current treatment with Oral Anticoagulation Therapies ● Implants that preclude MRI examinations, e.g. DBS, pacemaker
Sample Size:	80 patients (20 in placebo group, 60 in treatment groups)
Efficacy Assessments:	Primaryendpoint: Cerebral NAD levels as measured by ^{31}P-MRS (see details under Endpoints).
Safety Assessments:	Biochemistry: Routine blood analysis (see Lab-manual). Vital signs: pulse, blood-pressure. Registration of adverse events.

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LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation or special term	Explanation
AD	Alzheimer's Disease
ADL	Activities of Daily Living
ADAS-Cog	The Alzheimer's Disease Assessment Scale
ADRP	Alzheimer's disease related pattern
AE	Adverse Event
CDR	Clinical Dementia Rating
CRF	Case Report Form (electronic/paper)
CSF	Cerebrospinal fluid
CSA	Clinical Study Agreement
CTC	Common Toxicity Criteria
CTCAE	Common Terminology Criteria for Adverse Event
DAE	Discontinuation due to Adverse Event
DE-group	Dose-escalation group
DS-group	Dose-stable group
EC	Ethics Committee, synonymous to Institutional Review Board (IRB) and Independent Ethics Committee (IEC)
GCP	Good Clinical Practice
HPLC-MS	High performance liquid chromatography-mass spectrometry
IB	Investigator's Brochure
ICF	Informed Consent Form
ICH	International Conference on Harmonization
IP	Investigational Product (includes active comparator and placebo)
IND	Investigational New Drug
LC-MS	Liquid chromatography-mass spectrometry
MoCA	Montreal Cognitive Assessment

MRS-responder	An individual susceptible to NR-induced increase in cerebral NAD levels, detectable by ^{31}P-MRS
MRS-non-responder	An individual showing no NR-induced increase in cerebral NAD levels, detectable by ^{31}P-MRS
NAD	Nicotinamide adenine dinucleotide
NPI-Q	Neuropsychiatric Inventory Questionnaire
NR	Nicotinamide Riboside
NRRP	NR-related metabolic pattern
OBD	Optimal biological dose
PD	Parkinson's Disease
PDRP	Parkinson's Disease-Related Pattern
NAD	Nicotinamide adenine dinucleotide
SAE	Serious Adverse Event
SAM	S-denosyl methionine
SOP	Standard Operating Procedure
TMT	Trail Making Test

1 INTRODUCTION

1.1 Background – Disease

1.1.1. Alzheimer's disease (AD) is a major societal challenge

Alzheimer's disease (AD) is the most common neurodegenerative progressive dementia and a leading cause of disability, loss of independence and mortality. The pathological hallmarks of Alzheimer's disease are extracellular accumulation of amloid β (Aβ) peptide in the brain ("senile plaque"; SP) and by intraneuronal accumulation of hyperphosphorylated tau protein in neurofibrillary tangles (NFT). This is accompanied by synaptic and neuronal losses[1]. The prevalence of Alzheimer's disease is about one percent at age 65 and increases dramatically to between 20-50% in those older than 85 years[1]. AD will have a dramatic increasing emotional and economic impact as the median population age increases, yet available treatments remain largely ineffective.

1.1.2. NAD-replenishment therapy shows promise as neuroprotective therapy for AD

Increasing evidence supports that boosting cellular levels of nicotinamide adenine dinucleotide (NAD) confers neuroprotective effects in both healthy aging and neurodegeneration[2]. NAD, which constantly shuttles between its oxidized (NAD^+) and reduced (NADH) state, is an essential cofactor for metabolic redox reactions, including mitochondrial respiration. Furthermore, NAD^+ is substrate to vital signaling reactions involved in DNA repair, histone- and other protein deacylation, and second messenger generation[3]. These reactions consume NAD^+ at high rates, requiring constant replenishment via NAD biosynthesis. NAD levels

have been shown to decline with age and this is believed to contribute to age-related diseases[3,4]. Increasing the NAD replenishment rate (e.g., via supplementation of precursors), and/or enhancing the NAD^+/NADH ratio (e.g., via caloric restriction) have shown beneficial effects on life- and healthspan in multiple model systems, and evidence of neuroprotection in models of neurodegeneration and other age-related diseases[3–5]. Enhancing NAD replenishment could potentially help ameliorate several major processes implicated in the pathogenesis of AD, including mitochondrial respiratory dysfunction[6], neuroinflammation[7], epigenomic dysregulation[8] and increased neuronal DNA damage[9].

NAD can be replenished via supplementation of **nicotinamide riboside (NR)**, a vitamin B3 molecule and biosynthetic precursor of NAD[3,10]. NR has undergone extensive preclinical testing[11] and is Generally Recognized as Safe (GRAS) for use in food products by the United States Food and Drug Administration[12] and by the European Food Safety Authority[13]. NR is well tolerated by adult humans, showing no evidence of toxicity with doses up to at least 2000 mg daily[14]. Trials in healthy individuals have shown that oral intake of 1000 mg NR daily substantially elevates total levels of NAD and related metabolites in blood and muscle, boosts mitochondrial bioenergetics and decreases circulating inflammatory cytokines[11,15–17]. Moreover, evidence from cell and animal studies suggests that NR supplementation promotes healthspan and has neuroprotective effects in models of Cockayne syndrome[18], noise-induced injury[19,20], amyotrophic lateral sclerosis[21], Parkinson's disease[22], and AD[23,24].

1.1.3. Phase-I evidence for NR-therapy in neurodegenerative diseases

Two phase I studies of NR in PD have been completed, the NADPARK[25] study and the NR-SAFE study[26]. These are described briefly below.

I. The **NADPARK study (ClinicalTrials.gov: *NCT03816020*)**, is a phase I randomized, double blinded trial, aiming to assess the tolerability, cerebral bioavailability and molecular effects of NR therapy in Parkinson's disease (PD)[25]. A total of 30 individuals with newly diagnosed, drug-naïve PD were randomized to NR 500 mg x2/day or placebo for 30 days. The study showed promising results, which are briefly summarized below:

1) NR is well-tolerated: NR 1000 mg per day has excellent compliance, tolerability and no signs of toxicity or adverse side effects in neurodegenerative disease.

2) NR achieves brain penetration: *In vivo* measurement of cerebral NAD levels using phosphorus magnetic resonance spectroscopy (31P-MRS) of the brain showed a highly significant (paired t-test: P = 0.016) **increase in cerebral NAD levels** in the NR group, while no change was observed in the placebo group. Cerebral penetration was further validated by detecting the metabolite Me-2-PY in the CSF of participants receiving NR, but not placebo. While a significant NR-induced increase in cerebral NAD levels was detected at the group level, this effect was not uniform at the individual level. The magnitude of the cerebral NAD-increase showed high interindividual variation. Moreover, three out of 13 patients showed no evidence of cerebral NAD response, despite a clear peripheral metabolic response, confirming treatment compliance and an impact on the NAD metabolome, in CSF, blood, and muscle. Thus, the variable cerebral NAD response observed by ^{31}P-MRS may reflect interindividual variability in cerebral penetration and/or cerebral NAD metabolism. **3) NR is associated with clinical improvement of PD:** NR was associated with a significant decrease in the total MDS-UPDRS (I-III) score between visits (mean decrease: 2.33 ± 2.35; paired t-test: p = 0.017). **4) NR has a major impact on cerebral metabolism:** ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET), performed at baseline and 30 days of treatment, revealed that NR altered cerebral metabolic activity. The analysis revealed a significant ordinal trend pattern (i.e., metabolic network), which was represented by the first principal component (PC1), accounting for 20.6% of the variance in the paired data. This novel **NR-related metabolic pattern (NRRP)** was characterized by multiple regional metabolic changes, including bilateral metabolic reductions in the caudate and putamen, extending into the adjacent globus pallidus, and in the thalamus. Interestingly, the NRRP overlapped spatially with the Parkinson's Disease-Related Pattern (PDRP)[27], and changes in NRRP expression in the NR group resulted in partial normalization of the striatal and thalamic hypermetabolism, typically characterizing the PD brain[27]. Furthermore, changes in NRRP expression in the NR group correlated significantly (r=-0.59, p = 0.026) with a decrease of the UPDRS ratings recorded at the time of PET **(Fig 1F)**. These results indicate that NR ameliorates the cerebral metabolic pattern of PD, and this is associated with significant clinical improvement.

5) NR has widespread metabolic and regulatory effects: Metabolomics revealed a highly significant increase in NAD-related metabolites in blood, muscle and CSF **(Fig 1G)**, indicating that NR supplementation boosts NAD metabolism across tissues. Intriguingly, RNA-sequencing in blood and muscle biopsy showed a highly significant (FDR < 10^{-8}) upregulation of the mitochondrial, proteasomal and lysosomal pathways in the NR group. These findings indicate that NR supplementation increases both mitochondrial respiration and proteostasis – two hallmark pathogenic processes involved in AD[9,28].

II. The NR-SAFE study (ClinicalTrials.gov: NCT05344404)[21] is a phase I randomized, double blinded trial, aiming to assess the safety, tolerability, and bioavailability of NR in PD at an oral dose of 3000 mg daily. A total of 20 individuals with PD were randomized, in a 1:1 ratio, to NR 1500 mg x2/day or placebo for 30 days. The study was concluded in July 2022 and is currently in preparation. The main results are summarized below:

1) NR at a dose of 3000 mg daily is safe and well-tolerated: the treatment had excellent compliance, tolerability, and no signs of toxicity or clinically significant adverse effects in PD.

2) NR, 3000 mg daily, augments NAD-metabolism: oral NR intake at a dose of 3000 mg daily induced a potent (up to 5-fold) and highly significant augmentation of NAD^+ and $NADP^+$ levels, as well as a clear increase of the redox ratio (NAD^+/NADH) and the $NADP^+$/NADPH ratio (Fig. 2A).

3) NR, 3000 mg daily, is associated with clinical improvement of PD: the treatment was associated with a significant and substantial decrease in the total MDS-UPDRS (I-IV) score between visits (mean decrease 14 ± 13.7; paired t-test: P = 0.01). No significant change was seen in the placebo group (Fig. 2B). The UPDRS change (delta) in the NR-group was significantly higher than that of the placebo (t-test, P =0.02) (Fig. 2C).

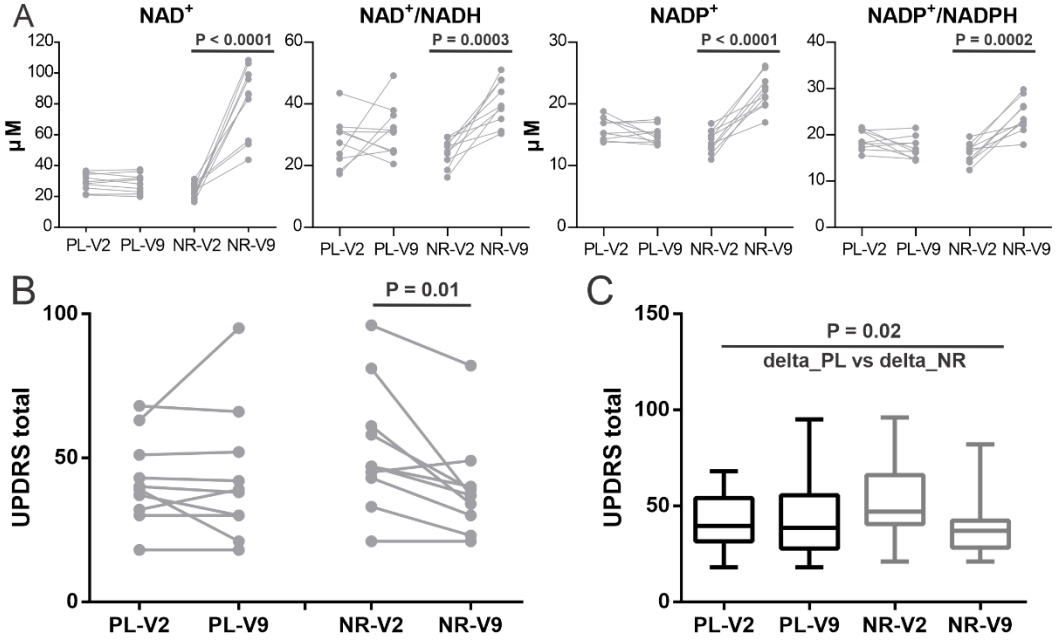

Fig 1. Results of the NR-SAFE trial. A: Metabolomics were performed in snap-frozen blood from the NR-SAFE participants at baseline (V2) and after 30 days of treatment (V9). Oral NR intake at a dose of 3000 mg daily induced a potent (up to 5-fold) and highly significant augmentation of NAD^+ and $NADP^+$ levels, as well as a clear increase of the redox ratio (NAD^+/NADH) and the $NADP^+$/NADPH ratio. P-values indicate the results of paired t-tests in the NR-group. There were no significant changes in the placebo group. **B:** Oral NR intake at a dose of 3000 mg daily was associated with a significant and substantial decrease in total MDS-UPDRS in the NR (mean decrease 14 ± 13.7; paired t-test: p = 0.01), but not in the placebo (mean decrease 1 ± 12.7; paired t-test: p = 0.81). **C:** At the group level, the change (delta) in total MDS-UPDRS in the NR group was significantly (t-test, P = 0.02) larger than that of the placebo group (B).
Key: V2: baseline; V9: after 30 days of treatment; NR: the group receiving NR 3000 mg daily; PL: the placebo group.

The results from our phase I trials suggest that NR holds promise as a neuroprotective therapy against neurodegenerative diseases. These results must now be replicated in AD. Several essential knowledge gaps (KG) must now be addressed in order to further develop NR towards an AD-drug, so that we may harness its full therapeutic potential and maximize clinical benefit and impact.

KG1 – What is the Optimal Biological Dose (OBD) of NR in AD? We define the OBD of NR as the dose required to achieve an optimal neurometabolic response in AD, i.e., maximal cerebral NAD increase or optimal change in cerebral metabolic pattern, in the absence of toxicity.

While our trials show that the treatment responses to NR in neurodegenerative diseases are clearly dose-dependent, the OBD of NR in AD remains undetermined. Based on the results of NR-SAFE, it is likely that improved biological and clinical responses can be achieved by escalating the dose. Moreover, the NR OBD may not be universal. NADPARK showed that the NR-mediated increase in cerebral NAD-levels, and accompanying metabolic and clinical response,are not universal and vary across individuals[25]. The fact that all NR-recipients showed a robust metabolic response in blood, muscle and CSF, suggests that the variable cerebral NAD response may reflect interindividual variability in cerebral NAD metabolism (i.e., variation in the rate of NAD-synthesis or consumption). It is likely that such differences can be modulated by varying the substrate concentration (i.e., the intake dose of NR). This question is critical to address, so that NR-therapy can be correctly dosed and tailored to individual patients to achieve an optimal neurometabolic response.

KG2 – Investigate whether NR has a symptomatic clinical effect in AD and assess its dose-responsiveness. The NADPARK and NR-SAFE studies showed that NR was associated with a clinical improvement, and this correlated significantly with the increase in cerebral NAD levels and brain metabolic network (NRRP) change. Furthermore, the clinical improval was more pronounced with mg NR in the NR-SAFE study, compared to 1000 mg in the NADPARK study. These findings suggest that NR may be ameliorating neuronal function in neurodegenerative disease resulting in symptom improvement. Exploring the dose-responsiveness of this effect will allow us to: **1)** confirm the clinical impact of NR, **2)** determine the optimal clinical dose of NR and **3)** allow us to account for symptomatic effects in neuroprotection trials.

KG3 Determine the dose-dependence of the metabolic response to NR therapy. The NADPARK study showed that a dose of 1000 mg NR daily augmented the NAD metabolome in PBMC, muscle and CSF. The NR-SAFE study suggested that a dose of 3000 mg NR daily leads to a more potent augmentation of the NAD metabolome in blood. The relationship between NR dose and metabolic response needs to be further explored in order to determine optimal dosing regimens**.**

KG4 – Determine whether NR therapy enhance proteostasis in AD and characterize the dose dependence of this effect. Impaired proteostasis plays a central role in AD and other neurodegenerative disorders, including Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Our transcriptomic analyses in the NADPARK study indicated that NR therapy may enhance proteostasis by inducing the expression of both proteasomal and lysosomal pathways. If confirmed, this would suggest that NR targets multiple major processes implicated in the pathophysiology of AD, including mitochondrial respiratory dysfunction, oxidative damage, lysosomal and proteasomal impairment, and neuroinflammation.

KG5 – Does NR therapy influence histone acetylation status? Histone hyperacetylation has been shown in the brain of individuals with AD[8]. Increasing neuronal NAD levels would boost the activity of the NAD-dependent histone deacetylases of the sirtuin family, potentially ameliorating histone hyperacetylation in AD.

KG6 – Does NR decrease neuroinflammation? While it is known that NR has anti-inflammatory properties in peripheral tissues[21], the results of the NAD-PARK trial suggest it also downregulates multiple inflammatory cytokines in the central nervous system. If confirmed this would be of importance for AD and other

neurodegenerative and neuroinflammatory disorders.

KG7 – Determine whether NR therapy alters methylation metabolism and characterize any dose dependence of such an effect. In theory, NAD replenishment via NR administration could decrease/deplete the cellular methylation capacity. NR boosts the NAD-metabolome, leading to increased production of the degradation product nicotinamide (NAM), which is eliminated via methylation to MeNAM, Me-2-PY, and Me-4-PY, and excreted in the urine. Synthesis of Me-Nam requires the methyl-donor S-adenosylmethionine (SAM). This, in turn, could limit SAM availability for other essential methylation reactions, such as DNA and histone methylation, and neurotransmitter synthesis, including dopamine[29]. Thus, in theory, NR would cause an increased consumption of SAM, limiting methylation reactions and generating higher levels of homocystein. Such a phenomenon would be a particular concern for the ~60% of the population that carries MTHFR variants that reduce the efficiency of methylation. The NADPARK study showed no change in serum homocystein levels, or any other evidence of methylation depletion associated with NR 1000 mg daily. It is, however, unknown whether such effects may occur with higher NR doses.

KG8 – Determine how orally ingested NR interacts with the microbiome in AD. Current evidence suggests that the gut microbiome may be involved in the pathogenesis of AD and that individuals with AD host a different microbiome composition compared to neurologically healthy individuals. It is possible that NR therapy may beneficially affect the gut microbiome in AD restoring normal patterns. On the other hand, it is also possible that variation in the gut microbiome may affect local metabolism of NR and absorption in the bloodstream. These effects have not been studied.

KG9 – Determine whether NR therapy influences gastrointestinal (GI) motility in AD. GI dysmotility due to dysfunction and degeneration of both central and local neuronal populations controlling GI motility occurs in AD. It is possible that NR may improve the function of these neurons and preserve them against the neurodegenerative process.

To address these pertinent questions, we will conduct N-DOSE AD, a multi-center randomized double-blinded placebo-controlled trial to assess the optimal biological dose for NR in AD.

1.2 Background - Therapeutic Information

NR (Niagen®, Chromadex) is fully approved for human use and no evidence of toxicity has been found. NR has undergone extensive preclinical testing[16] and is Generally Recognized as Safe (GRAS) for use in food products by the United States Food and Drug Administration[31] and by the European Food Safety Authority[32]. As mentioned above multiple animal studies suggest that NR has strong neuroprotective effects as well as our NADPARK study showing possible therapeutic effects in neurodegenerative disease. NR is well tolerated with no evidence of toxicity in adult humans with doses up to at least 3000 mg daily[11,13,14,25,26,30]. This evidence includes our recently concluded safety and tolerability trial NR-SAFE (clinicaltrials.gov: NCT05344404), which revealed no adverse events of clinical relevance with 3000mg NR daily for 30 days (manuscript in preparation). We therefore propose that dosages up to 3000 mg is highly unlikely to cause toxicity. Niagen and placebo will be provided from Chromadex, https://chromadex.com/. Active study drug capsules will contain 250 mg NR. Placebo will contain microcrystalline cellulose, which will be identical in appearance and taste.

1.3 Pre-Clinical & Clinical Experience with Investigational Product (IP)

NR (Niagen®, Chromadex) is fully approved for human use and no evidence of toxicity has been found. Dose selection is based on pharmacokinetic studies of oral bioavailability in humans[33]. See details under section 1.1.2 above.

1.4 Rationale for the Study and Purpose

Our phase I trial (NADPARK) showed that NR holds great promise as a potential neuroprotective, disease-modifying therapy for neurodegenerative diseases. Encouraged by these findings, this project aims to determine the optimal biological dose of NR in AD and to further explore its neuroprotective potential. We propose that oral administration of the NAD precursor NR (Niagen®, Chromadex) in increasing dosages up to 3000 mg will show a dose-responsive improvement in the cerebral metabolism in AD patients and show signs of improved mitochondrial function, histone acetylation, neuroinflammation and clinical symptoms.
The outcomes of this project will take us several steps closer to developing NR into an AD-drug, so that we may harness its full therapeutic potential and maximize its clinical benefit and impact.

2 STUDY OBJECTIVES AND RELATED ENDPOINTS

DS-group refers to the dose-stable group, i.e. participants receiving NR 1000 mg daily for 3 months. DE-group refers to the dose-escalation group, i.e. participants receiving NR 1000 mg in an escalating dose with 1000 mg from baseline to week 4, 2000 mg from week 4 to week 8 and 3000 mg from week 8 to week 12. PL-group refers to the placebo group.

Objective		**Endpoint**
Primary	To compare the effect of orally administered nicotinamide riboside (NR), escalated to 1500 mg twice per day (3000 mg/day) in the dose-escalation group (DE-group) - versus stable dosing of 500 mg twice per day (1000 mg/day) in the dose-stable group (DS-group) on cerebral NAD-levels, at week 12.	Change in cerebral NAD/ATP-α ratio measured by 31 Phosphorus magnetic resonance spectroscopy (31P-MRS) in the posterior brain (encompassing the occipital, parietooccipital and posterior parts of the temporal cortex).
Secondary	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in cerebral NAD levels from baseline to weeks 4, 8 and 12.	Change in cerebral NAD/ATP-α ratio measured by 31 Phosphorus magnetic resonance spectroscopy (31P-MRS) in the posterior brain (encompassing the occipital, parietooccipital and posterior parts of the temporal cortex).
	To compare the effectiveness of orally administered nicotinamide riboside (NR) 1500 mg twice per day versus 500 mg twice per day in augmenting the NAD-metabolome in the central nervous system (CNS) at week 12.	Change in the cerebrospinal fluid (CSF) levels of NAD or other metabolites of the NAD metabolome*, measured by LC-MS.
Exploratory	**Neuroimaging**	
	To compare the effect of orally administered NR in the DE-group versus DS-group on the NR related metabolic pattern (NRRP) expression at week 12.	Change in NRRP expression, measured by FDG-PET.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in NRRP expression from baseline to weeks 4, 8 and 12.	Change in NRRP expression, measured by FDG-PET.
	To compare the effect of orally administered NR DE-group versus DS-group on the AD-related pattern (ADRP) expression at week 12.	Change in ADRP expression, measured by FDG-PET.

	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in ADRP expression from baseline to weeks 4, 8 and 12.	Change in ADRP expression, measured by FDG-PET.
	Metabolism & molecular markers	
	To compare the effect of orally administered NR in the DE-group versus DS-group on the NAD metabolome* in the blood, urine and central nervous system (CNS) at week 12.	Change in levels of NAD metabolites in blood, urine and CSF, measured by HPLC-MS and/or the NADMed method.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in the NAD metabolome* in blood and urine from baseline to weeks 4, 8 and 12.	Change in levels of NAD metabolites in blood and urine, measured by HPLC-MS and/or the NADMed method.
	To compare the effect of orally administered NR in the DE-group versus DS-group on serum and CSF inflammatory markers at week 12.	Change in inflammatory cytokines in serum and CSF, measured by ELISA.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in serum inflammatory markers from baseline to weeks 4, 8 and 12.	Change in inflammatory cytokines in serum, measured by ELISA.
	Clinical – cognitive & non motor symptom severity, quality of life	
	To compare the effect of orally administered NR in the DE-group versus DS-group on clinical severity of AD symptoms at week 12.	Change in the total ADAS-Cog 13 score, CDR sum of boxes, MoCA score, TMT.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in clinical severity of PD symptoms from baseline to weeks 4, 8 and 12.	Change in the total ADAS-Cog 13 score, CDR sum of boxes, MoCA score, TMT.
	To compare the effect of orally administered NR in the DE-group versus DS-group on ADL severity in AD at week 12.	Change in IADL and PADL scores.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in ADL scores in AD from baseline to weeks 4, 8 and 12.	Change in IADL and PADL scores..
	To compare the effect of orally administered NR in the DE-group versus DS-group on depressive symptoms in AD at week 12.	Change in MADRS score.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and depressive symptoms in AD from baseline to weeks 4, 8 and 12.	Change in MADRS score.
	To compare the effect of orally administered NR in the DE-group versus DS-group on neuropsychiatric symptoms in AD at week 12.	Change in the NPI-Q score.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and change in neuropsychiatric symptoms in AD from baseline to weeks 4, 8	Change in the NPI-Q score.

	and 12.	
	Hypothesis-generating or resource-dependent endpoints (may be reported in follow-up or secondary publications).	
	To compare the effect of orally administered NR in the DE-group versus DS-group on gene expression at week 12.	Change in gene expression, measured by RNA sequencing (RNAseq).
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in gene expression from baseline to weeks 4, 8 and 12.	Change in gene expression, measured by RNA sequencing (RNAseq).
	To compare the effect of orally administered NR in the DE-group versus DS-group on protein expression at week 12.	Change in protein levels, measured by LC-MS.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in protein expression from baseline to weeks 4, 8 and 12.	Change in protein levels, measured by LC-MS.
	To compare the effect of orally administered NR in the DE-group versus DS-group on serum and CSF inflammatory markers at week 12.	Change in inflammatory cytokines in serum and CSF, measured by ELISA.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in serum inflammatory markers from baseline to weeks 4, 8 and 12.	Change in inflammatory cytokines in serum, measured by ELISA.
	To compare the effect of orally administered NR in the DE-group versus DS-group on histone acetylation in AD at week 12.	Change in histone panacetylation, measured by immunoblotting.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in histone acetylation in AD from baseline to weeks 4, 8 and 12.	Change in histone panacetylation, measured by immunoblotting.
	To compare the effect of orally administered NR in the DE-group versus DS-group on H3K27 and H4K16 histone acetylation in AD at week 12.	Changes in levels of H3K27 and H4K16 acetylation, measured by immunoblotting.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in on H3K27 and H4K16 histone acetylation in AD from baseline to weeks 4, 8 and 12.	Changes in levels of H3K27 and H4K16 acetylation, measured by immunoblotting.
	To compare the effect of orally administered NR in the DE-group versus DS-group on the genomic distribution of H3K27 and H4K16 histone acetylation in AD at week 12.	Change in the genomic distribution of H3K27 and H4K16 acetylation, measured by chromatin immunoprecipitation sequencing (ChIPseq).
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in the genomic distribution of H3K27 and H4K16 histone acetylation in AD from baseline to weeks 4, 8 and 12.	Change in the genomic distribution of H3K27 and H4K16 acetylation, measured by chromatin immunoprecipitation sequencing (ChIPseq).

	To compare the effect of orally administered NR in the DE-group versus DS-group on folate and one-carbon metabolism in AD at week 12.	Change in folate and one-carbon metabolites in blood and CSF, measured by HPLC-MS.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in folate and one-carbon metabolism in AD from baseline to weeks 4, 8 and 12.	Change in folate and one-carbon metabolites in blood, measured by HPLC-MS.
	To compare the effect of orally administered NR in the DE-group versus DS-group on methyl donors in AD at week 12.	Change in methyl-donors (e.g., SAM), measured by HPLC-MS, in the blood and/or CSF.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in methyl-donors in AD from baseline to weeks 4, 8 and 12.	Change in methyl-donors (e.g., SAM), measured by HPLC-MS, in the blood.
	To compare the effect of orally administered NR in the DE-group versus DS-group on DNA methylation at week 12.	Change in level and genomic distribution of DNA methylation, measured by Illumina Infinium MethylationEpic kit.
	To assess the dose-response relationship between NR dose (1000 mg, 2000 mg, 3000 mg per day) and changes in methyl-donors in AD from baseline to weeks 4, 8 and 12.	Change in level and genomic distribution of DNA methylation, measured by Illumina Infinium MethylationEpic kit.
	To compare the effect of orally administered NR in the DE-group versus DS-group on synthesis of neurotransmitters in AD at week 12.	Change in neurotransmitters in CSF, measured by HPLC-MS.
	Determine whether NR-therapy affects the gut microbiome in a dose-responsive manner at week 12.	Change in gut microbiome composition, measured by metagenomics in fecal samples.
	To compare the effect of orally administered NR in the DE-group versus DS-group on the gut metabolome at week 12.	Change in fecal metabolomics, measured by LC-MS in fecal samples.
Safety	To determine the safety and tolerability of NR at a dose of 1000 mg, 2000 mg, and 3000 mg per day in AD.	Number and severity of adverse events from baseline to week 12 across treatment groups and NR dose levels.

***The NAD metabolome is comprised of:** Nicotinamide adenine dinucleotide oxidized (NAD^+), Nicotinamide adenine dinucleotide reduced (NADH), NAD^+/NADH ratio, total NAD (sum of NAD^+ and NADH), Nicotinamide adenine dinucleotide phosphate oxidized ($NADP^+$), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), $NADP^+$/NADPH ratio, total NADP (sum of $NADP^+$ and NADPH, 1-methyl nicotinamide (Me-Nam), nicotinic acid-adenine dinucleotide (NAAD), N1-methyl-2-pyridone-5-carboxamide (Me-2-PY), Nicotinamide (Nam), Nicotinamide N-oxide (Nam N-oxide), ADP-ribose (ADPR), Nicotinic acid riboside (NAR), Nicotinamide riboside (NR), Nicotinamide mononucleotide (NMN), Nicotinic acid (NA).

2.1 Primary Endpoint Measure

Change in cerebral NAD level, measured by ^{31}P-MRS.

^{31}P-MRS assesses the levels of key-energy metabolites in the brain, including NAD, ATP, free inorganic phosphate (Pi), and phosphocreatine, and others, as previously shown[25]. Using this method, we will assess

total brain NAD levels normalized to ATP-α levels (i.e., the NAD/ATP-α ratio) in the posterior brain - encompassing the occipital, parietooccipital and posterior parts of the temporal cortex.

2.2 Secondary Endpoint Measure

Change in the CSF level of NAD or other metabolites of the NAD metabolome in CSF, measured by HPLC-MS.

Using **high performance liquid chromatography mass spectrometry (HPLC-MS)**, we will measure the NAD metabolome in CSF, including the following specific measures: Nicotinamide adenine dinucleotide oxidized (NAD^+), Nicotinamide adenine dinucleotide reduced (NADH), NAD^+/NADH ratio, total NAD (sum of NAD^+ and NADH), Nicotinamide adenine dinucleotide phosphate oxidized ($NADP^+$), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), $NADP^+$/NADPH ratio, total NADP (sum of $NADP^+$ and NADPH, 1-methyl nicotinamide (Me-Nam), nicotinic acid-adenine dinucleotide (NAAD), N1-methyl-2-pyridone-5-carboxamide (Me-2-PY), Nicotinamide (Nam), Nicotinamide N-oxide (Nam N-oxide), ADP-ribose (ADPR), Nicotinic acid riboside (NAR), Nicotinamide riboside (NR), Nicotinamide mononucleotide (NMN), Nicotinic acid (NA).

2.3 Exploratory Endpoint Measures

Change in the following parameters listed below:

NR related pattern (NRRP), an ordinal trend pattern (i.e., metabolic network), associated with NR treatment identified in the NADPARK study[25], measured by ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET).

AD related pattern (ADRP), an ordinal trend pattern (i.e., metabolic network), associated with AD, measured by ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET).

The ADAS-Cog 13 (Alzheimer's Disease Assessment Scale – Cognitive Subscale, 13-item version) is a widely used tool for assessing cognitive function in individuals with Alzheimer's disease and other forms of dementia. The total score ranges from 0 to 85, with higher scores indicating greater cognitive impairment.

Clinical Dementia Rating scale sum of boxes (CDR-SB). The CDR integrates assessments from 3 domains of cognition (memory, orientation, judgment/problem-solving) and 3 domains of function (community affairs, home/hobbies, personal care). Following caregiver interview and systematic participant examination, the rater assigns a score describing the participant's current performance level in each of these domains of life functioning. The "Sum of boxes" scoring methodology (CDR-SB) sums the score for each of the 6 domains and provides a value ranging from 0 to 18 with higher scores indicating greater impairment. Positive change from baseline indicates greater impairment.

The Montreal Cognitive Assessment (MoCA) scale. The MoCA scale (minimum score = 0, maximum score = 30) and a dichotomized cut-off score for normality of 26 or over. High scores indicate less cognitive impairment than low scores.

The Trail Making Test (TMT). The TMT is a timed test and the goal is to complete the test as accurately and as quickly as possible. Raw scores are reported in seconds to complete the test. For Part B, an average score is 75 seconds and a deficient score is greater than 273 seconds.

The Lawton Instrumental Activities of Daily Living (IADL) Scale. The IADL scale consists of 8 items providing information about telephone use, preparing food, shopping, doing daily household chores, doing laundry, using transport, medication managing, and managing money. Scores range from 0 (dependent) to 8 (independent).

The Physical Self-Maintenance Scale (PSMS). The PSMS includes 6 items, testing the following areas: toilet use, eating, dressing, physical appearance, deambulation and bath. The PSMS ranges from 1 to 30, with higher scores indicating WORSE functioning.

The Neuropsychiatric Inventory brief questionnaire form (NPI-Q). The NPI-Q measures the burden of 12 neuropsychiatric symptoms of dementia: delusions, hallucinations, agitation/aggression, depression/dysphoria, anxiety, elation/euphoria, apathy/indifference, disinhibition, irritability/lability, motor disturbance, nighttime behaviors, and appetite/eating. Symptom severity is rated on a 3-point scale with higher scores indicating worse symptoms. Minimum score would be 0 and maximum score would be 36.

The Montgomery-Asberg Depression Rating Scale (MADRS). MADRS is a clinician-rated scale designed to measure depression severity and detects changes due to antidepressant treatment. The scale consists of 10 items, each of which is scored from 0 (item not present or normal) to 6 (severe or continuous presence of the symptoms), for a total possible score of 60. Higher scores represent a more severe condition.

NAD metabolome. Using liquid chromatography mass spectrometry (HPLC-MS) and/or the NADMed method, we will measure the NAD metabolome in whole blood (and/or PBMCs), CSF and urine including the following specific measures: Nicotinamide adenine dinucleotide oxidized (NAD^+), Nicotinamide adenine dinucleotide reduced (NADH), NAD^+/NADH ratio, total NAD (sum of NAD^+ and NADH), Nicotinamide adenine dinucleotide phosphate oxidized ($NADP^+$), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), $NADP^+$/NADPH ratio, total NADP (sum of $NADP^+$ and NADPH, 1-methyl nicotinamide (Me-Nam), nicotinic acid-adenine dinucleotide (NAAD), N1-methyl-2-pyridone-5-carboxamide (Me-2-PY), Nicotinamide (Nam), Nicotinamide N-oxide (Nam N-oxide), ADP-ribose (ADPR), Nicotinic acid riboside (NAR), Nicotinamide riboside (NR), Nicotinamide mononucleotide (NMN), Nicotinic acid (NA).

Gene and protein expression levels in PBMC, measured by RNA sequencing (RNAseq) and proteomics (HPLC-MS), respectively.

Levels of inflammatory cytokines in serum and CSF, measured using enzyme-linked immunosorbent essay (ELISA).

Levels of histone panacetylation, and levels and genomic distribution of H3K27 and H4K16 acetylation in PBMC, measured by immunoblotting and chromatin immunoprecipitation sequencing (ChIPseq).

Levels of methyl-donors (e.g., SAM) in blood and/or CSF, measured by HPLC-MS.

Levels of neurotransmitters in CSF, measured by HPLC-MS.

Levels of one carbon metabolism metabolites, measured by HPLC-MS metabolomics in PBMC and CSF.

Levels and genomic distribution of DNA methylation, measured by the Illumina Infinium MethylationEPIC Kit.

Gut microbiome composition, assessed by metagenomics in fecal samples.

Fecal metabolomics, including fatty acid profiling, assessed by HPLC-MS in fecal samples**.**

- Determine whether NR-therapy affects increase in the NRRP in a dose-responsive manner.
- Determine whether NR-therapy affects increase in the ADRP in a dose-responsive manner.
- Determine whether NR-thearpy augments the NAD metabolome in the blood, urine and CSF in a dose-responsive manner.
- Determine whether NR-therapy improves cognitive dysfunction in AD in a dose-responsive manner.
- Determine whether NR-therapy improves ADL in AD in a dose-responsive manner.
- Determine whether NR-therapy affects neuropsychiatric symptoms in AD in a dose-responsive manner.

3 OVERALL STUDY DESIGN

This is a multi-center, phase II, double blinded, randomized, placebo controlled dose-optimization study.

Study Period	Estimated date of first patient enrolled: 01.10.2022
	Anticipated recruitment period: 01.10.2022 – 14.03.2025
	Estimated date of last patient completed: 30.06.2025
Treatment Duration:	12 weeks.

3.1 Study design

N-DOSE AD is a multi-center, phase II, double blinded, randomized, placebo-controlled dose-optimization clinical trial with a dose escalation design. Patients with AD (n = 80) who fulfill participation criteria at screening (see section 4) will be randomized (1:3) into one of two three groups and followed for a total of 3 months: **1) The placebo group** (PL-group, n = 20) will receive placebo for the duration of the study. **2) The NR 1000 mg group** (SD-group, n=20) will receive NR 1000 mg (500 mg x 2) per day for the duration of the study. **3) The NR dose escalation group** (DE-group, n=40) will receive first 1000 mg (500 mg x 2) NR per day for 30 days, then 2000 mg (1000 mg x 2) NR per day for 30 days, and finally 3000 mg (1500 mg x 2) NR per day for 30 days (Fig. 3). The selected dose range is within safety limits for healthy humans (see section 1.3). After eligibility screening, eligible participants will be randomly assigned to one of two study groups and assessed at baseline (Visit-1, V1) and three more visits (Visit-2, Visit-3 and Visit-4; V2, V3, V4) spaced 30 days apart (Fig. 3). Participants and investigators will be blinded to treatment group. Participants will be followed for a total of 90 days. All participants will be recruited from the Neuro-SysMed Center, HUS and Haraldsplass Deaconess Hospital.

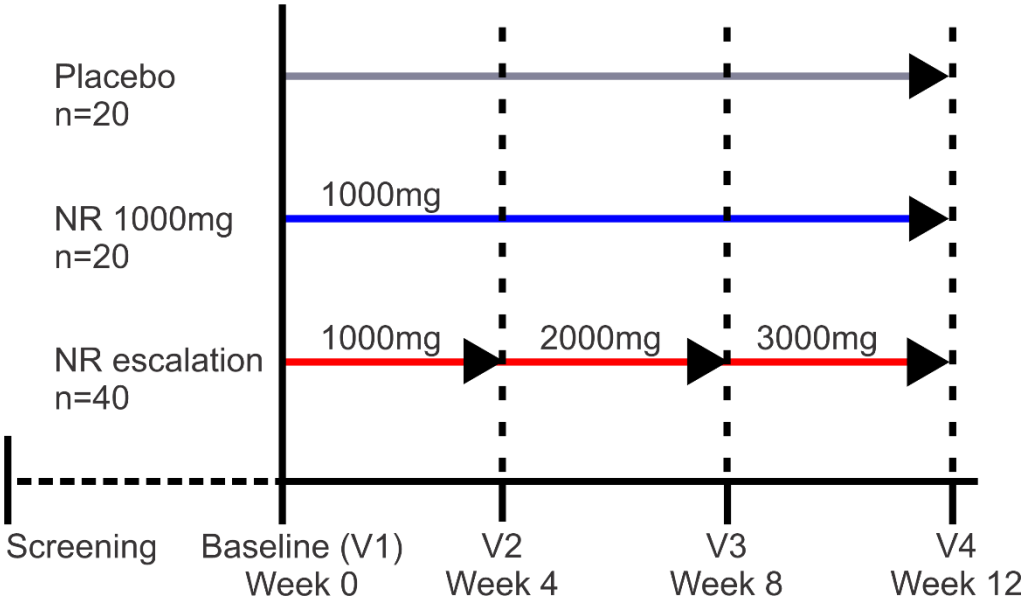

Fig 2. N-DOSE AD study design. A total of 80 patients will be recruited in three arms. Patients will be assessed at baseline (V1) and three additional visits, at 4 (V2), 8 (V3) and 12 (V4) weeks.

4 STUDY POPULATION

4.1 Selection of Study Population

The study setting is at the Geriatrics outpatient clinic at Haraldsplass Deaconess Hospital.

4.2 Number of Patients

A total of n=80 patients will be included in this study and randomized to placebo (PL-group, n=20), NR 500 mg twice per day (DS-group, n=20), or NR escalating dose (DE-group, n=40), starting at 500 mg twice per day, increasing to 1000 mg twice per day, and finally increasing to 1500 mg twice per day.

4.3 Inclusion Criteria

The following condition must apply to the prospective patient at screening prior to receiving study agent:

- Diagnosis of probable AD according to the core clinical criteria updated in the NIA and Alzheimer's Association guidelines.
- Biomarker evidence consistent with AD neuropathologic change, defined by CSF markers[31].
- Diagnosed with AD within two years from enrolment.
- CDR[32] 0.5-1 (inclusive) at enrolment.
- Age 50 to 85 years (inclusive) at the time of enrollment.
- A study partner (i.e. a family member or a friend) able to study data and assist the participant in the study drug administration, i.e. contact ≥ 3 times weekly.
- Capacity to provide written informed consent for study participation defined as Montreal Cognitive Assessment (MoCA)[33] score ≥ 16 or Mini Mental State Evaluation. (MMSE) score ≥ 20[34]. MMSE or MoCA must have been performed within 6 months prior to baseline. If there is any doubt regarding the participants capacity to give informed consent we will ask for an independent evaluation by a consultant clinician who is not associated with the study.
- Cholinesterase inhibitors and memantine can be used if stable for 8 weeks prior to baseline visit.
- Able to undergo lumbar punction.
- Able to undergo MRI

4.4 Exclusion Criteria

Patients will be excluded from the study if they meet any of the following criteria:

- Diagnosis of dementia other than probable AD.
- Comorbidity that precludes study participation or data interpretation.
- Any psychiatric disorder that would interfere with compliance in the study.
- Any severe somatic illness that would interfere with compliance and participation in the study.
- Use of high dose vitamin B3 supplementation within 30 days of enrolment.
- Metabolic, neoplastic, or other physically or mentally debilitating disorder at baseline visit.
- Current treatment with Oral Anticoagulation Therapies
- Implants that preclude MRI examinations, e.g. DBS, pacemaker

5 TREATMENT

For this study NR (Niagen®, Chromadex) is defined as the Investigational Product(s) (IP). IP includes also active comparator and placebo.

5.1 Drug Identity, Supply and Storage

NR (Niagen®, Chromadex) and placebo will be manufactured and provided from Chromadex. Both NR and Placebo will be prepared as identical capsules. The drug has marketing consent in Norway but is not registered by Statens legemiddelverk (SLV)/Direktoratet for medisinske produkter (DMP) as a medical drug, but as a supplement. The NR and placebo have a 1-year expiry date. Both the NR and placebo will be stored in room temperature with temperature <25 degrees (according to instructions given by Chromadex).

5.2 Dosage and Drug Administration

Each NR capsule contains 250mg.. To keep the study fully blinded, all participants will receive the same number of daily capsules irrespective of which treatment group they belong to. To achieve this, NR capsules will be combined with placebo capsules as necessary (see below):

- Patients in the NR 1000mg group will administer orally [2 NR capsules (500mg) + 4 placebo capsules] x 2 times daily (1000mg NR daily in total).
- Patients in the NR dose escalation group will administer orally the following doses:
    - Weeks 0-4: [2 NR capsule (500mg) + 4 placebo capsules] x 2 times daily (1000mg NR daily in total).
    - Weeks 5-8: [4 NR capsule (1000mg) + 2 placebo capsule] x 2 times daily (2000mg daily total).
    - Weeks 9-12: [6 NR capsules (1500mg) + 0 placebo capsule] x 2 times daily (3000mg daily total).
- The placebo group will administer orally [6 placebo capsules] x 2 times daily.

There is no specified time of day the dosages should be taken, only that they should be taken with about 12 hours apart, if possible. If a dose is missed, the patient can take the missed dose as soon as it is remembered, provided it is shorter time to the missed dose than the next scheduled dose. There are no restrictions with respect to combining the dose with other medication and/or food. **The study medication is to be taken every day during the treatment period, including prior to study visits.**

5.3 Duration of Therapy

Therapy duration for the study is 3 months (12 weeks).

5.4 Cognitive Therapy During Screening and IP treatment period

Eligible and consenting men and women with AD may use standard treatments with Cholinesterase inhibitors and memantine can be used if stable for 8 weeks prior to
screening and baseline visits. The treatment regime will then be frozen and remain unchanged for the study period (3 months). At the end of study visit (month 3), the physician determines (yes/no) whether the patient is still adequately treated for his/her AD with their current standard treatment.

5.5 Concomitant Medication

There are no restrictions on any other use of medications. All patients should use medications prescribed prior to enrollment in the study. There are no restrictions with respect to starting new medications that are necessary for the patient.

The Patient should not take any vitamin B3 supplements for the duration of the study.

All concomitant medication (incl. vitamins with the exception of vitamin B3, herbal preparation and other "over-the-counter" drugs) used by the patient will be recorded in the patient's file and CRF.

5.6 Subject Compliance

Patient compliance will be determined based on self-report at study visits (using information from the study participant and study partner). A pill count of remaining medication will be performed when providing new study medication and at the end of the study.

5.7 Drug Accountability

The responsible site personnel will confirm receipt of study drug and will use the study drug only within the framework of this clinical study and in accordance with this protocol. Receipt, distribution, return, and destruction (if any) of the study drug must be properly documented according to the sponsor's agreed and specified procedures.

Study drugs are stored locally at the study site and distributed by the study nurse upon registration in CRF. Remaining study drug is returned for pill count.

5.8 Drug Labeling

The investigational product will have a label permanently affixed to the outside and will be labeled according with ICH GCP and national regulations, stating that the material is for clinical trial / investigational use only and should be kept out of reach of children.

Label will include:

- Patient's initials
- Study number (CRF number)
- Date dispensed

5.9 Subject Numbering

At the screening visit, patients will be sequentially allocated a patient study number. The subject will be identified by this patient study number for the remainder of the study. Once a patient number has been assigned, no attempt will be made to use that number again.
The patient study number will be in the following format: XX-YYY where XX is the study site number and YYY is the patient id number. If a patient number is allocated incorrectly, no attempt will be made to remedy the error once study treatment has been dispensed. Any replacement patients will be given the next patient number in the sequence.

6 STUDY PROCEDURES

6.1 Flow Chart

Table 1. Trial flow chart

	Screening Period		Treatment Period Study Visit:			End of study visit
Event	**First Screening**	**Next/Last screening[1]**	**Visit-1 Baseline[1]**	**Visit-2**	**Visit-3**	**Visit-4**
Time			**Week 0**	**Week 4**	**Week 8**	**Week 12**
Informed consent	X					
Informed consent biobank, optional	X					
AD Clinical diagnosis Criteria	X		X			
Inclusion/exclusionEvaluation	X		X			
Medical history[1]	X		X			
Neurologic exam[2]	X		X	X	X	X
Physical Examination[3]	X		X	X	X	X
Body height			X			
Body weight and BMI			X	X	X	X
Vital signs[4]			X	X	X	X
Record of concomitant medication	X		X	X	X	X
MoCA[5]	X		X	X	X	X
CDR[5]	X		X	X	X	X
ADAS-COG 13			X	X	X	X
Trail Making Test (TMT)[5]	X		X	X	X	X
31P-MRS, 1H-MRSand FDG-PET imaging			X	X	X	X
MADRS[5]	X		X	X	X	X
NPI-Q, and IADL and PADL[5]	X		X	X	X	X
Dietary registration (3 days prior to visit)			X			X
Routine blood tests[6]			X	X	X	X
hCG[7]	X					

Blood for biobanking[8]			X	X	X	X
Cerebrospinal fluid collection[9]	X					X
Fecal sample collection			X			X
Urine sample collection			X	X	X	X
Treatment (IP) dispensation[10]			X	X	X	
Cognitive treatment stable[11]			X			X
Adverse event				X	X	X

Superscripts refer to the specifications below.

Specifications

1. Medical history includes: family history of neurological illness (what and who), family history of dementia (what and who), smoking history (period, pack-years), first AD symptoms, months since first AD symptoms,.
2. General Neurological examination
3. Heart and lung auscultation, abdominal palpation, any other examination dictated by patient's condition/symptoms
4. Blood pressure, pulse, temperature
5. Not repeated at baseline if conducted at screening within 4 weeks prior to the baseline visit.
6. CRP, ALAT, ASAT, GT, bilirubin, ALP, creatinine, urea, RBC, Hb, WBC with differential, platelets, CK, FT4, TSH, B12, folic acid, homocysteine, methylmalonic acid, sodium, potassium, glucose.
7. Women of childbearing potential will also have a pregnancy test performed.
8. EDTA blood, snap-frozen EDTA blood, PAXgene, platelet isolation, serum (see lab manual for details).
9. CSF, including Amloid-β and Tau protein levels to verify the AD diagnosis.
10. To ensure correct dosages during dose escalation and if necessary to resupply.
11. The patient has to be stable if on a standard cognitive treatment for 8 weeks prior to baseline. There should not be more than 3 months from last screening to baseline visit.

6.2 By Visit

6.2.1 Screening Visits/ Before start of Investigational Product (IP)

The first screening visit aims to determine if the patient is eligible to be included in the study. A full physical examination, anamnestic medical history, cognitive testing and lumbar puncture to establish AD pathology is performed. If the patient fulfills the inclusion/exclusion criteria and gives informed consent, standard treatment with cholinesterase inhibitors and memantine must be stable for 8 weeks prior to screening and baseline visits.

If the patient fulfills the inclusion/exclusion criteria, the patient is called in for the baseline study visit (week 0 study visit). The Baseline study visit should be within 8 weeks from the time the lumbar puncture was performed. There should not be more than 3 months from last screening to baseline visit.

Screening checklist:

1. Patient signed informed consent for N-DOSE AD
2. Patient signs informed consent for consent for storage and analysis of biological material ("Samtykke for lagring av biologisk material i biobank for demens og aldring")
3. Physical examination (general neurological examination) at first screening
4. MoCA
5. Clinical AD diagnosis Criteria
6. Record current use of medication. Advise to stop any use of vit B3 supplements
7. Plan/conduct Lumbar puncture to verify AD diagnosis
8. If the patient is ready for enrollment, they are referred to baseline study visit (including MRI and PET).
9. The study partner is given the 3-day dietary registration form along with instructions on how to fill it out and deliver to the study nurse at baseline.
10. Give instructions on how to collect urine and fecal sample before the baseline visit.

6.2.2 Baseline/Week 0

1. At the first study visit the investigator needs to verify the informed consent for the study and offer the subject to sign the informed consent for storage and analysis of biological material (Samtykke for lagring av biologisk materiale i FORSKNINGSBIOBANKEN FOR ALDRING, DEMENS OG NEVROLOGI).
2. Investigator verifies anamnestic information gathered at screening, current use of medication and medical history.
3. Investigator verifies fulfillment of inclusion and exclusion criteria.
4. The study identification number is assigned (see section 5.9).
5. Study medication is dispensed to the subject by the study nurse and the patient is reminded to take the study medication every day including at the morning of each visit.
6. The following clinical examination are performed by the study nurse or investigator (see flowchart in section 6.1):
    a. Vital signs
    b. Hight, body weight and BMI
    c. MoCA
    d. ADAS-COG
    e. CDR
    f. Trail Making Test (TMT)
    g. MADRS
    h. NPI-Q
    i. IADL and PADL
7. Routine blood tests are done (see flowchart in section 6.1)
8. Samples for biobanking are taken (see flowchart in section 6.1):
    a. EDTA whole blood
    b. Snap-frozen whole blood
    c. PAXgene blood for RNA
    d. Platelets

    e. Serum
    f. Fecal sample
    g. Urine sample
9. Imaging is conducted
    a. 31P-MRS & 1H-MRS
    b. FDG-PET
10. All data gathered during study visits is either written into the electronic journal or filled out in paper format with appropriate date and signatures. Each patient will have their own folder with gathered clinical data. The clinical tests are recorded in paper format.
11. The patient's study partner is given the 3-day dietary registration form along with instructions on how to fill it out and deliver to the study nurse at the next visit.
12. The patient and study partner is instructed to take the study medication every day for the remainder of the study. The study medication or placebo is to be taken as outlined in section 5.2. The capsules are taken in the morning and evening. There is no specified time of day the dosages should be taken, only that they should be taken with about 12 hours apart if possible. If a dose is missed, the patient can take the missed dose as soon as it is remembered, provided it is shorter time to the missed dose than the next scheduled dose. There are no restrictions with respect to combining the dose with other medication and/or food.

6.2.3 During treatment (visits 2-4)

See flowchart (section 6.1) for which clinical examinations are performed at each study visit. Some particular considerations follow:

- Should the patient need replenishment of study medication, it will be logistically handled by the study nurse.
- Points 6-12 from section 6.2.2 are repeated.

6.2.4 End of study (Visit 4)

- End of study (Week 12): Patients bring with them remaining study medication which is gathered by the study nurse for a pill count. Clinical examinations are performed as listed in section 6.1.
- Points 6-10 from section 6.2.2. are repeated.
- At the end of the study visit, consider changes in standard treatment with cholinesterase inhibitors or memantine.

6.3 Criteria for Patient Discontinuation

Patients may be discontinued from study treatment and assessments at any time. Discontinuation and the reason for discontinuation (withdrawn from the study) will be registered. Specific reasons for discontinuing a patient for this study are:

- Voluntary discontinuation by the patient who is at any time free to discontinue his/her participation in the study, without prejudice to further treatment.

- Any form of expression, both verbal and non-verbal, by a participant opposing participation, either before or during the study, will be respected.

- Safety reason as judged by the Principal Investigator.

- Incorrect enrollment, i.e. the patient does not meet the required inclusion/exclusion criteria for the study.

- Deterioration in the patient's condition which in the opinion of the Principal Investigator warrants study medication discontinuation (to be recorded as an AE or under Investigator Discretion).

6. 4 Procedures for Discontinuation

6.4.1 Patient Discontinuation

Patients who are withdrawn from the study before start of treatment, will be replaced. Withdrawn patients are not followed up.

6.4.2 Trial Discontinuation

The whole trial may be discontinued at the discretion of the PI or the sponsor in the event of any of the following:

- Occurrence of AEs unknown to date in respect of their nature, severity and duration.
- Medical or ethical reasons affecting the continued performance of the trial.
- Difficulties in the recruitment of patients.

The sponsor and principal investigator will inform all investigators and the Ethics Committees of the termination of the trial along with the reasons for such action. If the study is terminated early on grounds of safety and Ethics Committees will be informed within 15 days.

7 ASSESSMENTS

The schedule of assessments is indicated in Section 6 (see section 6.1, Flow Chart).

7.1 Safety and Tolerability Assessments

Safety will be monitored by the assessments described below as well as the collection of AEs at every visit. Significant findings that are present prior to the signing of informed consent must be included in the relevant medical history/ current medical condition page of the e-CRF. For details on AE collection and reporting, refer to Section 8.

For the assessment schedule refer to study flow chart in Section 6.1.

7.2 Clinical Assessments

1. **Medical history:**
   Performed by: investigator
   a. Family history of parkinsonism:
      i. Who
      ii. Which illness.

b. Family history of Dementia:
    i. Who
    ii. Was the diagnosis certain, probable or possible
c. Family history of cardiovascular disease
d. Smoking history:
    i. Active smoker, previous smoker, or never smoker?
    ii. Years since smoking cessation (if applicable)
    iii.
    iv.
e. Alcohol use
    i. Average alcohol units per week (if applicable)
f. First AD symptoms:
    i. Which symptoms: memory, orientation, language, behavior, other (if other = free text comment).

2. Vital signs:

Performed by: study nurse

a. Blood pressure.
b. Pulse.
c. Temperature.

3. Body metrics:

Performed by: study nurse

a. Body weight.
b. Height.
c. BMI.

4. Physical examination:

Performed by: investigator

a. Heart and lung auscultation.
    i. Heart: normal / findings (free field)
    ii. Lungs: normal / findings (free field)
b. Abdominal palpation: normal / findings (free field)
c. Any other examination dictated by patient's condition/symptoms: free field.

5. General neurological examination

Performed by: investigator

a. Register any findings not related to the subject's parkinsonism: free field.

6. Clinical scales & registrations:

Performed by: see individual tests below

a. MoCA:
   Performed by: investigator or study nurse
b. ADAS-COG
   Performed by: investigator or study nurse
c. CDR:
   Performed by: Investigator or study nurse
d. Trail Making Test (TMT)
   Performed by: Investigator or study nurse
e. MADRS:

Performed by: investigator study nurse

f. NPI-Q:
Performed by: study nurse
g. IADL and PADL
Performed by: study nurser
h. 3-day dietary record:
Performed by: self-filled by the patient and patient's study partner

7.3 Routine Laboratory Tests

These will include: CRP, ALAT, ASAT, GT, bilirubin, ALP, creatinine, urea, RBC, Hb, WBC with differential, platelets, CK, FT4, TSH, B12, folic acid, homocysteine, methylmalonic acid, sodium, potassium, glucose. Women of childbearing potential will also have a pregnancy test performed.
Laboratory tests and biosampling are listed and described in detail in Appendix A.

7.4 Imaging studies

1. **31P-MRS** (CSI, multinuclear coil – 15 min) will be conducted on a 3T Biograph mMR MR-PET scanner (Siemens Healthcare, Germany) to assess the intracerebral concentration of NAD, as we have done previously[25].
2. **FDG-PET** imaging will be performed on the same MR-PET scanner and in the same session, to assess the metabolic response to NR treatment, as we have previously shown[25]. Following standard preprocessing protocols and spatial normalization, the NRRP will be assessed using using ordinal trends/canonical variates analysis (OrT/CVA), a supervised form of principal component analysis (PCA). This multivariate approach is designed to detect and quantify regional covariance patterns (i.e., metabolic networks) for which expression values (i.e., subject scores) increase or decrease with treatment in all or most of the subjects.

The MRI protocol is also summarized in Appendix B.

7.5 Molecular analyses

1) Metabolomics analyses will be performed in snap-frozen blood, PBMC, and CSF, using liquid chromatography-mass spectrometry (LC-MS) as described[37]. Absolute metabolite concentrations will be determined using in house standards. We will assess the entire NAD-metabolome, and key-metabolites involved in the Krebs' cycle, fatty acid beta-oxidation, and methylation reactions (e.g., SAM, homocystein, folate).

2) Gene and protein expression. The transcriptome will be mapped in PBMC and/or PAXgene samples by RNA-sequencing, using ribosomal depletion and sequencing at 125 bp paired-end and 100 million read pairs per sample, as we have previously described[38]. Quantitative proteomics will be performed in PBMC, muscle and CSF, using TMT (Tandem Mass Tags) labeling and mass spectrometry (LC-MS/MS Q-Exactive HF).

3) Histone acetylation profiling. As in our previous work[12], we will first assess quantitative changes in global histone acetylation status in PBMC and muscle, by immunoblotting with a pan-acetyl-lysin antibody. Next, acetylation levels of specific lysine residues (e.g., H3K27 and H4K16) will be assessed with targeted immunoblotting. Finally, genome-wide changes in the acetylation status of histone lysine residues found to be quantitatively altered by the treatment, will be assessed by chromatin-immunoprecipitation sequencing (ChIP-Seq)[12].

4) Inflammatory cytokine concentration will be determined in CSF using ELISA as in the NADPARK trial[18].

5) DNA methylation will be mapped in PBMC and/or snap-frozen blood using the Illumina Infinium Epic Chip.

6) Neurotransmitter levels. Monoamine levels will be determined in the CSF using HPLC-MS.

7) Gut microbiome. Using fecal samples, we will assess the microbiota profile (i.e., estimates of composition and abundance) by metagenomics, and function by microbial metabolomics. Metagenomics analyses will comprise 16S-rRNA sequencing-based count of operational taxonomic units (OTUs), Illumina NovaSeq short-read sequencing, and long-read sequencing by MinION technology. For microbial metabolomics, feces will be analysed for short-chained fatty acids (SCFA) and, if feasible, NAD-related metabolites.

8 SAFETY MONITORING AND REPORTING

The investigator is responsible for the detection and documentation of events meeting the criteria and definition of an adverse event (AE) or serious adverse event (SAE). Each patient will be instructed to contact the investigator immediately should they manifest any signs or symptoms they perceive as serious.

The methods for collection of safety data are described below.

8.1 Definitions

8.1.1 Adverse Event (AE)

An AE is any untoward medical occurrence in a patient administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment.

An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product, whether or not related to the investigational product.

The term AE is used to include both serious and non-serious AEs.

If an abnormal laboratory value/vital sign is associated with clinical signs and symptoms, the sign/symptom should be reported as an AE and the associated laboratory result/vital sign should be considered as additional information that must be collected on the relevant CRF.

Only intensity 2 and 3 is registered as AE in eCRF, see section 8.3. An AE has to interfere with everyday life to be of intensity 2 or 3.

8.2.1 Serious Adverse Event (SAE)

Any untoward medical occurrence that at any dose:

- Results in death
- Is immediately life-threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability or incapacity
- Is a congenital abnormality or birth defect
- Is an important medical event that may jeopardize the subject or may require medical intervention to prevent one of the outcomes listed above.

Medical and scientific judgment is to be exercised in deciding on the seriousness of a case. Important medical events may not be immediately life-threatening or result in death or hospitalization, but may jeopardize the subject or may require intervention to prevent one of the listed outcomes in the definitions above. In such situations, or in doubtful cases, the case should be considered as serious. Hospitalization for administrative

reasons (for observation or social reasons) is allowed at the investigator's discretion and will not qualify as serious unless there is an associated adverse event warranting hospitalization.

8.2 Time Period for Reporting AE and SAE

Recording AE and SAEs will begin after baseline (week 0) and continue to be monitored and registered throughout the duration of the study up until 7 days after the last study visit.

During the course of the study all AEs and SAEs will be proactively followed up for each patient; events should be followed up to resolution, unless the event is considered by the investigator to be unlikely to resolve due to the underlying disease. Every effort should be made to obtain a resolution for all events, even if the events continue after discontinuation/study completion.

8.3 Recording of Adverse Events

If the patient has experienced adverse event(s), the investigator will record the following information in the e-CRF:

- The nature of the event(s) will be described by the investigator in precise standard medical terminology (i.e. not necessarily the exact words used by the patient).
- The duration of the event will be described in terms of event onset date and event ended data.
- The intensity of the adverse event: **Only intensity 2 and 3 is registered as AE in eCRF.**

Assessment of Intensity
The investigator will make an assessment of intensity for each AE and SAE reported during the study and assign it to 1 of the following categories: ● 1 Mild: An event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities. ● 2 Moderate: An event that causes sufficient discomfort to interfere with normal everyday activities. ● 3 Severe: An event that prevents normal everyday activities. An AE that is assessed as severe should not be confused with an SAE. Severe is a category utilized for rating the intensity of an event; and both AEs and SAEs can be assessed as severe.

The Causal relationship of the event to the study medication will be assessed as one of the following:

Unrelated:

There is not a temporal relationship to investigational product administration (too early, or late, or investigational product not taken), or there is a reasonable causal relationship between non-investigational product, concurrent disease, or circumstance and the AE.

Unlikely:

There is a temporal relationship to investigational product administration, but there is not a reasonable causal relationship between the investigational product and the AE.

Possible:

There is a reasonable causal relationship between the investigational product and the AE. Dechallenge information is lacking or unclear.

Probable:

There is a reasonable causal relationship between the investigational product and the AE. The event responds to dechallenge. Rechallenge is not required.

Definite:

There is a reasonable causal relationship between the investigational product and the AE.

- It will be recorded in the eCRF the outcome of the adverse event, the action taken and whether the event is resolved or still ongoing.
- It is important to distinguish between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined by the criteria in Section 8.1. An AE of severe intensity need not necessarily be considered serious. For example, nausea that persists for several hours may be considered severe nausea, but is not an SAE. On the other hand, a stroke that results in only a limited degree of disability may be considered a mild stroke, but would be an SAE.

8.4 Reporting Procedure

8.4.1 AEs and SAEs

All adverse events and serious adverse events that should be reported as defined in section 8.1.1 will be recorded in the patient's CRF.

SAEs must be reported by the investigator to the sponsor, (PI Kristoffer Haugarvoll, see contact information) within 24 hours after the site has gained knowledge of the SAE. Every SAE must be documented by the investigator on the SAE pages to be found in e-CRF. The initial report shall promptly be followed by detailed, written reports if necessary. The initial and follow-up reports shall identify the trial subjects by unique code numbers assigned to the latter.

The sponsor keeps detailed records of all SAEs reported by the investigators and performs an evaluation with respect to seriousness, causality and expectedness.

8.5 Clinical Study Report

The adverse events and serious adverse events occurring during the study will be discussed in both the main results and the safety evaluation part of the Clinical Study Report.

9 DATA MANAGEMENT AND MONITORING

9.1 Electronic - Case Report Forms (e-CRFs)

The study nurse/investigator will enter the data required by the protocol into the electronic Case report forms (e-CRF) online. The electronical CRF that will be used is Viedoc. The Investigator is responsible for assuring that data entered into the e-CRF for his/her patient is complete, accurate, and that entry is performed in a timely manner. The signature of the investigator will attest the accuracy of the data on each CRF. If any assessments are omitted, the reason for such omissions will be noted on the e-CRFs.

At study visit the following source date should be registered either on paper or hospital records. See section 6.1 and 6.2 for when the relevant information is gathered. This data will then be transferred to eCRF by the study nurse/investigator.

- That the patient is participating in the study, e.g. by including the enrollment number and the study code or other such study identification
- Date when Informed Consent was obtained from the patient and statement that patient received a copy of the signed and dated Informed Consent
- Results of all assessments confirming a patient's eligibility for the study
- Diseases (relevant past and current with date; both the disease studied and others, as relevant)
- Medical history
- Laboratory data
- Results of clinical assessments performed during the study
- Treatment given
- Non-Serious Adverse Events and Serious Adverse Events (if any) including causality assessments
- Date of, and reason for, discontinuation from study treatment
- Date of, and reason for, withdrawal from study

9.2 Study Monitoring

The investigator will be visited on a regular basis by the Clinical Study Monitor, who will check the following:

- Informed consent process
- Reporting of adverse events and all other safety data
- Adherence to protocol
- Maintenance of required regulatory documents
- Study Supply accountability log
- Data completion on the e-CRFs including source data verification (SDV).

The monitor will review the relevant e-CRFs for accuracy and completeness, based on a risk assessment, and will ask the site staff to adjust any discrepancies as required.

9.3 Confidentiality

The investigator shall arrange for the secure retention of the patient identification and the code list. Patient files shall be kept for the maximum period of time permitted by each hospital. The study documentation (e-

CRFs, Site File etc) shall be retained and stored during the study and for 5 years after study closure. All information concerning the study will be stored in a safe place inaccessible to unauthorized personnel.

9.4 Database management

- Access to study after the study completion is granted by Kristoffer Haugarvoll.
- Data for each patient will be recorded on the eCRF. Data collection must be completed for each patient who signs an informed consent form and receives at least one dose of study treatment.
- eCRFs will be designed and produced by the Investigator and should be completed in accordance with instructions. The Investigator is responsible for maintaining adequate and accurate medical records from which accurate information will be transcribed directly into the eCRFs using a secure internet connection. The eCRFs should be filled out completely by the Investigator or designee as stated on the delegation of responsibilities form.
- The eCRFs must be reviewed, signed and dated by the Investigator.
- Data entered into the eCRF will be validated as defined in the data validation plan. Validation includes, but is not limited to, validity checks (e.g. range checks), consistency checks and customised checks (logical checks between variables to ensure that study data are accurately reported) for eCRF data and external data (e.g. laboratory data). A majority of edit checks will be triggered during data entry and will therefore facilitate efficient 'point of entry' data cleaning.
- Data management personnel will perform both manual eCRF review and review of additional electronic edit checks to ensure that the data are complete, consistent and reasonable. The electronic edit checks will run continually throughout the course of the study and the issues will be reviewed manually online to determine what action needs to be taken.
- Manual queries may be added to the system by clinical data management or study monitor. Clinical data managers and study monitors are able to remotely and proactively monitor the patient eCRFs to improve data quality.
- Pharmacokinetic data will be transferred electronically into the study database. Discrepancies will be queried to the site and/or the laboratory until the electronic data and the database are reconciled.
- All updates to queried data will be made by authorised study centre personnel only and all modifications to the database will be recorded in an audit trail. Once all the queries have been resolved, eCRFs will be locked by password protection. Any changes to locked eCRFs will be approved by the Investigator.
- Once the full set of eCRFs have been completed and locked, the Sponsor will authorise database lock and all electronic data will be sent to the designated statistician for analysis. Subsequent changes to the database will then be made only by written agreement.
- Adverse events and medical history will be coded from the verbatim description (Investigator term). Prior and concomitant medications and therapies will be coded according to the World Health Organization drug code.

10 STATISTICAL METHODS AND DATA ANALYSIS

10.1 Determination of Sample Size

Our primary null hypothesis (H_0) is that the NR-induced increases in cerebral NAD levels (measured by 31P-MRS) are not dose-responsive. The alternative hypothesis (HA) is that this measure is dose responsive. In the NADPARK study, all three measures showed a highly significant increase in the group

receiving 1000 mg NR (n = 15) compared to the placebo group (n = 15). In the NADPARK study25, treatment with 1000mg of NR led to an increase in cerebral NAD-levels by a factor of 1.27 from baseline in the treatment group, whereas the change in the placebo group was negligible at -0.43%. Under the HA, we assume that cerebral NAD levels will increase in a linear fashion in the 2000 mg NR and 3000 mg NR groups, respectively. Based on these assumptions and given a type I error rate of 5% ($\alpha = 0.05$) and a type II error rate of 10% ($\beta = 0.1$, power = 90%), we estimated that a sample size of 30 individuals will be required in the dose escalation group. Accounting for drop-out and statistical safety margin, we estimate that the study requires 40 subjects in this group.

For the secondary and exploratory outcomes, we assume that the metabolomic, transcriptomic and inflammatory cytokine analyses will have sufficient power, since they produced very large effect sizes and highly significant results in the NADPARK study with 15 individuals per group. Since no pilot data exist for the proteomic and epigenomic analyses, these will be exploratory in nature. However, in our previous experience, a sample size of 20 per group should be sufficient to detect treatment-induced differences of biological relevance.

10.2 Randomization

Randomization is done by e-CRF upon enrollment to the study. Participants will be randomized into one of three groups (Placebo, NR 1000 mg for the duration of the study, NR dose escalation group).

10.3 Population for Analysis

Intention to treat (ITT)/Full analysis set (FAS) population: All participants, regardless of protocol adherence.

The Safety Analysis Set (SAS) will include all patients having received at least one study treatment infusion after randomisation.

The Per Protocol Analysis Set (PPS) will include all randomized patients meeting the study eligibility criteria and with no major protocol deviations affecting the treatment efficacy.

The following are pre-defined major protocol deviations regarded to affect the efficacy of the intervention:

- Entering the trial when the eligibility criteria should have prevented trial entry.
- *Discontinuation of intervention prior to 84 ± 7 days.*
- Received or used other intervention than allocated.
- *Adherence to allocated treatment below 80%.*
- *Visit date interval larger than 28 ± 7 days between individual visits (i.e.: V1 and V2, V2 and V3 and V3 and V4)*.*
- *Visit date interval larger than 84 ± 7 days from V1 to V4*.*
- *Not fasting before neuroimaging.*

*Due to updated findings from a pharmacokinetic study (ClinicalTrials.gov, NCT: NCT05698771, in revision) that NAD levels increase and reach a plateau within 1-2 weeks of NR treatment both in healthy

individuals and persons with Parkinson's disease. A 3 week period should therefore be sufficient to assess the effects of increasing NR dosage.

10.4 Planned analyses

All statistical analysis is planned after the completion of the study.

All randomized patients will be included in the primary analyses. All randomized patients will be included in the primary analyses and sensitivity analyses will be carried out comparing results from the ITT, SAS and PPS data sets. In the case of missing assessments, the subject will be included if possible.

The ***primary analysis*** of the primary endpoint will be performed using analysis of covariance (ANCOVA) between the dose stable (DS-group) and dose escalation group (DS-group), i.e. the regression of NAD levels measured by ^{31}P-MRS at week 12 depending on randomization group and adjusted for ^{31}P-MRS at baseline. No additional adjustments/covariates will be used. As this is the single primary outcome, alpha will be set at 0.05. No correction for multiple testing will be performed.

The ***secondary analysis*** of the primary endpoint will be the comparison of change over time from baseline to week 4, week 8 and week 12, between the DE-group and the DS-group. This will be assessed using a linear mixed model (LME), i.e. NAD (^{31}P-MRS) at weeks 4,8 and 12 depending on randomization group, time and their interaction adjusted for random individual intercept.

The ***primary analysis*** of the secondary endpoint will be performed using ANCOVA between the dose stable (DS-group) and dose escalation group (DS-group), i.e. the regression of NAD or NAD metabolite levels measured by HPLC-MS at week 12 depending on randomization group and adjusted for NAD or NAD metabolite at baseline. No additional adjustments/covariates will be used.

The analysis of exploratory endpoints will be performed in a similar manner as for the primary and secondary endpoints. ANCOVA will be used to compare endpoints at week 12, and LME will be used to compare change from baseline to week 4, week 8 and week 12.

In addition, for PET analyses, changes in network scores with treatment will be evaluated for each group separately using permutation tests. Relationships between network values, brain NAD levels and cognitive ratings or between treatment-related changes in these variables will be evaluated using Pearson's product-moment correlations, whereas Spearman rank-order correlation coefficients will be computed for non-normally distributed variables.

Omics data will undergo rigorous quality control and filtering according to established best practice procedures. The between visit change in this data will be assessed by a pairwise comparison between each NR group and the placebo group, using linear models with appropriate covariates. Comparison of adverse events and abnormal laboratory test results between the treatment and placebo groups will be analyzed descriptively.

Further details for the analysis are outlined in the statistical analysis plan (SAP) for the trial, and we refer the

reader to this document.

10.5 Statistical Analysis

Dependent variable

Primary analysis:

- Cerebral NAD levels (measured by 31P-MRS)

Secondary, exploratory and safety analysis:

- Adverse effects, categorized as either moderate or severe.
- Levels of CSF NAD and NAD-metabolites (measured by LS-MS)
- Level of ADRP expression (measured by FDG-PET)
-
- Routine blood tests.
- Total ADAS-COG. Assessed by the ADAS-COG 13 scale.
- CDR sum of boxes. Assessed by the CDR questionnaire.
- ADL score. Assessed by the IADL and PADL questionnaires.
- MoCA total score. Assessed by the MoCA questionnaire.
- MADRS score. Assessed by the MADRS scale.
- NPI-Q score. Assessed by the NPI-Q questionnaire.

- Levels of NAD-related and other metabolites in, frozen blood, urine and CSF
- Expression of RNA and protein of genes and pathways involved in proteasomal and lysosomal biogenesis and function.
- Histone panacetylation levels
- Levels of specific lysine residues H3K27 and H4K16 acetylation
- Genome-wide distribution of histone lysine residues found to be quantitatively altered by the treatment (measured by ChIP-Seq).
- Level of inflammatory cytokines in patient serum and CSF.
- Gene and protein expression levels in PBMC and/or PAXgene blood.
- Levels of one carbon metabolism metabolites, measured by HPLC-MS metabolomics in PBMC and CSF.
- Levels of monoamine neurotransmitters in CSF.
- Levels and genomic distribution of DNA methylation.
- Gut microbiome composition.
- Fecal metabolomics, including fatty acid levels.

Statistical hypothesis

Primary analysis:

- Our primary null hypothesis (H_0) is that the NR-induced increases in cerebral NAD levels (measured by 31P-MRS) is not dose-responsive. The alternative hypothesis (H_A) is that this increase is dose responsive.

Secondary analysis:

- Mean change between theNR dose escalation arm, NR 1000 mg arm and placebo arm for the dependent variables mentioned above.
- Mean change between and within the NR dose escalation arm, NR 1000 mg arm and placebo arm for the dependent variables mentioned above.

11 STUDY MANAGEMENT

11.1 Investigator Delegation Procedure

The principal investigator is responsible for making and updating a “delegation of tasks” listing all the involved co-workers and their role in the project. He will ensure that appropriate training relevant to the study is given to all of these staff, and that any new information of relevance to the performance of this study is forwarded to the staff involved.

11.2 Protocol Adherence

Investigators ascertain they will apply due diligence to avoid protocol deviations.
All significant protocol deviations will be recorded and reported in the Case Report Form (CRF).

11.3 Study Amendments

If it is necessary for the study protocol to be amended, the amendment and/or a new version of the study protocol (Amended Protocol) must be notified to the Ethics Committee according to EU and national regulations.

11.4 Audit and Inspections

Authorized representatives of the Ethics Committee may visit the centre to perform inspections, including source data verification. Likewise the representatives from sponsor may visit the center to perform an audit. The purpose of an audit or inspection is to systematically and independently examine all study-related activities and documents to determine whether these activities were conducted, and data were recorded, analyzed, and accurately reported according to the protocol, Good Clinical Practice (ICH GCP), and any applicable regulatory requirements. The principal investigator will ensure that the inspectors and auditors will be provided with access to source data/documents.

12 ETHICAL AND REGULATORY REQUIREMENTS

The study will be conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with ICH/Good Clinical Practice and applicable regulatory requirements. Registration of patient data will be carried out in accordance with national personal data laws.

12.1 Ethics Committee Approval

REK has approved the study (REK 428878). The investigator is responsible for informing the ethics committee of any serious and unexpected adverse events and/or major amendments to the protocol as per national requirements.

12.2 Other Regulatory Approvals

Statens legemiddelverket has deemed that the project is not a clinical trial due to the fact that NR is not classified as a drug, but as a nutritional supplement.

12.3 Informed Consent Procedure

All subjects will be presented verbally and with written informed consent to be signed prior to enrollment to the study. The informed consent will be presented at the first screening. Informed consent will be handled according to GCP principles. A copy of the informed consent will be given to the subject.

12.4 Subject Identification

Upon entry in screening, each subject is given a patient study number, this study number is used for the remaineder of the study. The Patient study number is in the format XX-YYY where XX is the study site number and YYY is the patient ID number.

The investigator is responsible for keeping a list of all patients (who have received study treatment or undergone any study specific procedure) including patient's date of birth and personal number, full names and last known addresses.

The patients will be identified in the CRFs by the patient study number and initials.

13 TRIAL SPONSORSHIP AND FINANCING

Research Council of Norway (RCN), Neuro-SysMed, (ES633272) 2020-2028

14 TRIAL INSURANCE

The Patients are insured by the government through the "Norsk Pasientskadeerstatning" (NPE).

15 PUBLICATION POLICY

Upon study completion and finalization of the study report the results of this study will either be submitted for publication and/or posted in a publicly accessible database of clinical study results.

The results of this study will also be submitted to the Ethics Committee according to EU and national regulations.

All personnel who have contributed significantly with the planning and performance of the study (Vancouver convention 1988) may be included in the list of authors.

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17 LIST OF APPENDICES

A. Lab Manual
B. Imaging Manual

APPENDIX A

LABORATORY MANUAL FOR N-DOSE AD

Helse Bergen, Haukeland University Hospital

Protocol:
N-DOSE AD

REK Number: 428878

N-DOSE SAFE: A randomized, double blind, dose optimization trial of nicotinamide riboside in Alzheimer's disease.

STANDARD OPERATING PROCEDURE (SOP) FOR SAMPLING AND PREPARATION OF BIOLOGICAL MATERIAL (LAB MANUAL)

Project Management Committee for the study

Kristoffer Haugarvoll

Ragnhild Eide Skogseth

Charalampos Tzoulis

1 GENERAL INFORMATION

The purpose of this document is to give an overview of the biological material collected for biobanking in the N-DOSE AD clinical trial. At different time points during the clinical trial the following biological samples are collected, processed and stored based on standard operating procedures: whole blood, serum, plasma, PBMCs, blood cells. The samples are stored at Biobank Haukeland and at the Neurology Department. Some of the biological material is collected for specific analysis. Samples in the general biobank may be distributed after approval of the application to the Project Management.

In the following chapters you will find a detailed description on how to collect, prepare, and store samples at HDS and HUS. It is critically important that the samples are prepared correctly and standardized, and that all deviations from the protocol are documented. This information will be registered in LabVantage when the Biobank Information system is in operation and at the Geriatrics and Neurology Department.

2 HEMATOLOGY, BIOCHEMISTRY, HORMONE, SEROLOGY AND SAFETY LABORATORY PARAMETERS

This chapter describes the biological material that should be collected for hematology, biochemistry, hormone and serology analyses and for serum hCG pregnancy test. The collection should be done as specified in the Flow Chart. The local study site laboratory will be used for the analyses of these components, as indicated in the Study Protocol. Protocols for collection of biological material for research and biobanking are described in Chapter 3 and 4.

Flow chart					**TUBES**
Visits	**V0 / Baseline**	**V2**	**V3**	**V4**	
Week	**0**	**4**	**8**	**12**	
Routine:					
hCG[1]	X				
CRP	X	X	X	X	
ALAT	X	X	X	X	
ASAT	X	X	X	X	
GT	X	X	X	X	
Bilirubin	X	X	X	X	
ALP	X	X	X	X	
Creatinine	X	X	X	X	
Urea	X	X	X	X	
RBC	X	X	X	X	
Hb	X	X	X	X	
WBC with differential	X	X	X	X	
Platelets	X	X	X	X	
CK	X	X	X	X	
FT4	X	X	X	X	
TSH	X	X	X	X	
B12	X	X	X	X	
Folic acid	X	X	X	X	
homocystein	X	X	X	X	
Methylmalonic acid	X	X	X	X	
Cobalamin	X	X	X	X	
Sodium	X	X	X	X	
Potassium	X	X	X	X	

Flow chart					**TUBES**
Visits	**V0 / Baseline**	**V2**	**V3**	**V4**	
Week	**0**	**4**	**8**	**12**	
Glucose	X	X	X	X	
Biobank:					
EDTA whole blood	X				FluidX, 0,7ml, 8 aliquotes
Snap-frozen whole blood	X	X	X	X	
PAXgene blood for RNA	X	X	X	X	PAXgene tubes
Platelet isolation and cryopreservation	X	X	X	X	VACUETTE® TUBE 9 ml ACD-A
Serum	X	X	X	X	FluidX, 0,7ml, 8 aliquotes
Fecal sample	X			X	
Urine sample	X	X	X	X	
Cerebrospinal fluid	X			X	

2.1.1 Safety laboratory (blood)

All safety laboratory parameters will be collected at the timepoints as indicated in the Flow Chart (page 4), and include hematology, liver enzymes/parameters, clinical chemistry, thyroid status. All safety parameters evaluated during the study are listed below. The samples will be analysed at the local laboratory at each study site. The respective reference ranges must be provided to the central study administration for uploading into the eCRF.

Hematology	
● Hemoglobin ● WBC/leukocytes	● Platelet count / thrombocytes ● Differentials: neutrophils, eosinophils, basophils, monocytes, lymphocytes
Liver enzymes/parameters	
● ALAT (alanine transaminase, SGPT) ● ASAT (aspartate transaminase, SGOT)	● Alkaline phosphatase ● GT (glutamyl transferase) ● Bilirubin total, fractionated if increased
Clinical chemistry	
● Creatinine ● CRP	● Potassium ● Sodium
Thyroid status	
● TSH (thyroid stimulating hormone) ● Free T4	

3 SAMPLING FOR BIOBANK AND RESEARCH

3.1Mandatory research and biobank samples

Chapter 3.2 gives an overview of all mandatory samples that should be collected for research and biobanking.The sample processing protocols are in Chapter 4.

3.1.1 Whole blood EDTA

EDTA blood will be collected at HDS

3.1.2 Whole blood EDTA – snap-frozen

Snap-frozen EDTA blood will be collected at HDS

3.1.3 RNA PAXgene tubes

Blood in PAXgene tubes for RNA extraction will be collected at HDS

3.1.4 Platelet isolation and cryopreservation

Platelet isolation and cryopreservation will be conducted at HUS

3.1.5 Whole blood for Serum

Serum will be collected at HDS

3.1.6 Cerebrospinal fluid (CSF)

3.1.7 Fecal sample biobanking

3.1.8 Urine sample biobanking

4 SAMPLE PROCESSING AND STORAGE

4.1 Whole blood (EDTA): standard

Performed at: ALL CENTERS

Material and instrumentation:

Item	How many	Supplier	Cat no	Comment
VACUETTE® TUBE 2 ml K2E K2EDTA	2	Greiner Bio-One International	454024	
0.7 ml FluidX tubes	8	Pedro Consulting	68-0702-11N	
Pipettes & tips				
Barcode reader				
-80°C freezer		Study center		

Collection, preparation and storage:

1. Collect whole blood into Vacuette tubes for EDTA blood
2. Aliquot 500 µL blood into 8 x 0.7 ml FluidX tubes.
3. Freeze the aliquots at -80°C within 60 min after sample collection.
4. Use the barcode reader for registration of the sample and log all deviation on the same registration form.
5. Sample aliquotes are stored at -80C and shipped on dry ice in the original FluidX boxes (x 48 tubes).
6. Samples must **never** be allowed to warm or thaw except from when to be used in analyses. Any deviation from this must be **registered**. Number and date of thawing an aliquote for analyses must be registered.

Contact person for biosampling and laboratory preparation:
Cesilie Dahll, HDS / Siri Hinteregger, MBF

Contact person for Biobank Haukeland:
Hilde Kristin Garberg

4.2 Whole blood (EDTA): snap-frozen

Performed at: ALL CENTERS

Snap-frozen whole blood will be used for the analysis of NAD+. NAD degrades very rapidly after sample collection and the concern is that measurements in any sample that requires extensive processing may not prove reliable.

Material and instrumentation:

Item	How many	Supplier	Cat no	Comment
VACUETTE® TUBE 2 ml K2E K2EDTA	1	Greiner Bio-One International	454024	
Screw cap micro tubes, 0.5 ml, sterile	8	Sarstedt	72.730.006	
Multi dispenser pipette & tips				
Liquid nitrogen in a thermos				
-80°C freezer		Study center		
Timer				

NB! The time from blood drawing (i.e. moment the blood starts flowing into the tube) and freezing MUST be 2 min!

Collection, preparation and storage:

1. Print labels. For one patient: 8x
2. Mark 8 x 0.5 ml micro tubes with labels
3. Laboratory technician must be next to the patient at the blood drawing with all equipment ready.
4. Laboratory technician must be wearing gloves and lab coat.
5. Collect whole blood into the Vacuette tube.
6. **START THE TIMER when blood starts flowing into the tube.**
7. Gently invert the EDTA tube at least 10 times. **DO NOT SHAKE**
8. Aliquot 8 x 200 µl of blood from the 2 ml EDTA tube into 8 x 0.5 ml micro tubes (easiest using a multi dispenser pipette).
9. Close micro tubes
10. **When the timer shows 2min:** immerse all the micro tubes simultaneously in liquid nitrogen.
11. Transfer the frozen EDTA-aliquots -80^{o}C freezer for storage.
12. Sample aliquots are stored at -80C in freezer-compatible cardboard or plastic storage boxes.
13. Samples are shipped on dry ice.
14. Samples must **never** be allowed to warm or thaw except from when to be used in analyses. Any deviation from this must be **registered**. Number and date of thawing an aliquot for analyses must be registered.

If the 2 min interval is not respected – still collect the samples but register the time interval.

Responsible:

Hanne Linda Nakkestad

4.3 RNA PAXgene tubes

Performed at: ALL CENTERS

Rationale:
The Paxgene Blood RNA collection system is intended for the purification of intracellular RNA from whole blood and is optimized for the stabilization of 4.8 x10e6 – 1.1 x 10e7 leukocytes/ml. This protocol describes the collection of whole blood in Paxgene RNA tubes from Qiagen for longterm storage at -80° C.

Important: Paxgene collection tubes **must** be at room temperature prior to collection. Follow standard procedure for venipuncture for tubes with stabilizing agents e.g. butterfly collection

Material and instrumentation:

- PAXgene blood RNA tubes (PreAnalytix, Cat. No. 762165)
- BD Vacutainer SafetyLok Blood collection set (BD, Cat. No. 367281) or similar Butterfly and safety lock or similar
- Sample labels capable of storage at -80 °C
- Phlebotomy materials: Tourniquet, alcohol swabs, gauze
- Barcode reader
- -80°C freezer

Collection, preparation, storage and shippment:

Before starting, ensure PAXgene tubes are at ambient temperature and labeled appropriately with temperature resistant labels. Draw PAXgene tubes last, after other blood tubes. If the PAXgene tubes are the only tubes, draw a small amount of blood into a discard tube.

1. Using the Blood Collection set, collect blood into the PAXgene tube using standard venipuncture techniques. Ensure that the donor's arm is in a downward position, and that the PAXgene tube is **held vertically** below the donor's arm.
2. Collect 2.5 ml of blood into each PAXgene tube. so that the tube gets filled with **exactly** 2.5 ml blood. This is essential so that the final concentration of the reagents will be correct. If PAXgene is the first sample to be taken, collect some blood in another tube first, to get rid of the air in the collection system, so that the PAXgene tube gets exactly 2.5 ml blood.
3. Allow at least 10 s for the blood draw to occur and ensure that blood has stopped flowing into the tube before removing the needle from the tube.
4. Immediately after blood collection mix the tube by gentle inversion (180°) 10 times
5. Temporartily store the PAXgene upright at RT for between **2-24 hrs**.
6. Temporarily store the PAXGene tube for at least **24 hrs** at -20°C
7. After at least 24 hrs at -20°C, move the PAXgene tube for **long-term storage at -80 °C**
8. Sample aliquotes are stored at -80C in freezer-compatible cardboard or plastic storage boxes.
9. Samples are shipped on dry ice.
10. Samples must **never** be allowed to warm or thaw except from when to be used in analyses. Any deviation from this must be **registered**.

Responsible:

Contact person for biosampling and laboratory preparation: Cesilie Dahll, HDS / Siri Hinteregger, MBF
Contact person for Biobank: Hilde Kristin Garberg

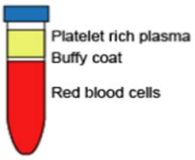
4.4 Platelet isolation and cryopreservation

Performed at: ALL CENTERS

Material and instrumentation

Item	How many	Supplier	Cat no	Comment
VACUETTE® TUBE 9 ml ACD-A	1	Greiner Bio-One	456055	
10 ml Falcon centrifugation tubes				
DMSO				
Pipettes & tips				
-80°C freezer		Study center		

Collection, preparation and storage:



1. Collect whole blood in vacutainer ACD tubes (yellow cap) 9ml?
2. Mix gently by slowly inverting the tube
3. Spin at room temperature at 200g for 20 min, no brake
4. After the spin, three distinct layers can be observed:
    a. the top: straw-colored layer contains platelets
5. Transfer about two thirds of the **top layer** into a new 10 ml Falcon tube
6. Freeze the cells in their own plasma adding DMSO at a final concentration of 5-6%
7. Store at -80

Responsible:
Contact person for biosampling and laboratory preparation: Cesilie Dahll, HDS / Siri Hinteregger, MBF
Contact person for Biobank: Hilde Kristin Garberg

4.5 Serum for biobanking

Performed at: ALL CENTERS

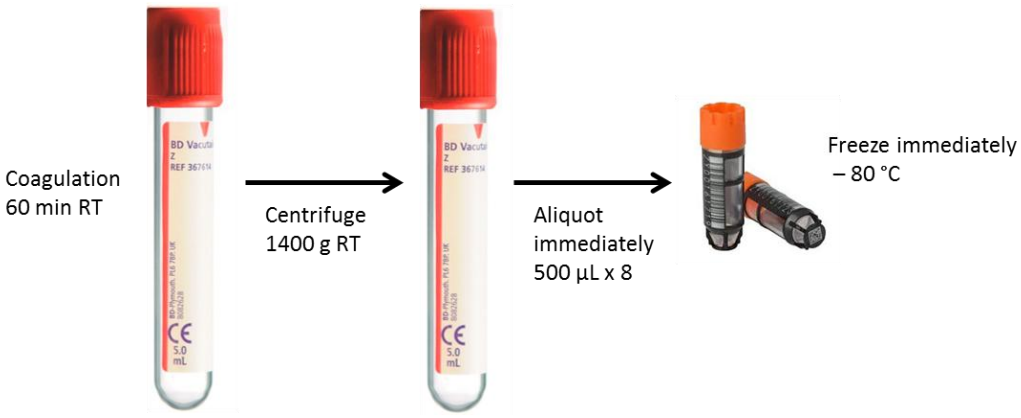
It is important to let the blood coagulate in an upright position at RT for 60 minutes. Centrifuge the sample and immediately aliquot and freeze the aliquots within 90 minutes after sample collection. Register the samples according to the local protocol and log all deviation on the same registration form.

Material and instrumentation:

- 3 x 5 ml BD VACUTAINER serum glass tube with no additive (367614)
- 8 x 0.7 ml FluidX (68-0702-11)
- Centrifuge for vacutainer tubes
- Barcode reader
- -80°C freezer

Collection, preparation and storage:

7. Collect whole blood into BD VACUTAINER glass tubes containing no additive.
8. Incubate in an upright position at room temperature for 60 min (no longer than 60 min) to allow clotting.
9. Centrifuge for 12 min at 1400 x g at room temperature.
10. Inspect serum for turbidity. Turbid samples should be centrifuged again to remove remaining insoluble matter. A new centrifugation must be indicated as a deviation.
11. Aliquot 500 µL serum into 8 x 0.7 ml FluidX tubes.
12. Freeze the aliquots at -80°C within 90 min after sample collection.
13. Use the barcode reader for registration of the sample. Register the samples according to the local protocol and log all deviation on the same registration form.
14. Sample aliquotes are stored at -80C and shipped on dry ice in the original FluidX boxes (x 48 tubes).
15. Samples must **never** be allowed to warm or thaw except from when to be used in analyses. Any deviation from this must be **registered**. Number and date of thawing an aliquot for analyses must be registered.



Responsible:

Contact person for biosampling and laboratory preparation: Cesilie Dahll, HDS / Siri Hinteregger, MBF
Contact person for Biobank: Hilde Kristin Garberg

4.6 Collection, of cerebrospinal fluid (CSF)

Performed at: ALL CENTERS

Material and instrumentation:

Item	How many	Supplier	Cat no	Comment
Standard lumbar puncture equipment		Study center		

5 ml PolyPropylene (PP) cryo-s tube with red PP cap	2	Greiner Bio-One via VWR	479-4154	
0.7 ml FluidX tubes (PP)	15		68-0702-11N	
5 ml PP tube for abeta-42, t-tau and p-tau181	1	Sarstedt	63-504-027	
Barcode reader				
Centrifuge for centrifugation tubes		Study center		
PP PIPETTE TIPS (ART 1000ML REACH)		VWR	732-2215	
Pipettes		Study center		
-80°C freezer		Study center		

4.6.1 Collection of cerebrospinal fluid

Absolute contraindications for the procedure:

- Thrombocytes less than 40 x 109/L
- Use of anticoagulation or antiplatelet drugs (except ASA)
- INR >1.7 (warfarin users)
- Local infection at the biopsy site
- Any other center-specific clinical routines and/or guidelines MUST be followed

During lumbar puncture the following procedures should take place:

1. If noticeably bloody tap, discard the first 1-2 ml until CSF is clear
2. 10 drops spinal fluid collected in sterile tube for cell count (erythrocytes and leukocytes)
3. A total of 10 ml is collected, e.g 5 ml x 2 is collected in 5 ml sterile PolyPropylene tubes (479-4154) for biobanking (see materials and preparation below)
4. 2 ml is collected in 5 ml PP tube (63-504-027) for clinical analysis of abeta-42, t-tau and p-tau181.
5. 10 drops spinal fluid collected in sterile tube for cell count (erythrocytes and leukocytes)

NB! Do not use pressure measurements tubing – collect CSF DIRECTLY into the tubes

NB! Only polypropylene (PP) pipette tips should be used to collect and handle the CSF!

4.6.2 Processing and storage of cerebrospinal fluid

NB! The sample has to be centrifuged within 1 hour from collection!

1. The collected 5ml x 2 CSF in "5 ml PolyPropylene (PP) cryo-s tube with red PP cap"

a. Centrifuge within 1 hour at 2,000 g, at room temperature, for 10 min

b. Transfer the supernatant to 2 x new "5 ml PolyPropylene (PP) cryo-s tube with red PP cap" – use PP pipette tips

c. Mix carefully by inverting the tube x 7-10 times (or mild vortexing)
d. Aliquot 500 µl CSF per tube into 0.7 ml FluidX tubes - use PP pipette tips
e. Freeze the aliquots at -80°C.
f. Register the samples according to the local protocol and log all deviation on the same registration form
g. The collected 2 ml CSF in 5 ml PP tube for abeta-42, t-tau and p-tau181 will be sent to Akershus University Hospital according to their specifications.

Responsible:
Cesilie Dahll, HDS / Hanne Linda Nakkestad, HUS

4.7 Fecal sample biobanking

Performed at: ALL CENTERS

Fecal samples from the last 24h will be collected according to standard clinical routines and stored at -80C.

4.8 Urine for biobanking

Performed at: ALL CENTERS

Morning urine will be collected according to standard clinical routines, aliquoted to 2.0 ml FluidX (65-9001) (total 8 tubes), frozen and stored at -80C.

5 CONTACT PERSONS

The following chapter summarizes the contact persons and responsible investigators for the sub-studies:

5.1Helse Bergen

5.1.1 Biobank Haukeland

Contact persons regarding biobanking, sample shipment, sample registration

Ann Cathrine Kroksveen	55971970 / 92031413, ann.cathrine.kroksveen@helse-bergen.no
Hilde Garberg	55975854 / 55973149, hilde.kristin.garberg@helse-bergen.no

5.1.2 Laboratory Clinic

Contact person regarding safety laboratory, serology and hormone analyses

Siri Hinteregger	55973124, siri.hinteregger@helse-bergen.no

5.1.3 Haraldsplass (HDS) Laboratory

Cesilie Dahll	55979291/947 83 350, cesilie.dahll@haraldsplass.no

5.1.4 Department of Neurology

Contact person for cells, CSF collection

Kristoffer Haugarvoll	55975045 / 98266741, kristoffer.haugarvoll@helse-bergen.no
Charalampos Tzoulis	55975045 / 94392305, chtzoulis@gmail.com
Hanne Linda Nakkestad	97571149, hanne.linda.nakkestad@helse-bergen.no

5.2 HDS

Ragnhild Eide Skogseth	99298171, ragnhild.eide.skogseth@haraldsplass.no

APPENDIX B

MRI and FDG-PET – PROTOCOL, N-DOSE AD

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Contact persons:

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Equipment :

Scanner:	Siemens Biograph mMR 3T (PET/MR)
Software:	E11P
Coils:	RAPID Biomedical 31P – H_Head Coil 3T Human head V-XQ-HQ-030-01921 V01 for SIEMENS Biograph mMR 3T

To PACS:	Clinical protocol
To fPACS:	All sequences (entire examination)

Protocol:

Participant preparation:

The participant should fast a minimum of 6 hours before injection of 18F-FDG. A peripheral venous catheter (PVC) is then placed in the medial cubital vein. Blood glucose is measured. The required blood glucose level is below 8 mmol/L. 30 minutes before the scan 250 MBq of 18F-FDG is injected into the PVC. The participant is required to lie in a quiet, dark environment during the interval after injection and before scanning to minimize brain activity. The participants should not use mobile phones or headphones during this period. Eyes must be closed for fMRI sequences. Imaging is to be taken up to 72 hours before or after physical examination of participants.

Positioning: Localizer Auto align if possible

Scan parameters: Scan labelled by study ID.

MRI sequences:

- Localizer for planning of MR and PET – 1.0 min.
- 3D T1 (sagital, 1x1x1 mm, 3D-BRAVO eller MPRAGE) – 5.5 min.
- Attenuation correction map, generated using DeepMRAC[1] – 2.0 min.
- 31P-MRS calibration: FID, multinuclear coil, generated using xnuccalc2 from NMproc-Dockers - 2 min.
- 31P-MRS sequence: CSI, multinuclear coil – 15 min.
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Total imaging time: 25,5 min imaging time - 35 min including changing the coil for CSI.

Specific procedures for 31P-MRS image analysis:

Spectra from the occipital region will be aligned using an adaption of the Spectral Registration implementation from Gannet 3.022[2,3], subject to thresholding on SNR (>=3) to eliminate the majority of out-of-brain voxels.

Voxels will then be averaged before being processed in Matlab 9.5 (the MathWorks, Natick, MA) using the OXSA toolbox[4] utilizing first order phase correction and fitting with AMARES.

Custom prior information was created based on literature values for membrane phospholipids (MP), glycerophosphocholine (GPC), glycerophosphoethanolamine (GPE), inorganic phosphate (Pi), phosphocoline (PC), phosphoethanolamine (PE) as well as alpha-, beta- and gamma resonances of adenosine triphosphate (ATP-α, -β, and –γ, respectively) in reference to the phosphocreatine (PCr) peak[5–7].

Additional information for the properties of nicotinamide adenine dinucleotide (NAD) was added based on the framework developed by Lu and colleagues[8] by calculating field-strength dependent chemical shift differences, relative amplitudes and frequency separations for oxidized and reduced NAD (NAD^+ and NADH, respectively).

Linewidths will be fixed to be equal for NAD^+, NADH and ATP-α. At 3T, and to comply with normal-mode specific absorption rate (SAR) restrictions, peak separation for NAD^+ and NADH is likely to be limited and therefore only combined values of total NAD (NAD^+ and NADH together) will most likely be reported.

Fitted peak amplitudes and areas are used to calculate total NAD in proportion to a normalisation constant such as ATP-α or PCr. This normalised NAD-value will be used for the planned statistical analysis defined in the trial SAP.

Specific procedures for NRRP and PDRP image analysis of FDG-PET images

FDG-PET scans will be transferred electronically to the Center for Neurosciences at The Feinstein Institutes for Medical Research (Manhasset, NY, USA) and analyzed using automated computing pipelines implemented in MATLAB R2023b (MathWorks, Natick, MA). Images will first be pre-processed using Statistical Parametric Mapping (SPM12) software (http://fil.ion.ucl.ac.uk/spm; Wellcome Centre for Human Neuroimaging, London, UK). FDG-PET scans acquired at visits V1, V 2, V3 and V4 will be aligned to produce a mean image, which will be spatially normalized in standard Montreal Neurological Institute (MNI) anatomic space along with the individual scans from each time point. The normalized images will then be smoothed with a 10-mm Gaussian filter in three dimensions to enhance the signal to noise ratio.

In the previous NADPARK phase-I study[9], we identified a specific NR-related metabolic pattern (NRRP) from paired metabolic scan data from participants in the NR 1000 mg group analyzed using ordinal trends/canonical variates analysis (OrT/CVA)—a supervised form of principal component analysis (PCA)[10]. This multivariate approach was designed to detect and quantify regional covariance patterns (i.e., metabolic networks) for which expression values (i.e., subject scores) increase or decrease with treatment in all or most of the subjects[11–15]. The significance of the resulting OrT/CVA topographies, i.e., the NRRP[9], was assessed using nonparametric tests, i.e., permutation testing of the

subject scores to show that the observed ordinal trend does not occur by chance. Likewise, the reliability of the voxel loadings (i.e., region weights) on the resulting NRRP network topography was assessed using bootstrap resampling procedures[12,16].

For this study protocol, pre-processed FDG-PET scans of individual subjects will be used to compute the expression values (subject scores) of NRRP at baseline and the follow-up timepoints, using the GCVA PCA software (available at https://www.nitrc.org/projects/gcva_pca) for Ordinal Trend (OrT/CVA) Analysis[10]. NRRP subject scores will be standardized (*z*-scored) to computed expression values for this pattern in an age-matched group of healthy volunteers scanned at the Feinstein Institutes.

FDG-PET scans will also be used to compute the expression values of the AD-related metabolic pattern (termed ADRP) which was identified and validated previously in several independent patient populations. Subject scores for PDRP and ADRP of individual subjects will be computed automatically on a single-scan basis at each study visit and in a blinded fashion, using in-house Scan Analysis and Visualization (ScAnVP) software (available at http://feinsteinneuroscience.org). The subject scores of each pattern will be standardized (*z*-scored) in reference to corresponding scores of age-matched healthy controls.

Outcome measures of the NRRP and ADRP z-scores for individual subjects at each study visit will be reported in tabular format and transferred electronically to the Norway group for the planned statistical analysis defined in the trial SAP.

References

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