STUDY PROTOCOL SYNOPSIS

The impact of N-PEP-12 dietary supplementation on attentional performance, cognition, and mental wellbeing of middle-aged and older healthy adults with subjective cognitive function complaints

Study Code	N-HANCE
Protocol Number	FSNANO08032022
Version	2.0 – amendment 1
Date	March 20, 2023
Coordinator	Foundation for the Study of Nanoneurosciences and Neuroregeneration (FSNN)

This protocol has been written in accordance with the ICH-GCP guidelines and the *Declaration of Helsinki* in current versions.

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1. PROTOCOL SUMMARY / SYNOPSIS

Coordinator	FSNN – Foundation for the Study of Nanoneurosciences and Neuroregeneration
Title	The impact of N-PEP-12 dietary supplementation on attentional performance, cognition, and mental wellbeing of middle-aged and older healthy adults with subjective cognitive function complaints
Study Code	N-HANCE
Study Locations	Single-center
Dietary Supplement	MemoProve/Cebrium
Name of Active Substance	N-PEP-12
Indication	Middle and older age healthy adults
Study Design	Randomized, placebo-controlled, double-blind
Study Duration	Study start: 10/2022 Study end: 06/2024
Sample Size Calculation	• K=**2** experimental treatments will be included in the trial. • A significance level of α=**0.05** will be used, in combination with **Dunnett's correction**. • The standard deviations of the responses will be assumed to be: $(\sigma 0,\ldots,\sigma K)$=**(1,1,1)**. • The **minimum marginal power** will be controlled to level $1-\beta$=**0.9** under **each of their respective least favourable configurations**. • The interesting and uninteresting treatment effects will be: δ_1=**0.5** and δ_0=**0** respectively. • The target allocation to each of the experimental arms will be: **the same as the control arm**. • Therefore, the realised allocation ratios to the experimental arms are: $(r_1,\ldots,r_K)$=**(1,1)**. • The maximum familywise error-rate is: **0.05**. • The **minimum marginal power** is: **0.9**. • The following critical threshold should be used with the chosen multiple comparison correction: **0.028**. • 10% adjustment for dropouts The total required sample size is: N=**270**. The required sample size in each arm is: $(n_0,\ldots,n_K)$=**(90,90,90)**. Source: Grayling MJ, Wason JMS (2020) A web application for the design of multi-arm clinical trials. *BMC Cancer* **20:**80. DOI: 10.1186/s12885-020-6525-0
Randomization	1:1:1 ratio, 3 blocks, ICH E9

CRF	Electronic, open-source, self-developed, ICH E6
Primary Objectives	To assess the efficacy of N-PEP-12 on the Test of Attentional Performance outcomes in healthy middle-aged and older adults at 90 days after baseline.
Secondary Objectives	To assess the efficacy of N-PEP-12 on individual outcomes (cognitive function, functional outcome, mental wellbeing, mood, and quality of life) in healthy middle-aged and older adults at 30, 90 and 180 days after baseline. To assess the efficacy of N-PEP-12 on the Test of Attentional Performance outcomes in healthy middle-aged and older adults at 30 and 180 days after baseline.
Screening Variables	• Medical exam • Demographic variables
Primary Variables	Test of Attentional Performance (TAP) Subtests: • Alertness (phasic alertness) • Working Memory (difficulty level 3) • Divided Attention
Secondary Variables	• Wechsler Adult Intelligence Scale (WAIS-IV) - Digit Span Forward, Backward (DSF, DSB) • Perceived Stress Scale (PSS) • Brief Mood Introspection Scale (BMIS) • Sleep Quality Scale (SQS) • EQ-5D-5L
Inclusion Criteria	• Sign the informed consent form and fully understand the test content, process and possible adverse reactions, and be able to complete the study according to the test plan requirements • Aged ≥50 years or ≤75 years, male or female (including the boundary value) • Male body weight≥50kg, female body weight≥45kg, and 18.0≤BMI≤29.9 kg/m2 • No clinically significant cognitive impairment (MoCA > 25) • Self-reported subjective cognitive complaints • Literacy to complete tests
Exclusion Criteria	• History of drug abuse or alcohol abuse (drank more than 14 units / week of alcohol: 1 unit =285ml beer, 25ml spirits or 100ml Wine) • Gastric sleeve patients • History of drug abuse within 5 years prior to screening, or urine drug screening test was positive • Other clinically major diseases, such as decompensated, in the investigator's judgement (such as neuropsychiatric system, cardiovascular system, urinary system, digestive system, respiratory system, metabolic endocrine system, blood system, skin diseases, immune diseases, tumors, etc.) • Participation in another clinical trial within 3 months prior to enrollment • Pre-existing and active major neurological disease • Injury of writing hand influencing cognitive or other outcome measures, in the investigator's judgment.
Visit Schedule	**Visit 1 - Screening** **Study day 0** • Demographic data • Medical history and risk factors • ICF • Randomization • Primary and secondary outcomes **Visit 2 - Efficacy Evaluation** **Study day 30** • Primary Outcomes • Secondary Outcome **Visit 3 - Efficacy Evaluation** **Study Day 90** • Primary Outcomes • Secondary Outcome

	Visit 4 - Efficacy Evaluation **Study Day 180** ● Primary Outcomes ● Secondary Outcome
Active dietary supplementation arms	N-Pep-12 (45 mg), daily (Day 0-Day 180) N-Pep-12 (90 mg), daily (Day 0-Day 180)
Reference Product	Placebo – Lactose (Day 0-Day 90)

*All treatment cycles and efficacy evaluations will be performed within a window of ±3 working days

2. INTRODUCTION

2.1 Background Information and Study Rationale

The global older population is growing, particularly in developed countries. The number of people aged 60 and up is expected to reach two billion by 2050, up from 900 million in 2015 (He, Goodkind, & Kowal, 2016). Normal mental performance is a vital aspect of quality of life and wellbeing. With greater longevity and shifting demographics, it is more important than ever to understand the characteristics that enable healthy aging (Simpson et al., 2014) and find solutions that can enhance the cognitive processes and overall mental wellbeing that come with the natural process of aging.

The basic mechanisms involved in attention, perception, memory, and learning are referred to as cognitive functions (Eysenck, 2018)**.** The majority of cross-sectional studies on healthy individuals suggest that attention, working memory, and information processing speed deteriorate gradually between the ages of 20 and 60 (Af, L, Sj, W, & Wt, 2004; Craik & Byrd, 1982; Salthouse, 2009)**.** Moreover, lower cognitive ability and greater decline in cognitive ability predict mortality in older people, even when other biomedical risk factors are controlled for (Batterham, Mackinnon, & Christensen, 2012; Connors, Sachdev, & Kochan, 2015)**.**

N-PEP-12 is a peptide-based nutritional supplement that has been proven in experimental studies and early clinical studies to have neuroprotective and pro-cognitive effects in patients with age-related cognitive impairments (Crook, Ferris, Alvarez, Laredo, & Moessler, 2005; Hutter-Paier, Reininger-Gutmann, Wronski, Doppler, & Moessler, 2015). After only 30 days of a once-daily intervention with 90 mg of N-PEP-12, it was found to considerably improve memory impairment in older persons with subjective memory complaints (Crook et al., 2005). Memoprove and Cebrium are two brands of the chemical that are available in film-coated pills or capsules.

Previous research has found that the peptides in N-PEP-12 exhibit qualities similar to those seen in naturally occurring peptide growth factors, such as increasing neurite outgrowth, improving neuronal survival, and protecting against metabolic stress (Windisch, Hutter-Paier, Grygar, Doppler, & Moessler, 2005). This study's rationale is based on these previously documented properties that have the potential to impact the attentional performance, cognition, and mental wellbeing of middle-aged and older healthy adults with subjective cognitive function complaints.

3. STUDY OBJECTIVES

This study shall assess the impact of N-PEP-12 dietary supplementation on attentional performance, cognition, and mental wellbeing of middle-aged and older healthy adults with subjective cognitive function complaints.

3.1 Primary Objectives

To assess the efficacy of N-PEP-12 on the Test of Attentional Performance outcomes in healthy middle-aged and older adults at 90 days after baseline.

3.1.1 Primary Variables

Test of Attentional Performance (TAP) subtests:

- Alertness
- Working Memory
- Divided Attention

3.2 Secondary Objectives

To assess the efficacy of N-PEP-12 on individual outcomes (cognitive function, functional outcome, mental wellbeing, mood, and quality of life) in healthy middle-aged adults at 30, 90 and 180 days after baseline.

To assess the efficacy of N-PEP-12 on the Test of Attentional Performance outcomes in healthy middle-aged and older adults at 30 and 180 days after baseline.

3.2.1. Secondary Variables

- Wechsler Adult Intelligence Scale (WAIS-IV) - Digit Span Forward, Backward (DSF, DSB)
- Perceived Stress Scale (PSS)
- Brief Mood Introspection Scale (BMIS)
- Sleep Quality Scale (SQS)
- EQ-5D-5L

4. STUDY DESIGN

Randomized, placebo-controlled, double-blind

N-PEP-12 groups shall be tested against Placebo. The 3 groups are:

Study Group 1: 45mg N-PEP-12

Study Group 2: 90mg N-PEP-12

Study Group 3: Placebo (Lactose)

The trial will be conducted in a sample of healthy middle-aged and older adults, with subjective cognitive function complaints. The study extends over an observation period of 180 days. Four visits for clinical evaluation are planned as follows:

Visit 1 - (Study day 0) - Screening - will consist of the baseline assessment - demographic data and the patient's medical history and risk factors will be collected. Also, the informed consent will be signed and the patient will be randomized. **Visit 2 - (Study day 30)** will be an efficacy evaluation. Between Visit 1, Visit 2 and **Visit 3 - (Study day 90)** patients shall receive their treatment, based on the group they have been allocated at baseline: Placebo, 45mg N-PEP-12 or 90 mg N-PEP-12. Between Visit 3 and **Visit 4 - (Study day 180)** the patients shall continue the treatment with the two N-PEP-12 groups unchanged and the initial Placebo group receiving 90 mg N-PEP-12.

All treatment cycles and efficacy evaluations will be performed within a window of ±3 working days.

5. SELECTION AND WITHDRAWAL OF PATIENTS

5.1 Patient Inclusion Criteria

- Sign the informed consent form and fully understand the test content, process and possible adverse reactions, and be able to complete the study according to the test plan requirements
- Aged ≥50 years or ≤75 years, male or female (including the boundary value)
- Male body weight≥50kg, female body weight≥45kg and 18.0≤BMI≤29.9 kg/m2
- No clinically significant cognitive impairment (MoCA > 25)
- Self-reported subjective cognitive complaints
- Literacy to complete tests

5.2 Patient Exclusion Criteria

- History of drug abuse or alcohol abuse (drank more than 14 units/week of alcohol: 1 unit =285ml beer, 25ml spirits or 100ml Wine)
- Gastric sleeve patients
- History of drug abuse within 5 years prior to screening, or urine drug screening test was positive
- Other clinically major diseases, such as decompensated, in the investigator's judgement (such as neuropsychiatric system, cardiovascular system, urinary system, digestive system, respiratory system, metabolic endocrine system, blood system, skin diseases, immune diseases, tumors, etc.)
- Participation in another clinical trial within 3 months prior to enrollment
- Pre-existing and active major neurological disease
- Injury of writing hand influencing cognitive or other outcome measures, in the investigator's judgment.

5.3 Stopping and Discontinuation Criteria

5.3.1 Discontinuation Criteria related to the Study

- Insufficient recruitment
- Continuous serious protocol violation and deviation

5.3.2 Discontinuation Criteria related to the Patient

Patients will be informed in the Informed Consent Forms that they have the right to withdraw from the study at any time without prejudice, and that they may do so at the discretion of the Investigator / Coordinator. The withdrawal/study termination page in the CRF must be completed whenever a patient drops out or is pulled from the study. In the withdrawal section, the Investigator should record the date of the withdrawal, the person who initiated the withdrawal, and the reason for the withdrawal. To finish evaluations and collect any outstanding data and research materials, reasonable efforts should be taken to reach any patient who went missing during the study for follow-up.

Withdrawn by the Investigator due to:

- Serious Adverse Drug Reaction
- Lack of efficacy
- Consent withdrew
- Administrative reasons

The patient or his/her representative requested withdrawal due to:

- An Adverse Event for which the Investigator did not consider removal from the study
- Perceived insufficient therapeutic effect.
- Withdrawal of consent for any other reason (data recorded until withdrawal will be kept in the database if not explicitly denied by the patient)

5.4 Randomisation, Blinding and Unblinding

This study will be conducted under double-blind conditions in order to keep investigators, other study personnel, and patients unaware of treatment allocation. A unique randomization number will be assigned to patients who fulfill the inclusion and exclusion criteria (patient number). This number is the next available randomization number in ascending order from 001 to, for example, 999 of a predefined randomization plan, and it identifies the treatment assigned to a unique patient in a double-blind manner, from a random list generated in advance by a biometrician chosen by the coordinator. Patients will be randomly allocated the study groups in a 1:1:1 ratio.

A sealed random code list and sets of sealed envelopes are prepared. Based on the random list sealed, opaque randomization/emergency envelops will be provided as follows:

- To the study center to break blinding if reasonable suspicion of harm to the patient exists
- To the person assigned to prepare the ready-to-use-infusion
- To the study coordinator

When the randomization envelopes are opened, the person who opened them will date (day, hour) and sign them. Any premature unblinding of the Investigational Product should be noted as soon as possible and explained to the Coordinator. The complete study will be unblinded after the database is closed and the analysis populations are identified.

6. INVESTIGATIONAL PRODUCTS

The Investigational Products will be made available by the study coordinator (FSNN).

6.1 Name and Description of the Investigational Product

N-Pep-12 (Cebrium/MemoProve)

6.2 Dosage, Formulation and Administration

Active Treatment - N-Pep-12: 45 mg / 90mg
Placebo: Lactose

6.3 Packaging and Labelling

The investigational product and the reference product will be packaged and labeled for use in clinical trials according to GMP Annex 13 and local legislation.

6.4 Storage

The study medication will be stored at room temperature in a dry place. All supplies must be kept in a locked place, inaccessible to unauthorized persons until they are delivered to the individual patient.

6.5 Investigational Product Accountability and Destruction

The amount of used medication will be recorded in the CRF. All unused medication will be counted and documented and unused investigational products will be destroyed upon completion of accountability.

7. CONCOMITANT MEDICATION

All previous and concomitant medication must be included in the CRF, including the starting date, stop date (if applicable), highest total daily dose, and method of administration. Moreover, the reason (diagnosis) for receiving a specific concomitant treatment must be documented. Finally, if a concomitant drug is administered as a preventive treatment, it must be noted on the CRF page for that medication.

8. DEFINITION OF THE PRIMARY AND SECONDARY VARIABLES

8.1 Primary Variables

8.1.1 Test of Attentional Performance (TAP)

The Test of Attentional Performance (TAP) is a software package that may be used to test attention skills in adults and children, as well as aspects of visual perception. The test battery comprises of a number of different tests that allow for a more detailed examination of the various aspects of attention. Norm values are provided for the interpretation of the results, taking into account age, gender, and education. The design of these tests was primarily driven by the needs of neuropsychological diagnostics, which place unique demands on appropriate test procedures due to the sometimes high specificity of the deficits as well as the patients' frequently present numerous impairments (Zimmerman & Fimm, 2022).

8.1.1.1 Alertness Subtest

For the Alertness (phasic alertness) subtest, the reaction time is tested under two conditions. The first

condition involves simple reaction time measurements in which a cross appears on the monitor at irregular points and the subject must respond as quickly as possible by pressing a key (Zimmerman & Fimm, 2022).

8.1.1.2 Working Memory Subtest

The task investigates information flow control and the updating of information in working memory. On the monitor, the subject is shown a series of numbers. The subject must determine whether each number corresponds to the previous number or the one before that, depending on the condition (Zimmerman & Fimm, 2022).

8.1.1.3 Divided Attention Subtest

A visual and an auditory task must be completed concurrently in this test. The tasks focus on attentional selectivity, focused attention and visuo-spatial attention (Zimmerman & Fimm, 2022).

8.2 Secondary Variables

8.2.1 Wechsler Adult Intelligence Scale (WAIS-IV) - Digit Span Forward, Backward (DSF, DSB)

The Digit Span task exercises a patient's verbal working memory. Attention and comprehension also contribute to performance. The digit span task is also closely related to language learning abilities. The procedures for assessing working memory are widely accepted. A list of numbers is read aloud at a rate of one per second, and the participant is then asked to recall the numbers in the correct order. The first list starts with three numbers and grows until the person starts making mistakes. plus or minus two numbers This test can be distributed both backward and forwards. Scores are thought to correlate with age and not intelligence (Wechsler, 2008).

8.2.2 Perceived Stress Scale (PSS)

The Perceived Stress Scale (PSS) is the most commonly used psychological tool for assessing stress perception. It's a metric for how stressful certain situations in one's life are regarded. Items were chosen to reflect how unexpected, unmanageable, and overburdened respondents' lives are. A number of direct questions about current levels of experienced stress are also included on the scale. The PSS was created with at least a junior high school education in mind for use in community samples. The items are straightforward, and the response options are concise. Furthermore, the questions are of a general nature and hence lack the substance that is specific to any demographic group. The PSS questions inquire about feelings and ideas from the previous month. Respondents are asked how often they feel a certain way in each situation (Cohen, 1994).

8.2.3 Brief Mood Introspection Scale (BMIS)

The Brief Mood Introspection Scale (BMIS) is an open-source mood scale that uses 16 mood adjectives like "glad" and "fed up" to score participants' present mood state. The 16-item Brief Mood Introspection Scale (BMIS) was created as a subset of the Mood-State Introspection Scale, which has 62 items. Both subtractive and reverse scoring are used to score the BMIS. Although subtractive scoring was indicated in the original article containing the BMIS, reverse scoring is now the preferred method of scoring the test. Reverse scoring is the process of locating items on a scale that measure the polar opposite of the dominant end (for example,

"Unpleasant" Items on a Pleasant Unpleasant Mood scale) and then reversing the scale values for those opposite items (Mayer & Gaschke, 1988).

8.2.4 Sleep Quality Scale (SQS) / Subjective, patient questionnaire

The SQS is a 28-item questionnaire that assesses six different aspects of sleep quality: daytime symptoms, sleep restoration, issues initiating and maintaining sleep, trouble waking, and sleep enjoyment. The goal of the developers was to construct a scale that could be used as a broad, efficient metric for measuring sleep quality in a variety of patient and research populations. The scale has been validated in individuals aged 18–59 years and it only requires 5-10 minutes to complete, being a straightforward self-reporting, pencil-and-paper test (Shahid, Wilkinson, Marcu, & Shapiro, 2011).

8.2.5 EQ-5D-5L

The EQ-5D-5L is divided into two sections: the description system and the visual analog scale. The five dimensions of the descriptive system are mobility, self-care, typical activities, pain/discomfort, and anxiety/depression. For each dimension, there are five levels: no issues, mild issues, moderate issues, severe issues, and extreme issues. The patient is asked to check the box next to the most appropriate statement in each of the five aspects to reflect his or her health status. A one-digit number reflecting the level chosen for that dimension is the result of this selection. The digits of the five dimensions can be added together to create a five-digit number that indicates the patient's health state (Herdman et al., 2011).

9. ASSESSING AND REPORTING OF ADVERSE EVENTS

Throughout the course of the clinical study particular attention is paid to the Adverse Events and Adverse Drug Reactions mentioned below.

9.1 Adverse Events (AE)

Any adverse medical event in a patient or clinical trial subject who has received a pharmaceutical product that does not necessarily have a causal relationship with this treatment is referred to as a Serious/Adverse Event (S/AE). Any undesirable and unanticipated sign (including an abnormal laboratory finding), symptom, or disease that is temporally connected with the use of an Investigational Product, whether related or not, is referred to as an adverse event (AE).

9.2. Adverse Drug Reaction (ADR)

All unanticipated and undesirable reactions to an Investigational Product caused by any application or dose given. The phrase "responses to an Investigational Product" refers to a causal relationship that has been determined by either the Investigator or the Coordinator. Reasonable implies that there is a hint or argument that a causal relationship exists. In the case of commercialized Investigational Products, an unpleasant and unanticipated reaction to a product happens in applications that are often utilized in man for disease prevention, diagnosis, or therapy, or to affect physiological function.

9.2.1 Serious Adverse Event or Serious Adverse Reaction (SAE/SAR)

Serious Adverse Events will be considered for AE documentation due to the patient's underlying constitution. Serious Adverse Drug Reactions will be handled as outlined below.

Expedited Reporting is required if the following criteria apply (ICH E2A):

1. Serious
2. Unexpected
3. A reasonable causal relationship to study treatment.

An Adverse Drug Reaction is considered serious if it:

- Results in Death
- Is life-threatening
- Requires additional inpatient hospitalization or prolongation of existing
- Hospitalization
- Results in persistent or significant disability/incapacity
- Results in a congenital anomaly or birth defect
- Other medically significant events that require immediate medical or surgical
- Intervention

Unexpected means:

- Not consistent with Investigators Brochure or SmPC

Causal Relationship means:

- There are facts/evidence to suggest a causal relationship
- As judged by the reporting health care professional to have a reasonable suspected causal relationship

NOTE

Death: is the result of an Adverse Event The medical condition that caused death, such as an underlying disease or an accident, must be reported in detail

Life-threatening: in the definition of a Serious Adverse Event or Adverse Reaction refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it was more severe

In some circumstances, medical judgment should be utilized to determine the seriousness of an Adverse Event / Reaction. Important Adverse Events / Reactions that are not immediately life-threatening or do not result in death or hospitalization but may put the patient at risk or demand intervention to avoid one of the other outcomes specified in the description above may also be deemed serious.

9.3. Suspected Expected Serious Adverse Reaction (SESAR)

Any adverse reaction that is classed in nature as serious and which is consistent with the available information on the medicinal product in question set out in the SmPC.

9.4. Suspected Unexpected Serious Adverse Reaction (SUSAR)

Any adverse reaction that is classed in nature as serious and which is **not** consistent with the available information on the medicinal product in question set out in the SmPC.

9.5. Recording of Adverse Events

All adverse events, whether serious or not, will be documented and reported in compliance with the definitions previously provided.

The Investigator must record all adverse signs and symptoms that are either volunteered by patients or seen during or after the administration of the Investigational Product on the corresponding CRF page.

The description should include the nature of the sign or symptom, the date of onset, the date of resolution (duration), the severity/intensity, the relationship to study treatment or other therapy, the action taken (if any), and the outcome.

9.5.1 Definition of Adverse Event intensity

Intensity	Definition
Mild	Patient is aware of signs and symptoms, but they are easily tolerated
Moderate	Signs/symptoms cause sufficient discomfort to interfere with usual activities
Severe	Patient is incapable to work or perform usual activities

9.5.2 Definition of Adverse Event causality

The following criteria are used to indicate the degree of causation (all points should be reasonably complied with) based on the WHO-UMC system for standardized case causality assessment (www.who-umc.org):

Definite

- Event or laboratory test abnormality, with plausible time relationship to drug intake
- Cannot be explained by disease or other drugs
- Response to withdrawal plausible (pharmacologically, pathologically)
- Event definitive pharmacologically or phenomenologically (i.e. an objective and specific medical disorder or a recognized pharmacological phenomenon)
- Re-challenge satisfactory, if necessary

Probable

- Event or laboratory test abnormality, with reasonable time relationship to drug intake
- Unlikely to be attributed to disease or other drugs
- Response to withdrawal clinically reasonable (for details refer to WHO-UMC)
- Re-challenge not required

Possible

- Event or laboratory test abnormality, with reasonable time relationship to drug intake
- Could also be explained by disease or other drugs
- Information on drug withdrawal may be lacking or unclear

Unlikely

- Event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible)
- Disease or other drugs provide plausible explanations

Not related

The event does not follow a reasonable temporal sequence from the administration of the IMP and is clearly related to other factors, such as clinical state, therapeutic intervention or concomitant therapy.

Not assessable

- Report suggesting an adverse reaction
- Cannot be judged because the information is insufficient or contradictory
- Data cannot be supplemented or verified

All cases judged by any or both assessors as having a “reasonable causal relationship” to the IMP qualify as ADR. This corresponds to the categories “definite”, “probable” and “possible”.

9.6 Reporting of Serious Adverse Events

All Serious Adverse Reactions and Unexpected Serious/Adverse Reactions must be reported to the Coordinator within 24 hours (one working day) of the Investigator becoming aware of them. Preference is given to the SAE report via e-mail at research@ssnn.ro.

9.7 Exemption from expedited reporting

Not applicable.

9.8 Adverse Event/Reaction follow-up procedure

Adverse Events/ Reactions will be followed up throughout the course of the clinical study and any changes will be recorded in the CRF.

10. STUDY SCHEDULE

10.1 Procedures at Each Visit

Visit 1 - Screening - Study day 0
- Demographic data
- Medical history and risk factors
- ICF
- Randomization
- Primary and secondary outcomes

Visit 2 - Efficacy Evaluation -Study day 30
- Primary Outcomes
- Secondary Outcome

Visit 3 - Efficacy Evaluation -Study Day 90
- Primary Outcomes
- Secondary Outcome

Visit 4 - Efficacy Evaluation - Study Day 180
- Primary Outcomes
- Secondary Outcome

10.2 Assessment of Compliance

Compliance will be documented in the CRF by recording the date and time of administration. The number of IV infusions actually given to each patient will be calculated as a percentage of the total number of IV infusions planned per protocol, providing a measure of treatment adherence.

10.3 Risk assessment and Precautionary Measures

The investigational medicinal product is in clinical use for many years and has demonstrated a very benign safety profile.

The safety information for the IMP is provided in the SmPC in Appendix 1.

11. STUDY AND TREATMENT DURATION

Study/Treatment start: 10/2022
Study/Treatment end: 11/2024

12. STATISTICAL METHODS

The final statistical analysis of the study will be performed by a qualified biometrician and will fulfill all ICH/GCP requirements for handling clinical study data. The statistical analysis, including any subgroup analysis will be agreed upon prior to data evaluation and the results will be fixed in a statistical analysis plan (SAP) which will be established prior to unblinding of the data. The study data will be analyzed and

the Statistical Report written as soon as all study data are entered into the study database and the entered data are validated.

The analysis will be based on pre-specification of the primary analyses and control of multiple level alpha. The power for this study is determined based on the following design specifications:

- K=2 experimental treatments will be included in the trial.
- A significance level of α=0.05 will be used, in combination with Dunnett's correction.
- The standard deviations of the responses will be assumed to be: (σ0,…,σK)=(1,1,1).
- The minimum marginal power will be controlled to level 1−β=0.9 under each of their respective least favourable configurations.
- The interesting and uninteresting treatment effects will be: δ1=0.5 and δ0=0 respectively.
- The target allocation to each of the experimental arms will be: the same as the control arm.
- Therefore, the realised allocation ratios to the experimental arms are: (r1,…,rK)=(1,1).
- The maximum familywise error-rate is: 0.05.
- The minimum marginal power is: 0.9.
- The following critical threshold should be used with the chosen multiple comparison correction: 0.028.
- 10% adjustment for dropouts
- The total required sample size is: N=270. The required sample size in each arm is: (n0,…,nK)=(90,90,90).

Source: Grayling MJ, Wason JMS (2020) A web application for the design of multi-arm clinical trials. BMC Cancer 20:80. DOI: 10.1186/s12885-020-6525-0

It is the primary endpoint of this study to assess the efficacy of N-PEP-12 on the Test of Attentional Performance outcomes in healthy middle-aged and older adults at 90 and 180 days after baseline.

Missing data

Missing data of the type ´missing completely at random´ (MCAR), which in principle will not bias the results; the analysis procedure should be able to cope with partially missing data of such a type. In many studies this type of data is treated by LOCF replacement (Last Observation Carried Forward) as far as there exist follow-up measurements at previous visits.

In a study like the one planned there might also be informatively missing data (missing not at random, MNAR): participants of the study died or are unable to complete the tests because of brain-related impairment. Neglecting these missing data might introduce bias.

In order to identify each type of missing data, outcome scales will be coded for every patient and visit according to the following scheme:

1 = valid (complete task)
2 = unable to complete (TBI-related neurological reason) [describe reason]
3 = not completed (different reasons, not TBI related) [describe reason].

For outcome scales with code "2" a worst rank imputation will be introduced for the corresponding patients since these data are informatively missing (missing not at random, MNAR). These missing data are replaced by the worst possible score of the corresponding outcome scale.

For outcome scales with code "3" a LPCF replacement will be introduced (Last Percentile Carried Forward) as far as previous follow-up evaluations exist. This method carries forward the actual status information of

the patient population, using the percentile value with back transformation to raw scale, instead of the last value carried forward. This approach was recently developed and recommended by O´Brien, Zhang and Bailey (2005) for the analysis of data from chronic, progressive diseases such as dementia. According to their simulation study the calculated estimators should be negligibly biased by missing data. If no general change of patients over time occurs the method is more or less identical with LOCF (Last Value Carried Forward), if change occurs bias is minimized. If no previous follow-up measurement exists, the outcome scale remains missing.

Study populations and protocol violations

Before the study is unblinded, a blind review will be performed. In this process, possible protocol violations will be classified as “severe”, “major”, “minor“, or “none”. Patients will be allocated to the individual data sets with regard to the classification of possible protocol violations. The analysis populations (ITT, and PP) will be listed individually in the final statistical analysis plan.

The safety population includes all patients who have had at least one dose of study medication and one contact with the Investigator afterwards. It will be used for safety analysis.

ITT population is defined as all patients who have no “severe” violation of entry criteria, had at least one dose of medication and at least one post-baseline observation of at least one primary efficacy criterion (definition according to ICH E9 § 5.2. Analysis Sets). ITT population will be used for all efficacy analyses.
A sensitivity analysis will be performed for a per protocol (PP) data set as an exploratory approach. The PP population includes all patients who are eligible for ITT evaluation and who additionally do not show major protocol deviations. As noted in section 13.9, the supportive analysis by means of the per-protocol set will be regarded as of equal scientific importance as the ITT analysis, since it most closely reflects the scientific model underlying the protocol (see ICH E9, section 5.2.2).

Homogeneity analyses for baseline shall be performed based on the ITT population. In addition to descriptive analyses (e.g., two-sided 95% confidence intervals), baseline comparability analyses shall present an overview on demographic-anamnestic variables and on the primary efficacy criteria at baseline. This allows comparison of baseline variables across different scales and data types. In the case of heterogeneities, stratified analyses will be performed as second line analyses. Patients with compliance for the entire study below 80% for the treatments will be considered protocol violators and will not be included in the per protocol analysis.

A blind review of the data shall be performed within the framework of the requirements of the ICH Guideline E9. The statistical analysis plan will be finalized by the statistician before the decoding takes place. The analysis populations (Safety, ITT, and PP) will be listed individually in the final statistical analysis plan.
Formal records will be kept of when the statistical analysis plan was finalized as well as when the blind was subsequently broken.

Sample size re-estimation

Sample size estimation (SSE) is an important issue in the planning of clinical studies. While larger studies are likely to have sufficient power, it may be unethical to expose more patients than necessary to answer a scientific question. Calculating the sample size based on the estimated nuisance parameter may not be stable. Sample size re-estimation (SSR) at the interim analysis will be performed to provide an opportunity to

re-evaluate the uncertainties using accrued data and continue the trial with an updated sample size (Wu, Lin, & Chow, 2016).

The data analysis will be performed in a validated working environment according to the requirements of the ICH-Guidelines E3 (1995). The software to be used for data evaluation will be described in the final statistical analysis plan.

13. ACCESS TO SOURCE DATA / DOCUMENTS

The Investigator will allow study-related monitoring, audits, IRB / IEC review and regulatory inspections, providing direct access to primary patient data (i.e. source data) which supports the data on the CRFs for the study, i.e. general practice charts, appointment books, original laboratory records, etc.

13.1 Source Data

Source data is defined as all information in original records and certified copies of original records of clinical results, observations, or other activities in a clinical study that is essential for the study's reconstruction and evaluation. Source data can be found in source documents (original records or certified copies).

13.2 Source Documents

Source documents are the original documents, data, and records (e.g. hospital records, clinical and office charts, laboratory notes, memoranda, patient diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies of manuscripts certified after verification as being accurate copies, microfiches, photographic negatives, microfilm or magnetic media, patient files, records kept at the pharmacy, at the laboratories and at medico technical departments involved in a clinical study).

13.3 Direct Access

Direct access is defined as the right to inspect, analyze, verify, and reproduce any records or reports relevant to the evaluation of a clinical study. Any party with direct access (e.g., domestic and foreign regulatory authorities, the Coordinator, and/or authorised representatives of the Coordinator such as monitors and auditors) should take all reasonable precautions within the constraints of the applicable regulatory requirements to protect patient identities and the Coordinator proprietary information.

14. QUALITY CONTROL AND QUALITY ASSURANCE

14.1 Quality Control

Quality control is described as the operational approaches and activities used inside the quality assurance system, such as monitoring, to guarantee that the quality requirements of study-related activities are met.

Quality control should be conducted at each stage of data processing to guarantee that all data is reliable and has been processed correctly.

14.2 Study Monitoring

A qualified and authorized Clinical Trial Monitor will visit the investigational site at regular intervals determined by the project's needs to verify protocol adherence and local legal requirements, perform source data verification, and assist the Investigator in his study-related activities.

14.3 Quality Assurance

The planned and systematic actions put in place to guarantee that the study is carried out and that the data is generated, documented (recorded), and reported in compliance with Good Clinical Practice (GCP) and the necessary regulatory standards are characterized as quality assurance.

14.4 Inspection

An inspection refers to the act of an authority (IRB/IEC) conducting an official review of documents, facilities, records, and any other resources that the authorities deem to be related to the clinical study and that may be located at the study site, at the Coordinators' and/or clinical research organization's facilities, or at any other establishments deemed appropriate by the authorities.

14.5 Audit

An audit is a systematic and independent review of study-related activities and documents to determine whether validated study-related activities were carried out and data were recorded, analyzed, and reported accurately in accordance with the protocol, designated Standard Operating Procedures (SOPs), Good Clinical Practice (GCP), and applicable regulatory requirements. An independent audit of the study site may occur at any time during or after the study.

15. ETHICAL AND LEGAL CONSIDERATIONS

15.1 Ethical Considerations

The Investigator will have received and dated permission / favorable opinion from the relevant IRB / IEC for the study protocol and any revisions before initiating the study. To signify consent in writing, the final protocol number and date shall be utilized. The IRB / IEC's constitution should be made accessible for inclusion in the Trial Master File, together with the names of its members and their roles in the committee (e.g., chairman, specialist, lay-member). During the study, the Investigator shall provide all documents that are subject to review by the IRB / IEC.

15.2 Independent Ethics Committee (IEC) / Institutional Review Board (IRB)

Before the study begins, the study protocol, including all amendments, and the study CRF will be submitted to the study center's IRB/EC. Prior to the start of any study-specific procedures, IRB/EC approval for the study protocol and all amendments will be obtained.

15.3 Informed Consent

Patients will be informed about the study procedures, as well as the potential risks and benefits. Before any study-specific procedures are performed, their consent to participate in this study will be obtained.

15.4 Modification of Protocol

The Investigator or Coordinator should not deviate from or change the protocol unless there is mutual agreement, prior review, and documented approval from the IEC of a respective amendment. The only exceptions are when changes are required to eliminate an immediate hazard to study patients or when changes involve only logistical or administrative aspects of the study (e.g., change in monitor(s), change in telephone number(s)). The party initiating the amendment must confirm it in writing and have it signed and dated by the Coordinator and the Principal Investigator. Protocol amendments that are required will be submitted to the relevant IECs.

15.5 Conduct of Study

This clinical study will be carried out in accordance with the Helsinki Declaration and in accordance with this protocol, Good Clinical Practice (2001/20/EC, CPMP/ICH/135/95), designated Standard Operating Procedures, and local laws and regulations applicable to the use of investigational new drugs in the country of conduct.

15.6 Personal Data and Data Protection

All data gathered throughout the course of the clinical investigation is subject to data protection. The investigator and the investigating physicians are not to reveal the patient's name or any other personal information (with the exception of the patient's date of birth/age and gender). The latter must ensure that the case report forms or other documentation (for example, copies of reports on exceptional findings) sent to the FSNN do not include names, but rather another identification. The patient identity must be used to store data for statistical analysis. Personal data can only be assigned an identification by the Investigator and the investigating physicians.

During the course of the study, if it becomes necessary to identify a patient's name for medical reasons, everyone engaged is obligated by a duty of confidentiality. Data protection legislation requires that personal data be stored and processed in a secure manner.

15.7 Data Handling and Record-Keeping

15.7.1 Completion of Case Report Forms

Any information that will be entered directly into the CRFs will be identified at the start of the trial. The investigator must ensure that the CRF data and all related reports are accurate, complete, legible, and timely. Any change or correction to a paper CRF must be dated, initialed, and explained (eCRF data entries are already audited) and must not obscure the original entry; this rule applies to both written and electronic alterations. Data derived from source documents should be consistent with the original documents, or inconsistencies should be justified. Within two weeks of the completion of the study, the investigator should have completed and signed CRFs accessible for a thorough inspection by the clinical monitor.

15.7.2 Archiving

The research documentation, including the emergency envelopes, must be returned to the Coordinator when the study is completed. These records must be kept for the time periods specified by ICH-GCP, i.e. until at least 2 years have passed since the last approval of a marketing application in an ICH region and there are no pending or contemplated marketing applications in an ICH region, or until at least 2 years have passed

since the formal discontinuation of clinical development of the Investigational Product (CPMP/ICH/135/95), or for the time period specified by national legal requirements, whichever is longer, but not less than 15 years.

After the Investigational Products have been removed from the market, the final report must be retained for at least two years. The clinical trial director or the investigating physicians must keep the informed consent papers and all original (raw) data for at least 15 years.

15.8 Confidentiality

The aim and contents of the study, in addition to its results are to be treated as confidential by everyone involved in the clinical study.

15.9 Responsibilities

The responsibilities of the Investigator, Monitor, and Coordinator of the clinical study in terms of data handling, storage, planning, assessment, and quality assurance are governed by the "International Conference on Harmonisation" (ICH) recommendations on "Good Clinical Practice" and apply to this clinical study.

16. FINAL REPORT AND PUBLICATION POLICY

The final study report must be agreed upon by both the Coordinator and the Investigator. The latter must be signed by both the investigator and the physicians who are conducting the investigation.

The findings of the investigation will be published in scientific journals. The findings could also be utilized in regulatory filings. The following restrictions are in place to protect commercially sensitive information (patents, for example) rather than to restrict disclosure.

All information about the Investigational Product (such as patent applications, formulae, manufacturing processes, basic scientific data, or formulation information provided to the Investigator by the Coordinator that has not been previously published) is considered confidential and shall remain the Coordinator's sole property. Without the Coordinator's explicit consent, the Investigator agrees not to use it for any other reason.

The Investigator knows that the information gathered in this clinical trial will be used by the Coordinator in the development of the Investigational Product, and that it may be shared with other investigators or international regulatory authorities as needed. To enable the utilization of information produced from this clinical study, the Investigator agrees that all test results and data created throughout the study must be provided to the Coordinator.

17. SIGNATURES

The undersigned have read this protocol and agreed to conduct this study in accordance with all stipulations of the protocol and in accordance with the Declaration of Helsinki.

Date: **Signature:**

_____ **Prof. Dr. Dafin F Muresanu**

(Coordinating Investigator)

_____ **Dr. Nicoleta Jemna**

(Principal Investigator)

_____ **Dr. Olivia Verisezan Rosu**

(Co Investigator)

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