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**Treating Mild Cognitive Impairment and sleep disturbances by
Medium Chain Triglycerides supplementation: a study protocol
for patients with a recent diagnosis**

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SUMMARY

This thesis presents a clinical study protocol investigating the potential benefits of medium-chain triglycerides (MCTs) as a nutritional intervention for individuals with Mild Cognitive Impairment (MCI) and sleep disturbances. The work begins by analyzing the biological and cognitive aspects of aging, with a focus on the pathophysiology and diagnosis of MCI and Alzheimer's disease. It highlights the role of brain-derived neurotrophic factor (BDNF) in cognitive function and circadian rhythm regulation, emphasizing the impact of sleep disorders on neurodegeneration. The study explores how MCTs may serve as an alternative energy source for the brain in states of glucose hypometabolism, which are characteristic of MCI and early Alzheimer's. A detailed methodology outlines patient selection, dietary protocols, neuropsychological assessments, biomarker analysis, and imaging techniques. The thesis also addresses broader issues such as the sustainability of healthcare systems in managing dementia. The ultimate goal is to evaluate whether MCT supplementation can improve cognitive and metabolic parameters and slow the progression of neurodegeneration.

Riassunto

Questa tesi presenta un protocollo di studio clinico volto a valutare i benefici di un intervento nutrizionale con trigliceridi a media catena (MCT) in soggetti affetti da decadimento cognitivo lieve (MCI) e disturbi del sonno. Dopo un'analisi dei meccanismi biologici e cognitivi dell'invecchiamento, viene approfondita la fisiopatologia e la diagnosi dell'MCI e del morbo di Alzheimer. Un'attenzione particolare è dedicata al ruolo del fattore neurotrofico BDNF nella funzione cognitiva e nella regolazione del ritmo circadiano, evidenziando l'influenza dei disturbi del sonno sulla neurodegenerazione. La tesi esplora il potenziale degli MCT come fonte energetica alternativa per il cervello in condizioni di ipometabolismo glucidico, tipico dell'MCI e delle prime fasi dell'Alzheimer. Il lavoro descrive in dettaglio la metodologia dello studio: selezione dei pazienti, protocolli dietetici, valutazioni neuropsicologiche, analisi dei biomarcatori e tecniche di imaging. Infine, viene affrontato il tema della sostenibilità del sistema sanitario nella gestione delle demenze. L'obiettivo è valutare se la supplementazione con MCT possa migliorare i parametri cognitivi e metabolici, rallentando la progressione della neurodegenerazione.

1. INTRODUCTION

The aging of the Italian population, as well as that of developed countries in general, represents one of the major challenges of the 21st century, with consequences across various social areas, including the economy, politics, healthcare, and social welfare. Due to socio-economic development, advances in medicine and education, and the increased availability of food and hygiene resources, Western countries have witnessed an increase in life expectancy over the past few decades. A population is considered aging when the average age surpasses the typical working-age reference point (Skirbekk, Staudinger & Cohen, 2019). However, the concept of aging should not be reduced merely to the chronological progression of life; it must be seen as a complex process involving biological, genetic, environmental, and behavioral dimensions (Ferrucci et al., 2020). Dementia is among the major causes of disability and dependency related to aging, representing an economic challenge both for the families of affected individuals and for national healthcare systems. Dementia is, in fact, the fifth leading cause contributing to the global burden of disease, with associated costs exceeding \$1 trillion as early as 2018 (Grande et al., 2019).

The World Health Organization (WHO) and Alzheimer's Disease International (ADI) have estimated that there are currently about 47 million cases of dementia worldwide, a figure expected to triple by 2050 (Prince, 2017). One of the most studied aspects in the context of aging is the transition phase between normal cognitive decline and pathological forms of cognitive deterioration. In this context, Mild Cognitive Impairment (MCI) is recognized as an intermediate condition between normal aging and dementias such as Alzheimer's disease (AD) (Petersen, 1999). Given the increasing prevalence of dementing conditions in the elderly population, screening programs based on validated and effective evaluation tools are urgently needed (Grande, Qiu & Fratiglioni, 2020)

1.1 AGING: BIOLOGICAL MECHANISMS

Aging is a biological process that involves morphological, physiological, and psychological changes (Bengtson & Settersten, 2016). Already after the age of thirty, the human body undergoes changes that manifest at the physical level (skin, hair, weight, voice), the functional level (cardiovascular system, gastrointestinal system), and the sensory level (vision, hearing, taste, smell, temperature perception) (De Beni & Borella, 2015). It is important to emphasize that each biological change corresponds to an adaptive response, involving a phase of accommodation, recovery, and plateau (Flatt, 2012). The elderly individual experiences reduced physiological compensation (Ferrucci & Fabbri, 2018), leading to the accumulation of biological damage that compromises systemic and cellular functionality.

Several mechanisms have been identified that contribute to aging:

- **Genomic Instability:** Over time, the accumulation of somatic mutations and DNA damage accelerates cellular senescence, contributing to tissue degeneration. Lopez-Otin et al. (2013) stated that genomic instability is closely linked to distinctive signs of aging, including mitochondrial dysfunction and cellular senescence.
- **Telomere Shortening:** Telomeres preserve the genetic information contained in chromosomes, playing a crucial role in genome integrity. They are located at the ends of chromosomes and shorten with each cell division. After continuous divisions, when the telomeric region reaches a critical point, cell division stops. Consequently, cells activate genetic programs such as replicative senescence. The accumulation of senescent cells accelerates organismal aging (Ovadya et al., 2014). Thus, telomeres are often considered a "molecular clock" (Lin et al., 2015).
- **Accumulation of DNA Damage:** Genomic and mitochondrial DNA suffer multiple damages from endogenous factors like reactive oxygen species (ROS) and exogenous factors like UV radiation or X-rays. Persistent, unresolved damage promotes genomic instability, potentially leading to apoptosis (Roos WP et al., 2006) or cellular senescence (d'Adda di Fagagna, 2008). DNA damage is associated with epigenetic changes that accelerate aging (Dabin J et al., 2016), and defective DNA repair mechanisms are linked to protein aggregation in the brain, contributing to cognitive decline and neurodegenerative diseases like Alzheimer's and Parkinson's (Weissman L et al., 2007; Sepe S et al., 2016; Obulesu M et al., 2010).
- **Mitochondrial Dysfunction:** Mitochondria are responsible for cellular respiration and ATP production, the primary energy source for cells. Altered mitochondrial function initially triggers a compensatory response by increasing mitochondrial mass. However, dysfunctional mitochondria exhibit low membrane potential and fail to generate ATP effectively (Miwa S et al., 2022). This leads to increased ROS production, promoting cellular senescence and organismal aging (Passos JF et al., 2010).
- **Loss of Heterochromatin:** Chromatin, the complex of DNA and proteins (mainly histones) within eukaryotic nuclei, can be classified as transcriptionally inactive heterochromatin and active euchromatin (Allshire RC et al., 2018). Aging is associated with a progressive loss of global heterochromatin, resulting in transcriptional silencing, genomic instability, and cellular senescence (Tsurumi A et al., 2012; Cheung P et al., 2018).
- **Stem Cell Depletion:** Stem cells are unspecialized primitive cells capable of differentiating into various cell types. With aging, they lose their regenerative capacity, leading to decreased tissue and organ repair. This phenomenon has been observed across hematopoietic,

mesenchymal, and neural stem compartments (Culig L et al., 2022; Zhong X et al., 2023). Stem cell depletion is associated with increased age-related frailty (Sharpless & DePinho, 2004).

- **Chronic Inflammation:** Aging is associated with chronic low-grade inflammation, known as "inflammaging," as demonstrated by Franceschi et al. (2014).

All these mechanisms are interconnected and collectively contribute to the aging process. Understanding them is crucial to developing strategies aimed at slowing aging and preventing related diseases

1.2 AGEING AND COGNITIVE DECLINE

Aging is not a single physiological process, but a complex phenomenon stemming from the evolution of adaptation. It inevitably involves a gradual decline in an individual's ability to respond to external environmental demands (Rose et al., 2012). This difficulty in adaptation can be analyzed both from a biological and cognitive point of view. From the biological standpoint, for example, sensory changes can manifest as a reduction in vision and hearing, with a consequent impairment of interaction with the surrounding environment. In addition, the circadian rhythm may also be altered, resulting in an inability to synchronize the sleep-wake cycle.

At the brain level, with advancing age there are different changes, as studied by Temnes et al, in 2013, in fact, over time the brain tends to atrophy resulting in a decrease of cortical gray matter and loss of neurons. Brain atrophy, however, is not necessarily indicative of a cognitive disorder. According to the “use it or lose it” theory cited by Swaab in 1991, constant exercise of cognitive functions and activities aimed at consolidating memory promote new neural connections even in old age.

This is because from the cognitive point of view, thanks to the aforementioned, compensatory plasticity mechanisms can be activated that guarantee a good cognitive and behavioral functioning even in elderly subjects (Cordella&Poiani, 2021). On the other hand, prolonged inactivity leads to a degeneration of some brain circuits (Cordella&Poiani, 2021).

The World Health Organization (WHO) has highlighted that dementia is one of the main causes of disability in the elderly, and it differs from normal age-related cognitive decline due to its progressive and disabling course. The key to distinguishing a physiological detuning from a pathological one, therefore, lies in the evolution of the clinical picture: in the first case, the change is gradual without serious impairment of quality of life, whereas in the second case, the deterioration appears to be progressive, resulting in pathological cognitive decline (Papagno, 2007; Cahill S, 2019).

In the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the term dementia has been replaced by the concept of neurocognitive disorder (NCD) (APA, 2013). NCD is recognised when there is a decline in at least one of the following cognitive domains:

- 1) learning and memory
- 2) Attention
- 3) Executive functions
- 4) Language
- 5) Motor perception
- 6) Social cognition

The emergence of these deficits can affect both healthy and pathological subjects, and in this sense, the clinician's task is to use validated neuropsychological batteries in order to accurately identify the pathological subject and thus make an accurate diagnosis (Sachdev et al., 2014, Blazer, 2013).

1.3 MILD COGNITIVE IMPAIRMENT

Mild Cognitive Impairment (MCI) was introduced to describe the intermediate area between intact cognitive functioning and clinical dementia (Petersen, 2014). The term was first introduced by Reisberg et al. in the 1980s and was used to identify patients who were in an intermediate stage of pathology according to the scores obtained from the Global Deterioration Scale. In 1999, Petersen further defined the concept of MCI based on clinical findings showing that some subjects with memory deficits did not meet the criteria for a diagnosis of dementia, although they exhibited a high risk of progression to it. Initially, the term MCI was closely associated with episodic memory impairment, but over time it became evident that cognitive decline could also involve other cognitive areas such as language or executive functioning. In 2003, at a Consensus Conference in Stockholm, new criteria were established for MCI by distinguishing between Amnesic-MCI (a-MCI), where the primary deficit is memory decline, and Non-amnesic-MCI (na-MCI), where the primary deficit concerns cognitive domains other than memory (Winblad et al., 2004). The inclusion of MCI in the DSM-5 (APA, 2013) consolidated the distinction made at the Stockholm conference, equating MCI to the broader category of mild neurocognitive disorders (NCDs). However, although MCI is often considered a pre-dementia condition, recent studies have shown that not all individuals with MCI progress to dementia. Koepsell and Monsell (2012) showed that about 16% of individuals with MCI can return to a normal or near-normal cognitive condition, especially in cases of less severe MCI, characterized by higher scores on cognitive tests, less functional impairment, and the absence of the APOE4 allele. Nevertheless, even those who regain normal cognitive function remain at risk of redeveloping MCI or dementia within the following three years (Koepsell & Monsell, 2012).

1.3.1 MCI AND CLASSIFICATIONS

The growing focus in research on aging and neurodegenerative diseases has brought to light the need to further explore the topic of MCI. This need arises from the recognition that individuals with MCI have a high risk of progression to dementia in a relatively short time (Roberts & Knopman, 2013). Initially, MCI was described as a pre-dementia symptomatic phase, characterized by greater cognitive decline than expected by age and level of education (Petersen et al., 1999). However, the need for a more accurate characterization has led to the definition of sub-categories of MCI, which are essential for better understanding its evolution.

As already described in the previous chapter, MCI was initially classified into:

- 1) Amnesic MCI (a-MCI): the primary deficit concerns the cognitive domain of memory
- 2) Non-Amnesic MCI (na-MCI): the deterioration concerns another cognitive domain such as language or visuo-spatial ability.

These two categories are further subdivided according to the number of compromised cognitive domains:

- 1) Single-domain MCI: cognitive impairment relates to a single area
- 2) Multiple domain MCI: the deficit affects more cognitive areas (Winblad et al., 2004).

The sub-categorization of MCI is fundamental for identifying the risk of conversion to a specific form of dementia and optimizing intervention and monitoring strategies (Luck T et al., 2012).

A study by Manly et al. (2008) examined the incidence of MCI and the risk of progression to Alzheimer's disease in a multi-ethnic population living in the United States. The study found that:

- Advanced age and lower educational attainment are predictors of the onset of MCI.
- Subjects with aMCI have a higher risk of developing AD.

These findings have further reinforced the importance of early identification and classification of MCI, which are key elements for dementia risk management and implementation of preventive strategies.

1.3.2 MCI AND DIAGNOSTIC CRITERIA

Tackling cognitive decline is a challenge starting from the diagnostic phase. The formulation of a timely, accurate and specific diagnosis allows planning targeted interventions and appropriate care and support paths, involving the person and their family members, in all stages of the disease. The diagnosis of MCI is a gradual process, starting with the recognition of the condition in primary care centers or community settings. In these primary care settings, initial screening tests are typically administered, while more in-depth investigations are carried out in specialized neurological centers after the patient's referral (Langa KM. et al., 2020). According to the updated guidelines of the American Academy of Neurology (AAN), it is necessary to assess modifiable risk factors and monitor

cognitive progression over time, considering both neuropsychiatric symptoms and patient function (Petersen et al., 2018). There are numerous cognitive assessment tools available, which must be combined to obtain an accurate diagnostic picture. Initial cognitive screening often involves tests with high sensitivity but lower specificity, such as:

- 1) IQCODE TEST, a questionnaire that evaluates memory, orientation and executive functions, with a sensitivity of 97.9% and a specificity of 71.4% (Quinn et al., 2014).
- 2) AD8 TEST, a self-assessment test with eight questions that investigate different cognitive domains, with 80% sensitivity and 70% specificity (Galvin JE. et al., 2007).
- 3) Montreal Cognitive Assessment (MOCA): is a test administered by a trained clinician, such as a neuropsychologist or a neurologist. In particular, the MOCA is designed to investigate 8 cognitive domains such as memory, executive functions, attention, language, visuospatial abilities, and orientation in time and space. The maximum score is 30 points but the cutoffs for identifying MCI may vary according to educational level:
 - 13 points: For illiterate individuals.
 - 19 points: For those with between 1 and 6 years of education.
 - 24 points: For those with 7 or more years of education.

The degree of education, in fact, can influence cognitive performance and it is precisely for this reason that cutoffs are established to adapt the cognitive assessment. Patients who receive scores below the cutoff may have mild cognitive impairment. The strength of this test lies in its sensitivity, as it allows for the detection of even mild cognitive deficits but must be accompanied by a more specific test (Carson N et al., 2017).

4) Addenbrooke's Cognitive Examination Revised (ACE-R): a test of 15-20 minutes administered to the patient by an expert neuropsychologist. The test investigates different cognitive areas and has a maximum score of 100 divided into 18 points for the cognitive area of attention, 26 points for memory, 14 points for verbal fluency, 26 points for language area and 16 points in visuospatial abilities. Cutoff values may vary according to level of education and generally, scores below 88 suggest the presence of cognitive decline (Mioshi E et al., 2006; Siciliano M et al., 2016).

5) Attentional Matrices Test: this test uses the equivalent scoring system (ES). Once the test is completed, the equivalent scores are transformed into raw scores (number of correct answers in the test) taking into account also the age and level of education of the patient. An ES equal to 1 suggests mild or initial cognitive decline while an ES equal to 0 indicates dementia. If the patient has an ES score of 2, it should be monitored as the subject performs slightly below average.

The tests described above should be implemented as mentioned, tests of greater specificity among them including:

- 1) Memory Impairment Screen (MIS), which evaluates episodic memory through word recall and has a specificity of 93%-97% (Buschke H et al., 1999).

- 2) Six Item Screener (SIS), which tests memory and orientation with a sensitivity of 86%-89% for dementia, but only 34% for MCI (Wilber ST et al., 2008).
- 3) Clock Drawing Test (CDT), which measures visuospatial and executive functions, with sensitivity between 67%-98% for Alzheimer's disease and 41%-85% for MCI (Rakusa M. et al., 2018).
- 4) Mini-Cog, which combines CDT with a word recall test, has a sensitivity between 76%-100% for dementia, but lower for MCI (Borson S. et al., 2000).
- 5) Mini Mental State Examination (MMSE): This test is a 30-point questionnaire that evaluates different cognitive areas including orientation in time and space, memory, language and computational ability. Scores below 18 are indicators of significant cognitive impairment while scores between 18 and 24 indicate moderate to mild cognitive decline. Usually this test, more specific, is accompanied by the MOCA which, instead, has a higher sensitivity (Jia X et al., 2021).

The tests most commonly used in clinical practice are MOCA compared to ACE-R and MMSE. In the case where, thanks to the use of neurocognitive tests, an impairment of the mnemonic area is ascertained, it can be chosen according to the criteria of Petersen (2004), for a single or multiple domain a-MCI based on the presence or absence of an involvement of a cognitive area other than memory. If the result of the cognitive test shows an impairment of a cognitive area other than memory, it can be determined the presence of single or multiple domain na-MCI. The task of the neuropsychological examination is therefore to determine the cognitive picture of the subject by distinguishing MCI from both physiological aging and other pathological conditions. The neuropsychological evaluation thus plays a crucial role in differentiating MCI from both normal aging and other pathological conditions. In addition to cognitive tests, imaging techniques play a crucial role in diagnosis. Magnetic resonance imaging (MRI) may reveal cerebral atrophy, especially of the hippocampus and medial temporal cortex, while fluorodeoxyglucose PET (FDG-PET) can identify hypometabolism in affected brain regions (Zacková et al., 2021; Chandra et al., 2019). Transcranial magnetic stimulation (TMS), combined with EEG, allows patients with MCI to be distinguished from those with Alzheimer's by assessing synaptic plasticity and cortical excitability (Chou et al., 2022). The cerebrospinal fluid (CSF) analysis may provide further diagnostic information. Biomarkers such as beta-amyloid 42, reduced in Alzheimer's patients, and phosphorylated tau protein, increased in neurodegeneration, may support differential diagnosis (Lefort-Besnard et al., 2023).

1.3.3 RISK FACTORS

The intermediate condition between normal aging and dementia, attributed to MCI, requires early diagnosis as well as identification of risk factors. As evidenced in numerous literature studies the risk factors can be classified as modifiable or non-modifiable (Roberts R et al., 2013). Non-modifiable risk factors are linked to genetics, sex and age while modifiable risk factors are clinical factors that can be treated to reduce the risk of developing MCI.

Among the non-modifiable risk factors related to genetics, we find the polymorphism of allele 4 of apolipoprotein E (APOE4), a protein involved in lipid transport and neuronal repair. The mechanism by which ApoE4 increases the risk of MCI is altered brain network organization. Patients with polymorphism of allele 4 have reduced overall brain connectivity, increased hippocampal atrophy and greater β -amyloid deposition than patients with MCI but not ApoE4 carriers. The ability to increase the deposition of β -amyloid makes carrying patients more likely to develop Alzheimer's disease (AD) (Sanabria-Diaz G et al., 2021). While we know that age can lead to cognitive decline, there is another non-modifiable factor, namely sex, there are conflicting views whether there is a higher prevalence of MCI in males than females but more scientific studies seem to lead to the hypothesis of a higher prevalence of mild cognitive decline in males (Lopez OL et al., 2003; Petersen RC et al., 2010).

Some clinical conditions may increase the risk of incurring MCI, these concern metabolic, vascular, inflammatory and lifestyle aspects (Menegon et al, 2024). Conditions such as hypertension, dyslipidemia, diabetes and obesity play a significant role in causing insulin resistance and systemic inflammation by promoting cognitive decline (Menegon et al., 2024). Recent studies have investigated the role of neuroinflammation by highlighting how a chronic inflammatory state leads to deleterious effects causing neuronal death (Comi C et al., 2017). The meta-analysis conducted by Bradburn S et al., in 2019, reports the presence of neuroinflammation in patients with MCI where it has been highlighted by PET, the Translocator Protein TSPO, a mitochondrial protein highly expressed in activated microglial cells. The presence of TSPO is therefore an indication of neuroinflammation and oxidative stress. Psychological factors also play a key role in the development of MCI. Depression is considered one of the major predictors of cognitive decline. Studies in the literature show that using certain imaging techniques, it is possible to find in patients with MCI and depression a common atrophy in brain regions such as the insula, the upper temporal gyrus, The amygdala and hippocampus or all those areas of the brain important for memory consolidation and cognitive processing (Chan JYC et al., 2019). In addition, social isolation resulting from depression reduces cognitive stimulation by increasing vulnerability to cognitive decline (Brailean A et al., 2017). Finally, two risk factors often underestimated are hearing loss which leads to social isolation and sleep disturbances which can lead to accumulation of proteins at the brain level resulting in neurodegeneration (Hahn EA et al., 2014; Menegon et al, 2024).

1.4 ALZHEIMER'S DISEASE: HISTORICAL BACKGROUND AND CLINICAL CLASSIFICATION

In 1907, the neurologist Alois Alzheimer first identified a devastating neurodegenerative disease that later took its proper name of Alzheimer's disease. The discovery of the pathology was based on studying the clinical picture of a 52-year-old woman who had a severe cognitive impairment of memory (Alzheimer, 1995). Today, AD is recognized as one of the most common dementias: more than 50 million people worldwide suffer from this disease. Due to advances in medicine and socio-economic development, the aging population has increased. As a result, it is expected, that by 2050 the number of individuals affected by AD will triple (Mantzavinos V et al., 2017).

Initially the diagnosis of Alzheimer's was limited to the stage of dementia, when the patient presented a cognitive deterioration of sufficient severity to significantly impact on daily life, then they were given the diagnosis of Alzheimer's (Jack CR et al., 2018). Subsequently, thanks to autopsic studies and biomarkers, the clinicians have highlighted as distinctive signs of the pathology, neurofibrillary tangles, senile plaques and loss of neurons (Dos Santos P et al., 2018). AD can be subdivided into:

- Genetics: affects a minority of people, and is linked to three genes that encode the amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2). The genetic form usually has an early onset with clinical symptoms that may appear before age 65 (Long JM et al., 2019)
- Sporadic: with a late onset mainly because of environmental and metabolic factors such as cerebrovascular diseases, traumatic brain injury, smoking, obesity (Hrelia P et al., 2020).

Among the sporadic forms a relevant role is played by apolipoprotein E (ApoE) or a glycoprotein involved in lipid metabolism and neuronal repair. The APOE gene exists in three main allelic variants such as E2, E3 and E4. The last mentioned, is associated with a significant increase in the risk of developing AD. Subjects carrying the E4 allele, especially in homozygous condition, have a higher risk and an earlier clinical onset than non-carriers (Karch&Goate., 2015). This occurs because the E4 allele promotes extracellular accumulation of β -amyloid, interferes with neuronal clearance and contributes to mitochondrial dysfunction (Rostagno, 2023; Liu et al., 2013). Although the presence of ApoE4 is not a *conditio sine qua non* for the development of the disease, it represents a significant genetic risk factor for the sporadic form of the disease. (Liu CC et al., 2013). AD is a multifactorial neurodegenerative disease in which several elements, including genetic, environmental and behavioral factors, are involved. In recent years, it has become clear that the environment and lifestyle can play a key role in modulating the risk of disease onset and progression (Livingston G et al., 2020). A healthy diet, regular physical activity and an adequate sleep-wake rhythm are now recognized as modifiable preventive factors, potentially able to delay the onset of the disease (Chan X et al., 2022).

1.4.1 PATHOGENIC MECHANISMS IN AD

Alzheimer's disease is a progressive neurodegenerative disease characterized by a complex interaction of biological events that develop over time, even years before the onset of clinical symptoms. The two main distinctive neuropathological signs are the extracellular accumulation of β -amyloid ($A\beta$), which leads to the formation of senile plaques, and the intracellular aggregation of hyperphosphorylated tau protein, responsible for the formation of neurofibrillary tangles (NFTs) (Breijyeh Z et al., 2020).

The amyloid hypothesis suggests that an increase in production or a reduced clearance of the peptide $A\beta$ -especially of the $A\beta_{42}$ form-is the origin of a neurotoxic process involving synaptic dysfunction, microglia activation and neuronal damage. In parallel, the tau hypothesis highlights the role of neuronal cytoskeleton disintegration and axonal transport impairment in cell death (Ferrari et al., 2018).

These processes are aggravated by concomitant phenomena, including chronic neuroinflammation, increased oxidative stress and mitochondrial dysfunction. Chronic activation of glial cells, especially microglia, contributes to a persistent inflammatory response that accelerates neurodegeneration (Zhang XX et al., 2021). In addition, the production of reactive oxygen species (ROS) and altered brain energy metabolism reduce neuronal efficiency and responsiveness to physiological stimuli.

These molecular mechanisms do not develop uniformly in all patients, but are modulated by genetic, environmental and systemic factors. Current and experimental treatments aim to address these processes, which is why understanding the pathogenic mechanisms of Alzheimer's is an essential basis for developing effective therapies.

1.4.2 DIAGNOSTIC CRITERIA

In recent years, the way of diagnosing Alzheimer's disease has evolved considerably from a purely clinical view to an integrated approach that includes biological markers, neuroimaging techniques and functional evaluation (Jack et al., 2011). The first diagnostic criteria, developed in 1984 by the NINCDS-ADRDA group, were based on a rigid clinical pathological model, where the definitive diagnosis could only be made post-mortem (McKhann et al., 1984). Due to the advancement of the scientific field, it has emerged that the correspondence between clinical picture and biological alterations is not always linear: asymptomatic subjects may still show a significant amyloid deposition typical of AD, while other subjects with cognitive symptoms may not exhibit the typical pathology of Alzheimer's (Jack et al., 2011). This observation has led researchers to distinguish between clinical diagnosis, based on observable symptoms, and biological diagnosis, based on the direct identification of neuropathological alterations through biomarkers, even in subjects still asymptomatic (Jack et al., 2018). Traditionally, clinical diagnosis was based on the observation of obvious cognitive and

functional symptoms, assessed by neuropsychological examinations. The biological diagnosis, on the other hand, allows detecting the presence of the disease before the onset of symptoms, through specific markers detected with imaging techniques or analysis of cerebrospinal fluid. This paradigm shift has made it possible to anticipate the diagnosis, making thus enabling to identify those at risk and to intervene early.

The expansion of neuropathological knowledge and the availability of biomarkers led the National Institute on Aging (NIA) and the Alzheimer's Association (AA) to propose a first revision of the criteria in 2011 (Jack et al., 2011), followed by a substantial update in 2018, where Alzheimer's disease is defined according to a biological structure based on three categories: A (amyloid), T (tau), N (neurodegeneration) (Jack et al., 2018). Specifically, category A refers to the presence of amyloid-beta substance detectable via PET or through the assay of the A β 42 peptide in cerebrospinal fluid; the T refers to the presence of phosphorylated tau protein, which can also be measured by cerebrospinal fluid analysis or PET imaging; while N, or neurodegeneration, is observed through markers such as hippocampal atrophy on structural MRI or hypometabolism on FDG-PET (Jack et al., 2018). This system, known as AT(N), represented a fundamental change: it not only allows the disease to be identified even in asymptomatic individuals, but above all offers a more solid basis for differential diagnosis, distinguishing Alzheimer's more precisely from other forms of dementia and prodromal conditions such as Mild Cognitive Impairment (MCI) (Jack et al., 2024). For example, a subject who is positive for A and T but does not yet show obvious signs of neurodegeneration (N-), could be in an amnesic stage of MCI at evolutionary risk, while the addition of N+ would strengthen the hypothesis of concluded Alzheimer.

In parallel with the evolution of diagnostic criteria, research has moved towards the evaluation of less invasive peripheral biomarkers. Although cerebrospinal fluid (CSF) and PET examinations provide excellent diagnostic accuracy, their use in clinical practice is still limited by high costs and logistical difficulties (O'Brien JT et al., 2015). For this reason, in recent years there has been increased interest in blood biomarkers such as the A β 42/A β 40 ratio, plasma phosphorylated tau (p-tau), neurofilament light chain (NfL) and β secretase activity (BACE1) (Zetterberg H et al., 2013; Hampel et al., 2018). The combined use of these markers, together with clinical and neuropsychological evaluation, could represent a breakthrough in the early identification and monitoring of disease progression, especially in general medicine or prevention settings.

1.5 OVERVIEW: OTHER DEMENTIAS

As described above, Mild Cognitive Impairment (MCI), the prodromal state of dementia, can be either mnesic or non-mnesic. Mnesic MCI is more likely to evolve into Alzheimer's disease, while the non-mnesic form may anticipate other dementias such as vascular dementia, Lewy body dementia and frontotemporal dementia. These conditions, which occur frequently in old age, can coexist in the same individual, generating mixed clinical pictures (Gale et al., 2018). As mentioned above, the prodromal state of dementia, defined as Mild Cognitive Impairment (MCI), can manifest itself in a mnesic or non-mnesic form, with a different profile of evolution. The mnesic form is most likely associated with progression to Alzheimer's disease, while the non-mnesic form may precede other forms of dementia. In this context, it is useful to distinguish between primary dementias, which include Alzheimer's (AD), Dementia with Lewy Bodies (DLB) and frontotemporal dementia (FTD), and secondary dementias, such as vascular dementia. Alzheimer's represents about 50-60% of dementia cases and is characterized by insidious onset, episodic memory deficit and progressive global cognitive deterioration (McKhann et al., 2011). Lewy body dementia is the second most common cause and is characterized by marked cognitive fluctuations, recurrent visual hallucinations and early extrapyramidal signs, sharing many clinical and neuropathological features with Parkinson's Disease (McKeith et al., 2017). Frontotemporal dementias, more frequent in younger, presenile individuals, are heterogeneous both for clinical manifestations and for pathogenic mechanisms, and include variants with predominance of behavior and forms with primary language disorder. At the pathological level, severe atrophy of the frontal and temporal lobes is observed, involving the insular cortex (Rascovsky et al., 2011). Vascular dementia, on the other hand, cannot be classified as neurodegenerative because it arises from ischemic or hemorrhagic cerebrovascular events that compromise the integrity of brain tissue. It can occur as a result of a single acute event, multiple infarctions or a chronic process related to small vessel disease, and often coexists with other neurodegenerative diseases, generating mixed clinical pictures (O'Brien & Thomas, 2015). The growing complexity of clinical profiles, combined with the heterogeneity of underlying etiopathogenic mechanisms, requires an increasingly integrated and personalized diagnostic approach. In a clinical landscape oriented towards early diagnosis and precision medicine, a clear distinction between the different forms of dementia is a key element to improve diagnostic accuracy, optimize therapeutic strategies and plan a care path adapted to the specific characteristics of the patient (Gale et al., 2018).

1.6 DEMENTIA AND SUSTAINABILITY OF THE NATIONAL HEALTH SYSTEM: AN INCREASING WEIGHT

The increasing aging of the population and the consequent increase in the prevalence of neurocognitive pathologies, determine an increasing pressure on the National Health System (NHS). If in 2019 over 57 million people worldwide were living with a diagnosis of dementia, by 2050, according to the Global Burden of Disease Study, 152 million will be reached (GBD 2019 Dementia Forecasting Collaborators, 2022). This demographic-health trajectory is likely to result in a significant economic impact, both for public healthcare facilities and for the family care fabric especially regarding AD which represents 50% of dementia. Due to a modelling study, Velandia et al in 2022, estimated that the global health expenditure directly attributable to dementia amounted to 263 billion dollars in 2019, a figure that is expected to increase since the average annual growth in 2000 was 4.5%. It can be estimated that by 2050, health expenditure on dementia could reach 1600 billion dollars corresponding to 11% of the world's health expenditure and, in scenarios with high intensity of treatment, up to 17%. Although home care is still the predominant modality, 80% of the total expenditure is absorbed by residential services, which are significantly more expensive than community-based services. These data highlight the urgent need for health policies that encourage sustainable home-based care models, in line with the World Health Organization's Global Action Plan on the Public Health Response to Dementia 2017-2025 (WHO, 2017). In the Italian context, where the incidence of Alzheimer's and dementia is constantly increasing and the population over 80 accounts for a growing share of the total, NHS faces a structural sustainability challenge. The high rate of underdiagnosis, estimated at up to 50% in early stages, the chronicity of clinical evolution and the constant increase in long-term care demand make strategic medium- to long-term planning essential (Bruno G et al., 2018; WHO, 2021). In the absence of curative treatments, the economic burden is determined not only by direct costs related to diagnosis, drug management and health facilities, as well as by indirect costs such as loss of productivity, Unpaid informal care and the psychological burden on caregivers (Wimo et al., 2017). These burdens, often invisible in the official budgets, constitute a substantial component of the overall socio-economic weight of dementia, and place the issue of its sustainability at the forefront of the national health debate. Current projections show that, in high-income countries, per capita expenditure on dementia exceeds \$180 a year, while in low-income countries it is less than \$1. However, it is in middle-income settings, including Italy, that we observe the fastest growth, driven by an aging population and improved diagnostic capabilities (Velandia et al., 2022). For the NHS, this translates into a progressive pressure which, if not addressed with structural interventions, appropriate investments and long-term strategies, risks compromising equity, The quality and sustainability of care services for people with dementia. In the light of these considerations, it is clear that the strengthening of primary and secondary prevention policies,

focusing on the control of modifiable risk factors and early diagnosis, It is an essential lever not only to contain the impact of dementia on public health, as well as to ensure the long-term sustainability of the system. Due to the numerous studies carried out on the human brain in recent decades, we now know that this complex organ is composed of about 100 billion neurons and over 100 trillion synapses, through which travel signals that constitute the cellular basis of memories, emotions, thoughts, feelings, movements and abilities (Pacitti et al., 2019). Neurotrophins, a family of proteins essential for growth, survival, differentiation and neuronal plasticity, play a key role in these processes. These molecules regulate both the development of the nervous system during embryogenesis and the maintenance of its structural and functional integrity in adulthood, acting at both central (CNS) and peripheral (PNS) levels (Skaper, 2018).

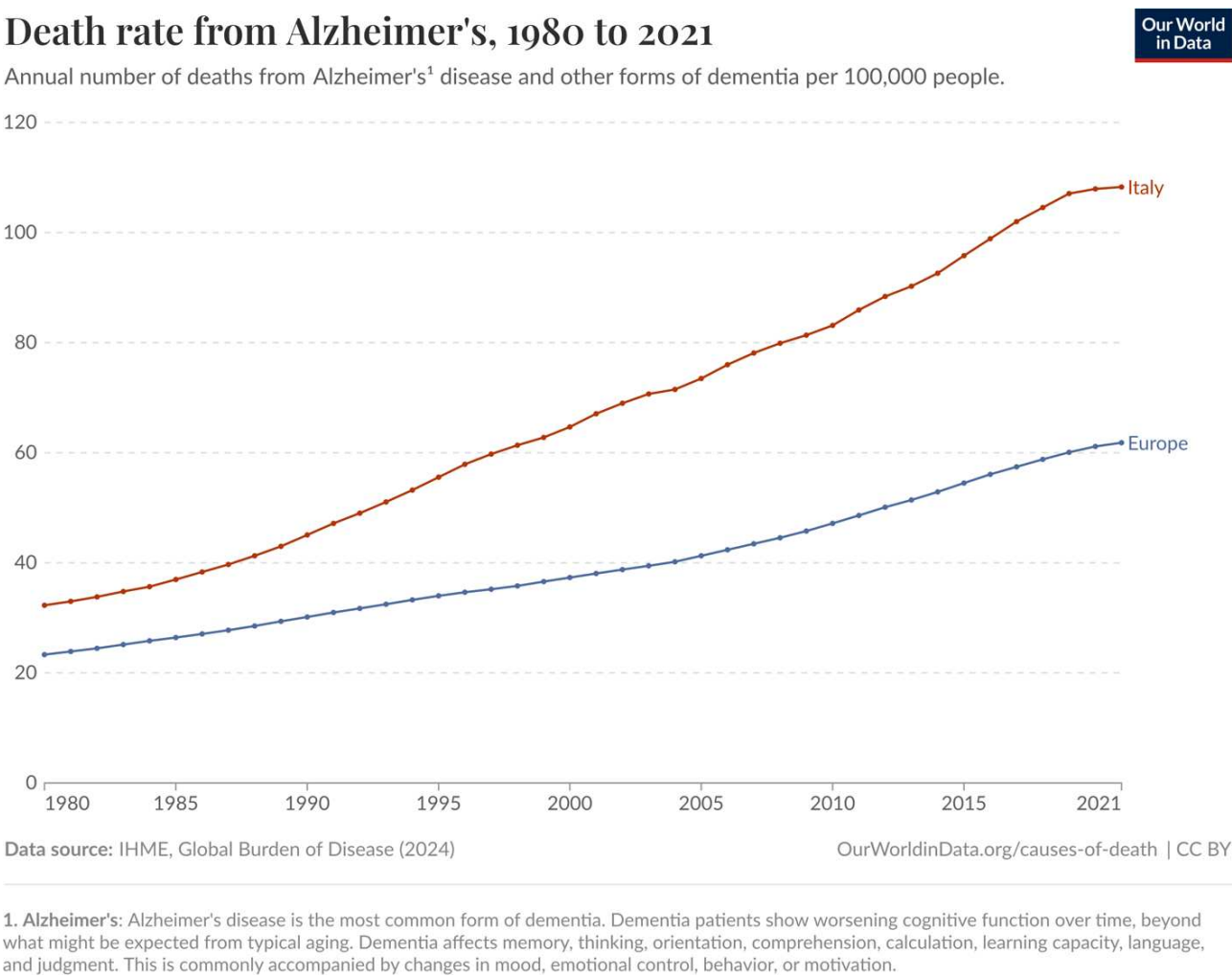


FIG.1 Death rate from Alzheimer’s disease in Italy and Europe between 1980 and 2021 (per 100,000 inhabitants). The graph illustrates a continuous and significant increase in mortality due to Alzheimer’s and other forms of dementia across the decades. In particular, Italy shows a steeper rise compared to the European average: from approximately 32 deaths per 100,000 people in 1980 to over 110 in 2021. In contrast, the European average increased more moderately, reaching about 60 per 100,000. This divergence highlights the growing impact of Alzheimer’s in Italy, likely influenced by demographic aging, improved diagnostic capabilities, and national reporting differences. The trend underscores the urgency of implementing preventive strategies and long-term care models, especially in countries facing accelerated population aging (https://ourworldindata.org/grapher/death-rate-from-alzheimers-other-dementias-gbd?tab=chart&country=OWID_EUR~ITA)

2. NEUROTROFINS: GENERAL STRUCTURE AND FUNCTIONS

The pathogenic mechanisms underlying Mild Cognitive Impairment (MCI) and its progression to dementia are complex and multifactorial. A central element in these processes is neuroplasticity, defined as the brain's capacity to reorganize itself by forming new neural connections. A key family of proteins, known as neurotrophins, plays an essential role in regulating neuronal growth, survival, and plasticity. Understanding their function provides a crucial link between the biological mechanisms of aging and potential intervention strategies. The first neurotrophin identified was the Nerve Growth Factor (NGF), discovered and characterized in the early 1950s by Rita Levi-Montalcini and Stanley Cohen, in one of the fundamental experiments of modern neurobiology (Levi-Montalcini, 2000). NGF plays a key role in the regulation of neuronal differentiation and modulation of the release of neurotransmitters such as acetylcholine, which is essential for the maintenance of the cholinergic neurons of the basal forebrain, involved in learning and memory processes (Mufson et al., 2019). In addition to NGF, other neurotrophins play specific and complementary roles. In 1989, Yves-Alain Barde and Hans Thoenen isolated the Brain-Derived Neurotrophic Factor (BDNF).

This important neurotrophin is particularly abundant in the CNS, with high expression in structures such as the hippocampus, prefrontal cortex and thalamus. It is involved in the regulation of synaptic plasticity, neurogenesis and long-term potentiation mechanisms (LTP), fundamental for learning and mnemonic consolidation (Lu et al., 2014; Allen et al., 2013). Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5), on the other hand, are involved in the development of spinal connections and the regulation of myelination (Skaper, 2018). The neurotrophins exert their biological action through interaction with specific receptors of the cell surface: in particular, the tyrosine-kinase receptor family Trk (TrkA, TrkB and TrkC) and the common receptor p75^{NTR}. Trk receptor binding activates intracellular pathways such as Ras/MAPK, PI3K/Akt and PLC γ , promoting neuronal survival, axonal growth and synaptic plasticity. In contrast, stimulation of p75^{NTR}, in the absence of concomitant trophic signals, can induce apoptosis and dendritic remodeling (Allen et al., 2013; Kowianski et al., 2018). The expression of neurotrophins is finely regulated by environmental and behavioral factors. Physical activity, nutrition, sleep, cognitive stimuli and stress levels can modulate gene expression and the release of neurotrophins, in particular BDNF, demonstrating the close interaction between experience and brain plasticity (Marosi et al., 2014). Not surprisingly, alterations in the levels of these molecules have been found in numerous pathological conditions, including neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD), as well as major psychiatric disorders, including depression, anxiety and schizophrenia (Castrén & Monteggia, 2017). To date, neurotrophins therefore play a key role in understanding the cellular mechanisms underlying cognitive function and neurological and psychiatric vulnerability. Among these, the Brain-Derived Neurotrophic Factor (BDNF) is distinguished by its wide involvement in the processes of synaptic

plasticity, memory and circadian regulation, key elements in the context of neurocognitive alterations such as Mild Cognitive Impairment.

2.1 BRAIN DERIVED NEUROTROPHIC FACTOR

BDNF is a neurotrophine crucial for neuronal survival, synaptic plasticity and modularity of synaptic efficacy, it is therefore involved in the brain development and cognitive processes of adults (Lu et al., 2014). It is particularly expressed in the hippocampus, prefrontal cortex and thalamus, regions used for cognition and emotional regulation. BDNF expression is particularly high in brain regions key to cognition and emotional regulation, such as the hippocampus, prefrontal cortex and thalamus.

At the molecular level, BDNF is first synthesized as pre-proBDNF in the endoplasmic reticulum subsequently translocated to the Golgi complex where it is converted into the precursor form proBDNF. Following specific cellular signals, proBDNF may be further split into the mature biologically active form (mBDNF) (Mowla et al., 2001; Mizui et al., 2016). This maturation process can occur through two distinct mechanisms: constitutive release and activity-dependent release.

In the constitutive release, the proBDNF cleavage occurs at the intracellular level, in the trans-Golgi network, by furin, or within secretory vesicles by regulated convertases. This mechanism ensures a continuous supply of BDNF and is crucial for basal neuronal survival (Lu et al., 2005; Foltran & Diaz, 2016). In activity-dependent release, proBDNF is secreted into the extracellular space and converted to mBDNF there by the action of plasmin activated by tissue plasminogen activator (tPA), or of the extracellular matrix metalloproteases, in particular MMP-2 and MMP-9 (Pang et al., 2004; Hwang et al., 2005; Huntley GW et al., 2012; Vafadari et al., 2016; Mizui et al., 2016).

The two BDNF isoforms have different functions; mBDNF binds to the tyrosine-kinase TrkB receptor, activating intracellular signaling pathways such as PI3K/Akt, MAPK/ERK and PLC γ , which promote neuronal survival, axonal growth and long-term enhancement (LTP) (Reichardt, 2006; Allen et al., 2013). In contrast, proBDNF interacts preferentially with the p75 NTR receptor, promoting long-term synaptic depression (LTD) and, in some contexts, cell apoptosis.

The BDNF gene is highly regulated and has a complex structure consisting of 11 exons and at least 9 functional promoters, which respond to different physiological stimuli including synaptic activity, calcium input and oxidative stress (Lu B et al., 2014). The expression of BDNF is activity-dependent: learning and intense synaptic activity activate the promoter associated with exon III through the transcription factor CREB, thus increasing local synthesis of BDNF (Lu, B et al., 2003). In addition, BDNF mRNA can be transported into dendrites and translated locally into active synapses, in line with the "synaptic tagging" mechanism, which allows for the synaptic selectivity of plasticity (Lu, B et al., 2003). Another clinically relevant element is the polymorphism Val66Met, which alters vesicular transport and regulated secretion of BDNF, resulting in a reduction in hippocampal volume and episodic memory impairment, even in healthy subjects (Castrén & Monteggia, 2017).

2.2 ROLE OF BDNF IN MCI AND AD

BDNF regulates neuroplasticity, neuronal survival and synaptic transmission playing a key role in the cognitive process (Lu et al., 2014). Its action, in fact, is more pronounced in the brain areas related to memory and learning, such as the hippocampus and prefrontal cortex. The BDNF gene is highly regulated but the regulatory system is extremely sensitive to conditions of oxidative stress, systemic inflammation and sleep deprivation, Frequently observed factors, in the context of age-related cognitive decline and neurodegenerative diseases (Miranda M et al., 2019; Angelucci et al., 2010). Common in elderly people and patients with cognitive impairment, interfere with BDNF promoter activation mediated by the CREB (cyclic AMP response element-binding protein), reducing its expression and altering the hippocampal synaptic circuits (Miranda M et al., 2019; Angelucci et al., 2010; Leßmann & Brigadski, 2009). This reduction contributes to early synaptopathy, which precedes and accompanies neurodegeneration. In the context of Mild Cognitive Impairment (MCI) and Alzheimer's disease (AD), numerous studies have documented reduced levels of BDNF, both in brain tissue and peripheral fluids. Kim et al. (2017), in a meta-analysis of over 3,000 subjects, observed that in patients with AD, the peripheral levels of BDNF are significantly lower than controls, Whereas in subjects with MCI there is a variability related to the stage and the probability of progression. In particular, in subjects with MCI that later evolve into Alzheimer's, peripheral levels of BDNF are significantly reduced, suggesting the possibility of using this neurotrophine as a predictive biomarker for conversion in the early stages of neurodegeneration (Kim et al., 2017). From the functional point of view, this reduction is associated with impairment of synaptic plasticity, lower dendritic spine density and altered long-term potentiation (LTP), as demonstrated in mouse models with dysfunction of the SIRT1-BDNF axis, in which the recovery of BDNF expression restores neuronal activity and improves cognitive performance (Wu et al., 2024). In addition, functional polymorphisms of the BDNF gene, such as Val66Met, are associated with deficits in mBDNF secretion and reduced activation of TrkB, factors that increase vulnerability to cognitive decline even in healthy subjects (Lim YY et al., 2013; Egan et al., 2003). In parallel, non-pharmacological therapeutic approaches such as multi-component exercise have been shown to be effective in increasing BDNF levels in patients with MCI and mild dementia. Wang et al. (2020) reported, through a systematic review, that such interventions are associated with an increase in BDNF and significant improvements in executive function and attention. Overall, the available data suggest that a reduction in BDNF levels, central to maintaining synaptic plasticity and hippocampal function, is a recurrent feature in patients with MCI and AD. The plasma and serum variations of neurotrophine are related to cognitive impairment and, in some cases, to the likelihood of conversion from MCI to dementia, highlighting the potential of BDNF as a biological indicator of pathological progression.

2.3 CIRCADIAN RHYTHM AND SLEEP: BDNF IMPLICATION

Every living being has a circadian rhythm, an internal physiological cycle that repeats approximately every 24 hours and regulates vital activities such as sleep, hormonal secretion, body temperature, metabolism, and blood pressure (Xu et al., 2005; Takahashi JS., 2017). In humans, the main regulatory center of this system is the suprachiasmatic nucleus (SCN), a group of neurons located in the anterior hypothalamus, just above the optic chiasma. This central pacemaker coordinates the circadian rhythm through environmental signals, mainly light, received by the retina via a subpopulation of ganglion cells containing melanopsin (melanopsin-expressing retinal ganglion cells, mRGCs), which transmit light information to the Suprachiasmatic Nucleus (SCN) through the retinohypothalamic tract (Scammell TE et al., 2017; Herzog et al., 2017). At the molecular level, the generation of circadian rhythm is based on a self-regulating transcriptional and translational feedback (TTFL) circuit. The CLOCK-BMAL1 heterodimeric complex activates the transcription of the Period (PER1/2) and Cryptochrome (CRY1/2) genes, which encode proteins capable of forming repressive complexes. Once accumulated in the cytoplasm and translocated in the nucleus, PER and CRY proteins inhibit the activity of the CLOCK-BMAL1 complex, closing the transcriptional cycle with a periodicity of about 24 hours (Panda, 2016).

The synchronization between the central clock (SCN) and the peripheral clocks in almost all cells of the organism takes place through neural, hormonal and metabolic signals. This integration ensures the temporal consistency of biological functions at a systemic level (Herzog et al., 2017). A crucial role, among the functions regulated by the circadian system, is played by sleep. In response to the dark, the pineal gland secretes melatonin which promotes relaxation and sleep. Sleep is a physiological mechanism that is divided into two main phases, the REM phase (Rapid Eye Movement) and the no-REM phase (NREM), which alternate cyclically in ultraadian sequences of 90 minutes (Meyer et al., 2022; Casagrande et al., 2022). These phases are characterized by 4 types of waves, first high frequency then low frequency. Literature studies have shown that during NREM sleep, particularly in deep phases characterized by low frequency delta waves (slow-wave activity, SWA), significant memory consolidation and synaptic maturation is observed (Mason et al., 2021). During the NREM phase, and in particular during slow-wave activity (SWA), synaptic activity is observed which aims at stabilising functional neuronal connections. Preclinical studies have shown that BDNF plays a key role in this process, facilitating the transformation of transient synaptic activity into stable and lasting modifications (Tononi & Cirelli, 2006). In contemporary society, prolonged exposure to light and artificial sources, particularly blue light emitted by electronic devices, together with the increase of stress factors related to lifestyles, contributed to a progressive deterioration of sleep quality in the general population (Brunborg GS et al., 2011). In sleep deprivation conditions, BDNF production is drastically reduced, resulting in impaired synaptic plasticity and cognitive functions, as demonstrated by reduced expression of BDNF mRNA and deficit in memory

consolidation processes (Vecsey et al., 2009). This relationship is particularly evident in the context of chronic stress and insomnia, where there is a deregulation of the hypothalamo-pituitary-surrene axis (HPA) with a consequent increase in cortisol and a reduction in serum levels of BDNF, both in animal models and in patients with insomnia (Ditmer M et al., 2023). BDNF is also a key mediator between synaptic activities induced by wakefulness and the subsequent homeostatic response of sleep: the degree of expression of BDNF during wakefulness is directly related to the intensity of slow-wave activity (SWA) observed in the NREM phase, suggesting that BDNF contributes to the regulation of "sleep pressure" (Huber et al., 2007). Genetic studies indicate that the polymorphisms of the BDNF gene Val66Met also affect sleep architecture: Met variant carriers show a significantly altered SWA compared to Val/Val homozygotes, both at baseline and after sleep deprivation (Bachmann et al., 2012). Finally, the most recent results support a biphasic model of stress response on BDNF: whereas acute stress or short-term deprivation may induce a compensatory increase in BDNF, chronic stress and persistent sleep loss lead to a marked reduction in its levels, with long-term negative effects on mental and cognitive health (Duman RS et al., 2006).

2.4 SLEEP DISORDERS IN THE MCI

During the aging process, a physiological alteration of the circadian rhythm occurs, resulting in fragmentation and reduction of sleep efficiency (Naismith et al., 2010). As described in the previous paragraph, slow-wave activity (SWA) represents the typical electrophysiological manifestation of the slow-wave sleep phase (SWS), and is commonly used as an index of sleep depth and homeostatic pressure (Riedner BA et al., 2007). In particular, it has been shown that slow wave sleep (SWS), which is essential for the consolidation of memory and synaptic plasticity, tends to decrease significantly after age 60, almost disappearing (Carskadon & Dement, 2011). Numerous evidence suggest that sleep quality is closely related to cognitive function in the elderly, and in particular to the diagnosis of Mild Cognitive Impairment (MCI). The study by Rodrigues et al. (2022), conducted on a sample of 289 uninstitutionalized elderly subjects (41.5% with MCI), showed through path analysis that poor sleep quality is significantly associated with a higher probability of receiving a diagnosis of MCI. Winer JR et al. (2019) have also shown that a selective slow wave sleep reduction is already observable in MCI, suggesting an early involvement of homeostatic sleep mechanisms in cognitive impairment. In recent years, the role of SWS has also attracted increasing attention for its function in the clearance of amyloid beta ($A\beta$). Studies on animal and human models have shown that increased deep sleep is associated with reduced levels of $A\beta$ in the interstitial and cerebrospinal fluid, while sleep deprivation (both acute and chronic) increases amyloid load (Kang JE et al., 2009; Shokri- Kojori E et al., 2018). Significantly higher levels of cerebral $A\beta$ were found in elderly subjects with shorter sleep duration and subjectively worse quality (Spira AP et al., 2013), suggesting that sleep represents an active modulator in the pathophysiology of Alzheimer's disease. The relationship between sleep and

A β appears to be bidirectional: the accumulation of A β may in turn impair sleep quality by reducing time spent in NREM and REM phases, as observed in mouse models with overproduction of APP/A β (Roh JH et al., 2012). The interruption of SWS is associated with an increased expression of A β , while the loss of diurnal fluctuation of A β levels is considered to be an early signal of glioblastic system dysfunction, a brain clearance system that uses perivascular channels to remove waste products, including A β , mainly during deep sleep (Xie et al., 2013). Alterations of the SWS have also been observed in subjects with Subjective Cognitive Decline (SCD), that is those who perceive a cognitive decline even in the absence of objective deficits. Jo KJ et al., in 2024 showed that, subjects with A β -positive SCD have a significant reduction of time spent in SWS compared to A β -negative subjects, confirming the early vulnerability of deep sleep to early neurodegenerative processes. Overall, these data reinforce the hypothesis that SWS is a critical stage in slowing cognitive decline by acting as a biological barrier against toxic accumulation of misfolded proteins. Maintaining a healthy sleep architecture, in particular the SWS, could therefore be an important protective factor in preventing MCI and Alzheimer's, emphasizing the value of sleep as a modifiable risk factor within multidimensional preventive strategies (Livingston G et al., 2020; Ngandu T et al., 2015).

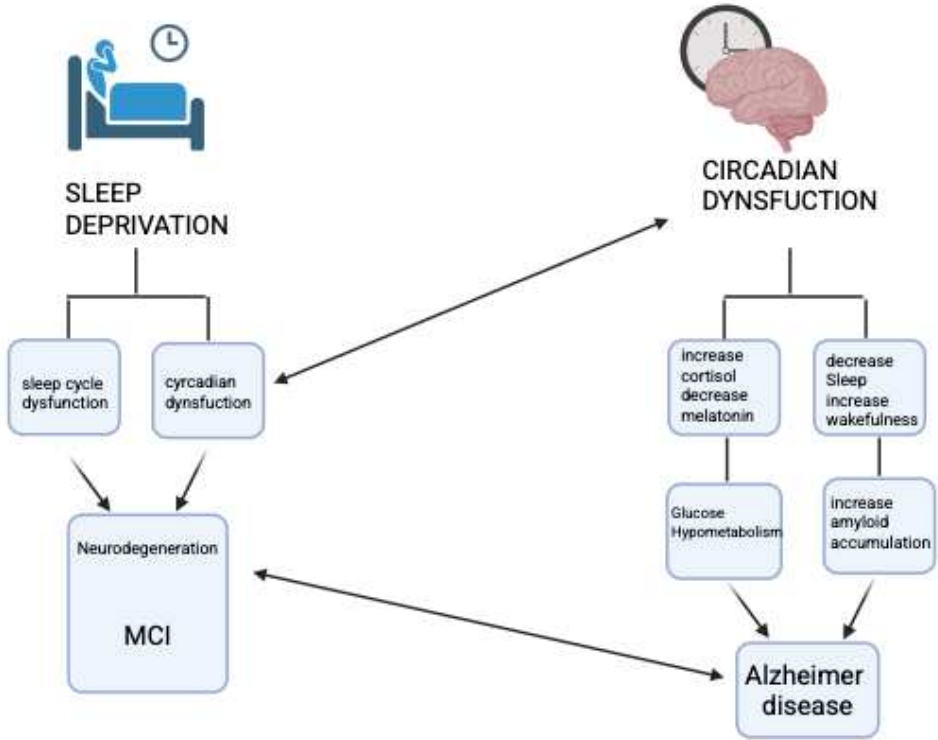


FIG.2 The diagram illustrates the connection between sleep deprivation, circadian dysfunction, and the development of Alzheimer's disease. Sleep deprivation leads to disruptions in the sleep cycle and circadian rhythms, contributing to neurodegeneration. Circadian dysfunction, characterized by increased cortisol, decreased melatonin, reduced sleep, and heightened wakefulness, results in glucose hypometabolism and increased amyloid accumulation, both of which are linked to Alzheimer's pathology. This visual highlights the critical role of sleep and circadian regulation in neurodegenerative processes

3. MEDIUM-CHAIN TRIGLYCERIDES

Medium chain triglycerides (MCTs) consist of a glycerol molecule esterified with three medium chain fatty acids (MCFA), characterized by hydrocarbon chains with a length between 6 and 12 carbon atoms (St-Onge MP et al., 2002). The most representative MCFA in MCTs include caproic acid (C6), caprylic acid (C8), capric acid (C10) and lauric acid (C12) (Heydinger & Galante, 2020). Compared to long-chain triglycerides (LCTs), MCTs show higher water solubility, lower molecular weight and better fluidity at body temperature, characteristics that facilitate absorption and digestion. At the intestinal level, MCTs are rapidly hydrolysed by gastric and pancreatic lipase, without the need for preliminary emulsification by bile salts, unlike LCTs (Bach & Babayan, 1982). The released MCFAs are absorbed by the intestinal mucosa and transported directly to the liver through the portal vein. This bypass of the lymphatic system accelerates the metabolic availability of MCTs. Within hepatocytes, MCFAs enter the mitochondria without requiring carnitine-dependent transport, making their β -oxidation extremely rapid and efficient (McCarty & DiNicolantonio, 2016). Hepatic catabolism of MCTs leads to the production of ketone bodies, including β -hydroxybutyrate, acetoacetate and acetone, which can act as an alternative energy substrate for the brain, heart and skeletal muscle. This process is particularly relevant in conditions of low carbohydrate brain metabolism, such as those observed early on in subjects with MCI and Alzheimer's (Cunnane et al., 2016). Unlike LCTs, MCTs are poorly re-esterified in triglycerides and tend not to accumulate in adipose deposits, resulting in less lipogenic (McCarty & DiNicolantonio, 2016). In addition to the energy role, some MCFA show complementary biological properties. It has been observed that lauric acid and its derivatives, such as monolaurin, possess antimicrobial activity and may affect the intestinal microbiota (Wang Yet al., 2022). In addition, recent studies suggest that MCTs may improve mitochondrial function and contribute to the reduction of oxidative stress and systemic inflammation, which are relevant factors in the pathophysiology of neurodegenerative diseases (Neth et al., 2020).

Overall, MCTs can be said to represent a lipid source that is metabolized in an atypical way compared to other dietary fats. Their rapid conversion into ketone bodies and limited tissue deposition make them particularly attractive for nutritional and clinical applications, In particular in the conditions associated with cellular energy dysfunction such as cerebral hypometabolism in MCI and AD conditions (Fortier et al., 2021).

3.1 GLUCOSE HYPOMETABOLISM IN NEURODEGENERATION

The adult human brain is made up of about 100 billion neurons, each with long branches forming synaptic connections with other neurons. These synapses allow rapid transmission of signals and enable the formation of functional circuits which form the cellular basis for memory, thought, sensory perceptions, emotions, movements and learned skills. Although the brain only accounts for 2% of total body weight, it consumes about 20% of the body's metabolic energy (Raichle, M. E., & Gusnard, D. A. 2002). This energy is critical for processes such as nerve impulse transmission, protein synthesis and maintenance of membrane potential (Oyarzabal et al., 2019; Falkowska A et al., 2015). Glucose is the main energy substrate of the brain (Mergenthaler et al., 2013). It crosses the blood-brain barrier (BBB) via specific transporters such as GLUT1 and GLUT3. Once entered the brain, glucose is metabolized through glycolysis, with production of ATP, which represents the main source of energy for neuronal activity (Burillo et al., 2021). In many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease and epilepsy, there is a brain-level insulin resistance condition. This resistance compromises the use of glucose at the neuronal level (Adlimoghaddam et al., 2019; Castellano et al., 2017). One of the main consequences is the increase in activity of glycogen synthase kinase 3 β (GSK-3 β), an enzyme involved in tau protein phosphorylation, which under insulin resistance conditions becomes hyperphosphorylated (Lauretti et al., 2020). Under physiological conditions, tau contributes to microtubule stabilization; if hyperphosphorylated, it alters the organization of the cytoskeleton, favoring synaptic disconnection. In addition, the accumulation of hyperphosphorylated tau can be aggravated by an altered function of the autophagic-lysosomal system, responsible for its degradation (Hamano et al., 2008). Similar mechanisms also influence the metabolism of the amyloid precursor protein, leading to the accumulation of β -amyloid (Vidoni et al., 2016). These neurotoxic aggregates activate the immune response of the brain, especially the microglia, which tries to eliminate them. However, chronic stimulation of the microglia can induce neuroinflammation and brain atrophy (Hansen et al., 2018). In summary, the alteration of carbohydrate and protein metabolism leads to the accumulation of toxic aggregates, which increase oxidative stress and inflammation, compromising the homeostasis of nervous tissue. Hypometabolism of carbohydrates in Alzheimer's disease has been extensively documented by imaging techniques such as 18FDG- fluorodeoxyglucose (FDG-PET) (Drzezga A et al., 2008). As mentioned above, MCI represents a prodromal stage of Alzheimer's disease. Although patients remain autonomous in daily activities, neuropsychological tests show a clear decline in specific cognitive domains. Studies with FDG-PET have shown that, even in the early stages of MCI, the cerebral metabolism of glucose is already impaired (Morbelli S et al., 2015), particularly at the level of the medial temporal region (including the hippocampus), the entorhinal cortex and the precuneus (Mosconi et al., 2010).

3.2 WARBURG EFFECT AND ALZHEIMER

Glucose metabolism is the main mechanism by which eukaryotic cells generate energy. Glucose is initially processed in the cytosol by glycolysis, a universal metabolic pathway divided into ten enzymatic reactions leading to the formation of two pyruvate molecules, with a net yield of two ATP and two NADH per glucose molecule (Mergenthaler et al., 2013). Under normal conditions, pyruvate enters the mitochondria where it is converted into acetyl-CoA, entering the Krebs cycle and feeding the electron transport chain. This process, known as oxidative phosphorylation (OXPHOS), can produce up to 30-32 ATP molecules, making it the most efficient mechanism for generating energy (Papaneophytou, 2024).

However, some cells, such as erythrocytes, activated immune cells and especially cancer cells, show a preference for glycolysis even in the presence of oxygen, converting pyruvate into lactate through lactate dehydrogenase A (LDH-A). This phenomenon, known as the Warburg effect or aerobic glycolysis, enables rapid ATP production and maintenance of the redox balance through the regeneration of NAD⁺, also favouring the availability of metabolic intermediates for biosynthesis (Warburg, 1956; Vander Heiden et al., 2009; Liberti & Locasale, 2016). The Warburg effect is now recognized as a strategic adaptation in cellular contexts characterized by high proliferation, hypoxia or oxidative stress, also manifesting itself in non-cancerous cells under specific conditions (Abdel-Haleem et al., 2020; Barros et al., 2020).

While in the oncologic context aerobic glycolysis is functional to proliferation, in the neurodegenerative field we observe an opposite adaptation, which reflects a different balance between energy demand and oxidative capacity, called inverse Warburg effect. According to the entropic hypothesis, Alzheimer's involves a negative selection of neurons with high synaptic and metabolic efficiency, with the progressive survival of cells less performing but more resistant to stress (Demetrius & Simon, 2012; Demetrius et al., 2015). This compensatory mechanism, while it allows neurons to maintain energy homeostasis, amplifies the production of ROS and oxidative stress, contributing to neuronal degeneration (Burillo et al., 2021; Neth et al., 2020). Neuroimaging studies with FDG-PET have shown the presence of a hypometabolism of glucose already in the early stages of AD and Mild Cognitive Impairment (MCI), with an early involvement of the hippocampus, precuneus and entorhinal cortex (Mosconi et al., 2010; Morbelli et al., 2015). This pattern is consistent with a reduced efficiency of glucose transport and utilization at the neuronal level, aggravated by the dysfunction of the ANLS mechanism (astrocyte-neuron lactate shuttle), which under normal conditions provides lactate to neurons as an alternative energy substrate (Bélanger et al., 2011; Cunnane et al., 2020). The resulting lack of energy substrates and accumulation of mitochondrial damage induce a selective change in neuronal metabolic order. The dysfunctional neurons that survive because of OXPHOS upregulation become dominant, progressively contributing to the loss of highly functional neurons and synaptic impairment (Demetrius et al., 2015). In parallel, the energy imbalance

promotes the accumulation of β -amyloid and hyperphosphorylated tau, feeding a vicious circle involving neuroinflammation, oxidative stress and degeneration (Lauretti et al., 2020).

In summary, the paradigm of the inverse Warburg effect proposes an alternative reading of the physiopathology of AD, shifting attention from protein alterations to the impairment of brain bioenergetic circuits. This perspective opens new therapeutic scenarios based on the restoration of neuronal metabolism, such as the use of alternative substrates (e.g. ketones), mitochondrial support and glial metabolism modulation (Fortier et al., 2021).

3.3 EFFECTS OF THE WESTERN DIET ON ALZHEIMER'S PATHOGENESIS

In recent decades, the Western Diet (WD) has spread globally, becoming one of the predominant food models in industrialised countries. This diet is characterized by a high intake of simple sugars, saturated fats, refined carbohydrates, salt and ultra-processed foods, often low in fiber, antioxidants and essential micronutrients (Imamura F et al., 2010). Growing scientific evidence suggests that the Western Diet not only promotes metabolic conditions such as obesity, type 2 diabetes and metabolic syndrome, as well as acts as a potent low-intensity chronic systemic inflammation activator (inflammaging) (Więckowska-Gacek et al., 2021). This prolonged inflammatory state is a common pathogenic factor in many chronic-degenerative diseases, including neurodegenerative disorders. In particular, it has been shown that the Western Diet is associated with an increased risk of developing Alzheimer's disease (AD), acting through mechanisms involving metabolic alterations, neuroinflammation and dysfunctions of the gut-brain axis (Więckowska-Gacek et al., 2021; Dissanayaka et al., 2024). In addition to promoting the accumulation of amyloid and hyperphosphorylated tau plaques, a diet rich in processed foods and simple sugars was significantly correlated with an increase in the incidence of Mild Cognitive Impairment (MCI), especially in people with metabolic disorders such as type 2 diabetes (Shengdan Pu et al., 2024). WD alters the composition of bacterial flora, causing dysbiosis, increased intestinal permeability and systemic passage of toxic metabolites and pro-inflammatory cytokines, which can cross the blood-brain barrier (BBB) and trigger inflammatory responses at the brain level (Dissanayaka et al., 2024). In particular, there is a reduction of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Eubacterium*, and an increase of pro-inflammatory strains such as *Escherichia/Shigella* and *Alistipes* spp., related to markers of cerebral amyloidosis and cognitive alterations (Cattaneo et al., 2017; Vogt et al., 2017). The alteration of the intestinal microbiota linked to the western diet affects the production of short-chain fatty acids (SCFAs) and neurotransmitters such as GABA, disrupting the brain's neurochemical balance. In addition, the reduction of SCFAs, especially butyrate, compromises the health of the intestinal barrier and immune regulation, aggravating neuroinflammation (Sun et al., 2019). Recent studies show that alternative diets such as the Mediterranean or ketogenic diet, based on fiber,

unsaturated fats and polyphenols, are associated with increased microbial diversity, reduction of systemic inflammation and protection against cognitive decline (Dissanayaka et al., 2024; Fortier et al., 2021).

4. MATERIALS AND METHODS

In clinical practice, patients with suspected mild cognitive impairment (MCI) access specialist services following a referral by the general practitioner or after an initial assessment by the geriatrician or neurologist. Once the clinical suspicion has been detected, the patient is taken in by a multidisciplinary team specialised in cognitive impairment assessment, typically composed of neurologists, geriatricians, neuropsychologists and, when necessary, clinical nutritionists. The diagnostic path involves a detailed anamnestic and clinical framework, followed by a structured neuropsychological assessment through standardized tests. To support the diagnosis, laboratory tests and instrumental examinations are often required, such as positron emission tomography (PET) for brain metabolism. Management is not limited to the diagnostic aspect, as well as includes longitudinal monitoring of the patient, with personalized interventions aimed at slowing cognitive decline and functional support.

This work is part of the clinical research aimed at evaluating the impact of dietary supplementation with medium-chain triglycerides (MCT) in subjects recently diagnosed with mild cognitive decline (MCI). Patients involved in the study are usually referred to the Centre for Cognitive Disorders and Dementia (CDCD) of the SS Hospital. Antonio, Biagio and Cesare Arrigo from Alessandria, on the recommendation of the general practitioner or after a first geriatric and/or neurological evaluation. Once taken care of, patients follow a multidisciplinary diagnostic and therapeutic path, involving professionals such as neurologists, geriatricians, neuropsychologists and clinical nutritionists, To define the cognitive and metabolic framework and identify a possible personalized approach.

4.1 NUTRITIONAL COUNSELING IN THE PATIENT WITH MCI

Nutritional counselling represents a key moment in the management of patients with MCI, especially in terms of prevention and metabolic support. In line with the literature, the recommended diet is the Mediterranean diet, for its proven anti-inflammatory and neuroprotective properties. Patients undergo a thorough initial dietary assessment that includes: collection of food history using a detailed 24-hour recall method, anthropometric measurements (weight, height, BMI, abdominal circumference), and screening for malnutrition (NRS 2002). The daily energy requirement is estimated using the Harris-Benedict formula. The MedDiet Score is also calculated to assess adherence to the Mediterranean diet. Regarding the Nutrition Risk Screening 2002 (NRS-2002), validated for the identification of

nutritional risk in hospital settings, as well as applicable in outpatient settings in frail or elderly subjects (Kondrup et al., 2003). It evaluates two main components: the patient's nutritional status (weight loss, reduced food intake, BMI) and the severity of the acute or chronic disease, finally integrating an additional point for patients aged over 70 years. A total score of 3 indicates the presence of nutritional risk and justifies the initiation of specific nutritional intervention. In the context of MCI, where cognitive decline may influence self-management of nutrition, early identification of nutritional risk is crucial to avoid metabolic complications, loss of lean mass and worsening of cognitive performance (Rojer et al., 2023; Cederholm et al., 2019).

Another tool used in the nutritional assessment of patients with MCI is the MedDiet Score (MDs), a validated index that measures individual adherence to the Mediterranean dietary model. The score is based on the frequency of consumption of 11 key food groups: fruits, vegetables, legumes, whole grains, fish, olive oil, red meat, dairy products, wine, sweets and sugary drinks (Panagiotakos et al., 2007). For each component, a score of 0 to 5 is given, where the highest scores indicate an intake more in line with the Mediterranean model for healthy foods, or less frequent for those to be restricted. The total score can range from 0 to 55, with higher values associated with a higher degree of adherence. In the context of MCI, increased adherence to the Mediterranean diet has been associated with slower cognitive decline and a lower risk of progression to dementia, presumably because of the combined effect of antioxidant nutrients, mono- and polyunsaturated fatty acids, and bioactive compounds in plant foods (Scarmeas et al., 2006; Petersson & Philippou, 2022).

4.2 FOOD INTEGRATION WITH MCT: PRODUCTS USED

The clinical protocol includes nutritional supplementation with products containing medium-chain triglycerides (MCTs), supplied by Dr. Schär through its specific Kanso line. Dr. Schär is a leading European company in the field of medical and specialised nutrition with long-standing experience in the production of dietary foods for special clinical needs. The Kanso line is specially formulated to support patients with fatty acid oxidation disorders, lipid malabsorption, resistant epilepsy and neurological pathologies treated with a ketogenic diet.

In this study, three products from the Kanso range were selected, different in shape and taste, to facilitate adherence and food variety:

- Kanso MCT Oil 100%: Oil made exclusively of medium chain triglycerides, derived from palm fat and coconut. It is a pure source of MCT fatty acids (mainly C8 and C10), highly bioavailable and quickly converted into ketone bodies, offering alternative energy at the brain level. It is free of proteins and carbohydrates and suitable to enrich savory or sweet dishes.
- Kanso DelíMCT Tomato: A ready-to-use tomato sauce, with a 3:1 ketogenic ratio and containing approximately 28 g of MCT per 100 g. It consists of water, MCT oil, tomato pulp, vegetable fibers

and natural spices. It is particularly suitable for enriching first courses and savoury dishes, guaranteeing practicality and palatability without renouncing the functional lipid contribution .

- Kanzo DelíMCT CacaoBar: high-fat (ketogenic ratio 4.8:1) cocoa bar containing MCT, protein, fibre and sweeteners. Ideal as a sweet snack to support compliance, it is a welcome alternative especially in patients with reduced appetite or tendency to weight loss.

These products, formulated as foods for special medical purposes, must be used under medical supervision. Their dosage is defined by the dietitian or doctor according to individual needs, with a gradual progression to minimize gastrointestinal side effects (e.g. osmotic diarrhea or swelling). The variety of forms (oil, sauce, sweet snacks) is designed to promote the customization of nutritional intervention and improve the sustainability of the diet in the long term.

4.3 COGNITIVE EVALUATION IN THE MCI PATIENT

In the management of patients with Mild Cognitive Impairment (MCI), neuropsychological assessment is essential for defining the cognitive profile and determining clinical classification. Patients undergo a standardized battery of cognitive tests, typically administered by an experienced neuropsychologist. However, these assessments can also be conducted in clinical settings by trained neurologists or other qualified healthcare professionals. The test battery includes the Mini-Mental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), and specific memory tasks such as the Rey Auditory Verbal Learning Test (RAVLT). The Trail Making Test (TMT A and B) is used to assess attention and cognitive flexibility, and the Clock Drawing Test offers a quick, integrative evaluation of visuospatial and executive functions.

The MMSE, despite its widespread use in clinical practice for general cognitive screening, has limited sensitivity in detecting the subtle deficits typical of MCI. For this reason, it is supplemented by the MoCA, which has demonstrated greater accuracy in identifying early cognitive impairment (Nasreddine et al., 2005). The combined use of these tools ensures a more comprehensive and reliable cognitive profile, enabling better tracking of cognitive status over time.

Functional and behavioral aspects are evaluated using validated questionnaires. Sleep quality and daytime sleepiness are assessed through the Pittsburgh Sleep Quality Index (PSQI) and the Epworth Sleepiness Scale (ESS). Depressive symptoms, which may affect cognitive performance, are evaluated with the Geriatric Depression Scale (GDS). The patient's perceived quality of life is measured using the Quality of Life in Alzheimer's Disease (QoL-AD) scale.

In addition to quantitative results, the neuropsychological interview provides key qualitative insights, including the patient's awareness of deficits, impact on daily functioning, and any compensatory strategies employed. These elements are fundamental in designing tailored interventions aimed at preserving residual functions and promoting overall cognitive well-being (Clare & Woods, 2004).

4.4 HAEMATOCHEMICAL ANALYSIS AND DETERMINATION OF β -HYDROXYBUTYRATE

The protocol includes monitoring of specific haematochemical parameters (complete blood count (CBC) with formula, fasting blood glucose, hemoglobin A1c, basal insulinemia, total cholesterol, high-density lipoprotein (HDL), triglycerides, creatinine, blood urea nitrogen (BUN), uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), albumin, total protein, thyroid-stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4)), both for diagnostic purposes and to evaluate the effectiveness of nutritional intervention with medium chain triglycerides (MCT). In particular, special attention is paid to the determination of β -hydroxybutyrate (BHB), the main circulating ketone body, considered a direct marker for the activation of ketogenesis.

β -Hydroxybutyrate is determined from venous blood samples collected in tubes containing potassium fluoro-oxalate or EDTA, usually under fasting conditions of at least 8 hours. The sample is then analysed by automated spectrophotometric enzymatic methods, which are based on oxidation of BHB to acetoacetate by β -hydroxybutyrate dehydrogenase, with concomitant reduction of NAD to NADH. The intensity of the spectrophotometric variation (measured at 340 nm) is proportional to the concentration of BHB in the sample (Yang et al., 2019).

Physiological values of β -hydroxybutyrate in non-fasting or non-ketogenic subjects are generally <0.3 mmol/L. As part of dietary interventions containing MCTs, an increase up to 0.5-1.0 mmol/L is an indication of efficient activation of the alternative metabolic pathway, suggesting that MCTs have been converted into ketone bodies and made available as a brain energy substrate (Cunnane et al., 2016). This is particularly relevant in the context of Mild Cognitive Impairment (MCI), where cerebral hypometabolism can be partially compensated by using ketones as an alternative energy source. β -Hydroxybutyrate is measured at the screening visit and at the end of treatment (6 months), to assess stability and individual response to surgery. Any side effects or clinical deviations are recorded in parallel, in order to correlate the biochemical course with the functional and cognitive status of the patient.

4.5 BRAIN IMAGING USING FDG-PET

Positron emission tomography with ¹⁸F-fluorodeoxyglucose (FDG-PET) will be used as a functional brain imaging tool in the protocol. The examination is performed at baseline to assess glucose metabolism in the main cortical areas involved in cognitive disorders, particularly the hippocampus, precuneus, entorhinal cortex and medial temporal lobe (Mosconi et al., 2010; Morbelli et al., 2015). PET is performed at rest, after at least 6 hours of fasting, and after intravenous administration of 185-370 MBq (5-10 mCi) of FDG.

The images are acquired 30-45 minutes after injection using high resolution PET-CT tomography. The data obtained are analysed by means of dedicated software, with comparison with age-specific regulatory databases. The FDG-PET allows to identify patterns of brain hypometabolism typical of early stages of Alzheimer's, and is a validated biomarker useful also in monitoring the effect of nutritional interventions in patients with MCI (Drzezga et al., 2008; Pagani et al., 2015).

5. RESULT

This section presents in detail the experimental protocol carried out as part of this PhD project. The clinical trial, entitled "Effect of medium chain triglyceride supplementation on cognitive function and sleep quality in subjects with mild cognitive impairment: an open-label randomized clinical study", has been designed with the aim of investigating targeted nutritional strategies that can support brain function in the early stages of cognitive impairment when preventive treatment can offer maximum benefit. In recent years, several studies have pointed out that the brain of people with MCI has a reduced ability to use glucose for energy purposes, a condition known as cerebral hypometabolism (Cunnane et al., 2016; Mosconi et al., 2010). This deficit represents a potential therapeutic target that can be modified, and has paved the way for alternative strategies based on the administration of metabolic substrates capable of bypassing the glycolytic pathway. Among these, medium chain triglycerides (MCTs) are considered particularly promising for their ability to physiologically increase hepatic production of ketone bodies including β -hydroxybutyrate (BHB), which can be used by neurons as an alternative energy source to glucose (Fortier et al., 2021; Yang et al., 2019).

The protocol described here represents the main product of the research activity carried out during the PhD, and has the objective to test, in a controlled clinical context, the impact of MCT integration on two central domains for the well-being of the subject with MCI: cognitive function and sleep quality. The decision to include sleep assessment is because of a growing focus on the role of deep sleep in amyloid beta clearance and in the regulation of synaptic plasticity (Xie et al., 2013; Jo et al., 2024), relevant aspects in the pathophysiology of neurodegeneration.

ABSTRACT

Mild Cognitive Impairment (MCI) is an early stage of cognitive decline associated with an increased risk of developing Alzheimer's disease (AD). Among the modifiable risk factors, sleep disorders and metabolic dysfunctions play a key role in accelerating neurodegenerative processes. Medium chain triglycerides (MCTs) provide an alternative energy source for neurons, overcoming the difficulties in glucose metabolism often encountered in people with MCI. In addition, MCT supplementation could regulate circadian rhythms and improve sleep quality, both of which are key to cognitive health. On these grounds, we hypothesize that appropriate nutritional interventions in the early stage of MCI could delay the onset, mitigate the symptomatology and reduce the risk of progression toward AD. Here, we propose a randomized controlled trial to evaluate the impact of a Mediterranean diet enriched with MCTs on cognitive performance and sleep quality in MCI patients. The study involves 120 participants, divided into two groups: a control group to follow the Mediterranean diet and an intervention group that will follow the same diet supplemented with 30g/day of MCT. Primary outcomes include assessment of cognitive function and sleep quality. To this end, specific tests such as MoCA, ACE, and CDR will be employed to determine, among others, attention, memory, fluent speech, space-time orientation, problem-solving abilities, and conceptual thinking. Sleep quality will be assessed using Pittsburgh and Epworth scales. To corroborate the primary findings, secondary objective data will be collected that include biochemical markers of ketone metabolism, neuroimaging (FDG-PET) and regulation of circadian rhythms. Through the analysis of the interaction between MCT metabolism, sleep and cognitive function, the study aims to offer new perspectives on non-pharmacological strategies to slow cognitive decline and improve quality of life in the elderly population.

RATIONALE, HYPOTHESIS AND OBJECTIVES OF THE CLINICAL TRIAL

Mild Cognitive Impairment (MCI) represents an intermediate clinical phase between physiological aging and dementia and is now considered a crucial window for preventive strategies (Petersen et al., 2001). Several studies have shown that in the early stages of MCI, brain glucose metabolism is significantly impaired. This impairment is associated with regional hypometabolism and reduced mitochondrial oxidative efficiency, particularly in areas such as the hippocampus and entorhinal cortex (Mosconi et al., 2010; Morbelli et al., 2015). In this context, the use of alternative metabolic substrates, such as ketone bodies derived from medium-chain triglycerides (MCTs), has been proposed as a complementary strategy to support neuronal activity and mitigate the progression of cognitive decline. Once ingested, MCTs are rapidly hydrolyzed in the gastrointestinal tract and

metabolized in the liver to produce β -hydroxybutyrate (BHB), which can cross the blood–brain barrier and be utilized as an energy source by neurons, even in conditions of glucose deficiency (Fortier et al., 2021; Cunnane et al., 2016).

Despite growing evidence supporting the neuroprotective role of ketone bodies, controlled studies evaluating the combined effect of a Mediterranean diet and MCT supplementation on cognitive function and sleep quality in individuals with MCI are still lacking. This study was designed to address this gap by investigating the potential benefits of a Mediterranean diet enriched with MCTs in improving cognitive function and sleep quality in patients recently diagnosed with MCI. The working hypothesis is that MCT supplementation, by offering an alternative energy source to neurons, can help stabilize synaptic function, reduce oxidative stress, and enhance overall neuronal efficiency, thereby contributing to cognitive preservation (Neth et al., 2020; Yang et al., 2019).

Furthermore, sleep disturbances are commonly observed in MCI and are closely linked to neurodegenerative processes, in part due to impaired clearance of neurotoxic proteins such as amyloid-beta. MCT supplementation may positively influence circadian rhythm regulation and sleep quality, supporting neuronal homeostasis through multiple mechanisms. The outcomes of this study could provide useful insights for the development of personalized nutritional strategies for the early prevention of cognitive impairment.

The intervention consists of daily supplementation with 30 grams of MCTs in addition to a standard Mediterranean diet, administered over a six-month period. The primary objective is to evaluate the effect of this nutritional intervention on cognitive performance, measured through changes in composite scores derived from a standardized neuropsychological battery including MOCA and ACE-R. Secondary objectives include assessing the impact on sleep quality and daytime sleepiness through validated scales such as the PSQI and Epworth, evaluating changes in quality of life using the QoL-AD, and measuring the effects on metabolic markers including plasma β -hydroxybutyrate, lipid profile, and glycemic control. In a subgroup of participants, cerebral glucose metabolism will be assessed using FDG-PET imaging. The study will also explore correlations between cognitive outcomes and plasma levels of brain-derived neurotrophic factor (BDNF), as well as evaluate the safety and tolerability of daily MCT intake

ETHICAL ASPECTS

This study was approved by the International Territorial Ethics Committee of the AOU Maggiore della Carità on 11 June 2024 (Practice number: CE420/2023, Protocol number: 636/CE). The trial will be conducted in accordance with the principles of the Declaration of Helsinki (2013), Good Clinical Practice (GCP) and national regulations on clinical trials. All participants will be adequately informed about the objectives, modalities and potential risks of the study, and written informed consent will be required prior to enrollment. The protection of personal data will be guaranteed according to Regulation (EU) 2016/679 (GDPR).

DESIGN OF STUDY

The experimental protocol was designed as a randomized, open-label, two-arm parallel clinical trial aimed at evaluating the effect of medium chain triglyceride (MCT) supplementation on cognitive function and sleep quality in subjects with Mild Cognitive Impairment (MCI). A summary of the study timeline and assessments at each time point is provided in Figure 3. The open-label design was chosen for feasibility, although it is a limitation that will be addressed in the discussion. The study was designed to last a total of 28 months, divided into several operational phases: two initial months for administrative authorizations and ethical compliance, followed by a recruitment period of twelve months, six months of active nutritional intervention, six months of follow-up, and finally two months dedicated to the statistical analysis of the data and the drafting of the final report.

POPULATION AND RANDOMIZATION

A total of 120 participants will be enrolled and distributed equally between two groups (n=60 per group), by simple randomization. The sample size was calculated assuming a significance level of 0.05, a statistical power of 80% and an expected effect of 0.5 (Cohen's d), consistent with previous evidence on the potential impact of MCTs on cognitive performance (Fortier et al., 2021).

Participants will be assigned to one of the following groups:

- Control group: will follow a standard Mediterranean diet, developed and monitored by a team of qualified nutritionists;
- Intervention group: will follow the same Mediterranean diet, supplemented with 30 grams per day of MCT, administered through specially developed food products (MCT oil, tomato sauce enriched and chocolate with MCT).

The dietary intervention will be personalized through an individualized and monitored nutritional counseling path, with periodic assessments of dietary adherence, cognitive status, sleep quality and major metabolic parameters.

DIETARY AND RATIONAL NUTRITIONAL TREATMENT

The Mediterranean diet has been selected as a basis for nutritional treatment because of its established anti-inflammatory, antioxidant and neuroprotective properties. This food pattern is characterized by a high intake of fruits, vegetables, legumes, whole grains, fish, extra virgin olive oil and moderate consumption of red wine. The results of the randomized trial conducted by Hoscheidt et al. (2022) showed that a four-week isocaloric dietary intervention, based on this scheme, is associated with an improvement in cerebral perfusion and favorable changes in CSF biomarkers of Alzheimer's in cognitively healthy subjects. The protocol provides for 30 grams of medium chain triglycerides (MCT) to be added daily to the intervention group, as part of a Mediterranean-inspired diet. The choice of this dosage is based on evidence from clinical trials that have demonstrated its effectiveness in promoting ketogenesis and improving cognitive performance in subjects with Mild Cognitive Impairment (MCI) (Fortier et al., 2021). In particular, Fortier and colleagues administered a drink containing 30 g/day of MCT for six months, observing a significant improvement in cognitive scores compared to the control group. Subsequent studies have confirmed that similar doses (about 0.5 g/kg body weight) are well tolerated even in healthy subjects and induce a significant increase in plasma levels of β -hydroxybutyrate (BHB) (Sun L et al., 2023). Supplementation is carried out by means of functional foods developed in collaboration with the company Dr. Schär, including MCT oil-based formulations, enriched tomato sauce and chocolate snacks. The use of familiar and palatable foods helps to improve adherence to dietary therapy, reducing the risk of protocol abandonment. In addition to ensuring a constant supply of MCT, the objective is to evaluate the individual's ability to activate a ketogenic metabolic response, monitored through the plasma assay of BHB. This approach allows a personalized reading of the intervention, considering that the efficiency of ketogenesis can vary according to individual factors such as age, body composition, metabolic status and adherence to diet. The evaluation of ketone response allows therefore to explore with greater precision the potential role of MCTs in neuroenergetic support of patients with MCI.

INCLUSION AND EXCLUSION CRITERIA

To ensure sample homogeneity and the internal validity of the study, strict inclusion and exclusion criteria have been defined. These criteria are based on standardised diagnostic indications and international recommendations for studies of mild cognitive impairment.

Inclusion criteria:

- Aged between 18 and 85;
- Clinical diagnosis of mild cognitive impairment according to the criteria of Petersen (Petersen et al., 2001), confirmed by a thorough neuropsychological evaluation;
- Presence of relatively preserved overall cognitive function (MMSE 24);
- Independence in daily life activities;
- Absence of malnutrition, diagnosed using the NRS 2002 screening test.
- Willingness to follow the nutritional protocol for the duration of the study;
- Signature of informed consent.

Exclusion criteria:

- Diagnosis of dementia of any type (including Alzheimer's disease, vascular dementia, etc.) according to DSM-5 criteria.
- Relevant neurological or psychiatric comorbidities (e.g., Parkinson's disease, uncompensated major depression, epilepsy).
- Uncontrolled severe metabolic diseases (e.g., glycated hemoglobin >8%, patients on insulin therapy, severe dyslipidemia with total cholesterol >300 mg/dL or triglycerides >400 mg/dL).
- Severe hepatic insufficiency (MELD score >9) or renal insufficiency (eGFR <30 mL/min).
- Known allergies to MCT oil, nuts, or other ingredients in the study products.
- Participation in other clinical trials within the last 2 months.
- Any condition that, in the opinion of the researcher, may put the patient at risk or hinder compliance with the study.

Participants will be selected through multidisciplinary clinical screening, including neurological assessment, laboratory tests, brain imaging with FDG-PET and standardised neuropsychological tests. This approach will allow to exclude cases of unknown dementia or confounding conditions, ensuring the inclusion of a representative sample of the population with MCI.

INCLUSION CRITERIA	EXCLUSION CRITERIA
- Age: 18-85 years - Clinical diagnosis of mild cognitive impairment according to the criteria of Petersen (Petersen et al., 2001), confirmed by a thorough neuropsychological evaluation - Presence of relatively preserved overall cognitive function (MMSE 24) - Independence in daily life activities - Absence of malnutrition, diagnosed using the NRS 2002 screening test - Willingness to follow the nutritional protocol for the duration of the study - Signature of informed consent.	- Diagnosis of dementia of any type (including Alzheimer's disease, vascular dementia, etc.) according to DSM-5 criteria - Relevant neurological or psychiatric comorbidities (e.g., Parkinson's disease, uncompensated major depression, epilepsy) - Uncontrolled severe metabolic diseases (e.g., glycated hemoglobin >8%, patients on insulin therapy, severe dyslipidemia with total cholesterol >300 mg/dL or triglycerides >400 mg/dL) - Severe hepatic insufficiency (MELD score >9) or renal insufficiency (eGFR <30 mL/min) - Known allergies to MCT oil, nuts, or other ingredients in the study products - Participation in other clinical trials within the last 2 months - Any condition that, in the opinion of the researcher, may put the patient at risk or hinder compliance with the study

FIG.3 Summary table with inclusion and exclusion criteria for the protocol

NUTRITIONAL INTERVENTION

The nutrition plan for the intervention group is based on a Mediterranean-style regimen, which has been supplemented by daily administration of 30 grams of medium chain triglycerides (MCTs). The Mediterranean diet was chosen as a basis for its proven protective effect against cognitive decline, thanks to its wealth of unsaturated fats, polyphenols and dietary fiber (Petersson & Philippou, 2022). The supplementation with MCT takes place through functional foods developed by the company Dr. Schär, where a period of collaborative research has been carried out. In particular, products from the Kanso line were used. The distribution of MCTs throughout the day is monitored through a weekly food diary and periodic checks by the nutrition team. Adherence to diet is verified through regular interviews with nutritionists, MedDiet Score assessment, and estimation of daily intake of MCT. A crucial aspect of the protocol is the assessment of individual metabolic response to integration, with particular emphasis on endogenous production of ketone bodies, and in particular β -hydroxybutyrate (BHB). The plasma assay of BHB, carried out at pre-defined times during the study, will allow to verify if the daily intake of MCT is able to activate, at least partially, the ketogenic pathway. This is crucial to understand the subjective variability in response and the potential role of ketone bodies as alternative substrates in neuroenergetic support.

ADVERSE EVENTS

The administration of medium chain triglycerides (MCTs), while generally well tolerated, may be associated with some side effects, especially in the early stages of introduction. The most commonly reported disorders in the literature include nausea, bloating, abdominal cramps and diarrhea, usually of a mild and transient nature (Sun et al., 2023). These effects can be minimized by a fractional daily dose (30 g/day) and the intake of MCT-containing products during main meals. In patients with previous metabolic disorders, especially liver disease or insulin resistance, the use of MCT requires careful clinical and biochemical monitoring to prevent any metabolic overloads or changes in blood glucose. However, studies conducted in patients with MCI and healthy subjects did not report any serious adverse events or significant discontinuities of therapy, confirming the safety of the dosage used in this protocol (Fortier et al., 2021; Cunnane et al., 2016). Throughout the course of the nutritional intervention, participants will be monitored for possible side effects through scheduled visits and telephone contact. Adverse events will be recorded in a dedicated tab and classified according to their severity and possible relationship with the treatment. In case of persistent symptoms or poor individual tolerance, it will be possible to modulate the dose or temporarily suspend supplementation, in agreement with the doctor responsible for the study

DIAGNOSTIC ALGORITHM

The protocol provides for a total duration of six months for each patient, during which several face-to-face visits and telephone contacts are planned in order to monitor adherence to the intervention and evaluate clinical outcomes.

After the screening visit (T0), which includes clinical, neuropsychological, nutritional evaluation and PET with FDG to document the presence of brain hypometabolism, patients who meet the inclusion criteria are formally enrolled in the study. Randomization assigns them to one of two arms: standard Mediterranean diet or MCT-enriched Mediterranean diet. Patients in the intervention group are given a nutrition diary, products containing MCT (oil, tomato sauce, chocolate) and instructions for their daily intake (30 g/day). During the first month (T1) and second month (T2), patients are contacted by telephone to monitor adherence to the protocol, identify any adverse events and provide dietary support. In the third month (T3), a presence visit is carried out, with complete reassessment: hematochemical tests, cognitive tests, questionnaires on sleep and quality of life, anthropometric parameters and control of the food diary (with calculation of compliance). The follow-up continues with monthly calls to the fourth (T4) and fifth month (T5), useful to ensure continuity of assistance and promote the retention of participants. In the sixth month (T6), the final visit takes place, which follows the intermediate one but represents the concluding moment of the intervention. Neuropsychological tests (MoCA, ACE, CDR, Attentive matrices), sleep quality questionnaires (PSQI, Epworth) and quality of life (QoL-AD) are repeated, as well as blood tests including the dosage of β -hydroxybutyrate and acetate.

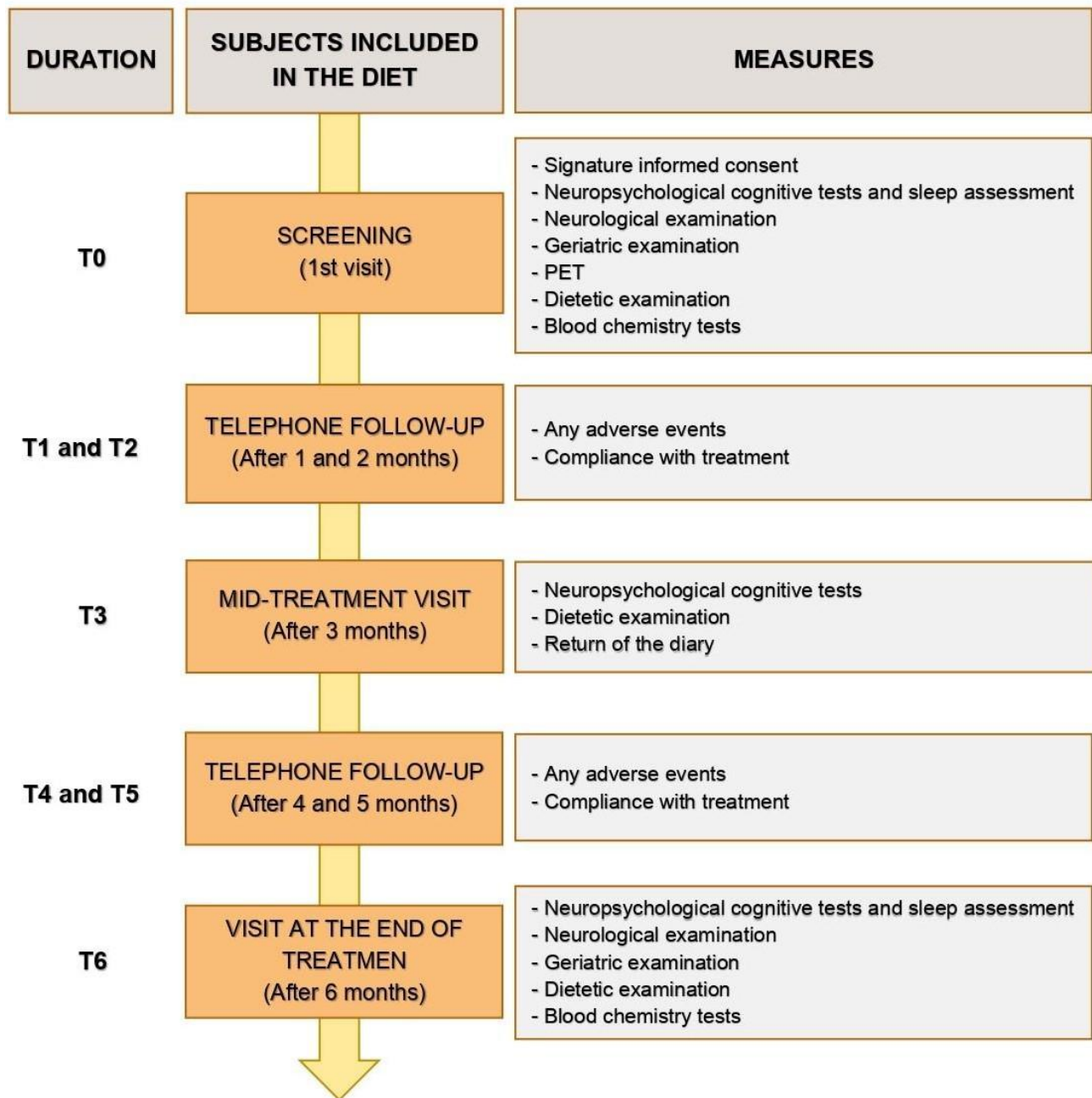


FIG.4: Illustration of the study timeline, detailing the assessment and follow-up schedule over six months.

STATISTICAL ANALYSIS

All statistical analyses will be carried out using STATA v.17 software. Analyses will primarily follow an intention-to-treat (ITT) principle, including all randomized participants in the group to which they were assigned. A per-protocol (PP) analysis will also be conducted on participants who completed the study with adequate adherence. Baseline demographic and clinical characteristics will be summarized using descriptive statistics. Continuous variables will be expressed as mean and standard deviation (SD) or median and interquartile range (IQR) after assessing for normality with the Shapiro-Wilk test. Categorical variables will be presented as absolute frequencies and percentages.

To evaluate the effect of the intervention on primary and secondary continuous outcomes (e.g., cognitive scores from MOCA and ACE-R, sleep scores from PSQI/Epworth, QoL-AD, and biomarkers like BHB and BDNF) over time, linear mixed-effects models will be used. This longitudinal approach is preferable to simple pre-post comparisons as it accounts for intra-individual correlations of repeated measures and can handle missing data more effectively. The model will include fixed effects for time, treatment group, and the time-by-treatment interaction term, which will be the primary indicator of the intervention effect. Baseline values of the outcome will be included as a covariate to adjust for any initial imbalances. Models will also be adjusted for potential confounding variables such as age, sex, and baseline BMI.

A value of $p < 0.05$ will be considered statistically significant.

DIPARTIMENTO ATTIVITA' INTEGRATE RICERCA E INNOVAZIONE

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SSD MEDICINA TRASLAZIONALE

Responsabile: Prof. Luigi Castello

Coordinatore: Dott.ssa Annalisa Roveta

***Valutazione dell'impatto degli acidi grassi a catena media
nell'evoluzione del decadimento cognitivo lieve di recente
diagnosi: studio prospettico randomizzato e controllato***

MCI-MCT

Sperimentatore Principale: Prof. Luigi Mario Castello, SCU Medicina Interna, SSD Medicina Traslazionale, Azienda Ospedaliero-Universitaria SS Antonio e Biagio e C. Arrigo di Alessandria

Promotore: Azienda Ospedaliero-Universitaria SS Antonio e Biagio e Cesare Arrigo di Alessandria

Titolo studio: Valutazione dell'impatto degli acidi grassi a catena media nell'evoluzione del decadimento cognitivo lieve di recente diagnosi: studio prospettico randomizzato e controllato

Acronimo: MCI-MCT

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5.1 FIRST RESULTS

As of April 2025, a total of 19 patients had been enrolled in the study. Of the remaining patients, seven were assigned to the control group and ten to the treatment group. At this stage, five patients in the treatment group and one patient in the control group have completed the full six-month protocol and are included in this preliminary analysis. While numbers are too limited for statistical analysis, preliminary observations suggest that the intervention may help stabilize cognitive and functional parameters in some individuals with MCI. For instance, two of the five patients in the treatment group showed an improvement or stabilization in their MOCA and ACE-R scores, and three reported notable improvements in their quality of life (QoL-AD). Outcomes, however, displayed significant inter-individual variability. Further data from ongoing participants are essential to draw any meaningful conclusions. A detailed summary of these preliminary results is provided in Table 1.

Patient	BMI T0	BMI T6	MOCA T0	MOCA T6	ACE-R T0	ACE-R T6	QoL- AD T0	QoL- AD T6	PSQI T0	PSQI T6	Epworth T0	Epworth T6	MEDAS T0	MEDAS T6	Adverse Events
1	30,9	31,2	24	25	76	88	40	41	18	27	8	5	7	9	NO
2	19,6	19,8	25	24	79	84	36	34	7	12	3	4	8	9	NO
3	28,3	27,6	28	26	86	83	29	37	7	7	16	10	8	8	NO
4 (Intention to Treat)	29,1	28,8	17	14	72	73	33	42	8	10	6	4	6	8	NO
5	32,5	32,1	22	25	84	93	26	34	30	24	17	10	8	7	YES (mild, unrelated)
6 (Mediterranean Diet)	25	25	24	24	85	83	35	34	6	8	5	5	9	9	NO

TAB.1 Individual patient data from the first five participants in the MCT treatment group and one patient in the control group (Mediterranean diet only). The table summarizes key clinical and functional parameters at baseline (T0) and after six months (T6), including BMI, cognitive function (MOCA and ACE-R), quality of life (QoL-AD), sleep quality (PSQI), daytime sleepiness (Epworth Sleepiness Scale), and adherence to the Mediterranean diet (MEDAS). Patient 4, although assigned to the treatment group, did not consume the MCT supplements and is included in the analysis according to the intention-to-treat principle. Control 6 represents the only control patient who had completed the full follow-up at the time of data collection. No major adverse events were reported. The table highlights interindividual variability and preliminary differences between the two groups

6. DISCUSSION AND FURTHER PROSPECTIVE

In recent years, interest in nutritional strategies to modulate brain metabolism has led to an increasing use of medium-chain triglycerides (MCTs) in the treatment of Mild Cognitive Impairment (MCI). The hypothesis behind this approach is based on evidence that, already in the early stages of cognitive impairment, the brain shows a progressive reduction in the use of glucose for energy purposes, a phenomenon detectable by functional imaging such as FDG-PET (Mosconi et al., 2010; Cunnane et al., 2016). Ketone bodies derived from MCTs, in particular β -hydroxybutyrate (BHB), represent an alternative energy source able to overcome this deficit, providing readily usable substrates by neurons and astrocytes even in the presence of insulin/mitochondrial resistance or dysfunction (Yang et al., 2019).

Numerous clinical and pre-clinical studies have suggested that regular MCT intake may improve several cognitive domains - in particular episodic memory and executive functions - through an increase in neuronal energy availability, reduction of oxidative stress and modulation of inflammation (Fortier et al., 2021; Sun et al., 2023). To these benefits is added the possibility that ketones stimulate autophagy, favoring the removal of toxic protein aggregates such as beta-amyloid and hyperphosphorylated tau, two hallmarks of neurodegeneration (Newman & Verdin, 2017).

In the protocol presented here, the nutritional approach is based on a standard Mediterranean diet, recognized for its protective effects on the central nervous system. Randomized trials have shown that this regimen improves cerebral perfusion, positively modulates CSF biomarkers of Alzheimer's and reduces systemic inflammation (Hoscheidt et al., 2022). The integration with 30 g/day of MCT has been adopted on the basis of clinical trials that have demonstrated its tolerability and efficacy in the production of BHB, with measurable effects on cognitive functions in subjects with MCI (Fortier et al., 2021; Sun et al., 2023).

A further innovation of this protocol is the assessment of the impact of the diet enriched with MCTs also on sleep quality. Deep sleep (slow-wave sleep) is essential for amyloid removal and synaptic plasticity. Numerous evidences show that the alterations of the circadian rhythm and the fragmentation of the sleep anticipate the appearance of cognitive deficits and favor the progression towards the dementia (Xie et al., 2013; Wang et al., 2023). In this context, the possibility that MCTs, through their influence on the regulation of energy metabolism and the release of neurotransmitters, help to stabilize sleep-wake cycles is an additional therapeutic potential to be investigated.

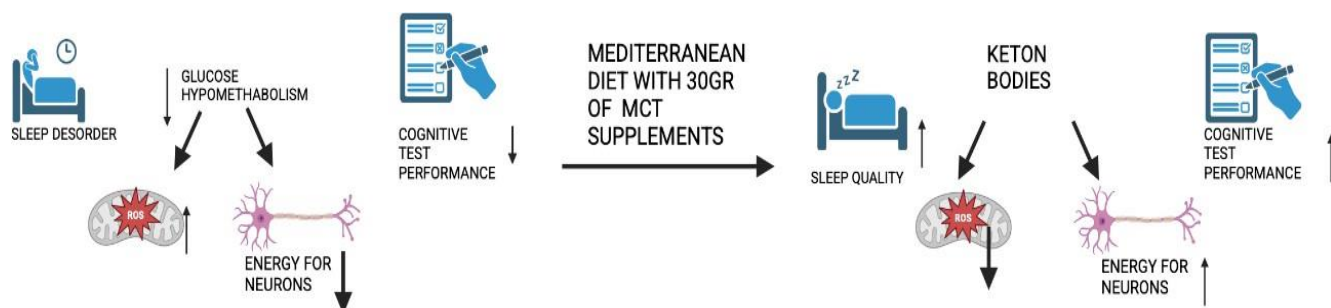


FIG.5 This figure illustrates the proposed mechanism linking sleep disorders, glucose hypometabolism, and cognitive decline, and how a Mediterranean diet supplemented with 30 grams of medium-chain triglycerides (MCTs) could mitigate these effects.

- Sleep disorders contribute to glucose hypometabolism, leading to increased reactive oxygen species (ROS) production and reduced neuronal energy supply.
- This results in impaired cognitive test performance.
- Implementing a Mediterranean diet with MCT supplementation improves sleep quality.
- MCTs are metabolized into ketone bodies, which provide an alternative energy source for neurons, reducing ROS levels and enhancing neuronal energy supply.
- These effects ultimately improve cognitive test performance.

6.1 STUDY LIMITATION

This study protocol has several limitations that should be acknowledged. First, the open-label design, while chosen for practical reasons, introduces a potential for bias, as knowledge of the treatment allocation could influence participant reporting and clinician assessment. Future studies should aim for a double-blind, placebo-controlled design to mitigate this bias, for example by using a placebo oil and food products matched for taste and texture. Second, the preliminary analysis is based on a very small number of participants, which is significantly lower than the planned sample size of 120 subjects. This severely limits the statistical power and the generalizability of the findings. The study should therefore be considered a pilot investigation aimed at assessing feasibility and gathering preliminary data. The recruitment challenges encountered highlight the difficulties of conducting nutritional intervention trials in this vulnerable population. Finally, while the Mediterranean diet provides a healthy baseline, individual dietary habits can introduce variability. Although monitored with food diaries, a more detailed nutritional analysis could provide deeper insights. Future research should focus on recruiting a larger, adequately powered sample to definitively test the study hypothesis. It would also be valuable to investigate the long-term effects of MCT supplementation beyond six months and explore its efficacy in different MCI subtypes (e.g., AD-related vs. non-AD-related MCI).

7.HEALTH SUSTAINABILITY OF THE INTEGRATED DIET WITH MCT

The growing social and health impact of neurodegenerative diseases, particularly Alzheimer's disease (AD), highlights the urgent need for preventive strategies that are both clinically effective and economically sustainable. Nutritional intervention stands out in this context as a promising, non-invasive, and accessible tool, especially suitable for elderly populations in both clinical and community settings. The protocol presented here is based on an integrated approach that combines a standardized Mediterranean diet with the addition of medium-chain triglycerides (MCTs). This model is easy to implement in practice, thanks to the availability of functional food products and the support of personalized dietary counselling. MCTs are administered through common food items (such as oil, sauces, and chocolate), improving patient adherence and reducing the need for pharmaceutical formulations. The chosen dosage of 30 g/day, already validated in previous studies (Sun et al., 2023; Fortier et al., 2021), has shown good tolerability with only minor and reversible side effects, making this approach scalable within public healthcare systems. From a public health perspective, this type of nutritional prevention could significantly reduce the economic burden associated with dementia. AD remains a leading cause of disability, dependency, and institutionalization among the elderly. Delaying even slightly the transition from mild cognitive impairment (MCI) to dementia could lead to substantial savings in medical, pharmacological, and social care costs (Cummings et al., 2022). Thus, early dietary interventions supported by structured nutritional education represent a high-value, cost-effective strategy. Recent data underscore the scale of the challenge: in Italy, the death rate from Alzheimer's disease has more than tripled since 1980, rising from just over 30 to more than 110 deaths per 100,000 inhabitants by 2021. This trend significantly exceeds the European average, which over the same period increased from 25 to around 60 deaths per 100,000 (Figure 4). This sharp rise reflects not only an aging population but also a growing diagnostic awareness and longer life expectancy. However, it also exposes the limitations of current management systems and reinforces the need to invest in sustainable prevention.

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9 LIST OF PUBLICATION

Randomized, double-blind, placebo-controlled trial of rapamycin in amyotrophic lateral sclerosis

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In preclinical studies rapamycin was found to target neuroinflammation, by expanding regulatory T cells, and affecting autophagy, two pillars of amyotrophic lateral sclerosis (ALS) pathogenesis. Herein we report a multicenter, randomized, double-blind trial, in 63 ALS patients who were randomly assigned in a 1:1:1 ratio to receive rapamycin 2 mg/m²/day, 1 mg/m²/day or placebo (EUDRACT 2016-002399-28; NCT03359538). The primary outcome, the number of patients exhibiting an increase >30% in regulatory T cells from baseline to treatment end, was not attained. Secondary outcomes were changes from baseline of T, B, NK cell subpopulations, inflammasome mRNA expression and activation status, S6-ribosomal protein phosphorylation, neurofilaments; clinical outcome measures of disease progression; survival; safety and quality of life. Of the secondary outcomes, rapamycin decreased mRNA relative expression of the pro-inflammatory cytokine IL-18, reduced plasmatic IL-18 protein, and increased the percentage of classical monocytes and memory switched B cells, although no corrections were applied for multiple tests. In conclusion, we show that rapamycin treatment is well tolerated and provides reassuring safety findings in ALS patients, but further trials are necessary to understand the biological and clinical effects of this drug in ALS.

With a life-long risk of 1:400, Amyotrophic Lateral Sclerosis (ALS) is the 3rd most common neurodegenerative disease, holding an estimated increase of 69% in the upcoming years¹. No specific pharmacological treatments modify the disease relentless course leading to death within 3–5 years from symptoms onset. The only approved drug in Europe, riluzole, extends survival for a few months at best². Despite extreme heterogeneity and intricate pathobiology, protein misfolding

and immune system dysfunction are two acknowledged pillars of ALS pathogenesis, which present in the early stages in all patients and constantly evolve to the terminal phases, therefore representing promising therapeutic targets³.

Rapamycin, a drug used to prevent renal transplantation rejection, inhibits mammalian Target of Rapamycin Complex 1, leading to regulatory T lymphocytes (Treg) expansion and autophagy

enhancement. In fact, mTOR inhibits the induction of Tregs, a specific cell population that down-regulates immune system activation and is found to be reduced and dysfunctional in ALS patients³. As a consequence, self-sustaining inflammatory cytokines are upregulated and peripheral immune cell migration into the brain is promoted⁴. In ALS patients, percentage of Tregs in the blood inversely correlated with progression rate⁵, and FoxP3 levels were early predictors of ALS progression and survival⁶. Thus, Tregs may be considered important therapeutic targets in ALS addressed by rapamycin.

Furthermore, several in vitro and in vivo studies also suggest that inhibition of mTOR by rapamycin may target autophagy, which is crucial not only for cell-autonomous clearance mechanisms, but also for limiting detrimental and uncontrolled activation of inflammasomes⁷. In ALS, accumulation of aggregates drives caspase-1-mediated proteolytic cleavage and secretion of proinflammatory cytokines, that further amplify inflammatory responses, resulting in chronic inflammation, tissue damage and cell death⁸. Rapamycin enhances the autophagic degradation of various aggregate-prone proteins with subsequent reduction of their toxicity in cellular or animal models not only in ALS, but also in other neurodegenerative diseases e.g., Huntington disease^{9–11}. Autophagy enhancement by rapamycin is mediated by the unc-51-like kinase 1 complex and the formation of autophagosome from the phagophore¹⁰.

In preclinical ALS studies, rapamycin reduced TDP-43 fragments accumulation and restored TDP-43 nuclear localization in

cell lines¹² and in human stem cell-derived neurons and astrocytes with mutant TDP43, where rapamycin enhanced survival⁹. A microfluidic approach using rapamycin rescued the ALS motor neuron phenotype in 2D and 3D environments from a TDP-43 mouse¹³. Early rapamycin administration determined phenotype amelioration in TDP-43 mouse¹⁴, *SQSTM1* knock-down zebrafish¹⁵ and in *Drosophila* with *VAPB* mutation¹⁶ (details in supplementary appendix, Section 1).

Here we report the results of RAP-ALS, a phase 2, multicenter, randomized, double-blind, placebo-controlled clinical trial (RCT), that evaluates safety, biological and clinical effects of oral rapamycin in persons with ALS¹⁷.

Results

Participants and follow-up

From 05/10/2017 to 02/01/2020 a total of 70 patients with ALS were screened for eligibility, of whom 63 were randomly assigned to a trial group: 21 to rapamycin 2 mg/m²/day, 21 to rapamycin 1 mg/m²/day and 21 to placebo (Fig. 1). Seven patients dropped out from the study during the treatment period, whereas 15 did not conclude the follow up (after treatment). Two patients did not take at least 80% of the study drug as planned per protocol. As these two patients dropped out between week 18 and 30, Per Protocol (PP) and Intention To Treat (ITT) analyses differ at week 8 and 18 only, whereas the results of the two analyses were identical at week 30 and 54.

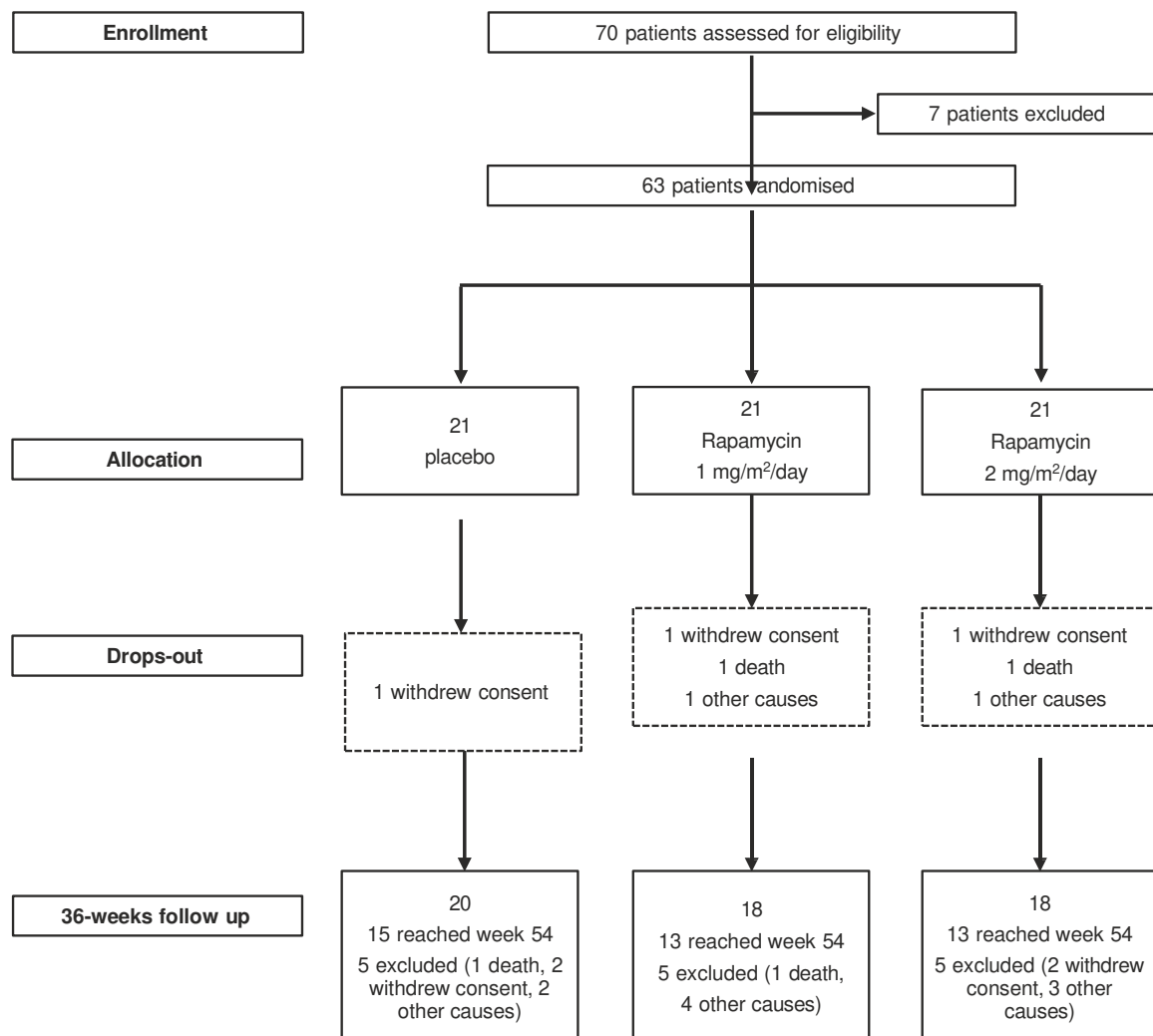


Fig. 1 | CONSORT diagram of the study reporting screening, randomization and follow-up of ALS patients enrolled in the trial. Source data are provided as a Source Data file. *Nature Communications* | (2023) 14:4970 | 2

Table 1 | Demographic and clinical characteristics of the participants at baseline (intention-to-treat population)

Characteristic	Rapamycin 2 mg/m ² /d (N = 21)	Rapamycin 1 mg/m ² /d (N = 21)	Placebo (N = 21)
Male sex – n. (%)	11 (52.4)	7 (33.3)	13 (61.9)
Age – years	56.04 ± 11.88	55.08 ± 10.99	55.55 ± 12.88
Body-mass index, kg/m ²	25.39 ± 4.43	25.63 ± 4.75	24.71 ± 2.73
BSA, m ²	1.84 ± 0.17	1.81 ± 0.23	1.83 ± 0.22
Bulbar onset – n. (%)	4 (19.0)	4 (19.0)	6 (28.6)
Upper limb onset – n. (%)	10 (47.6)	9 (42.9)	8 (38.0)
Lower limb onset – n. (%)	9 (42.9)	10 (47.6)	10 (47.6)
Months since ALS symptom onset	11.90 ± 3.95	14.10 ± 3.66	11.67 ± 4.25
ALSFRS-R total score	39.05 ± 4.17	38.29 ± 4.36	38.62 ± 5.36
Bulbar score	11.00 ± 1.61	10.71 ± 1.68	10.57 ± 1.72
Fine-motor score	8.29 ± 1.98	8.10 ± 2.47	8.38 ± 2.75
Gross-motor score	8.00 ± 2.86	7.67 ± 2.78	7.90 ± 2.79
Breathing score	11.76 ± 0.70	11.81 ± 0.40	11.76 ± 0.89
Prebaseline ALSFRS-R slope ^a	0.80 ± 0.42	0.75 ± 0.41	1.05 ± 1.00
Forced vital capacity — % of predicted normal value	95.47 ± 18.27	94.86 ± 17.85	95.04 ± 15.72
MRC upper-limb score — % of predicted normal value	86.79 ± 14.22	85.57 ± 10.94	85.27 ± 12.84
MRC lower-limb score — % of predicted normal value	85.44 ± 17.02	85.68 ± 18.29	84.90 ± 17.14
MRC neck score — % of predicted normal value	97.38 ± 5.15	92.62 ± 14.02	95.95 ± 9.17
ALSAQ40 total score	32.56 ± 14.51	30.67 ± 14.64	32.98 ± 17.72
Edaravone treatment – n (%)	0 (0.0)	2 (9.0)	5 (23.8)

Source data are provided as a Source Data file.

ALSFRS-R Amyotrophic Lateral Sclerosis Functional Rating Scale Revised, MRC Medical Research Council scale, BSA body surface area, ALSAQ40 Amyotrophic Lateral Sclerosis Assessment Questionnaire.

^aPrebaseline ALSFRS-R slope has been calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset.

Table 2 | Patients exhibiting a positive response (increase in Treg of at least 30%), comparing baseline and treatment end (week 18) between rapamycin and placebo arm. Intention to Treat analysis

	Positive response		Not positive response		Unadjusted analysis			Adjusted analysis ^a		
	n	%	n	%	RR	CI	p	OR	CI	P
Placebo	2	11.8	15	88.2	-	-	-	-	-	-
Rapamycin 1 mg/m ² /d	5	27.8	13	72.2	2.36	0.42–13.12	0.2365	3.53	0.31–40.80	0.2477
Rapamycin 2 mg/m ² /d	3	20.0	12	80.0	1.70	0.26–11.21	0.5220	2.76	0.20–37.58	0.3836
Rapamycin	8	25.0	25	75.8	2.06	0.49–8.65	0.2961	3.19	0.43–23.66	0.2567

Source data are provided as a Source Data file.

The unadjusted comparisons were carried out with a chi-square test without any correction and the adjusted comparisons with a logistic regression model. All statistical tests were two-tailed.

RR relative risk, OR odds ratio, CI confidence interval.

^aAdjusted for sex, months from onset of symptoms, ALSFRS-R slope at baseline and treatment with edaravone.

Baseline demographic and disease characteristics are summarized in Table 1. Baseline biological features of the trial participants are summarized in Supplemental materials, Table S1.

Rapamycin effect on Treg cells

Although 56 patients reached treatment end, only 50 blood samples from 50 participants were available for cells populations analysis at week 18 due to the COVID-19 pandemic. Among patients treated with rapamycin 1 mg/m²/d, 28% of patients presented an increase of Treg cells by at least 30%, versus 12% of patients in the placebo group (Relative Risk [RR] 2.36, 97.5% confidence interval [CI] 0.42 to 13.12; $p = 0.236$) (ITT analysis, Table 2); the positive response was observed in 20% of patients treated with rapamycin 2 mg/m²/d (RR 1.70, 97.5%CI 0.26 to 11.21, $p = 0.522$). PP analysis yielded similar results (Table S2).

In an adjusted analysis, after correction for sex, time from onset, ALSFRS-R slope at baseline, and edaravone treatment, based on the comparison of differences of clinical significance between groups at baseline, odds ratio (OR) of a positive response was non-significantly increased in Rapamycin treatment arms (Table 2). At week 18, 6/17 (35%) placebo-treated patients experienced any increase in Treg cells compared with 10/18 (56%) patients treated with rapamycin 1 mg/m²/d (RR 1.57, 97.5%CI 0.66–3.77; $p = 0.2291$) and 10/15 (67%) patients treated with rapamycin 2 mg/m²/d (RR 1.89, 97.5%CI 0.81–4.38; $p = 0.0765$).

At treatment end patients treated with rapamycin 1 mg/m²/d experienced a mean increase of Treg cells by 0.41, patients treated with 2 mg/m²/d showed stable values (–0.05), whereas patients in placebo showed a decrease in Treg cells by –0.46 from baseline (mean difference [MD] 0.87, 97.5% CI –0.36 to 2.09; $p = 0.109$ in rapamycin 1 mg/m²/d group and MD 0.41, 97.5%CI, –0.88 to 1.69; $p = 0.465$ in rapamycin 2 mg/m²/d group) (Table 3). While in the placebo arm Treg monthly variation during and after treatment showed a nearly constant decline (mean –0.10, 95%CI: –0.25 to 0.05, $p = 0.182$ during treatment; mean –0.04, 95%CI: –0.09 to 0.02, $p = 0.216$ after treatment), during treatment there was a trend towards an increase in patients who were treated with the lower dose of the drug compared to those who received the placebo (MD 0.17, 97.5%CI: –0.06 to 0.41, [Nature Communications](#) | (2023) 14:4970 3

Table 3 | Changes from baseline to week 8, 18, 30, and 54 in Treg cells across treatment arms. Intention to treat analysis

Time point	Arm	Absolute change from baseline			Unadjusted analysis			Adjusted analysis ^a				
		<i>n</i>	mean	SD	MD	CI	<i>p</i>	MD	CI	<i>p</i>		
Week 8	Placebo	19	-0.35	1.61	-	-	-	-	-	-	-	-
	Rapamycin 1 mg/m ² /d	19	-0.22	1.13	0.13	-0.92	1.18	0.7774	-0.13	-1.28	1.01	0.7868
	Rapamycin 2 mg/m ² /d	18	0.17	1.44	0.52	-0.55	1.58	0.2661	0.22	-0.92	1.35	0.6617
	Rapamycin	37	-0.03	1.28	0.32	-0.47	1.11	0.4232	0.04	-0.81	0.90	0.9187
Week 18	Placebo	17	-0.46	1.58	-	-	-	-	-	-	-	-
	Rapamycin 1 mg/m ² /d	18	0.41	1.56	0.87 ^b	-0.36	2.09	0.1088	0.53	-0.73	1.79	0.3327
	Rapamycin 2 mg/m ² /d	15	-0.05	1.55	0.41 ^b	-0.88	1.69	0.4655	0.09	-1.21	1.39	0.8757
	Rapamycin	33	0.20	1.55	0.66	-0.28	1.59	0.1645	0.32	-0.63	1.28	0.4985
Week 30	Placebo	12	-0.34	1.51	-	-	-	-	-	-	-	-
	Rapamycin 1 mg/m ² /d	15	0.06	1.21	0.40	-0.81	1.61	0.4455	-0.20	-1.60	1.21	0.7456
	Rapamycin 2 mg/m ² /d	13	0.12	1.30	0.45	-0.80	1.70	0.4028	-0.17	-1.59	1.26	0.7862
	Rapamycin	28	0.09	1.23	0.42	-0.50	1.34	0.3573	-0.18	-1.26	0.90	0.7342
Week 54	Placebo	9	-0.36	2.02	-	-	-	-	-	-	-	-
	Rapamycin 1 mg/m ² /d	11	-0.15	1.26	0.21	-1.46	1.88	0.7703	-0.57	-2.33	1.20	0.4514
	Rapamycin 2 mg/m ² /d	11	-0.62	1.44	-0.26	-1.94	1.41	0.7100	-0.80	-2.44	0.83	0.2521
	Rapamycin	22	-0.39	1.34	-0.03	-1.29	1.23	0.9634	-0.71	-1.98	0.56	0.2634

Mean absolute changes from baseline to week 8, 18, 30, and 54 are showed for each treatment group and comparison were performed using linear regression models that include indicator variables for treatment arms as the independent variables.

For the comparison of Rapamycin and placebo arms, a *P* value of 0.05 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 95% confidence interval (CI). For the comparisons between Rapamycin 1 mg/m²/d or 2 mg/m²/d arms and the placebo arm, a *P* value of 0.025 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 97.5% CI, to account for multiple arms comparison with the Bonferroni method. CIs were calculated based on the exact *t* distribution. All statistical tests were two-tailed.

MD mean difference, CI confidence interval.

^aAdjusted analyses for sex, ALSFRS-R slope at baseline, disease duration from onset to baseline and edaravone treatment.

^bWhile mean Treg cells percentage at baseline was similar in the treatment group (4.66 ± 1.86) with respect to the placebo group (4.39 ± 2.13), at week 18 Treg percentage were 4.69 ± 1.75 in the treatment group and 4.22 ± 1.62 in the placebo group. Source data are provided as a Source Data file.

p = 0.100 in rapamycin 1 mg/m²/d arm; MD 0.08, 97.5%CI: -0.16 to 0.33, *p* = 0.450 in rapamycin 2 mg/m²/d arm), that was not statistically significant (Table S3). In a post-hoc analysis on a limited number of samples, we found that after in vitro stimulation with anti-CD3/CD28 functionality of Treg cells resulted similar before and after therapy and not different to those from age and sex-matched healthy controls (Fig. S1).

Treatment impact on blood cell subpopulations We next examined the change from baseline to each time point (week 8, 18, 30, 54) of the activation and homing capabilities of different T, B, NK cell subpopulations, comparing treatment and placebo arms (Fig. 2, Table S4); no correction was applied for multiple tests (55 outcomes were examined), therefore the following results on secondary outcomes should be interpreted cautiously.

At week 18 patients treated with rapamycin 1 mg/m²/day showed a not significant trend toward a reduction of activated (CD38+, HLA-DR+) CD8 + T lymphocytes (MD -0.92, 97.5% CI -1.88 to 0.05; *p* = 0.032), and intermediate monocytes (CD14+, CD16dim monocytes, MD -13.48, 97.5% CI -28.17 to 1.22; *p* = 0.038), with respect to placebo group from baseline to treatment end; in the same frame time this group showed an increase of the percentage of memory switched B cells (defined as IgM-, IgD-, CD21+, CD24+, CD27+, CD38- B cells, MD 0.82, 97.5% CI 0.28 to 1.37; *p* = 0.002) and of classical monocytes (CD14+, CD16- monocytes, MD 17.76, 97.5% CI 0.91 to 34.60; *p* = 0.019) (Fig. 2a–d) (Table S4). Similar results were detected at week 30 (Fig. 2g–j). In a post-hoc analysis on a limited number of samples, we found that, with respect to controls, at baseline ALS patients showed a trend towards higher percentages of Th1 CD4 + T cells that remained similar after 18 weeks (Fig. S2).

Rapamycin effect on inflammasome

Patients treated with rapamycin showed lower mRNA relative expression of pro-inflammatory cytokine IL-18, which is a readout of inflammasome activation (MD -0.45, 97.5%CI -1.09 to 0.18; *p* = 0.101 for rapamycin 1 mg/m²/d group and MD -0.60, 97.5%CI -1.18 to -0.01; *p* = 0.022 for rapamycin 2 mg/m²/d group), and consistently a marked reduction in plasmatic IL-18 protein (MD -107.80, 97.5%CI -187.12 to -28.48, *p* = 0.002 for rapamycin 1 mg/m²/d group and MD -103.00, 97.5%CI -183.51 to -22.48; *p* = 0.004 for rapamycin 2 mg/m²/d group) from baseline to treatment end with respect to placebo-arm patients (Fig. 2e, f, Table S5). These effects were lost at subsequent measurements during follow up (week 30 and 54) (Fig. 2k, l). Due to the high number of tests (11 outcomes were examined) these results should be interpreted cautiously.

Longitudinal assessment of neurofilament

In the placebo arm an overall decrease in neurofilament levels was observed since the first follow-up by serial measurements in the serum (MD from baseline in serum pNfH at week 8: -174.95 ± 602.19; week 18:

-289.35 ± 788.33; week 30: -482.29 ± 927.19; MD from baseline of serum NfL at week 8: -11.61 ± 96.07; week 18: -25.76 ± 93.46; week 30: -33.21 ± 101.57) (Tables S6, S7). The decrease of serum pNfH and NfL levels from baseline to week 18 that was found in the placebo group

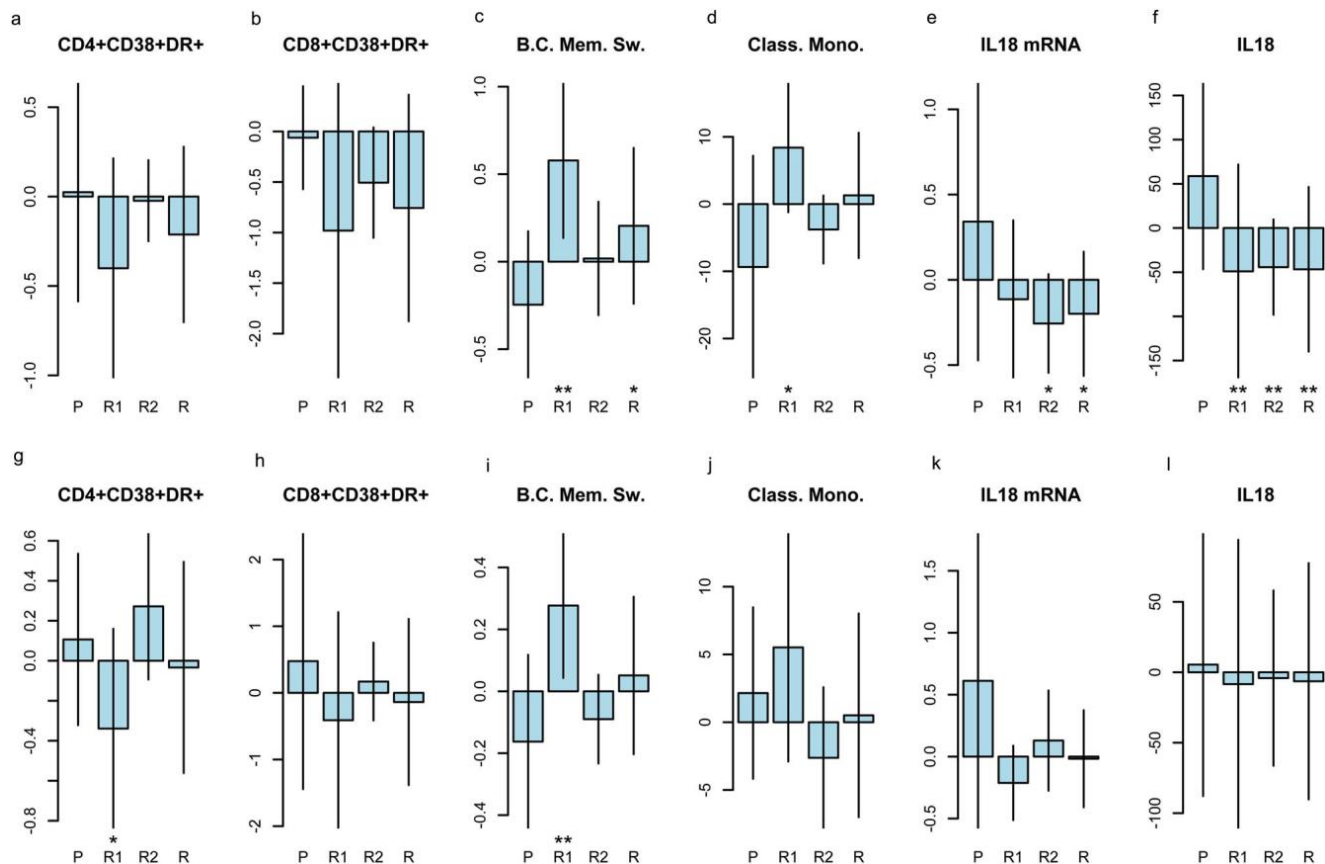


Fig. 2 | changes from baseline in blood cells population and inflammasome across treatment arms (P = placebo, R1 = rapamycin 1 mg/m²/d, R2 = rapamycin 2 mg/m²/d, R = combined rapamycin arm). The figure displays only a selection of the most interesting outcomes (55 cell subpopulation were examined and 11 inflammasome/cytokines, without accounting for multiple outcomes). In detail from left to right: changes from baseline to week 18 (a, $n = 32$ patients) and 30 (g $n = 26$ patients) in activated (CD38+, HLA-DR+) CD4 + T cells; changes from baseline to week 18 (b, $n = 29$ patients) and 30 (h, $n = 21$ patients) in activated (CD38 +, HLA-DR+) CD8 + T cells; changes from baseline to week 18 (c $n = 22$ patients) and 30 (i, $n = 19$ patients) in memory switched B cells (B.C. Mem. Sw.); changes from baseline to week 18 (d, $n = 19$ patients) and 30 (j, $n = 19$ patients) in classical monocytes (Class. Mono.); changes from baseline to week 18 (e, $n = 27$ patients) and 30 (k, $n = 20$ patients) in IL18 mRNA level (IL18 mRNA); changes from

baseline to week 18 (f, $n = 49$ patients) and 30 (l, $n = 39$ patients) of plasmatic IL18. Comparison were performed using linear regression models that include indicator variables for treatment arms as the independent variables. For the comparison of Rapamycin and placebo arms, a P value of 0.05 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 95% confidence interval (CI). For the comparisons between Rapamycin 1 mg/m²/d or 2 mg/m²/d arms and the placebo arm, a P value of 0.025 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 97.5% CI, to account for multiple arms comparison with the Bonferroni method. CIs were calculated based on the exact t distribution. All statistical tests were two-tailed. * means $p < 0.05$ for R and $p < 0.025$ for R1 and R2; ** means $p < 0.01$ for R and $p < 0.005$ for R1 and R2 arms. Error bars represent $\pm$ standard deviation. Source data are provided as a Source Data file.

was not observed in patients treated with rapamycin. This difference among treatment arms (pNfH MD 399.03, 95%CI 81.77 to 716.28; $p = 0.015$ and NfL MD 34.92, 95%CI 0.47 to 69.36; $p = 0.047$) was lost after treatment end (Tables S6, S7). Similar results were obtained from measurement of CSF pNfH and NfL at week 18 (pNfH MD 866.57, 95%CI -105.15 to 2345.72; $p = 0.075$ and NfL MD 4756.10, 95%CI 808.45 to 11531.5; $p = 0.051$, respectively) (Table S8).

Other biological outcome measures Monthly changes of selected biological outcome measures during and after treatment, across arms confirmed an increase of classical monocytes/CD14+ (MD 5.42, 97.5%CI 2.19 to 8.65, $p = 0.0003$) and memory switched B cells/CD45+ (MD 0.20, 97.5%CI 0.06 to 0.33, $p = 0.0018$) and a decrease of intermediate monocytes/CD14+ (MD -3.28, 97.5%CI -5.29 to -1.28; $p = 0.0004$) and IL-18 (MD -24.94, 97.5%CI -41.58 to -8.30; $p = 0.009$) in the rapamycin 1 mg/m²/day arm. The change from baseline to each time point of the phosphorylation of the S6RP was not different between rapamycin arms and placebo arms; this test was performed on a limited number of samples (Table S9). Changes from baseline to each time point in creatinine and albumin, CK, vitamin D, were not different in rapamycin and placebo arms.

Secondary clinical outcomes

Absolute changes from baseline to each time point in ALSFRS-R total score in patients treated with rapamycin or placebo is showed in Table S10, whereas ALSFRS-R variation before, during and after treatment is showed in Table 4. Patients treated with rapamycin 1 mg/m²/d showed a mean monthly difference of 0.50 points in the ALSFRS-R total score with respect to placebo during treatment (97.5%CI -0.32 to 1.32; $p = 0.172$), and of -0.12 after treatment (97.5%CI -0.75 to 0.51; $p = 0.671$). The difference between the rapamycin 1 mg/m²/d group and the placebo group in the change in monthly variations from before to during treatment was 0.46 (97.5%CI -0.21 to 1.13; $p = 0.174$). Figures 3 and S3 show the mean and individual rate of decline of the ALSFRS-R total scores.

Correlation analyses was performed on changes in ALSFRS-R and neurofilament levels to investigate whether a relation existed between clinical and biological outcomes. In the placebo arm, an inverse correlation was found between the change (week 18–baseline) in serum and CSF neurofilament light levels and the change (baseline – week 18) in ALSFRS-R (Pearson's r coefficient: -0.41, 95% CI 0.09 to -0.74, $p = 0.106$ between serum NFL changes and ALSFRS-R, and r coefficient: -0.58, 95% CI -0.01 to -0.86, $p = 0.049$ between CSF NFL changes and

Table 4 | Changes of ALSFRS-R monthly decline during and after treatment across treatment arms

Outcome	Period		Arm	MD	CI	p	
ALSFRS-R Total score	Before treatment	Monthly variation	Placebo	-0.80	-1.05	-0.56	0.0000
	During Treatment		Placebo	-1.39	-1.89	-0.89	0.0000
	After treatment		Placebo	-1.07	-1.45	-0.68	0.0000
	Before treatment		Rapamycin 1 mg	-0.77	-1.00	-0.53	0.0000
	During Treatment		Rapamycin 1 mg	-0.89	-1.40	-0.37	0.0008
	After treatment		Rapamycin 1 mg	-1.19	-1.58	-0.79	0.0000
	Before treatment		Rapamycin 2 mg	-0.83	-1.08	-0.59	0.0000
	During Treatment		Rapamycin 2 mg	-1.62	-2.14	-1.10	0.0000
	After treatment		Rapamycin 2 mg	-1.22	-1.62	-0.81	0.0000
	Before treatment	Between-groups analysis: comparison with placebo (monthly variation)	Rapamycin 1 mg	0.04	-0.35	0.43	0.8240
			Rapamycin 2 mg	-0.03	-0.42	0.37	0.8676
			Rapamycin	0.01	-0.29	0.30	0.9699
	During treatment		Rapamycin 1 mg	0.50	-0.32	1.32	0.1716
			Rapamycin 2 mg	-0.23	-1.06	0.60	0.5321
			Rapamycin	0.14	-0.48	0.76	0.6604
	After treatment		Rapamycin 1 mg	-0.12	-0.75	0.51	0.6706
			Rapamycin 2 mg	-0.15	-0.79	0.49	0.5948
			Rapamycin	-0.14	-0.62	0.34	0.5777
	During treatment	Intra-group analysis: comparison with before treatment period (monthly variation)	Placebo	-0.58	-1.05	-0.12	0.0146
			Rapamycin 1 mg	-0.12	-0.60	0.36	0.6238
			Rapamycin 2 mg	-0.78	-1.27	-0.30	0.0016
	After treatment		Placebo	-0.26	-0.61	0.08	0.1327
			Rapamycin 1 mg	-0.42	-0.77	-0.07	0.0177
			Rapamycin 2 mg	-0.39	-0.74	-0.03	0.0336
	During treatment	Between-groups analysis: comparison with placebo (difference in monthly variation compared to before treatment period)	Rapamycin 1 mg	0.46	-0.21	1.13	0.1742
			Rapamycin 2 mg	-0.20	-0.87	0.47	0.5581
			Rapamycin	0.13	-0.45	0.71	0.6512
	After treatment		Rapamycin 1 mg	-0.16	-0.65	0.33	0.5251
			Rapamycin 2 mg	-0.12	-0.62	0.37	0.6258
			Rapamycin	-0.14	-0.57	0.28	0.5125

Source data are provided as a Source Data file. Average monthly variations before, during and after treatment for the placebo group, as well as the comparisons between arms, are shown. Comparisons were performed using segmented repeated measures linear mixed models. Three segments of time were analyzed: before treatment (from onset to baseline), during treatment (after baseline and up to week 18), and after treatment (after week 18). The dependent variables were the raw measurements of the outcomes, whereas the independent variables were: arm, time (months from baseline), period (before, during or after treatment), and all their pairwise and three-way interactions. A random intercept term was also used to account for repeated measurements over the same individual, as well as a random slope term was used to account for individual linear variations over time. Random intercept and random slope terms were kept in the model if they improved the overall goodness-of-fit of the model. For the comparisons between Rapamycin 1 mg/m²/d or 2 mg/m²/d arms and the placebo arm, a *P* value of 0.025 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 97.5% confidence interval (CI), to account for multiple arms comparison with the Bonferroni method. For the comparison of Rapamycin and placebo arms, as well as for monthly variations and for the intra-group comparisons, a *P* value of 0.05 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 95% CI. CIs were calculated based on the exact *t* distribution using the Satterthwaite's method for degrees of freedom. All statistical tests were two-tailed.

MD mean difference, CI confidence interval.

ALSFRS-R), indicating a decrease in neurofilament while increasing disease progression. Correlation analyses among the change (week 18–baseline) in serum and CSF neurofilament light levels and the change (baseline - week 18) in ALSFRS-R within rapamycin arm showed no correlation: Pearson's *r* coefficient was 0.18 (95% CI -0.50 to 0.17, *p* = 0.308) between ALSFRS-R and serum NFL change (w18–w0), and 0.32 (95% CI -0.65 to 0.10, *p* = 0.131) between ALSFRS-R and CSF NFL changes (w18–w0). Similar results were obtained with pNfH (Fig. 4). Comparison among correlation coefficients showed a nearly significant difference between rapamycin and placebo arm in serum NfL (*p* = 0.0608); the difference was less pronounced for pNfH (*p* = 0.1429).

There were no statistically significant differences between patients treated with rapamycin and placebo as far as PEG (19.0% in the placebo group, 14.3% in the rapamycin group, *p* = 0.664) or NIV

positioning (28.6% in the placebo group, 19.0% in the rapamycin group, *p* = 0.468) are concerned. During the study 7.1% of patients treated with rapamycin and 4.8% of patients in the placebo group had died (*p* = 0.672). There was only one IV in the placebo group. The most common cause of death was respiratory failure, accounting for three of the four deaths, a finding consistent with the natural history of ALS.

A post-hoc analysis on tracheostomy-free survival with last observation set on 31st December 2021, showed that 52.4% of patients treated with rapamycin and 61.9% of patients in the placebo group had died or underwent tracheostomy, not a statistically significant difference (*p* = 0.356) (Table S11, Fig. S4). There were no significant differences in the mean absolute change from baseline to each time point in the FVC (at week 18, MD 3.20, 97.5%CI -9.798 to 16.19, *p* = 0.571 for rapamycin 1 mg/m²/d group, and MD -4.57, 97.5% CI -17.56 to 8.42, *p* = 0.419 for rapamycin 2 mg/m²/d group)

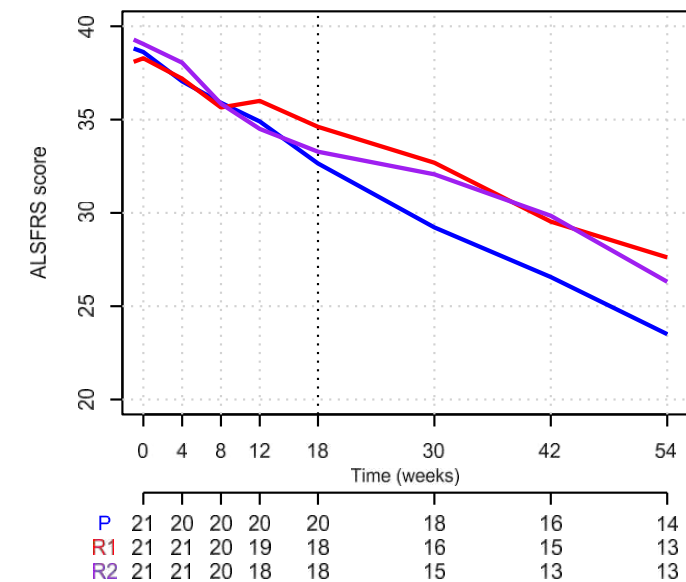


Fig. 3 | Mean rates of decline in Amyotrophic Lateral Sclerosis Functional Rating Scale Revised (ALSFRS-R) total score (Intention to Treat population) of patients enrolled in RAP-ALS over the study (baseline to week 54) based on treatment arm allocation (red = R1, rapamycin 1 mg/m²/d, violet = R2, rapamycin 2 mg/m²/d, blue = P, placebo). Source data are provided as a Source Data file.

(Table S12, Fig. S5). There were no significant differences in the mean absolute change from baseline to each time point in the ALSAQ40 scores (Tables S13, S14). ALSAQ40 main questions scores are represented in Fig. 5. Correlations among clinical outcome measures are presented in Table S15.

Safety and drug adherence

A total of 23 over 42 individuals (55%) in the rapamycin group and 11 over 21 individuals (52%) in the placebo group had one or more AEs during the trial (Table S16). The total number of reported AEs was 23 for placebo arm, and 46 for the rapamycin arms. Severe AEs (SAEs) were 7 in the placebo group (30.4% of total AEs in that group), and 9 in the rapamycin groups (19.6% of total AEs in those groups) (Table S17). Among the totality of AEs, four caused treatment discontinuation (one in the placebo group and three in the rapamycin groups) (Table S17).

Individuals with SAEs were 19% both in the placebo and in the rapamycin groups (Table S18).

Events occurring at a greater frequency in the rapamycin group were primarily skin and subcutaneous tissue disorders (erythema, pruritus, rash, conjunctivitis, dermatitis, eczema), then gastrointestinal disorders, injuries, respiratory disorders, headache and psychiatric disorders (Table 5). The majority of SAEs were represented by complications related to disease progression, such as dysphagia and hospital admissions to undergo PEG positioning, or respiratory failure/pneumonia. One subject committed suicide.

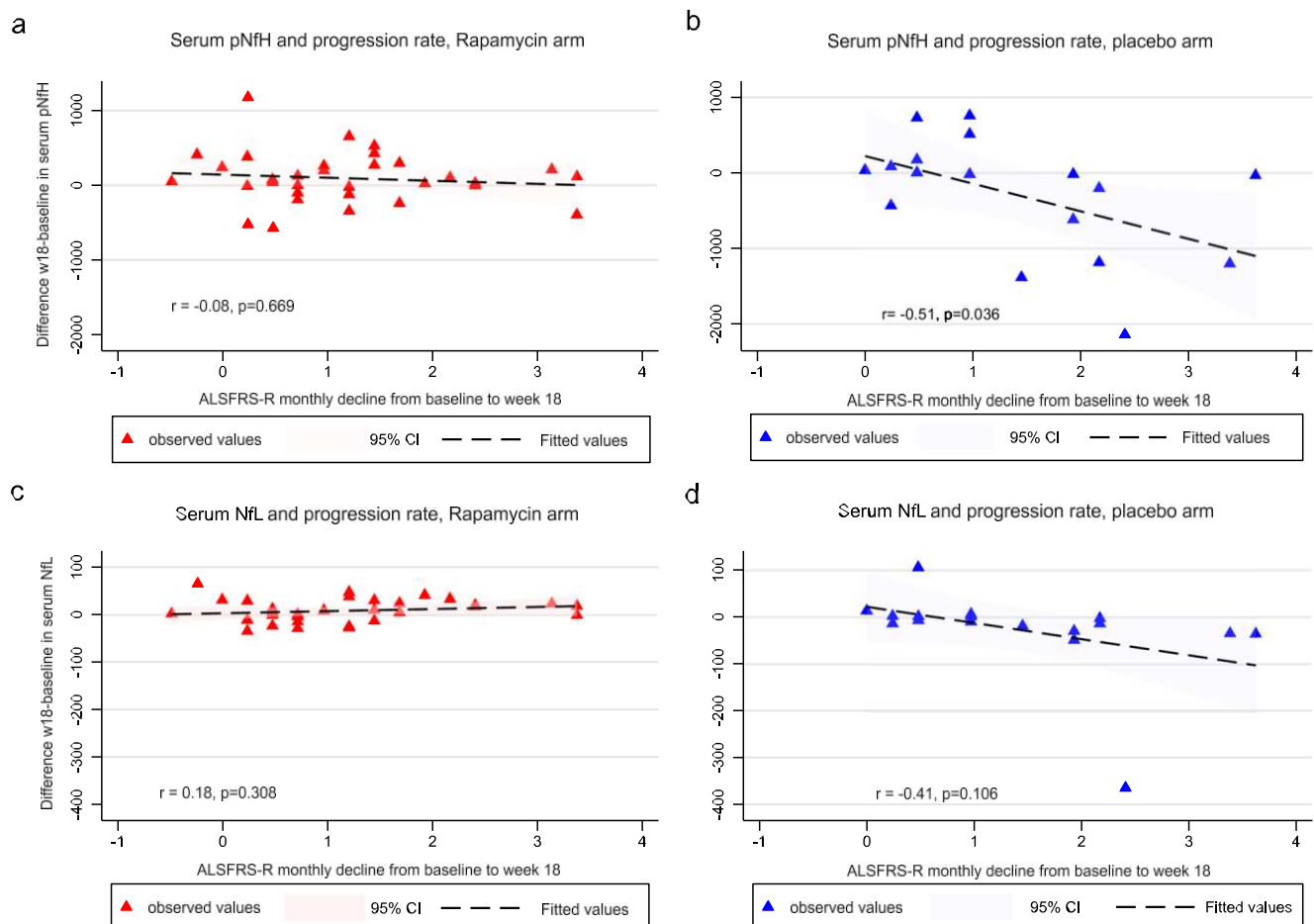


Fig. 4 | Changes from week 18 to baseline in serum Neurofilament Light (NfL) and phosphorylated Neurofilament Heavy (pNfH) in relation to progression rate across treatment arms. In detail from left to right, upper panels: changes from week 18 to baseline in serum pNfH in rapamycin (a) and placebo arm (b), in relation to progression rate calculated as the monthly decline in the ALSFRS-R from baseline to week 18. From left to right, lower panels: changes from week 18 to baseline in serum NfL in rapamycin (c) and placebo arm (d), in relation to progression rate calculated as the monthly decline in the ALSFRS-R from baseline to week 18. Individual differences in neurofilament concentration between week 18 and baseline are plotted as colored symbols (Rapamycin arms in red; placebo arm in blue). The shaded areas represent the 95% confidence intervals around the model estimates. The lines and confidence intervals are drawn from the actual distributions of linear model fits. All statistical tests were two-tailed. Source data are provided as a Source Data file.

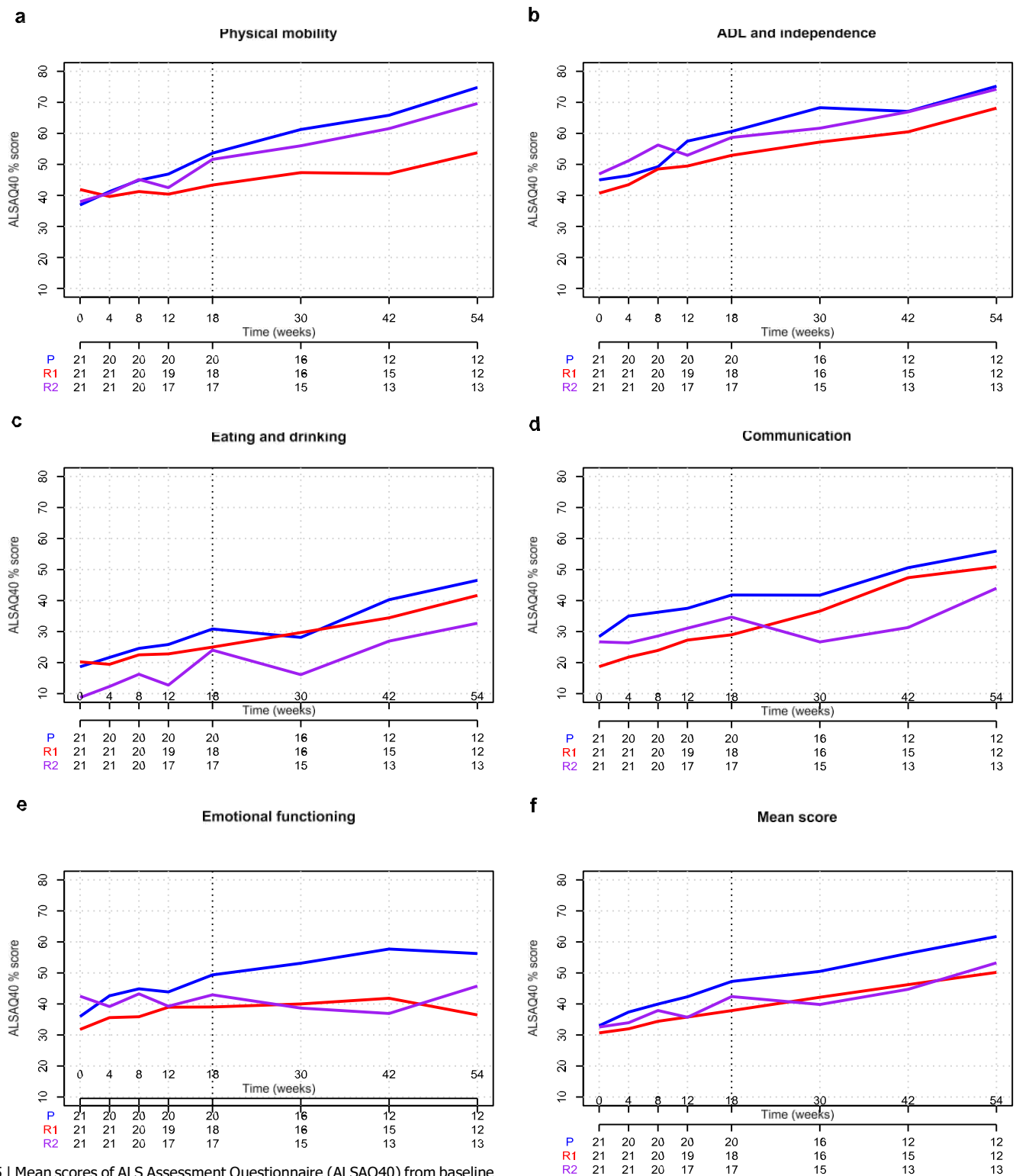


Fig. 5 | Mean scores of ALS Assessment Questionnaire (ALSAQ40) from baseline to study end across treatment arms. A–E These show the treatment-dependent mean scores of ALSAQ40 physical mobility, Activity Daily Living (ADL) and independence, eating and drinking, communication, and emotional functioning main

questions, respectively, from baseline to study end. Panel F shows ALSAQ40 mean total score from baseline to study end (red = R1, rapamycin 1 mg/m²/d, violet = R2, rapamycin 2 mg/m²/d, blue = P, placebo; Intention to treat population). Source data are provided as a Source Data file.

Of note, there was a case of acute hepatitis probably related to the study drug, that occurred in a subject allocated to the rapamycin 2 mg/m²/day treatment arm, who had a very high peak of rapamycin at first blood dosage and for whom dose reduction applied. No permanent consequences had been reported from this event.

A total of 11% of the participants dropped out during the study treatment, 5% in the placebo group and 14.3% in the rapamycin group. During follow up time, 24% of patients in each group abandoned the study. Events leading to discontinuation of the treatment are presented in Table S19. Data on adherence to the trial regimen are summarized in Table S20.

Table 5 | Treatment-emergent adverse events

Treatment-emergent adverse events, MedDRA preferred term	PLA-CEBO (n = 21)		Rapamycin 1 mg/m ² /d (n = 21)		Rapamycin 2 mg/m ² /d (n = 21)	
	n	%	n	%	n	%
Blood and lymphatic system disorders	0	0.0%	1	4.8%	0	0.0%
Depression and suicide/self-injury	0	0.0%	1	4.8%	0	0.0%
Ear and labyrinth disorders	1	4.8%	0	0.0%	0	0.0%
Eye disorders	1	4.8%	0	0.0%	0	0.0%
Gastrointestinal disorders	0	0.0%	1	4.8%	2	9.5%
General disorders and administration site conditions	1	4.8%	1	4.8%	0	0.0%
Infections and infestations	4	19.0%	3	14.3%	4	19.0%
Injury, poisoning, and procedural complications	3	14.3%	2	9.5%	6	28.6%
Investigation	0	0.0%	1	4.8%	0	0.0%
Metabolism and nutrition disorders	1	4.8%	0	0.0%	2	9.6%
Musculoskeletal and connective tissue disorders	2	9.6%	0	0.0%	0	0.0%
Nervous system disorders	1	4.8%	0	0.0%	1	4.8%
Psychiatric disorders	0	0.0%	0	0.0%	1	4.8%
Renal and urinary disorders	2	9.6%	0	0.0%	0	0.0%
Renal and urinary disorders—Investigation	1	4.8%	0	0.0%	0	0.0%
Respiratory, thoracic, and mediastinal disorders	2	9.5%	4	19.0%	5	23.8%
Skin and subcutaneous tissue disorders	1	4.8%	5	23.8%	6	28.6%
Vascular disorders	1	4.8%	0	0.0%	0	0.0%
Not classified	2	9.5%	0	0.0%	0	0.0%

Drug dosage assessment

Plasma levels of rapamycin at different time points for each treatment arm are displayed in Fig. S6. While patients who were allocated to rapamycin 1 mg/m²/day treatment had a quite stable plasma dosage, patients allocated to the other treatment arm, during the first dosages presented an initial peak, with plasma levels above the upper limit of therapeutic range and also of the safety threshold (15 ng/ml), that next required drug re-dosing either to 1 mg/m²/day arm or to the minimal dose of 1 mg/day. Finally, examining CSF samples of patients at week 18 by LC-MS/MS a clear peak corresponding to Sirolimus was not detected neither in the CSF of treated patients, nor in the CSF of placebo (Fig. S7).

Discussion

This clinical trial measured the biological, clinical and safety effects of rapamycin on patients affected by ALS. Unfortunately, the primary outcome measure could not be satisfied also due to the reduced number of samples that could be analyzed at week 18, to which the COVID-19 pandemic significantly contributed. Notwithstanding our expectations for an inevitable loss in samples at treatment end, this led to a final sample size of 50 on which the primary endpoint was assessed. Therefore, we could not demonstrate a significant effect of the study drug on Treg lymphocytes, and the question as to why some subjects did not show the expected Tregs increase in response to rapamycin remains.

Inter-individual variation amongst ALS patients in the ability to increase Tregs in response to treatment have been recently observed in other RCTs¹⁸. Since rapamycin effect on mTOR results from a delicate equilibrium between time and doses, many factors may affect the highly variable individual drug response including low bioavailability

caused by liver and gut metabolism, genetic changes in the enzymes (mainly CYP3A4 and CYP3A5) involved in rapamycin metabolism, transportation and pharmacodynamics¹⁹.

Furthermore, increased expression of the mTOR-interacting Raptor protein, increased phosphorylation of Akt, and activation of growth-related transcriptional factor AP-1 may induce rapamycin resistance²⁰, as well as mutant SOD1²¹. The complex and incompletely understood action of mTOR in both the regulation of lymphocyte differentiation and the enhancement of autophagy, may also explain why in a post-hoc analysis we did not find reduced Th1 markers in the treated group, besides the small sample analyzed; a previous study demonstrated that rapamycin yields an anti-apoptotic Th1/Tc1 effector phenotype by promoting autophagy²². In fact, mTOR blockade during human Th1/Tc1 cell generation induced autophagy, which promoted the survival of an anti-apoptotic T cell population that possesses the capacity to persist in vivo for prolonged intervals²². Finally, if rapamycin effect on immune response is dose-dependent, a high exposure to rapamycin may also cause inhibition of mTORC2 with detrimental immunologic and metabolic consequences²³. This may explain the different results obtained on several immunological parameters in the group treated with rapamycin 1 mg/m²/d.

Among secondary biological outcome measures we observed changes on B and T cell subpopulations, monocytes, and on IL-18, which could be suggestive of a rapamycin-mediated effect on neuroinflammation in ALS, where misfolded protein aggregates may activate a cascade of events that drives chronic inflammation and secretion of proinflammatory cytokines, among which IL-18, that finally leads to tissue damage and cell death²⁴. Nonetheless, these results should be interpreted cautiously and require confirmation in larger studies, considering that we did not apply corrections for multiple tests.

Together with the above mentioned limitation, the increase in classical monocytes and the reduction in intermediate monocytes in patients treated with rapamycin, is even more difficult to interpret because of the uncertainty on their role in ALS, and if monocyte subsets and activation profiles are altered depending on the stage of the disease (i.e., the changes are a response to disease and their changes suggest that the immune system becomes more activated as the disease progresses) or if they are instead pathogenetic deserves further studies^{25–27}. The role of B cells in ALS has to be established yet, but increase in switched memory B cells, a population that differentiate into plasma cells upon reactivation²⁸, may suggest a compensatory mechanism of controlling inflammation by B cells. Overall, our study shows that there is a minor impact of the treatment on the measured outcomes given the large number of small group differences, but further studies should be performed to look at other biomarkers, such as Treg suppressor function and the best dose, to establish if rapamycin deserves to be pursued in larger ALS trials.

Serial measurements of neurofilament showed a constant decrease in placebo-arm patients, not observed in the treatment arms, where we found stable neurofilament levels. The different behavior of neurofilament in treatment and placebo arms may have several explanations since longitudinal behavior of neurofilament during ALS and/or in clinical trials is still being studied with conflicting results. Some studies found fast disease progression to be associated with neurofilament decrease over time²⁹, other observed an absence of correlation between a drug clinical effect and repeated neurofilament measurements^{30,31}, others correlated a rise of serum NfL over time to a fast-progressing disease course³². Differently from other trials we followed up patients with regular serum collection for 54 weeks, ensuring a relatively long period of observation and revealing an unusual trajectory in the placebo-arm patients, with a constant decrease for serum NfL and an initial steep decrease later diminished for serum pNfH. This may suggest that neurofilaments concentration is affected not only by the rate of neuronal degeneration but also by the phase of the disease

and the share of surviving MNs (i.e., in the presence of massive MNs degeneration, neurofilament release diminishes as a consequence of the decreased number of surviving MNs)³³. As far as rapamycin arm is concerned, preclinical studies demonstrated that rapamycin reduces TDP-43 proteinopathy through autophagy enhancement and consequently relieves the pathogenic translational suppression of neurofilaments with increased protein synthesis^{6,34}. This hypothesis is supported by the reversibility of dose-related biological effects upon rapamycin discontinuation. Therefore, neurofilament concentrations in relation with highly active neurodegenerative processes such as ALS may uncover different pathobiological mechanisms and should be interpreted cautiously in the setting of experimental drug testing³⁵. In fact, we found an inverse correlation between disease progression and longitudinal change in NF only in the placebo group, whereas in rapamycin-treated patients there was no correlation between NF changes and disease progression.

This is a non-profit, exploratory study on rapamycin action in ALS patients, that was designed to investigate if this drug warrants further research in patients with ALS, considering also its safety profile. Rapamycin in combination with riluzole, was safe and well tolerated by ALS patients. AEs and SAEs were equally distributed between treatment and placebo arms, reassuring about safety, provided that a drug plasma dosage monitoring was performed. It is also reassuring that ~24% of the participants discontinued the trial, that is in line with recent data from other clinical studies³⁶. Besides demonstrating that rapamycin is safe in patients with ALS, we found that rapamycin dosage of 1 mg/m²/day ensured a better stability of plasma dosages (never overcoming toxicity threshold) that only seldom required dosages adjustment due to safety concerns.

This study was not powered to test an effect on clinical measures, but we could not observe a slowing in the ALSFRS-R decline nor an effect on quality of life, even after excluding SOD1 patients from enrollment. If the effect on Treg cells and other immunological outcome measures was more evident for patients treated with rapamycin 1 mg/m²/day, the absence of data on Treg function requires further studies to ascertain what dose has the more beneficial immunological effect in ALS.

Given the reported detrimental effects in SOD1-ALS mice¹¹, however, next studies should be again cautiously restricted to non-SOD1 ALS patients. A specific detrimental interaction between rapamycin and SOD1 may be hypothesized²¹, reinforcing the concept that clinical and pathological heterogeneity of ALS may require personalized therapeutic strategies.

The main potentiality of this trial stands on immediate availability of the drug for patients use, should an effect be found by larger trials, and by the existence of newer and interesting molecules i.e., rapalogs, who offer the possibility to cross the blood brain barrier perhaps deserving further properties towards autophagy and neuroinflammation. In fact, we could not find the drug in the CSF of patients treated for 18 weeks, and although we cannot exclude rapamycin free fraction values below the detection limit of LC-MS/MS, it is probable that rapamycin cannot cross blood brain barrier. If this may be of secondary importance for the drug immunological effects, the concentration at which rapamycin or any rapalog are found able to penetrate the blood-brain barrier might be a matter of uttermost relevance for the sake of fostering autophagy¹³.

Limitations of this early trial of rapamycin in ALS were the small number of participants and the short duration of treatment, that were mainly due to safety concerns. Furthermore, the expected effect size of the primary outcome measure was perhaps too optimistic and, together with the difficulties in obtaining blood samples at treatment end, this led to miss the primary outcome. The choice of the primary outcome measure, as a binary response, has to be acknowledged as the main drawback of this study, together with the fact that Treg function, rather than their number would have been relevant for treatment

effect. Indeed, we did not plan to study Treg suppressive function instead of their number alone and we were not able to assess Treg suppressor function as a post-hoc analysis due to lack of available samples. Before further studies in ALS, assessment of Treg suppressor function in response to different doses of rapamycin would be critical. In addition, some data on the primary outcome were not measured due to the COVID-19 pandemic, which may introduce a risk of bias into the results. However, the double-blind design of the study and the random missing mechanisms should have limited this risk. Other drawbacks are represented by the imbalance of some factors at baseline (such as in edaravone treatment), and the exploratory nature of the clinical outcomes. Finally, the results on biological outcomes should be interpreted cautiously and require confirmation in larger studies, considering that we did not apply corrections for multiple tests.

In conclusion, this trial demonstrated that treatment with a low dose of rapamycin is safe in patients with ALS, but it failed to demonstrate an effect of the drug on Treg cells. Further trials focused on different outcome measures are necessary to better understand rapamycin biological and clinical effects in ALS.

Methods

Study design

A randomized, double-blind, placebo-controlled trial was conducted at seven Italian ALS referral centers from 2017 through 2020. The trial was conducted in accordance with the Good Clinical Practice guidelines of the International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the ethical principles of the Declaration of Helsinki, as amended by the 64th WMA General Assembly, Fortaleza, Brazil, in October 2013. The study complies with ICMJE guidelines on reporting.

Protocol (EUDRACT 2016-002399-28 registration: 31st May 2016) approval was provided for the coordinating center and all trial sites by Ethical Committee of the coordinating center (Comitato Etico Provinciale di Modena) on 23th May 2017 (file number 95/17) and by AIFA (Agenzia Italiana del Farmaco) on 14th July 2017. All the participants provided written informed consent before screening (first and last patient enrollment: 05/10/2017 to 02/01/2020). The trial was a non-profit trial, financed by ARISLA (Fondazione Italiana di Ricerca per la SLA) with the “2015 Arisla Ice Bucket Call for Clinical Projects”. Pfizer Inc. provided the active drug. Participants received no compensation.

The study promoter was Azienda Ospedaliero-Universitaria (AOU) di Modena. The trial design, data analysis, and manuscript development were shared by the Steering Committee of the Study represented by all the local PIs (Supplementary Appendix, Section 2). The authors were responsible for writing the manuscript and making the decision to submit it for publication. Confidentiality agreements were set up between the authors and ARISLA. Data collected at each trial site were input in an online case report form (CRF) written in PHP version 7.2, jquery version 3.4.1 and PostgreSQL version 9.5 as a database backend, set up and managed by the Coordinator Center at AOU, Modena and only accessible to RAP-ALS investigators through password protected access.

An independent data and safety monitoring board was established at trial beginning and periodically reviewed unblinded safety data during the trial (Acknowledgements section). Statistical analyses were performed by the Unit of Statistical and Methodological Support to Clinical Research, AOU, Modena, Italy. All the authors guarantee for data completeness and accuracy, and for adherence to RAP-ALS study protocol (available at <http://www.nature.com/> as supplementary material of the present article and already published¹⁷).

Trial participants

The trial enrolled patients diagnosed with definite, clinically probable or probable with laboratory support ALS according to revised El

Escorial criteria who presented ALS symptoms onset not earlier than 18 months before screening. Inclusion criteria encompassed age between 18 and 75 years old, a forced vital capacity (FVC) exceeding 70% of the predicted value for sex, age, height, and weight, a Body Mass Index above 18 and a body weight over 50 kg, use of riluzole at a stable dose for at least 30 days before screening. Exclusion criteria covered a wide range of diseases and conditions that would make rapamycin use or immunosuppression contraindicated. Patients with known SOD1 mutation or with FALS and family members carrying SOD1 mutation were to be excluded as well, based on contrasting evidence of Rapamycin action in SOD1 models of ALS,^{37,38} quite the reverse on previous studies on models linked to TDP43 pathology^{14,39}. Inclusion and exclusion criteria are fully described in the published protocol¹⁷.

Randomization and masking

Eligible participants were randomly assigned in three treatment arms with a 1:1:1 ratio to receive rapamycin 1 mg per square meter (m²) of body surface area a day (1 mg/m²/day) (21 patients), rapamycin 2 mg/m²/day (21 patients), or placebo (21 patients). The randomization schedule was computer generated by an unblinded statistician using STATA software (StataCorp. 2017. Stata Statistical Software: Release 15). Randomization was stratified by rate of disease progression as measured by monthly decline of the Revised ALS Functional Rating Scale (ALSF_{RS}-R) from onset to screening visit, with a cut off set at ≤ 0.7 . Euromed Clinical Supply Services (ECLISSE) (Cantù, Como, Italy; <http://www.css.euromed.it/en/>) prepared the active formulation and the placebo complying the Good Manufacturing Practices of the European Union for active pharmaceutical ingredients and ICH Q7A guidelines. Trial drug was dispensed in kits with random four-digit identification numbers. Kits were sent in sequence to sites as each new participant was enrolled. Treatment under investigation and placebo were made indistinguishable to patients and neurologists.

Procedures

Treatment was administered orally, in the morning, at fast. Patients received 4 bottles, each containing 15 tablets of active drug or placebo depending on the assigned treatment arm, every 2 weeks, for a planned duration of 18 weeks. Rapamycin plasma levels were regularly measured by high-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) and made known only to an independent monitor, who input rapamycin levels on a separate eCRF, allowing dose reduction if rapamycin plasma levels exceeded 12 ng/mL. A dose reduction could be asked also by caring neurologist directly through eCRF in case of adverse events (AEs) or reactions that, on clinical judgment, could be attributed to the study drug. After treatment end patients had to be followed up for further 36 weeks (<https://www.clinicaltrialsregister.eu/ctr-search/trial/2016-002399-28/IT>; ClinicalTrials.gov, NCT03359538).

Outcomes

The primary efficacy outcome was the proportion of positive response (Tregs number increase of at least 30%) at treatment end (18 weeks) with respect to baseline, in patients treated with rapamycin compared to the placebo arm. This difference was established based on a previous study demonstrating that slowly progressing ALS patients presented a number of Tregs that was equal to healthy controls, whereas fast progressors had 31% fewer Tregs⁴⁰. Since Tregs % were demonstrated to be inversely correlated with the rate of disease progression,⁴¹ we considered a “positive response” as an increase of the proportion of Tregs by at least 30% at treatment end.

Secondary outcomes were:

- I. Assessment of rapamycin safety and tolerability through documentation of the occurrence of any AEs, changes on clinical examination including vital signs and weight, and laboratory

examinations (biochemistry, hematology and urinalysis) that were registered throughout the study duration. Symptoms consistent with disease progression, were recorded as AEs.

- II. Biological outcomes, assessed as the change from baseline to week 8, 18, 30, 54, comparing rapamycin and placebo arms, of the following biological variables: a) activation and homing capabilities of different T, B, NK cell subpopulations; b) relative expression of inflammasome genes and its activation status; c) phosphorylation of the S6 ribosomal protein (S6RP); d) plasma/CSF neurofilament heavy/light chain protein; e) creatinine and albumin, CK, vitamin D; f) the assessment of rapamycin in CSF samples at week 18 by LC-MS/MS. Laboratory methods are explained in Supplementary Appendix, Section 3.1.
- III. Clinical outcomes through comparison between placebo and treatment arms of: a) the changes in ALSFRS-R from baseline to weeks 4, 8, 12, 18, 30, 42, 54; b) overall survival from randomization to date of documented death or tracheostomy; c) survival rate at weeks 18, 30, 42, and 54; d) respiratory muscle function as assessed by FVC score from baseline to weeks 4, 8, 12, 18, 30, 42, 54 (Supplementary Appendix, Section 3.2).
- IV. Quality of life, measured through absolute and relative change from baseline in ALSAQ-40 at week 4, 8, 12, 18, 30, 42 and 54 comparing treatment and placebo arms.

Statistical analysis

Sample size was calculated using data from an Italian study showing that ALS patients have a decreased number of circulating Treg% (mean $\pm$ SD: 2.1 ± 0.7) if compared to healthy controls (2.6 ± 0.6), except for slow progressors⁴⁰. Considering normal values of total Treg of $71.5 \pm 17/\text{mmc}$ on a normal total lymphocytes count between 1000 and 4500/mmc, slowly progressing ALS patients presented a number of Tregs that was equal to healthy controls, whereas fast progressors had 31% fewer Tregs⁴⁰. Since Treg % were demonstrated to be inversely correlated with the rate of disease progression,⁴¹ we considered a “positive response” as an increase of the proportion of Tregs by at least 30% at treatment end. The null hypothesis was that Rapamycin could not significantly increase the proportion of positive responses in treated patients at week 18, compared to baseline and to placebo group. The alternative hypothesis was that Rapamycin could determine a positive response in at least 50% of treated patients compared to a maximum 5% of patients in the placebo group. The study was designed to reject the null hypothesis with an alpha error of 0.025 (in order to take into account multiple arms comparisons) and a power of 0.80. With a 1:1:1 randomization ratio among the three arms we calculated that 54 participants would provide 80% power to detect a 30% difference in the percentual of circulating Treg in at least 50% of treated patients versus less than 5% in the placebo group, using a chi-square test without any correction at a two-sided alpha level of 0.025. The study was planned to reach a sample size of 63 patients taking into consideration a possible 15% of drop out.

Safety analyses were performed including all patients who received at least one tablet of rapamycin or placebo. All AEs, SAEs, and AEs leading to treatment discontinuation were recorded according to ICH Guidelines, listed, and compared in the treatment arms at any follow-up visit and at the end of the study.

The primary population for analyses was the ITT population, which included all the participants who received at least one tablet of the investigation drug. PP analysis was performed after excluding patients as per major protocol deviations (i.e., patients who took <80% therapy) from the above-mentioned population. Descriptive statistics comparing the two groups of rapamycin treatment and placebo was performed using mean and standard deviation for continuous variables, counts and percentages for categorical variables. Immune response to rapamycin was analyzed as the difference in positive

response to rapamycin (mean Tregs increase exceeding 30%) between the placebo group and the rapamycin groups. Results were expressed as the relative risk (RR) comparing treatment arms. The comparison was carried out with a chi-square test without any correction. Mean absolute differences from baseline to week 18 and other time points among treatment arms for S6RP phosphorylation, of different T, B, NK cell subpopulations, of biomarkers, inflammasome, cytokines, were calculated and compared using linear regression models that include indicator variables for treatment arms as the independent variables. Results were expressed as the mean difference (MD) comparing treatment arms. Correlations between numerical variables were calculated with the Pearson's linear correlation coefficient. The time to death, tracheostomy or permanent ventilation, were compared by using the log-rank test. Adjustment for main prognostic or unbalanced factors (namely sex, months from symptoms onset, ALSFRS-R slope at baseline and edaravone treatment) has also been performed using logistic or linear regression models. Results of logistic models were expressed as the OR comparing treatment arms.

A segmented repeated measures linear mixed model analysis was carried out to assess whether the average monthly variation from baseline in selected numerical outcomes was different amongst treatment arms in two segments of time: during the treatment (after baseline and up to week 18), or after the treatment (after week 18). The dependent variables were the raw measurements of the outcomes, whereas the independent variables were: arm, time (months from baseline) $\times$ period (during or after treatment) interaction, and arm $\times$ time $\times$ period interaction. A random intercept term was also used to account for repeated measurements over the same individual, as well as a random slope term was used to account for individual linear variations over time. Random intercept and random slope terms were kept if they improved the overall goodness-of-fit of the models (assessed using the Akaike information criterion). Results of this analysis were expressed as: the monthly variation of outcomes in the placebo group; the MD in the monthly variations, comparing treatments arms vs placebo. Both these quantities were reported for the two segments of time (during and after treatment). In the analysis of average monthly variation of ALSFRS-R, three segments of time were analyzed: before, during and after treatment, by considering two additional timepoints such as the prebaseline screening visit and the disease onset. At disease onset, the ALSFRS-R was assumed to be equal to 48 for all patients. The repeated measures linear mixed model for ALSFRS-R included arm, time (months from baseline), period (before, during or after treatment), and all their pairwise and three-way interactions. The intra-group differences comparing ALSFRS-R monthly variations in the periods during and after treatment with those occurred before treatment were also reported for each arm, as well as the comparison between treatment arms and placebo in these quantities. A post hoc analysis on tracheostomy-free survival from baseline to 31st December 2021 was performed.

For the comparisons between Rapamycin 1 mg/m²/d or 2 mg/m²/d arms and the placebo arm, a *P* value of 0.025 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 97.5% confidence interval (CI), to account for multiple arms comparison with the Bonferroni method. For the comparison of Rapamycin and placebo arms and, when applicable, for all other statistical measures, a *P* value of 0.05 or less was considered to indicate statistical significance and uncertainty in results was expressed with the 95% CI. CIs for linear models were calculated based on the exact *t* distribution. For linear mixed models, the Satterthwaite's method for degrees of freedom was used. All statistical tests were two-tailed. Missing values were excluded from the analyses and the number of analyzed individuals in each arm was reported. Missing values were due to Covid-19 pandemic, and, for secondary biological outcome measures, also to the reduced amount of material of biological samples for difficulty in blood withdrawal; for FVC they were due to

increasing patients' difficulties to perform spirometry with advancing respiratory or bulbar impairment.

Analyses were performed using STATA software, version 15 (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC) and R software, version 3.6.3 (The R Foundation for Statistical Computing, Wien).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author (jessica.mandrioli@unimore.it) to external researchers who provide methodologically sound scientific proposals and whose proposed use of the data has been approved by an independent review committee identified for this purpose. All requests will be reviewed by corresponding author and Steering Committee of the Study and response to requests will be given in 2 months. A materials transfer and/or data access agreement with the study promoter will be required for accessing shared data. However, individual participant data will not be available because informed consent did not explicitly include this. Source data are provided as Source Data files with this paper. The study protocol, including the statistical analysis plan has been uploaded in the Supplementary Information file. Source data are provided with this paper.

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Author contributions

J.M., A.Co., R.D.A., M.P. had the idea for the study, did the literature searches and designed the study. E.Z., I.M., N.F., G.G., C.L., F.G., C.T., L.M., F.D.M., A.S., G.S., A.F., G.L., E.D.B., C.C., G.M., A.Ch., and A.Ca. were co-investigators, were responsible for patients recruitment, treatment, biological samples collection, data input in eCRF. Clinical oversight of the study was undertaken by J.M. and supported by R.D.A. The RAP-ALS Investigators provided feedback on the protocol and oversaw site recruitment and data collection for their respective sites. J.M., R.D.A., A.Co., E.Z., I.M., R.V., F.B. were responsible for development of protocols, site training, and validation and creation of the eCRF and database. The study conduct was overseen by J.M. and R.V., supported by R.D.A. and a local management team comprising I.M., E.Z., N.F. A.Co., S.D.B., M.P. developed standard operating procedures for immunological analyses. S.D.B., C.S., D.L.T., M.P., under the supervision of A.Co., performed immunological analysis of biological samples. Acquisition of the financial support for the project leading to this publication was performed by J.M., A.Co. and R.D.A. with participation of A.C., C.C., C.L., G.S., E.D.B., L.M. Project administration was performed by J.M., R.D.A. and A.Co. The final analysis of all the primary and secondary outcomes was undertaken by F.B. under the supervision of R.D.A., and both verified the raw data. The manuscript was drafted by J.M., E.Z., F.B., S.D.B. All authors had access to all the study data and read, contributed to, reviewed and approved the submission of the manuscript for final publication.

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Competing interests

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Additional information

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ORIGINAL ARTICLE

Effect of RNS60 in amyotrophic lateral sclerosis: a phase II multicentre, randomized, double-blind, placebo-controlled trial

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For RNS60-ALS Study Group—see [Appendix B](#).

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Abstract

Background and purpose: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with limited treatment options. RNS60 is an immunomodulatory and neuroprotective investigational product that has shown efficacy in animal models of ALS and other neurodegenerative diseases. Its administration has been safe and well tolerated in ALS subjects in previous early phase trials.

Methods: This was a phase II, multicentre, randomized, double-blind, placebo-controlled, parallel-group trial. Participants diagnosed with definite, probable or probable laboratory-supported ALS were assigned to receive RNS60 or placebo administered for 24 weeks intravenously (375 ml) once a week and via nebulization (4 ml/day) on non-infusion days, followed by an additional 24 weeks off-treatment. The primary objective was to measure the effects of RNS60 treatment on selected biomarkers of inflammation and neurodegeneration in peripheral blood. Secondary objectives were to measure the effect of RNS60 on functional impairment (ALS Functional Rating Scale—Revised), a measure of self-sufficiency, respiratory function (forced vital capacity, FVC), quality of life (ALS Assessment Questionnaire-40, ALSAQ-40) and survival. Tolerability and safety were assessed.

Results: Seventy-four participants were assigned to RNS60 and 73 to placebo. Assessed biomarkers did not differ between arms. The mean rate of decline in FVC and the eating and drinking domain of ALSAQ-40 was slower in the RNS60 arm (FVC, difference 0.41 per week, standard error 0.16, $p = 0.0101$; ALSAQ-40, difference -0.19 per week, standard error 0.10, $p = 0.0319$). Adverse events were similar in the two arms. In a *post hoc* analysis, neurofilament light chain increased over time in bulbar onset placebo participants whilst remaining stable in those treated with RNS60.

Conclusions: The positive effects of RNS60 on selected measures of respiratory and bulbar function warrant further investigation.

KEY WORDS

ALS, clinical trial, placebo-controlled, randomized, treatment

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease that affects motor neurons in the spinal cord, brainstem and motor cortex resulting in muscle atrophy and paralysis. Respiratory complications are the most common cause of death and develop due to progressive paralysis of the diaphragm and the accessory muscles that support ventilation [1]. Riluzole [2] and edaravone [3] are the only two available disease-modifying medications on the market and they both have only modest effects on disease outcomes. Thus, there is an urgent need to develop more treatments for people living with ALS.

In recent years, multiple lines of evidence have supported a critical role for neuroinflammation in ALS pathophysiology including *in vivo* imaging studies in mouse ALS models, *ex vivo* analyses revealing astrocyte and microglia activation even in pre-symptomatic phases, and recent discoveries showing involvement of several genes directly linked to the immune response [4]. Defects in mitochondrial bioenergetics have also been implicated in the pathogenesis of ALS and are being considered as potential therapeutic targets [5–7].

RNS60 is an investigational product generated by using modified Taylor–Couette–Poiseuille flow under elevated oxygen pressure, which is hypothesized to generate oxygen-filled charge-stabilized nanostructures (O₂ nanobubbles). Although at a molecular level the mechanism of action of RNS60 has not been fully elucidated yet, its immunomodulatory and cytoprotective properties have been demonstrated in animal models of neuroinflammation, neurodegeneration and brain injury [8–11]. Importantly, in SOD1G93A transgenic mice, a widely used model of ALS, RNS60 administered at the onset of the disease delayed the neuromuscular deficit and paralysis by virtue of its effects on multiple disease mechanisms in motor neurons, glial cells and peripheral immune cells [8]. RNS60 promotes mitochondrial function and biogenesis [12, 13] and activates the PI3K–Akt pro-survival pathway in neurons [14]. Furthermore it reduces glial inflammation [9] and increases regulatory T cells (Tregs) in multiple sclerosis and ALS mouse models [8, 11, 15]. Moreover, RNS60 has been shown to increase neurotransmission and reduce fatigability of nerve stimulated muscles in a murine phrenic nerve diaphragm *ex vivo* preparation [16]. Clinically, long-term RNS60 administration has been shown to be safe and well tolerated in a pilot open-label trial in ALS patients. No serious adverse events (AEs) related to RNS60 occurred and no participant withdrew from the trial due to drug-related AEs [17]. Here the results are reported of a phase II trial in people with ALS where the effects of RNS60 on a number of candidate biomarkers and clinical end-points were investigated. Biomarkers measured in this trial included markers known to be modified by RNS60 in preclinical studies (interleukin-17 [IL-17], Tregs and protein nitration) [8, 11], markers implicated in ALS pathogenesis (monocyte chemoattractant protein-1 [MCP-1] and cyclophilin A/peptidyl prolyl isomerase A [PPIA]) [18, 19] and neurofilament light chain (NfL), an established biomarker of neuroaxonal degeneration [20].

METHODS

Trial design

This was a multicentre, randomized, double-blind, placebo-controlled, parallel group, add-on trial.

The primary study objective was to measure the effect of RNS60 treatment on candidate markers of inflammation and neurodegeneration in the peripheral blood of people living with ALS. Candidate markers were MCP-1, PPIA, tyrosine-nitrated actin (ac-tin-NT), 3-nitrotyrosine (3-NT), IL-17, NfL and Tregs (measured via FOXP3 and CD25 mRNA). Secondary objectives were to evaluate the effect of RNS60 on functional impairment as measured by the ALS Functional Rating Scale—Revised (ALSFRS-R); the effect on respiratory function as measured by the forced vital capacity (FVC) (per cent predicted of normal value); the impact on quality of life as measured by ALS Assessment Questionnaire-40 (ALSAQ-40) (five domains: physical mobility, activities of daily living and independence, eating and drinking, communication, emotional reactions); the effect on self-sufficiency defined as having a score of 3 or 4 on all three selected ALSFRS-R questions (specifically swallowing, cutting food and handling utensils, and walking); the effect on survival; tolerability and safety as measured by the identification of AEs.

Study population

Trial participants were enrolled at 22 Italian Expert ALS Centres from May 2017 to January 2020. The trial enrolled adults (aged 18–80 years) with a diagnosis of definite, probable or probable laboratory-supported ALS according to the revised El Escorial criteria [21] whose symptom onset had occurred 6 to 24 months prior to enrolment. Additional eligibility criteria included geographic access to the enrolling centre (so that they could go to the site on a weekly basis for intravenous infusions); self-sufficiency (defined as having a score of 3 or 4 on all three selected ALSFRS-R questions, specifically swallowing, cutting food and handling utensils, and walking); satisfactory respiratory function (FVC ≥ 80% of predicted normal value); documented progression of ALS symptoms in the 3 months prior to screening as measured by a decrease of at least 1 point in the ALSFRS-R scale total score; stable treatment with riluzole at a dose of 50 mg twice a day.

Trial interventions and procedures

Participants were randomly assigned to receive either RNS60 or matching placebo whilst concomitantly taking riluzole. Treatment allocation was centrally managed using a computer-generated, permuted block (with a block size of 4), 1:1 randomization scheme. Any concomitant treatment could be continued as per medical indication. RNS60 or placebo was administered intravenously

(375 ml infused at a rate of 700 ml/h) once a week as well as inhaled via nebulization (4 ml RNS60 or placebo) every morning (6 days a week on non-infusion days) for 24 weeks (on-treatment period). The intravenous (IV) dose was scaled from animal studies using conventional formulae, although the dosing in animal studies (intraperitoneal route) was more frequent. To minimize the need for more frequent IV infusions, the earlier pilot trial had employed daily inhalations to bridge the interval between the higher volume weekly IV doses. As that combination dose had been well tolerated in the pilot trial, it was decided to use the same dose in this trial. Participants were also followed for an additional 24 weeks off-treatment period (through week 48) to investigate whether RNS60 effects persist after suspension of the treatment and, in the case of a positive effect, to evaluate their duration. Adherence with the assigned home treatment was measured by counting the number of used/unused syringes returned by the participant at each visit.

Nebulizer machines, RNS60 and placebo for infusion and inhalation were supplied by Revaesio Corporation in identical IV bags and syringes, respectively. Both participants and study staff were blinded to the treatment assignment. Assigned box (infusion and nebulizer) treatment was shown by an interactive web response system at each visit making investigators blinded for the entire study.

Data collection

Blood samples for biomarker analyses (RNA and proteins; for detailed methods see [Supplementary Material](#)) were collected on day 1 (before the first infusion) and at weeks 4, 12, 24 (on-treatment period) and 48 (off-treatment). Safety and efficacy were assessed by way of physical examination, vital signs and AEs. Changes in function and quality of life were assessed using the ALSFRS-R, FVC (on day 1 and at weeks 4, 12, 24, 36 and 48) and ALSAQ-40 (on day 1 and at weeks 4, 24 and 48).

Statistical analysis

Separate analyses were performed for (1) all randomized participants (intention-to-treat [ITT] population, primary analysis); (2) study completers and compliers (which included participants who completed the 24-week on-treatment period and took at least 75% of the assigned dose); (3) participants who did not have any major protocol deviation (PP population).

Descriptive statistics were performed comparing the two treatment groups. Continuous variables were described using mean and standard deviation or median and interquartile range; categorical variables were described as counts and percentages.

Pre-enrolment progression rate was calculated as follows: $(48 - \text{ALSFRS score at diagnosis})/(\text{disease duration at diagnosis})$, where disease duration at diagnosis is the time from onset to diagnosis in

months. Participants with a progression rate higher than or equal to 0.67 were classified as fast progressing, whilst those with a score lower than 0.67 were classified as slow progressing [22, 23].

Biomarker analyses

The mean changes in biomarker levels over the on-treatment period (weeks 0–24) and the follow-up, off-treatment period (weeks 24–48) were analysed using repeated measures analysis of variance (ANOVA) with an unstructured variance–covariance matrix (see [Supplementary Material](#) for details). The variation of the biomarkers over time was tested by the time effect, the difference between treatment groups at specific visit times was tested by the treatment main effect, and the difference between groups in the variation over time was tested by the treatment*time interaction term. In all models, time was expressed in weeks.

Clinical outcome analyses

The mean changes in FVC (per cent predicted), ALSFRS-R total score and the five domains of the ALSAQ-40 scale were analysed over the on-treatment period (weeks 0–24) and the follow-up, off-treatment period (weeks 24–48) using repeated measures linear mixed models with random intercept and slope and an unstructured variance–covariance matrix (see [Supplementary Material](#) for details). In these models, the treatment effect was measured by the difference between the slopes of the two treatment arms, which was tested by the treatment*time interaction term. Differences in baseline values between treatment groups were tested by the treatment main effect, whilst changes over time were tested by the time effect. In all models, time was expressed in weeks.

The cumulative proportions of participants remaining self-sufficient and survival were calculated in both treatment arms at 4, 12, 24, 36 and 48 weeks using Kaplan–Meier survival curves. Differences between survival curves were assessed with the log-rank test.

Safety

Total, mean number (with standard deviation) and the proportion of patients with at least one AE were reported separately for different types of AEs (mild, moderate, severe, serious AEs, AEs leading to treatment discontinuation and drug-related AEs) at the end of the study (week 48) and during the on-treatment period at weeks 4, 12 and 24, in each study group. Differences between treatment arms were tested with the Wilcoxon–Mann–Whitney test and Fisher's exact test. For classification of the different types of AEs, see the [Supplementary Material](#).

Significance level was set at 5% for all analyses and all tests were two-tailed. Missing data were handled including all available data,

using maximum likelihood estimations in repeated measures ANOVA and mixed models. All analyses were performed with SAS 9.4 (SAS Institute, Cary, NC, USA).

Post hoc analyses

Forced expiratory volume 1 (FEV1) (a measure of the amount of air that a person can exhale within the first second of forced expiration) and the three ALSFRS-R subscores (bulbar, motor, respiratory) were evaluated using the same statistical methods used for FVC and ALSFRS-R.

Subgroup analyses were performed stratifying participants by site of onset (bulbar or spinal), pre-enrolment progression rate (fast or slow) and NfL level at baseline (below the median or above the median). The outcomes analysed included all biomarkers, FVC and ALSFRS-R. These analyses were performed using the same models as in the total sample with the addition of the stratification variable main effect (in this case onset and, separately, progression rate) and its interactions with all other effects included in the model. Least squares means were estimated separately for each category of the stratification variable (bulbar and spinal onset, fast and slow progressing patients) at each time point and in each treatment group. Differences between categories of the stratification variable were tested by the stratification variable main effect, the treatment*(stratification variable) and the time*(stratification variable) interaction terms. The difference in the treatment effect between categories of the stratification variable was tested by the treatment*time*(stratification variable) triple interaction.

Determination of sample size

The sample size was determined making different assumptions for each biomarker included as primary outcome. A total of 142 patients was required to detect with 80% power a 44% decrease in the rate of progression of PPIA at a two-sided 5% level of significance, allowing for a 10% drop-out rate over the entire study period. The number of patients required for other biomarkers ranged from 8 to 68. In addition, with this sample size the study was also powered (80% at a 5% two-sided level of significance) to detect a 43% decrease in the rate of progression in ALSFRS-R over 24 weeks, and to show an absolute 25% reduction in the proportion of patients becoming non self-sufficient at 24 weeks assuming that this proportion in the placebo arm was 85%. See the [Supplementary Material](#) for a detailed explanation of sample size calculation and relative assumptions.

Ethical issues and quality assurance

The study and any amendments were approved by an independent ethics committee of each participating centre. This study was planned and

performed according to the principles of good clinical practice (ICH-GCP), the Declaration of Helsinki, and the national laws and regulations about clinical studies. Eligible patients were included in the study only after written IRB/IEC/REB-approved informed consent or, if incapable of doing so, after approval by a legally acceptable representative. Data review and management were performed by Mario Negri IRCCS.

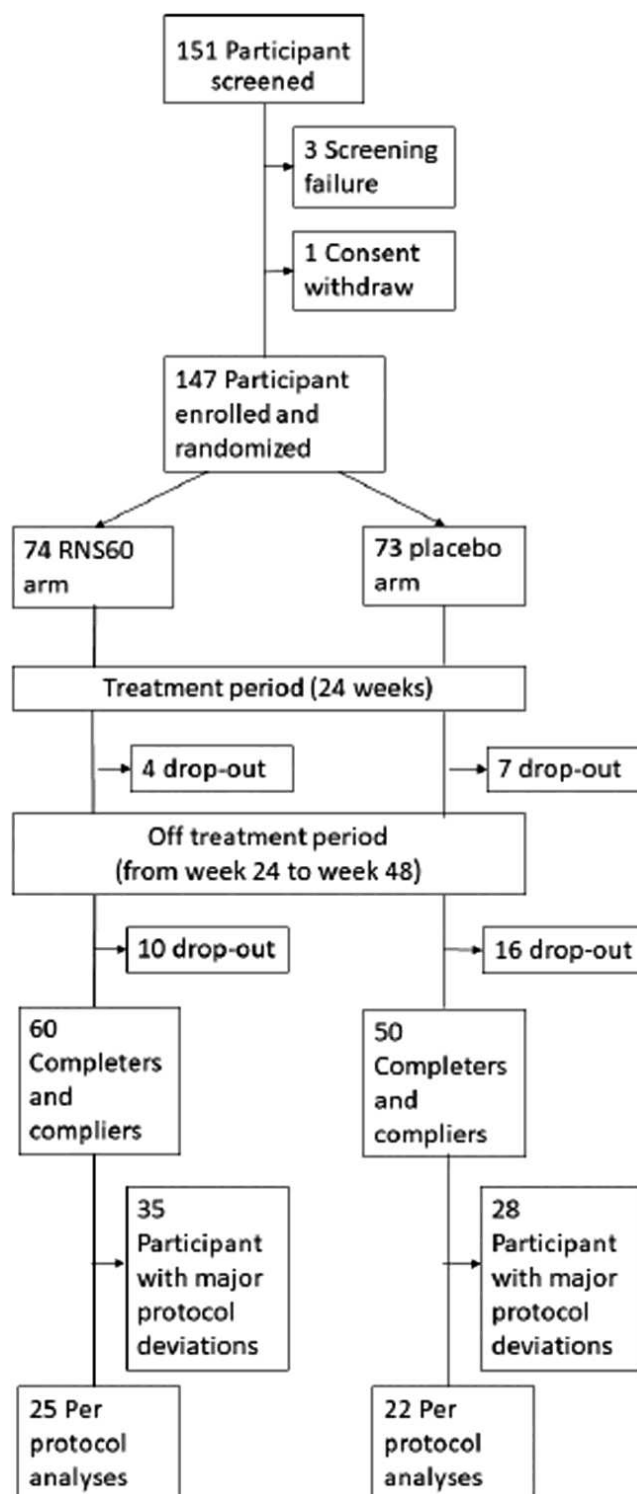


FIGURE 1 Study flow-chart: screening, randomization and follow-up

TABLE 1 Demographic and clinical characteristics of the trial population at baseline (ITT population)

	RNS60 (N = 74)		Placebo (N = 73)		p value
	Mean	SD	Mean	SD	
Age	59.3	10.4	56.0	10.0	0.0534
Disease duration (months)	15.5	5.8	14.3	5.8	0.2172
Diagnostic delay (months)	9.8	5.1	8.8	4.9	0.2292
BMI	25.0	3.3	24.8	4.1	0.7878
FVC (% of predicted normal value)	102.7	18.2	103.3	16.1	0.8317
ALSFRS-R total score	41.6	3.2	41.4	3.6	0.6871
ALSAQ-40					
Physical mobility	32.1	21.3	33.2	23.1	0.7540
ADL and independence	36.8	27.8	32.9	26.1	0.3762
Eating and drinking	14.5	23.9	13.5	23.9	0.8015
Communication	19.3	25.5	18.6	28.6	0.8742
Emotional reactions	33.6	18.5	33.1	22.2	0.8825
	n	%	n	%	
Sex					0.4467
Male	52	70.3	47	64.4	
Female	22	29.7	26	35.6	
Site of onset					0.6778
Bulbar	11	14.9	9	12.5	
Spinal	63	85.1	63	87.5	
Pre-enrolment progression rate					0.7232
Fast	18	27.3	18	27.3	
Slow	48	72.7	48	72.7	
Unknown	8		7		

Abbreviations: ADL, activities of daily living; ALSAQ-40, Amyotrophic Lateral Sclerosis Assessment Questionnaire-40; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised; BMI, body mass index; FVC, forced vital capacity; ITT, intention to treat.

The study was registered at [ClinicalTrials.gov](https://clinicaltrials.gov) number NCT03456882; EudraCT number 2016-002382-62.

RESULTS

A total of 147 participants were enrolled and randomly assigned to RNS60 (74 participants) or placebo (73 participants). Results are reported for the ITT population that included all randomized participants. The study flow-chart is illustrated in [Figure 1](#).

Demographic and other baseline characteristics

Baseline characteristics of the ITT population are shown in [Table 1](#). The baseline characteristics of the two treatment groups were fairly similar.

Biomarker levels

The levels of MCP-1 ($p = 0.0162$), NfL ($p = 0.0373$) and FOXP3 mRNA ($p = 0.0067$) changed significantly over the course of study

participation in both treatment arms. In both arms, MCP-1 and NfL levels increased over time, whilst FOXP3 mRNA decreased, without significant differences between the two arms, as indicated by the treatment main effect and treatment*time interaction effect that were not statistically significant. PPIA, actin-NT, 3-NT, IL-17 and CD25 mRNA did not significantly change over time, and no significant differences in their levels between treatment groups were observed. The mean estimates at each visit time for all analysed biomarkers and their graphical representation are shown in [Table S1](#), [Figures 2](#) and [S1](#).

Clinical outcomes

Forced vital capacity and FEV1

The RNS60 and the placebo groups showed similar FVC values at baseline. FVC decreased significantly over time in both arms ($p < 0.0001$) ([Table 2](#) and [Figure 3a](#)). During the on-treatment period (baseline to week 24), the estimated mean rate of change was lower in the RNS60 arm (-0.46 per week) compared to the placebo arm (-0.87 per week) ($p = 0.0101$). During the off-treatment follow-up

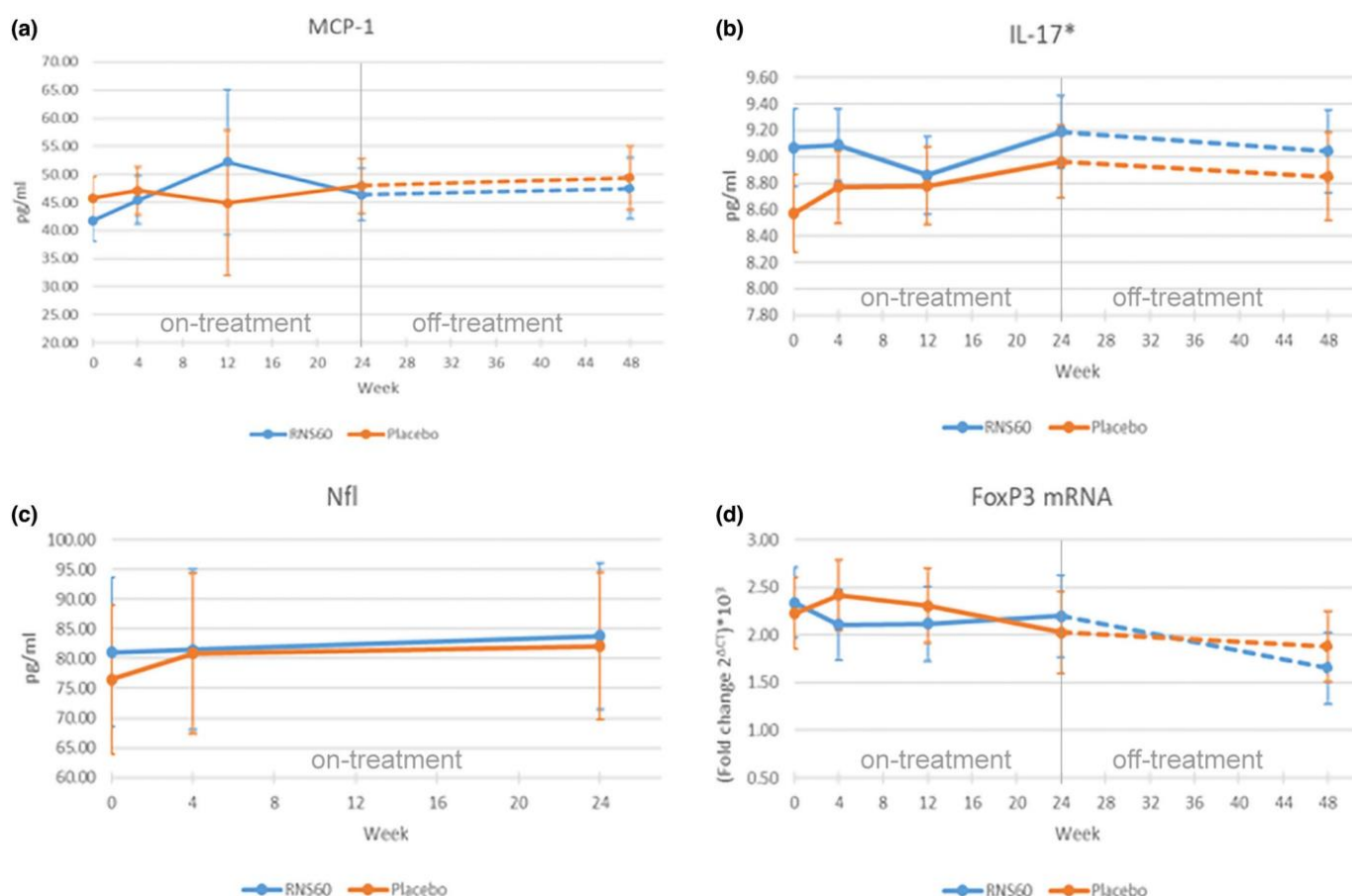


FIGURE 2 Effect plots for MCP-1, IL-17, NfL, FOXP3 mRNA at different visit times (in weeks) in each treatment arm (ITT population)

period (week 24 to week 48), no differences were observed (-0.45 per week for RNS60; -0.54 per week for placebo; $p = 0.5924$).

In a *post hoc* analysis, FEV1 was also evaluated. A significant difference in the rate of decrease of FEV1 between the two treatment groups was observed (-0.45 per week for RNS60; -0.52 per week for placebo; $p = 0.0146$) during the on-treatment period, with the RNS60 group showing a slower decline, in line with the findings on FVC (Table 2 and Figure 3b). There were no significant differences between groups during the follow-up, off-treatment period.

ALSFRS-R

The ALSFRS-R scores were not significantly different between groups at baseline. The ALSFRS-R total score declined significantly over time in both groups ($p < 0.0001$) (Table 2 and Figure 3c). The rate of change over time in ALSFRS-R scores did not differ between treatment arms.

ALSAQ-40

There were no significant differences between treatment groups at the baseline visit for any of the domains of the ALSAQ-40 (Table S2). The mean scores for each domain increased significantly over time

($p < 0.0001$), corresponding to a decrease in each aspect of quality of life. The mean rate of change in the eating and drinking domains of the ALSAQ-40 were significantly slower in the RNS60 arm (difference -0.19 per week, SE 0.1, $p = 0.0319$) (Figure 3d). No significant differences between treatment groups were detected for the other four domains of the ALSAQ-40 scale.

Self-sufficiency and survival

No significant differences in percentage of participants who remained self-sufficient were seen in the treatment arms throughout the study period. The cumulative probability of remaining self-sufficient in the RNS60 arm was 62% at 4 weeks, 29% at 12 weeks, 15% at 24 weeks and 7% at 48 weeks. The corresponding probabilities in the placebo group were 62%, 38%, 23%, 0% (Figure S2A). Survival was no different between treatment arms. The cumulative survival probability was 99% in both arms at 24 weeks, 88% in the RNS60 arm and 89% in the placebo arm at 48 weeks (Figure S2B).

Completers and compliers and PP population

Data for completers and compliers ($n = 110$, $n = 60$ in the RNS60 group; $n = 50$ in the placebo group) and PP populations ($n = 47$,

TABLE 2 Global tests of fixed effects, means and contrasts for FVC, FEV1, ALSFRS-R at different visit times in each treatment arm (ITT population)

FVC									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p value	<0.0001			0.9928			0.0101		
Effect	Follow-up			Follow-up*treatment					
p value	0.1387			0.1734					
Estimated means and contrasts									
Visit time (week)	RNS60			Placebo			Difference (RNS60 – placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	102.6	1.9	<0.0001	102.6	2.0	<0.0001	-0.02	2.8	0.9928
4	100.7	2.1	<0.0001	99.1	2.1	<0.0001	1.6	2.9	0.5778
12	97.0	2.5	<0.0001	92.1	2.5	<0.0001	4.9	3.5	0.1647
24	91.4	3.5	<0.0001	81.6	3.5	<0.0001	9.8	4.9	0.0474
Slope (treatment period)	-0.46	0.11	<0.0001	-0.87	0.1	<0.0001	0.41	0.16	0.0101
Follow-up period (off-treatment)									
36	86.0	3.7	<0.0001	75.1	3.8	<0.0001	10.9	5.3	0.0394
48	80.6	4.4	<0.0001	68.6	4.6	<0.0001	12.0	6.3	0.0571
Slope (follow-up period)	-0.45	0.11	<0.0001	-0.54	0.13	<0.0001	0.09	0.18	0.5924

FEV1									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p value	<0.0001			0.8723			0.0146		
Effect	Follow-up			Follow-up*treatment					
p value	0.4219			0.1148					
Estimated means and contrasts									
Visit time (week)	RNS60			Placebo			Difference (RNS60 – placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	96.7	2.2	<0.0001	97.2	2.2	<0.0001	-0.5	3.1	0.8723
4	95.2	2.2	<0.0001	94.1	2.2	<0.0001	1.1	3.1	0.7107
12	92.3	2.5	<0.0001	87.9	2.5	<0.0001	4.4	3.5	0.2128
24	87.9	3.4	<0.0001	78.6	3.5	<0.0001	9.3	4.9	0.0563
Slope (treatment period)	-0.36	0.1	0.0022	-0.77	0.1	<0.0001	0.41	0.2	0.0146
Follow-up period (off-treatment)									
36	82.6	3.7	<0.0001	72.3	3.8	<0.0001	10.3	5.3	0.0522
48	77.2	4.3	<0.0001	66.0	4.4	<0.0001	11.2	6.1	0.0678
Slope (follow-up period)	-0.45	0.1	<0.0001	-0.52	0.1	<0.0001	0.07	0.1	0.5758

TABLE 2 (Continued)

ALSFRS-R									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p value	<0.0001			0.9076			0.5725		
Effect	Follow-up			Treatment*follow-up					
p value	0.3181			0.3363					
Estimated means and contrasts									
Week	RNS60			Placebo			Difference (RNS60 – placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	41.4	0.4	<0.0001	41.3	0.4	<0.0001	0.1	0.6	0.9076
4	40.4	0.5	<0.0001	40.2	0.5	<0.0001	0.2	0.7	0.8010
12	38.3	0.6	<0.0001	37.9	0.6	<0.0001	0.4	0.9	0.6877
24	35.1	0.9	<0.0001	34.5	1.0	<0.0001	0.6	1.3	0.6280
Slope (treatment period)	−0.26	0.03	<0.0001	−0.28	0.03	<0.0001	0.02	0.04	0.5725
Follow-up period (off-treatment)									
36	32.0	1.1	<0.0001	31.6	1.1	<0.0001	0.4	0.6	0.8048
48	28.9	1.3	<0.0001	28.8	1.3	<0.0001	0.1	1.9	0.9493
Slope (follow-up period)	−0.26	0.03	<0.0001	−0.24	0.03	<0.0001	−0.02	0.04	0.5728

ALSAQ-40 (Eating and drinking)									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p value	<0.0001			0.5022			0.0319		
Estimated means and contrasts									
Week	RNS60			Placebo			Difference (RNS60 – placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
0	15.9	2.8	<0.0001	13.3	2.8	<0.0001	2.6	3.9	0.5022
24 (end of treatment)	20.4	3.2	<0.0001	22.4	3.2	<0.0001	−2.0	4.5	0.6524
48 (follow-up)	24.9	4.1	<0.0001	31.6	4.2	<0.0001	−6.7	5.8	0.2521
Slope	0.19	0.1	0.0032	0.38	0.1	<0.0001	−0.19	0.1	0.0319

Abbreviations: ALSAQ-40, Amyotrophic Lateral Sclerosis Assessment Questionnaire-40; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised; BMI, body mass index; FEV, forced expiratory volume; FVC, forced vital capacity; ITT, intention to treat.

$n = 25$ in the RNS60 group; $n = 22$ in the placebo group) are shown in the [Supplementary Material](#).

Results of the biomarkers' analyses are shown in Tables [S3](#) and [S4](#). For MCP-1, FOXP3 mRNA and NfL a significant change over the course of the treatment and follow-up periods was confirmed in completers and compliers. For MCP-1 and FOXP3 mRNA this effect was confirmed also in the PP population. A significant treatment main effect, entirely explained by differences at the baseline visit, was found for IL-17 only in the completers and compliers population. A significant difference in the evolution over time between treatment groups was found for PPIA only in the PP population. A change over time of borderline significance was found only in the PP population for actin-NT. No other significant effects were found for any biomarker in the completers and compliers nor in the PP population.

The results of the analysis of clinical outcomes are shown in Tables [S5](#) and [S6](#). The evolution over time was consistently significant in all clinical continuous outcomes (decrease of FVC, decrease of ALSFRS-R, increase of all five domains of the ALSAQ-40), both in the completers and compliers and in the PP population.

The significant difference between treatment groups in the evolution over time found in the ITT population for FVC was not confirmed in completers and compliers, nor in the PP population. The difference between treatment groups in the evolution over time for the eating and drinking domain of the ALSAQ-40 was consistently significant in completers and compliers and the PP population. A difference in the evolution over time for the emotional reactions domain of the ALSAQ-40 was found only in the PP population, with the RNS60 group showing a slower increase. No other significant effects were found for the clinical outcomes in the completers and compliers nor in the PP population.

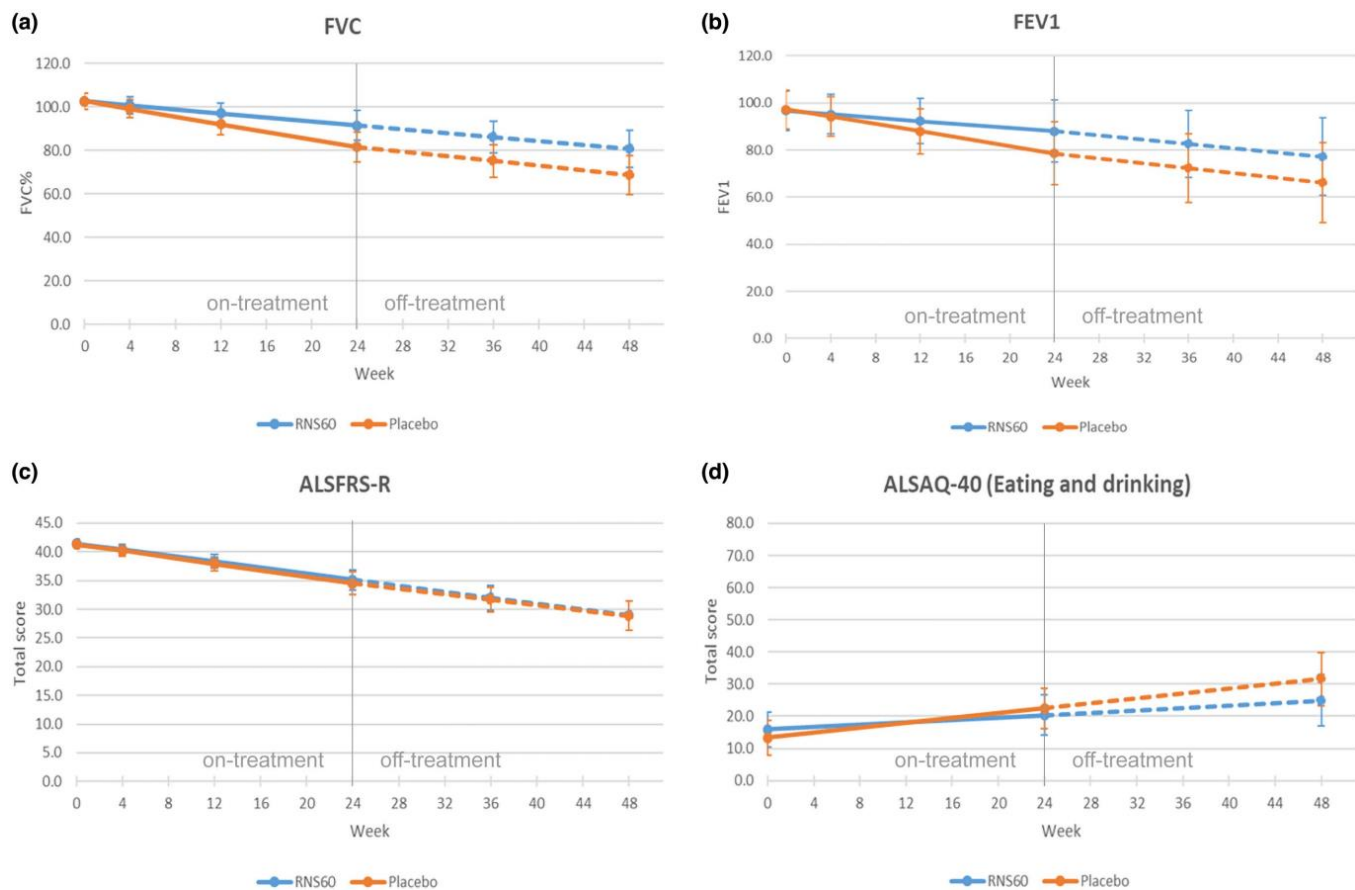


FIGURE 3 Effect plots for FVC, FEV1, ALSFRS-R, ALSAQ40 (eating and drinking) at different visit times (in weeks) in each treatment arm (ITT population)

Adverse events

The total number of AEs and the proportion of participants experiencing at least one AE were similar in the two groups, except for a higher number and, respectively, proportion of moderate AEs and AEs leading to study discontinuation in the placebo group (Table 3). The list of all AEs in each treatment group is shown in Table S7.

Post hoc subgroup analyses

Bulbar and spinal onset subgroup analyses

A significant difference in the evolution over time of NfL was found between bulbar and spinal onset in different treatment arms (significant triple interaction) (Figure 4a, Table S8). When considering participants with spinal onset, the baseline levels and the change over time were similar between the two treatment arms. When considering participants with a bulbar onset, those in the RNS60 arm showed higher values than those in the placebo arm at baseline; NfL levels were stable over the 24 weeks of treatment in the RNS60 arm, whilst in the placebo arm the levels increased. However, the difference between treatment arms in the bulbar onset subgroup was not statistically significant due to small sample size. A significant difference between the bulbar and spinal onset subgroups was also found for FVC: bulbar

onset participants had lower FVC values and a more rapid decrease over time compared to spinal onset participants. A slower decrease of FVC in the RNS60 arm compared with the placebo arm was also observed in both subgroups, particularly in bulbar onset; however, this difference was not statistically significant (Figure 4b, Table S8).

Fast and slow progressing subgroups

A significant difference in the evolution over time of NfL was found between fast and slow progressing participants included in different treatment arms (significant triple interaction). When considering participants with a slow progression rate, the baseline values and the change over time (increase) was similar between the two treatment arms. When considering participants with a fast progression rate, those in the RNS60 arm showed values that were higher than those in the placebo arm, and the change over time was different between the two treatment groups: participants in the RNS60 arm showed an increase in the first 4 weeks that was followed by a decrease between week 4 and week 24; in the placebo arm a progressive increase between baseline and week 24 was observed (Figure 4c, Table S9).

A significant difference between fast and slow progressing participants was found in the rate of decrease of FVC, with fast progressing participants showing a more rapid decrease compared to slow participants, but without significant differences between treatment groups (Figure 4d, Table S9).

TABLE 3 Adverse events occurring during the on-treatment period (up to week 24) and the entire study period (from baseline to week 48) (ITT population)

	Total number of AEs						Patients with at least one AE					
	RNS60			Placebo			p value	RNS60		Placebo		p value
	Sum	Mean	SD	Sum	Mean	SD		n	%	n	%	
AEs occurring during the on-treatment period (up to week 24)												
All AEs	128	1.7	2.6	144	2.0	3.6	0.6477	42	56.8	47	64.4	0.3442
Mild AEs	117	1.6	2.5	120	1.6	3.3	0.7427	42	56.8	40	54.8	0.8107
Moderate AEs	6	0.1	0.3	18	0.2	0.5	0.0166	5	6.8	15	20.6	0.0147
Severe AEs	3	0.04	0.2	6	0.1	0.3	0.4520	3	4.1	5	6.9	0.4940
SAEs	5	0.1	0.3	5	0.1	0.3	0.9882	4	5.4	4	5.5	0.9842
AEs leading to study/treatment discontinuation	0	0.0	0.0	3	0.04	0.2	0.0826	0	0.0	3	4.1	0.1199
Drug-related AEs	8	0.1	0.6	1	0.01	0.1	0.3140	3	4.0	1	1.4	0.6198
AEs occurring through week 48												
All AEs	142	1.9	2.8	158	2.1	3.6	0.6084	44	59.5	48	65.8	0.4304
Mild AEs	128	1.8	2.8	129	1.8	3.4	0.8810	42	56.8	40	54.8	0.8107
Moderate AEs	7	0.1	0.4	20	0.3	0.6	0.0101	5	6.8	16	21.9	0.0086
Severe AEs	7	0.1	0.3	9	0.1	0.4	0.8563	7	9.5	6	8.2	0.7912
SAEs	6	0.1	0.3	6	0.1	0.3	0.4122	5	6.8	5	6.9	0.9822
AEs leading to study/treatment discontinuation	0	0	0	5	0.1	0.3	0.0243	0	0.0	5	6.9	0.0224
Drug-related AEs	10	0.1	0.7	1	0.01	0.1	0.1728	4	5.4	1	1.4	0.3664

Abbreviations: AE, adverse event; ITT, intention to treat; SAE, serious adverse event.

A significant difference between fast and slow participants was observed in the progression rate of the ALSFRS-R score, with fast progressing patients showing lower values. However, no significant differences between treatment groups were found (data not shown).

Neurofilament light chain levels at baseline

A significant difference in the rate of change of FVC, ALSFRS-R and all ALSAQ-40 domains, with the exception of the emotional reaction domain, was found between patients with high (above the median) or low (below the median) baseline NfL levels. Participants with high baseline NfL levels showed a faster decline compared to patients with low baseline levels. However, no significant differences in RNS60 treatment effects were detected between the two subgroups (data not shown).

DISCUSSION

In this phase II randomized, double-blind, placebo-controlled trial, administration of RNS60 showed no effects on a number of biological markers of inflammation and neurodegeneration.

The biomarkers that were investigated in this trial were chosen based on previous preclinical data and/or relevance to ALS

pathophysiology. IL-17 was chosen because increased serum and cerebrospinal fluid levels of IL-17 have been observed in ALS patients [24] and, in a mouse model of multiple sclerosis, RNS60 treatment was associated with reduced expression of IL-17 [15]. Yet, measurement of IL-17 is limited by the fact that there is no prior information about the longitudinal pattern of IL-17 in ALS patients and by the very low levels of IL-17 in the blood (<1 pg/ml). FOXP3 mRNA was measured because, in a mouse model of multiple sclerosis and in SOD1G93A mice, RNS60 upregulated FOXP3 mRNA [8, 15]. FOXP3 mRNA is low in rapidly progressing ALS patients and its levels inversely correlate with progression rates [25]. 3-NT was evaluated because, in a previous study conducted in our laboratory, RNS60 reduced 3-NT levels in the spinal cord of the SOD1G93A mouse model, suggesting a possible role of RNS60 in suppressing nitric oxide (NO) production [8]. Increased levels of 3-NT and actin-NT were found in peripheral blood mononuclear cells (PBMCs) of ALS patients [26–28], implicating inflammation and oxidative/nitrative stress in disease pathogenesis. Decreased levels of PPIA in the PBMCs of ALS patients have been associated with an early onset of the disease [29] and with a short disease duration [30] indicating that reduced chaperone/foldase activity may be at the basis of a more severe phenotype. Increased levels of MCP-1 have been found in the central and peripheral nervous system of the SOD1G93A mouse model [19] and in serum and

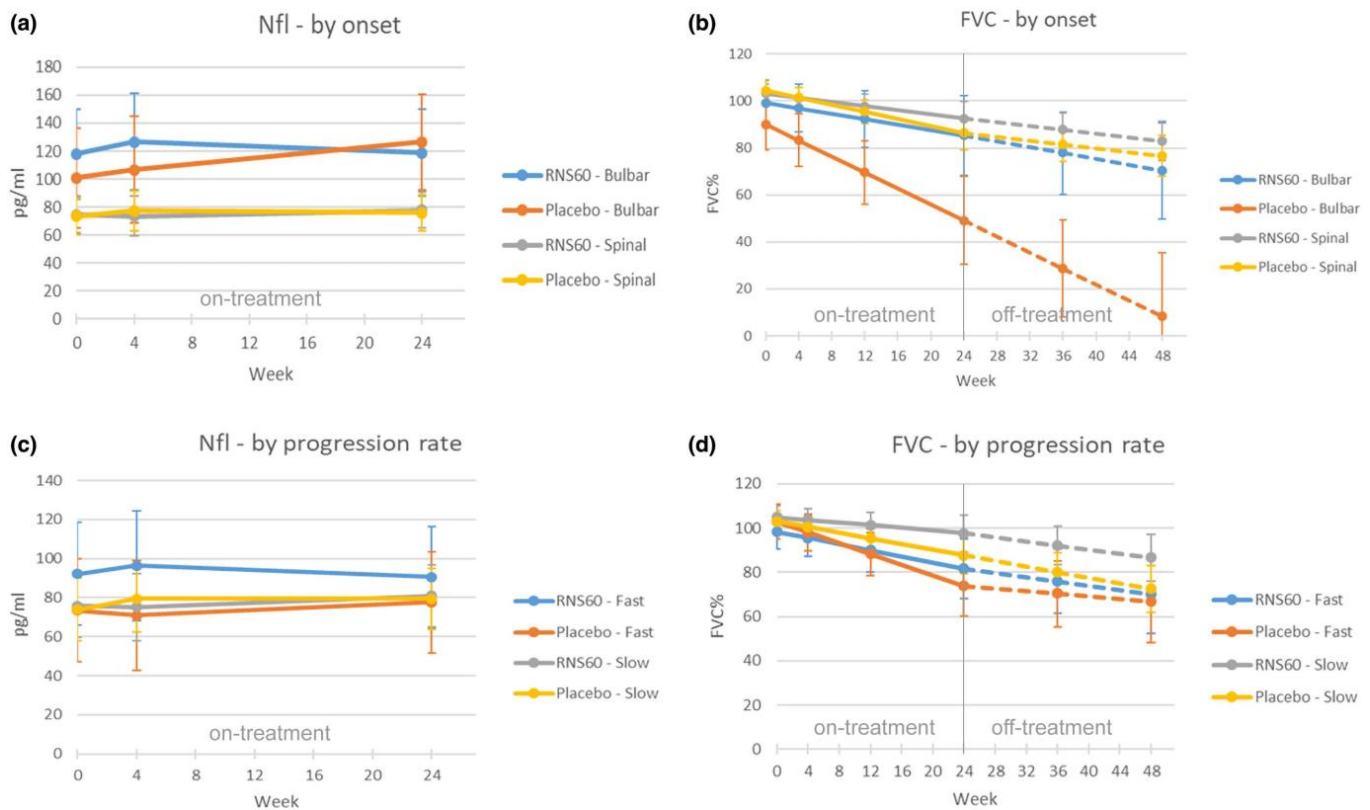


FIGURE 4 Effect plots for NfL and FVC at different visit times in each treatment arm by site of onset (ITT population)

cerebrospinal fluid of ALS patients [31–34], indicating central and peripheral immune activation. NfL levels were measured as there is growing evidence supporting their prognostic and pharmacodynamic potential [20]. In our study, NfL values were significantly higher in participants with bulbar onset, in agreement with recent studies showing an association between higher NfL serum levels and bulbar onset in ALS [35].

Although RNS60 did not affect NfL levels in the whole population of the study, a statistically different trajectory of NfL was found by the *post hoc* analysis in patients with bulbar onset, in which RNS60 seems to have a stabilizing effect.

Whilst RNS60 administration did not affect the levels of candidate biomarkers of neuroinflammation and neurodegeneration, encouraging results were obtained on selected clinical outcomes. Specifically, RNS60 administration was associated with slower rates of decline in respiratory function and in the bulbar domains of ALSAQ-40 compared to placebo over a 24-week treatment period. These effects were not accompanied by positive effects on global function or survival, although longer treatment periods may be needed to detect such changes. During the off-treatment follow-up period (weeks 24–48), the difference in the two treatment arms persisted, suggesting a protective effect of early treatment with the drug rather than a transient symptomatic effect. These findings were similar in *post hoc* analyses showing differences in FVC decline when comparing bulbar and spinal ALS onset, although differences did not attain statistical significance probably due to the small sample size of the subgroups. RNS60 treatment

slowed the decline of FVC in patients with bulbar onset and stabilized levels of NfL. Indirect evidence of a possible beneficial effect of the active treatment comes from the difference in the drop-out rate between active treatment and placebo (18.9% vs. 31.5%, respectively) over 48 weeks. The lower drop-out rate in the RNS60 arm could be explained by possible subjective perception of a positive effect of the treatment. It is worth noting that there was a steep rise of drop-outs in both treatment arms towards the end of follow-up. This might reflect the absence of treatment and perhaps disease progression in the absence of treatment during the second semester of the study. Our study also confirmed the benign safety profile of RNS60 expanding on previous open-label observations [17].

The positive effects of RNS60 on respiratory function are supported by an exploratory study on the functional consequences of tissue oxygenation with O₂ nanobubbles in murine phrenic nerve diaphragm *ex vivo* preparations [16]. Alternatively, there may be a symptomatic effect of the nebulized treatment, which may be particularly evident in bulbar patients. Future trials focused on clinical efficacy of RNS60 may need to include additional measures of respiratory and bulbar function. Spirometry manoeuvres in the present study were performed in the seated position. Future studies may include supine FVC which may be a more sensitive measure of decline in diaphragm function. Measures of orthopnoea and sleep disruption may also be considered. Hence it is thought that a larger clinical trial focused on clinical efficacy of inhaled/nebulized administration of RNS60 with respiratory and functional end-points in well-selected

populations of patients is necessary to conclude whether RNS60 can be used for the treatment of respiratory symptoms or for preventing and delaying disease progression [16]. Treatment with RNS60 for 24 weeks did not affect disease progression as measured by the ALSFRS-R or mortality. However, another compound with anti-inflammatory properties like palmitoylethanolamide was able to improve the pulmonary function in ALS patients without modifying the ALSFRS-R scale; the effect was attributed to a direct action of palmitoylethanolamide on the neuromuscular junction of the diaphragmatic muscle which are the last muscles to be paralysed [36]. RNS60 may also have directly affected the diaphragm muscle.

These results are in contrast with the positive effects of RNS60 reported in the animal model of ALS; however, in those mice the drug was administered intraperitoneally before overt symptom onset [8]. The motor deficit slowing effect observed in RNS60-treated SOD1G93A mice was accompanied by partial prevention of lumbar spinal motor neuron loss and neuromuscular junction denervation of skeletal muscle. Interestingly, the clinical studies with tofersen (VALOR, NCT02623699, NCT02623699) [37, 38], an antisense oligonucleotide targeting SOD1, have shown that those who started tofersen earlier had better outcomes than those who started later (<https://investors.biogen.com/static-files/4e9393b8-0f67-47a3-a331-05ef3ac7fc63>). A therapeutic intervention in earlier phases of ALS is under investigation in the ATLAS study (NCT04856982), in which tofersen is initiated in clinically presymptomatic SOD1 variant carriers [39]. It should be noted that in the 6-month phase 3 study there were positive results only with regard to neurofilaments and not to ALSFRS-R and survival, as occurred in the present study. Moreover, a recent clinical trial on high caloric nutrition diets has yielded positive results for fast progressing patients only [40], possibly because the duration of the investigation was also too short to detect effects in slower progressing patients, which could have been the case in our study, too. Therefore, it is possible that earlier and/or longer treatment with RNS60 might have a better clinical benefit in people living with ALS, which warrants further investigation.

The strengths of the study include the analysis of multiple candidate biomarkers by a centralized laboratory at the Mario Negri Institute IRCCS using standard operation procedures, thereby limiting analytical variation. Thus, this trial provided an excellent opportunity to characterize the pattern and longitudinal behaviour of these markers in the context of a well-defined trial population. Tertiary sites with broad national distribution were included to increase access and support the generalizability of study results. FVC measurements were performed using the same instrument at each site provided by the study sponsor. Finally, the study was sufficiently powered to detect clinically meaningful differences in measures of functional impairment.

The study was limited by the COVID-19 outbreak that started in Italy in February 2020. The pandemic caused restrictions in terms of participant access to trial sites and limitations in the performance of respiratory function tests. This context led to missing visits and missing data.

Also, a total of 155 protocol deviations occurred during the study (71 RNS60 group, 84 placebo group) in 100 patients (49 RNS60 group, 51 placebo group), of which 22 (10 RNS60 group, 12 placebo group) were due to the COVID-19 pandemic. The description of all protocol deviations that occurred during the study with number of occurrences in each treatment group is reported in Table S10. The third limit was the drug administration route that required weekly visits to the participating centre, with significant discomfort for disabled patients. For this reason, testing the efficacy of a full treatment via the inhalation route only at the patient's home would have been preferred.

CONCLUSION

In conclusion, administration of RNS60 to people living with ALS for 24 weeks showed no effects on candidate biomarkers of inflammation and neurodegeneration, ALSFRS-R scores or survival. Positive effects of RNS60 on selected measures of respiratory and bulbar function warrant further investigation, which might include earlier treatment, longer follow-up, additional measures of respiratory and bulbar function, and focus on dosage and route of administration.

AUTHOR CONTRIBUTIONS

See the [Appendix A](#).

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CONFLICT OF INTEREST

E. Beghi reports grants from the Italian Ministry of Health, grants from SOBI, personal fees from Arvelle Therapeutics, outside the submitted work. E. Beghi reports grants from American ALS Association for the conduct of the present study. E. Pupillo reports grants from the Italian Ministry of Health, grants from AIFA, outside the submitted work. A. Sherman reports research grants from the ALS Association, ALS Finding a Cure and the NIH, outside the submitted work. S. Paganoni reports research grants from Amylyx Therapeutics, Revalesio Corporation, UCB/Ra Pharma, Biohaven, Clene, Prilenia, Seelos, the ALS Association, the American Academy

of Neurology, ALS Finding a Cure, the Salah Foundation, the Spastic Paraplegia Foundation, the Muscular Dystrophy Association and reports personal consulting fees for advisory panels from Orion and Cytokinetics. All of them are outside the submitted work. M. Filosto reports serving on a scientific advisory board for Amicus, Sanofi, Sarepta; honoraria for speaking engagements for Sanofi, Alnylam. All of them are outside the submitted work.

A. Padovani serves on a scientific advisory board: Director of School of Neurology, University of Brescia; President-elect of the National Society of Neurology SIN; GE Healthcare, Lilly and Actelion Ltd Pharmaceuticals. Travel funded by a commercial entity: Roche, Ely-Lilly, Biogen, Zambon, Nutricia, Lundbeck. Serving as a journal editor, associate editor, or on an editorial advisory board: MDPI. Patents held or pending: Method of generation of a diagnostic index for Alzheimer's disease, electronic device for implementing the method and system, 2016. Honoraria for speaking engagements: He has received honoraria from Nutricia, PIAM, Langstone Technology, GE Healthcare, Lilly and Chiesi. Commercial research support: Advisory and served on the Scientific Advisory Board for GE Healthcare, Lilly and Actelion Ltd Pharmaceuticals. Government research support (including funding organization, grant number, and role): He has participated as PI in more than 30 research projects funded by the Ministry of Health and MIUR. Support from a non-profit foundation or society: CHDI Foundation, Fondazione Cariplo. All of them are outside the submitted work.

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All other authors report no disclosures.

DATA AVAILABILITY STATEMENT

Anonymised data are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

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Fabiola De Marchi, MD	ALS Expert Center 'Maggiore della Carità' Hospital and University of Piemonte Orientale, Novara	Identification of patients, data collection at study site, revision of final draft
Paolo Bongioanni, MD, PhD	Spinal Cord Injuries Section, Azienda Ospedaliero-Universitaria Pisana	Identification of patients, data collection at study site, revision of final draft
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APPENDIX A (Continued)

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APPENDIX A (Continued)

Name	Location	Contribution
Nilo Riva, MD, PhD	Neurology Unit, Neurorehabilitation Unit and Neurophysiology Unit, Vita-Salute San Raffaele University and San Raffaele Scientific Institute, Milan, Italy	Identification of patients, data collection at study site, revision of final draft
Massimo Filippi, MD, FEAN, FAAN	Neurology Unit, Neurorehabilitation Unit and Neurophysiology Unit, Vita-Salute San Raffaele University and San Raffaele Scientific Institute, Milan, Italy	Identification of patients, data collection at study site, revision of final draft
Isabella Laura Simone, MD	Neurology Unit, Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari	Identification of patients, data collection at study site, revision of final draft
Gianni Sorarù, MD, PhD	Motor Neuron Disease Center, Department of Neurosciences, Azienda Ospedale Università di Padova	Identification of patients, data collection at study site, revision of final draft
Cristina Spera, MD	Azienda Ospedaliera 'S Maria' Terni	Identification of patients, data collection at study site, revision of final draft
Lucia Florio, MD, PhD	Neurology Department, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo (FG)	Identification of patients, data collection at study site, revision of final draft
Sonia Messina, MD, PhD	Department of Clinical and Experimental Medicine University of Messina	Identification of patients, data collection at study site, revision of final draft
Massimo Russo, MD, PhD	Department of Clinical and Experimental Medicine University of Messina	Identification of patients, data collection at study site, revision of final draft
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Amelia Conte, MD	Centro Clinico NEMO-Fondazione Policlinico Universitario A. Gemelli IRCCS	Identification of patients, data collection at study site, revision of final draft
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Letizia Mazzini, MD	ALS Expert Center 'Maggiore della Carità' Hospital and University of Piemonte Orientale, Novara	Study conception, project coordinator, first and final draft

All authors contributed to the revision of the manuscript and accepted the final version.

APPENDIX B**RNS60-ALS Study Group**

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