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DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Laurea Magistralis in Neurobiology

Role of Lifestyle-Related Risk Factors in Cognitive Decline: A Non-Transgenic Mouse Model

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Experimental thesis by
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Ruolo dei fattori di rischio legati allo stile di vita nel declino cognitivo: un modello murino non transgenico

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Abstract

Alzheimer's disease (AD) is the leading cause of dementia worldwide and is increasingly recognized as a multifactorial disorder resulting from the interaction between genetic susceptibility, ageing, and environmental factors. Although the familial form accounts for only a small proportion of cases, the mechanisms underlying sporadic AD (SAD) remain incompletely understood. Increasing evidence suggests that modifiable lifestyle-related risk factors, including unhealthy diet, physical inactivity, and social isolation, contribute to disease onset by promoting early molecular alterations that precede overt neurodegeneration. The present study investigated the effects of prolonged exposure to combined environmental risk factors in a non-transgenic mouse model of SAD. Wild-type mice were exposed either to a protective condition, consisting of a standard diet, voluntary physical exercise, and group housing, or to a risk-factor (RF) condition characterized by a Western diet and social isolation. Both groups received either intracerebroventricular (ICV) administration of $A\beta_{1-42}$ or phosphate-buffered saline (PBS). Cognitive performance was evaluated using the Open Field Test (OFT) and the Novel Object Recognition (NOR) test, while molecular and histological analyses were performed to investigate inflammatory, oxidative, mitochondrial, neurotrophic and neuropathological markers. Exposure to the RF condition impaired recognition memory without affecting locomotor activity and was associated with molecular alterations suggestive of inflammatory activation, oxidative imbalance, and mitochondrial dysfunction. These changes occurred in the absence of detectable Aβ plaque deposition, marked astroglial activation, or significant alterations in BACE1 and BDNF expression, supporting the hypothesis that the experimental paradigm reproduces an early stage of disease progression before the appearance of the classical neuropathological hallmarks of AD. The findings indicate that prolonged exposure to lifestyle-related risk factors promotes early molecular and functional alterations consistent with the initial phases of SAD. The coexistence of cognitive impairment with inflammatory, oxidative, and mitochondrial changes, despite the absence of overt neurodegeneration, supports the concept that these processes precede irreversible neuronal damage. This experimental model therefore represents a valuable tool for investigating the early mechanisms underlying SAD and for evaluating preventive strategies targeting the earliest stages of disease development.

CHAPTER 1: INTRODUCTION

1.1 Dementia and Its Historical Background

Dementia has been documented in historical writings for centuries, whereas understanding of its biological and pathological foundations has emerged only relatively recently, within the past hundred years. During the past five decades, a major objective in neuropsychological research has been the characterization of the cognitive and behavioral alterations linked to dementia (Bondi et al., 2017). This task has become increasingly complex and relevant because of the progressive aging of the population and the strong association between aging and the neurodegenerative diseases of which dementia represents the main clinical manifestation. (Bondi et al., 2017). Alzheimer's disease (AD) represents the most common form of dementia, accounting for approximately two-thirds of dementia cases in individuals over the age of 65 (Safiri et al., 2024). The first detailed description of the disease was reported in 1906 by the German doctor Alois Alzheimer, who published the clinical case of a 51-year-old woman, Auguste Deter, hospitalized at the state asylum in Frankfurt, Germany (Bondi et al., 2017). Neuropathological examination of patients affected by AD revealed extensive cerebral degeneration accompanied by characteristic alterations within cortical cellular structures. Since these initial observations, considerable advances have been achieved in the understanding of the physiopathology of the disease. (Safiri et al., 2024). A major milestone was reached in 1984, when Dr. George Glenner and Dr. Cain Wong identified amyloid protein as the principal component of extracellular plaques (Glenner & Wong, 1984). Subsequently, in 1986, it was demonstrated that neurofibrillary tangles, one of the defining pathological hallmarks of AD, originate from the abnormal hyperphosphorylation of tau protein (Safiri et al., 2024). According to one of the most recent updates published in 2024, AD is currently considered to begin as an asymptomatic biological process characterized by AD neuropathologic changes (ADNPC), which progressively evolves into clinically evident symptoms as the neuropathological burden increases (Safiri et al., 2024).

1.2 Genetic Basis of Alzheimer's Disease

The progressive aging of the global population is expected to markedly increase the prevalence of dementia, which currently affects approximately 50 million individuals worldwide and is projected to triple by 2050. This substantial rise will consequently contribute to a higher risk of disability, an

increasing disease burden, and escalating healthcare costs (X.-X. Zhang et al., 2021). Among the different forms of dementia, AD represents the most common neurodegenerative disorder and has a complex genetic basis involving multiple genes, making it difficult to predict both clinical manifestations and age of onset during presymptomatic testing. However, the identification of causative genes is crucial, as it enables correlations between genetic alterations and pathological features, biomarkers, neuroimaging findings, and clinical presentation of the disease (Krishnamurthy et al., 2025). From a clinical and genetic perspective, AD is generally divided into familial AD (FAD) follows an autosomal dominant pattern of inheritance and typically presents as early-onset AD (EOAD) and late-onset AD (LOAD) (Krishnamurthy et al., 2025; Lo Vecchio et al., 2026). The majority of cases, approximately 90%, are sporadic and typically manifest between 60 and 65 years of age, corresponding to LOAD. EOAD develops earlier, usually between 30 and 65 years, and around 13% of familial EOAD cases follow an autosomal dominant inheritance pattern (Campion et al., 1999; Roses, 1995). The genetic determinants associated with these two forms of the disease differ considerably. The genes most commonly implicated in EOAD are APP, PSEN1, and PSEN2, whereas APOE represents the principal genetic factor associated with LOAD (Kang et al., 1987; Selkoe, 2001). Their biological functions and their involvement in disease pathogenesis are described in the following sections. Overall, these observations highlight the multifactorial nature of AD and emphasize the importance of understanding the interaction between genetic susceptibility and other contributing factors in order to improve disease prediction, diagnosis, and characterization (Krishnamurthy et al., 2025).

1.2.1 Amyloid Precursor Protein (APP)

The APP gene, located on chromosome 21, encodes the amyloid precursor protein (APP), which serves as the source of amyloid beta (Aβ) peptides that accumulate in amyloid plaques (APs) (Giaccone et al., 1989). APP is abundantly expressed at synaptic sites, where it participates in synaptic regulation and neuronal plasticity (Priller et al., 2006; Walter et al., 2001). The enzymatic cleavage of APP by β- and γ-secretases ultimately leads to the generation of Aβ peptides (Bird, 2008).

A number of APP mutations have been linked to early-onset familial AD (EOFAD), particularly those located in proximity to the Aβ sequence. These mutations alter APP processing and promote increased

production of Aβ42 (Krishnamurthy et al., 2025). Clinically, individuals carrying such mutations often develop symptoms at approximately 40 years of age (Goate et al., 1991; J. Hardy, 2001). Among the most extensively studied variants are the Swedish and London mutations, both of which are associated with elevated Aβ production and disease onset (Kwok et al., 2000; Mullan, 1992).

1.2.2 Presenilin 1 (PSEN1)

The PSEN1 gene, located on chromosome 14, encodes a membrane protein that forms part of the catalytic core of the γ-secretase complex, which is essential for APP processing (Krishnamurthy et al., 2025). γ-secretase also mediates the cleavage of other type-I transmembrane proteins, including NOTCH. PSEN1 missense mutations account for approximately 18%–50% of autosomal dominant EOFAD cases (Citron et al., 1997) and are known to increase the Aβ42/Aβ40 ratio (Lippa et al., 1998). PSEN1 mutations are associated with particularly aggressive disease forms, often with onset as early as 30 years of age, and are inherited in an autosomal dominant manner (Rudzinski et al., 2008). More than 300 PSEN1 mutations have been identified, most of which are missense variants that enhance Aβ42 production and promote amyloid deposition in the brain (Moehlmann et al., 2002). Experimental animal models further support the role of PSEN1 in both cognitive processes and Aβ metabolism (Krishnamurthy et al., 2025).

1.2.3 Presenilin 2 (PSEN2)

PSEN2, located on chromosome 1, encodes a protein that is also part of the γ-secretase complex (Bentahir et al., 2006; Wolfe et al., 1999). It is predominantly expressed in neuronal tissue, and, similarly to PSEN1, mutations in PSEN2 are associated with an increased Aβ42/Aβ40 ratio (Lippa et al., 1998). To date, 33 disease-associated PSEN2 mutations have been identified. These variants affect APP processing at the γ-secretase cleavage site, although their effects on Aβ production are heterogeneous, for example, the L166P mutation reduces Aβ production, whereas G384A increases Aβ42 levels. Overall, PSEN2 mutations tend to produce lower levels of Aβ42 compared with PSEN1 mutations (Sherrington, 1996). Unlike PSEN1, PSEN2 mutations are rarely associated with EOFAD, and disease onset can vary even within the same family, suggesting modulation by additional genetic and environmental influences (Levy-Lahad et al., 1995). The first PSEN2 mutation identified was

N141I (Lindquist et al., 2008), while more recently the V393M variant has been described within the seventh transmembrane domain (Krishnamurthy et al., 2023).

1.2.4 Apolipoprotein E (APOE)

The APOE gene, located on chromosome 19, encodes apolipoprotein E, the principal protein involved in lipid transport within the brain. It is expressed by astrocytes, microglia, and neurons (Corder et al., 1993). ApoE plays a key role in lipid and cholesterol transport by forming lipoprotein particles that distribute lipids to neurons (Krishnamurthy et al., 2025). Three major APOE alleles exist: ε2, ε3, and ε4. These alleles are defined by the rs7412 and rs429358 polymorphisms, with ε3 being the most common isoform (Krishnamurthy et al., 2025). Although the exact pathogenic mechanisms of ApoE in the brain are not fully understood, the ε4 allele is strongly associated with an increased risk of AD and has been linked to both tau hyperphosphorylation and amyloid aggregation (Krishnamurthy et al., 2025). It is also associated with an earlier age of disease onset (Liu et al., 2002). Neuroimaging studies have demonstrated that APOE ε4 is associated with increased Aβ deposition in individuals with mild cognitive impairment (MCI) and AD (Fleisher et al., 2013; Mishra et al., 2018), with a particularly strong effect during early amyloid accumulation (J. C. Morris et al., 2010). Conversely, the ε2 allele is associated with reduced Aβ deposition and slower cognitive decline (Krishnamurthy et al., 2025; Lim et al., 2017). However, the presence of ε4 appears to override the protective effect of ε2, as ε4/ε2 and ε4/ε3 genotypes still exhibit Aβ accumulation (Lim et al., 2017). Furthermore, APOE ε4 has been linked to increased vulnerability to TDP-43, α-synuclein, and tau pathologies (Krishnamurthy et al., 2025), although its precise contribution to AD risk remains unclear. Experimental studies in cellular and animal models also suggest that APOE ε4 contributes to neuroinflammation, synaptic dysfunction, reduced dendritic density and plasticity, decreased glutamate receptor expression, and accumulation of Aβ oligomers at synaptic sites (Krishnamurthy et al., 2025). Nevertheless, not all carriers of the APOE ε4 allele develop AD, indicating that additional genetic and environmental factors contribute to disease risk (Krishnamurthy et al., 2025).

1.3 Molecular Pathogenesis of Alzheimer's Disease

AD is a progressive neurodegenerative disorder characterized by the accumulation of Aβ and hyperphosphorylated tau, which give rise to neuritic plaques and neurofibrillary tangles (NFTs), respectively, primarily affecting the medial temporal lobe and neocortical regions (Krishnamurthy et al., 2025). Although these pathological features represent the defining hallmarks of AD, growing evidence indicates that the disease arises from a multifactorial interplay of mechanisms that extend beyond Aβ and tau pathology, including neuroimmune dysregulation, metabolic dysfunction, and systemic factors (Di Crescenzo et al., 2026). To better understand the molecular basis of AD, that drive neurodegeneration, it is essential to examine the mechanisms responsible for Aβ generation and tau hyperphosphorylation, but also the neuroinflammation, oxidative stress, lipid and metabolic dysregulation that contribute to the development and progression of the characteristic neuropathological alterations of the disease.

1.3.1 Amyloid beta pathway

APP, the precursor of Aβ, can be metabolized through two alternative pathways: the non-amyloidogenic and the amyloidogenic pathway (Krishnamurthy et al., 2025). Under physiological conditions, APP is predominantly processed via the non-amyloidogenic route, involving α-secretase and γ-secretase activity, which prevents Aβ generation and helps maintain the physiological balance between Aβ production and clearance in the brain (Vetrivel & Thinakaran, 2006). In this pathway, cleavage of APP by α-secretase occurs within the Aβ sequence, producing the soluble fragment sAPPα and the C83 fragment, which is subsequently processed by γ-secretase to generate the P3 peptide (Krishnamurthy et al., 2025). In contrast, under pathological conditions, APP processing is shifted toward the amyloidogenic pathway, leading to the accumulation of Aβ in the brain (Kunjathoor et al., 2004). Here, APP is sequentially cleaved by β-secretase and γ-secretase, resulting in the production of Aβ peptides, mainly Aβ40 and Aβ42, which represent the predominant species found in the human brain (Olsson et al., 2014; Takami et al., 2009). This processing also yields the APP intracellular domain (AICD), which is able to translocate to the nucleus and modulate gene expression, including genes involved in apoptosis (G. Chen et al., 2017). A shift toward the amyloidogenic pathway may be promoted by several factors, including mutations in APP and PSEN

genes, overexpression of APP and β-secretase, oxidative stress, inflammatory processes, impaired Aβ clearance, environmental influences, and interactions between tau and APP (Krishnamurthy et al., 2025).

1.3.2 Tau hyperphosphorylation and Neurofibrillary Tangles

Tau is a major microtubule-associated protein (MAP) expressed in neurons, where it plays an essential role in microtubule assembly and stabilization (Köpke et al., 1993; Weingarten et al., 1975). Six isoforms of tau have been identified (0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R), and under normal physiological conditions, tau phosphorylation is required for its binding to tubulin and for proper microtubule organization (Jicha et al., 1997). In AD, tau undergoes abnormal hyperphosphorylation, a process that disrupts its physiological role in microtubule stabilization and promotes its aggregation into NFTs. These intracellular aggregates represent one of the major pathological hallmarks of AD and are also observed in a group of neurodegenerative disorders collectively known as tauopathies. Therefore, tau hyperphosphorylation is considered a key event underlying tau dysfunction and NFT formation in AD (Krishnamurthy et al., 2025). In healthy individuals, tau is predominantly found in a soluble form, whereas in AD it can be detected in soluble, oligomeric, and fibrillar states (Jicha et al., 1997). Although total tau levels do not differ substantially between healthy and AD brains, hyperphosphorylated tau is increased approximately four- to eight-fold in AD (Alonso et al., 2006). Once incorporated into NFTs, tau loses its normal physiological function in microtubule stabilization (Li et al., 2007). In addition, a substantial proportion (around 40%) of hyperphosphorylated tau remains in the cytosol without aggregating into NFTs, where it contributes to microtubule destabilization and interferes with normal tau and other microtubule-associated proteins, including MAP1A/B and MAP2 (Krishnamurthy et al., 2025). Importantly, experimental evidence shows that tau dephosphorylation can restore its normal function, supporting the notion that abnormal phosphorylation is the key determinant of its toxic effects (Krishnamurthy et al., 2025).

1.3.3 Neuroinflammation and Glial Activation

Accumulating evidence indicates that chronic neuroinflammation represents a key mechanism in the pathogenesis of AD, linking immune dysregulation with genetic and metabolic alterations (Di Crescenzo et al., 2026). From a temporal perspective, AD has been proposed to progress through an initial biochemical phase, during which pathological processes remain largely confined within neurons, followed by a cellular phase characterized by dysfunction of local glioneuronal networks, promoting the spread of amyloid and tau pathology together with inflammatory responses (Di Crescenzo et al., 2026). As the disease advances into the clinical phase, these alterations become more widespread and ultimately disrupt large-scale neuronal networks. Throughout the cellular and clinical stages, inflammatory processes within the central nervous system (CNS) are closely regulated by a complex interaction between metabolic and immune pathways (Di Crescenzo et al., 2026). Microglia, the resident immune cells of the CNS, play a central role in AD-associated neuroinflammation. In response to the accumulation of Aβ and tau aggregates, microglia become activated and release pro-inflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Di Crescenzo et al., 2026), while also activating inflammasome pathways such as NLRP3. These responses contribute to neuronal injury, and prolonged activation promotes the transition of microglia toward dysfunctional and neurotoxic phenotypes (Di Crescenzo et al., 2026). Genetic evidence further supports the involvement of immune mechanisms in AD, as several risk loci associated with sporadic AD (SAD) encode genes involved in immune regulation, suggesting that immune dysregulation may actively contribute to disease development rather than simply represent a downstream consequence of pathology (Uddin et al., 2022). Astrocytes also participate in the neuroinflammatory response and, together with activated microglia, become chronically reactive in the presence of aggregated Aβ and tau (Di Crescenzo et al., 2026). In addition, transforming growth factor-β1 (TGF-β1), which is produced by astrocytes, neurons, and other cell types, modulates microglial activation by limiting the release of pro-inflammatory cytokines and reactive species, thereby exerting neuroprotective effects and enhancing Aβ clearance. However, prolonged microglial activation is associated with a diminished responsiveness to TGF-β1 signaling, resulting in increased cytotoxicity and a reduced capacity for Aβ uptake (Von Bernhardi et al., 2015). Under conditions of persistent activation, microglia and astrocytes promote a pro-inflammatory environment through the release cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS), which further exacerbate synaptic dysfunction and neuronal damage (Novoa et al., 2022). Persistent

neuroinflammation not only contributes directly to neuronal loss but also compromises several protective mechanisms, including microglial phagocytosis, glymphatic clearance, and the integrity of the blood-brain barrier (BBB). As a consequence, the accumulation of amyloid deposits and the propagation of tau pathology are further enhanced, creating a self-perpetuating cycle of neurodegeneration (Uddin et al., 2022). Recent studies have highlighted the close relationship between inflammation and cellular metabolism in the CNS (Di Crescenzo et al., 2026). The interaction between immune activity and metabolic processes, commonly referred to as immunometabolism, has emerged as a crucial component in understanding chronic neuroinflammation (Lonkar et al., 2025). In AD, maladaptive metabolic reprogramming impairs the ability of immune cells to efficiently clear Aβ and tau aggregates through phagocytosis. Furthermore, disruption of metabolic–immune homeostasis has been proposed as a contributing factor to the increased vulnerability of the aging brain to neurodegenerative processes (Di Crescenzo et al., 2026; Lonkar et al., 2025).

1.3.4 Oxidative Stress and Mitochondrial Dysfunction

Oxidative stress is widely recognized as a key contributor to the pathogenesis of several disorders, including neurodegenerative diseases such as AD (Angelova & Abramov, 2018; Tan et al., 2018). Under physiological conditions, ROS and RNS are continuously generated during oxidative metabolism and mitochondrial respiration (Sies, 2017). However, when their production exceeds the capacity of antioxidant defenses, oxidative damage accumulates, affecting lipids, proteins, RNA, and DNA, ultimately compromising cellular function and viability (Lovell & Markesbery, 2007; Mazon et al., 2017; L. Wang et al., 2017). The brain is particularly susceptible to oxidative stress because of its high oxygen consumption, elevated metabolic activity, abundance of polyunsaturated fatty acids, and relatively limited antioxidant capacity (Cobley et al., 2018). Although only a small proportion of oxygen consumed during oxidative phosphorylation is normally converted into ROS (Chance et al., 1979), this process is markedly enhanced under conditions of mitochondrial dysfunction, such as those occurring in AD. Furthermore, increased levels of redox-active metals, including copper and iron, have been reported in vulnerable brain regions, further promoting ROS generation through redox reactions (Lovell & Markesbery, 2007). Increasing evidence suggests that oxidative stress is an early event in AD pathogenesis. Elevated levels of RNA and protein oxidation, together with enhanced

lipid peroxidation, have been detected in vulnerable brain regions of individuals with MCI, indicating that oxidative damage precedes the onset of overt dementia (Butterfield et al., 2007; Lovell & Markesbery, 2007). In parallel, the accumulation of misfolded proteins further exacerbates oxidative stress by impairing mitochondrial function and promoting excessive ROS production (Selfridge et al., 2013; Zhao & Zhao, 2013). Mitochondria play a central role in this process, as they are not only the primary source of cellular ATP but also a major source of ROS (Bhat et al., 2015). Defects in oxidative phosphorylation, together with mutations in mitochondrial DNA (mtDNA), can impair mitochondrial function, increase ROS production, and establish a self-perpetuating cycle of oxidative damage that contributes to neuronal dysfunction and aging (Bhat et al., 2015; Tan & Norhaizan, 2019). As oxidative stress progresses, mitochondrial membrane integrity, Ca^{2+} homeostasis, and respiratory chain activity become progressively compromised, ultimately promoting neurodegeneration and cognitive decline (Swerdlow et al., 2014; Tan & Norhaizan, 2019). Among the cellular targets of oxidative injury, DNA, and particularly mtDNA, is especially vulnerable because of its limited repair capacity and close proximity to the electron transport chain (ETC). ROS, especially hydroxyl radicals, induce DNA strand breaks and DNA–DNA or DNA–protein cross-links, further amplifying genomic instability and neuronal damage during aging and AD (Lovell & Markesbery, 2007; Markesbery & Lovell, 2006).

1.4 Alzheimer's Disease Hypotheses

AD is considered a multifactorial disorder, and numerous hypotheses have been proposed over the years to explain its pathogenesis. These hypotheses have largely arisen from the progressive identification of the major neuropathological and neurochemical alterations observed in the AD brain, each highlighting a different mechanism potentially involved in disease onset and progression (Breijyeh & Karaman, 2020; Kametani & Hasegawa, 2018). Among the most extensively investigated are the amyloid, tau, and cholinergic hypotheses (Babic; et al., 1999; Kametani & Hasegawa, 2018; Paroni et al., 2019). Although each has significantly contributed to the current understanding of AD, none has been able to fully explain the complexity of the disease (Breijyeh & Karaman, 2020).

1.4.1 Amyloid Hypothesis

The amyloid hypothesis, also referred to as the amyloid cascade hypothesis, was first proposed in the early 1990s and has long provided a conceptual framework for understanding the pathogenesis of Alzheimer's disease (J. A. Hardy & Higgins, 1992; J. Hardy & Allsop, 1991; J. Hardy & Selkoe, 2002; Selkoe, 1991). According to this hypothesis, the abnormal accumulation of Aβ peptides in the brain represents an early event that initiates a cascade of pathological processes ultimately leading to synaptic dysfunction, neuronal loss, and cognitive impairment (Kametani & Hasegawa, 2018). Under physiological conditions, Aβ peptides generated from APP are efficiently removed through degradation and clearance mechanisms. However, aging and pathological conditions may impair these processes, resulting in progressive Aβ accumulation within the brain (Kametani & Hasegawa, 2018). In particular, increased production of Aβ42, or an elevated Aβ42/Aβ40 ratio, promotes the formation of insoluble amyloid fibrils that progressively aggregate into senile plaques. These aggregates are thought to exert neurotoxic effects and promote neuronal degeneration (Kametani & Hasegawa, 2018). Support for the amyloid hypothesis was strengthened by the identification of pathogenic mutations in the APP, PSEN1, and PSEN2 genes, all of which directly influence APP processing and Aβ production. These mutations increase Aβ42 generation and/or alter the Aβ42/Aβ40 ratio, providing a mechanistic link between abnormal Aβ metabolism and the development of familial AD (Kametani & Hasegawa, 2018; Paroni et al., 2019; Ricciarelli & Fedele, 2017). Similarly, individuals with Down syndrome, who carry an additional copy of chromosome 21 containing the APP gene, develop early Aβ accumulation and frequently exhibit AD-like neuropathological changes during adulthood (Kametani & Hasegawa, 2018). Although the amyloid hypothesis remains strongly supported by genetic evidence in FAD, its role in sporadic disease has been more extensively debated, as APs can also be detected in cognitively normal older individuals and the extent of plaque deposition does not always correlate with the severity of cognitive impairment (Paroni et al., 2019).

1.4.2 Tau Hypothesis

The tau hypothesis proposes that abnormal tau protein is the primary driver of neurodegeneration in AD. Under physiological conditions, tau is a microtubule-associated protein mainly localized in neuronal axons, where it stabilizes microtubules and supports axonal transport (Crowther et al., 1989).

In AD, tau undergoes abnormal hyperphosphorylation, leading to its dissociation from microtubules and subsequent aggregation into paired helical filaments (PHFs) and NFTs, which represent one of the major neuropathological hallmarks of the disease (Kametani & Hasegawa, 2018). Tau pathology follows a characteristic spatiotemporal progression described by Braak and Braak, initially affecting the transentorhinal cortex before spreading to limbic and neocortical regions (Braak & Braak, 1991). Importantly, the extent of tau pathology closely correlates with cognitive impairment and clinical disease severity, and several studies suggest that tau abnormalities may arise before extensive Aβ deposition during the early stages of AD (Kametani & Hasegawa, 2018). Hyperphosphorylated tau disrupts microtubule dynamics, impairs axonal transport, alters synaptic function, and compromises mitochondrial integrity, ultimately promoting neuronal dysfunction and degeneration (Kametani & Hasegawa, 2018). In addition, increasing evidence indicates that pathological tau can propagate between interconnected neurons in a prion-like manner, contributing to the progressive spread of neurodegeneration throughout the brain (Kametani & Hasegawa, 2018).

1.4.3 Cholinergic Hypothesis

During the 1970s, several studies identified neocortical and presynaptic cholinergic deficits associated with reduced activity of choline acetyltransferase (ChAT), the enzyme responsible for acetylcholine (ACh) synthesis, leading to the formulation of the cholinergic hypothesis of AD (Breijyeh & Karaman, 2020). ACh, synthesized from choline and acetyl-coenzyme A in cholinergic neurons, plays a crucial role in cognitive processes, including memory, learning, attention, and sensory processing (Breijyeh & Karaman, 2020). The cholinergic hypothesis is primarily based on three key observations: the reduction of presynaptic cholinergic markers in the cerebral cortex, the marked neurodegeneration of the nucleus basalis of Meynert (NBM), which provides the major cholinergic innervation to the cerebral cortex, and the opposite effects of cholinergic antagonists and agonists on memory function (Hampel et al., 2018). Accordingly, degeneration of cholinergic neurons in AD has been closely associated with cognitive impairment and memory decline. In addition, Aβ has been shown to disrupt cholinergic neurotransmission by reducing choline uptake and ACh release (Babic; et al., 1999). The neurotoxicity of Aβ oligomers has also been linked to cholinergic synaptic loss and to interactions between acetylcholinesterase (AChE) and Aβ peptides. Furthermore, reduced expression of presynaptic nicotinic and muscarinic (M2) ACh receptors, together with impaired

excitatory amino acid neurotransmission characterized by decreased glutamate levels and D-aspartate uptake in several cortical regions, may further contribute to disease progression (Breijyeh & Karaman, 2020). Experimental evidence further supports this hypothesis. Cholinergic antagonists, such as scopolamine, have been shown to induce reversible amnesia, whereas compounds that enhance ACh synthesis or cholinergic neurotransmission can attenuate these cognitive deficits (Babic; et al., 1999; Monczor, 2005).

1.5 Pharmacological Treatments for Alzheimer's Disease

Despite the major public health burden associated with AD, only two classes of drugs are currently approved for its treatment: cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists (Breijyeh & Karaman, 2020). Acetylcholinesterase inhibitors (AChEIs), act by inhibiting AChE and butyrylcholinesterase (BChE), thereby preventing ACh degradation and increasing its concentration within the synaptic cleft (Sharma, 2019; Singh & Patel, 2026). In contrast, NMDA receptor antagonists reduce glutamate-mediated excitotoxicity by preventing excessive Ca^{2+} influx, which is associated with synaptic dysfunction and neuronal death (Breijyeh & Karaman, 2020; R. Wang & Reddy, 2017). However, although these therapeutic approaches can alleviate symptoms and slow cognitive decline, they neither cure nor prevent disease progression (Breijyeh & Karaman, 2020) and despite evidence supporting the efficacy of drugs acting on the cholinergic system in the treatment of these forms of dementia, such therapies are still unavailable in many countries (Livingston et al., 2024). Furthermore, despite considerable research efforts over the past decades, relatively few therapeutic strategies have demonstrated clear clinical efficacy, and many clinical trials have produced disappointing results. Without treatment, individuals diagnosed with mild AD dementia typically progress to severe dementia within approximately three years (J. C. Morris et al., 1993). Nevertheless, treatment with cholinesterase inhibitors has been shown to preserve relatively adequate cognitive function for five to eight years in patients with mild AD dementia, resulting in a slower rate of cognitive decline (Rogers et al., 1998). More recently, the combination of anti-amyloid therapy with cholinesterase inhibitor treatment in early-stage AD has demonstrated the potential to maintain cognitive function for longer periods, extending over five to ten years (Van Dyck et al., 2023). Over the past two decades, therapeutic research has progressively shifted from symptomatic treatments toward disease-modifying approaches targeting the molecular mechanisms underlying AD,

particularly Aβ and tau pathology (Wu & Fuh, 2025). Since Aβ42 is the major component of senile plaques (SPs) and its abnormal accumulation has been strongly implicated in AD pathogenesis, considerable efforts have been directed toward the development of anti-amyloid therapies (Wu & Fuh, 2025). In parallel, increasing evidence supporting the role of tau pathology in neurodegeneration has promoted the development of therapeutic approaches targeting tau aggregation and propagation (Wu & Fuh, 2025). Parallel advances in NFT research have further clarified the role of tau, a microtubule-associated protein essential for neuronal function. The propagation of phosphorylated tau (p-tau) from limbic regions to the neocortex has been linked to worsening neurodegeneration, and several therapeutic strategies targeting tau pathology are currently under investigation, although no effective therapy has yet been successfully developed (Wu & Fuh, 2025). Among the most promising candidates are semorinemab, a monoclonal antibody designed to promote the clearance of extracellular tau; zagotenemab, a monoclonal antibody that neutralizes soluble tau aggregates; and BIIB080, an antisense oligonucleotide that reduces tau production by targeting its mRNA (Breijyeh & Karaman, 2020; Wu & Fuh, 2025). Current knowledge regarding therapies based on anti-Aβ antibodies remains limited. Nevertheless, some recent clinical trials have, for the first time, shown a modest reduction in cognitive decline in patients treated with anti-amyloid antibodies, accompanied by a marked decrease in cerebral Aβ levels. These therapies were associated with a 27–35% reduction in disease progression after 18 months of treatment. However, their use is associated with high costs, significant treatment burden, and the need for intensive monitoring and follow-up, in addition to the possibility of clinically relevant and occasionally severe adverse effects. Furthermore, evidence regarding their long-term efficacy and safety is still lacking (Livingston et al., 2024). Among recently developed disease-modifying therapies, aducanumab represents a novel human monoclonal antibody directed against Aβ pathology in AD. It received approval from the FDA in 2021 and became the first AD drug licensed since 2003 (Rahman et al., 2023). Clinical studies demonstrated that monthly aducanumab infusions reduced cerebral Aβ plaque burden in a dose- and time-dependent manner and were associated with a slower rate of cognitive decline in patients receiving the highest doses (Rahman et al., 2023). Aducanumab has also been reported to influence both amyloid and tau pathological hallmarks of AD (Rahman et al., 2023). However, clinical trials identified amyloid-related imaging abnormalities (ARIA) as the principal adverse event, including ARIA-E, characterized by cerebral edema or sulcal effusion, and ARIA-H, associated with microhemorrhages or hemosiderin deposition within the brain parenchyma (Budd Haeberlein et al., 2022). Severe

symptoms, including seizures, were observed in association with ARIA-E, although no fatal events were reported. (Budd Haeberlein et al., 2022). Although aducanumab represented a major milestone in the development of disease-modifying therapies for AD, its clinical development proved challenging. In January 2024, Biogen announced the discontinuation of the development and commercialization of aducanumab, together with the termination of the post-marketing phase IV ENVISION study. According to the company, this decision was not related to concerns regarding the drug's safety or efficacy, but rather reflected a strategic reallocation of resources toward other therapeutic candidates for AD, including lecanemab and additional pipeline programs (Biogen to Realign Resources for Alzheimer's Disease Franchise, 2024). Among these, lecanemab has shown promising results in phase III clinical trials and has been suggested to be more effective than previously developed anti-amyloid monoclonal antibodies (Avgerinos et al., 2021; Van Dyck et al., 2023). Nevertheless, current evidence indicates that lecanemab is effective primarily in reducing amyloid biomarkers and delaying cognitive decline during the early stages of AD, whereas efficacy and safety data for later disease stages remain unavailable (Van Dyck et al., 2023).

1.6 Modifiable Risk and Protective Factors in Alzheimer's Disease

AD has been considered a multifactorial disease associated with several risk factors such as increasing age, genetic factors, head injuries, vascular diseases, infections, and environmental factors The underlying cause of pathological changes in Alzheimer's disease (Aβ, NFTs, and synaptic loss) is still unknown (Breijyeh & Karaman, 2020). Increasing evidence indicates that lifestyle-related interventions play an important role in reducing AD risk and promoting cognitive health (Crous-Bou et al., 2017). Physical activity has been shown to enhance brain vascularization, neuroplasticity, and neurogenesis while simultaneously reducing inflammation and Aβ production, thereby contributing to improved cognitive function in older individuals (Breijyeh & Karaman, 2020). Likewise, adherence to the Mediterranean diet, intellectual engagement, and higher educational attainment have been associated with slower memory decline, preservation of cognitive performance, and a reduced risk of AD progression (Hardman et al., 2016; Petersson & Philippou, 2016). Furthermore, multidomain interventions combining dietary modifications, regular physical exercise, cognitive training, management of AD symptoms, and control of cardiovascular risk factors have been shown to preserve cognitive function and reduce the incidence of AD in older populations (Crous-Bou et al.,

2017). The 2024 update of the The Lancet Commission on dementia presents encouraging new evidence regarding dementia prevention, intervention, and patient care (Livingston et al., 2024). Several modifiable risk factors previously identified, including limited education, hearing loss, hypertension, smoking, obesity, depression, physical inactivity, diabetes, excessive alcohol consumption, traumatic brain injury, air pollution, and social isolation, have been associated with an increased risk of dementia (Livingston et al., 2020). More recent evidence additionally identifies untreated vision loss and elevated LDL cholesterol as further relevant risk factors (Livingston et al., 2024). Together, these findings support a comprehensive life-course model of dementia prevention based on 14 modifiable risk factors, suggesting that nearly half of dementia cases could theoretically be prevented through their elimination (Livingston et al., 2024). Current evidence suggests that reducing the risk of dementia may increase the number of years lived in good health while simultaneously shortening the duration of the disease in individuals who eventually develop it. Consequently, preventive strategies should aim to reduce exposure to risk factors as early as possible and maintain low risk levels throughout life (A. Armstrong, 2019).

1.6.1 High-Fat Diet as a Risk Factor for Alzheimer's Disease

Food represents an essential source of energy and plays a crucial role in brain function by directly supplying metabolic substrates (Tan & Norhaizan, 2019). In recent decades, dietary fat consumption has significantly increased, particularly between 1991 and 2008 (Vadiveloo et al., 2014). This increase has been attributed to progressive changes in dietary habits and the food environment over time, which have influenced population-wide eating patterns despite nutritional recommendations aimed at reducing fat intake (Vadiveloo et al., 2014). For instance, Vadiveloo et al. suggests that the intake of foods rich in animal fats increased over time; however, the percentage of energy consumed from animal fats did not markedly increase in men and women (16.3-17.4% in men and 15.8-17.1% in women, $p<0.01$), probably due to a reduction in the animal fat content of the food supply during this period. Vegetable fats increased substantively (13.8-15.5% in men and 14.1-16.0% in women, $p<0.01$) and appears to be driven by an increased intake of nuts and seeds in this population. However, we were unable to examine trends of total oil intake in this population, which may have also contributed to an increase in vegetable fats over time. The reduction in trans-fat intake seems to be driven by a decrease in margarine intake. Several studies have shown that excessive intake of high-

fat foods promotes the overproduction of circulating free fatty acids and contributes to systemic inflammation (Tan & Norhaizan, 2019). Considerable evidence has also demonstrated the detrimental effects of diets rich in saturated fats (DiNicolantonio et al., 2016). High dietary fat intake is strongly associated with obesity, which promotes a chronic inflammatory state through the expansion of white adipose tissue and the consequent release of pro-inflammatory mediators (Muñoz & Costa, 2013). In addition, high-fat diets (HFD) have been linked to increased oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, and impaired hippocampal neurogenesis and synaptic plasticity within the CNS (Lindqvist et al., 2006; Pipatpiboon et al., 2012). Consequently, diet-induced obesity may accelerate age-related neural damage and increase brain susceptibility to pathological insults involved in cognitive decline and dementia development (Bruce-Keller et al., 2009; Uranga et al., 2010). In humans, obesity has been associated with reduced cortical and hippocampal volume, accelerated brain atrophy, impaired memory performance, and deficits in executive functions (Więckowska-Gacek et al., 2021). Furthermore, increased caloric and fat intake during late midlife has been associated with a higher risk of dementia and AD (Kalmijn et al., 1997; Luchsinger et al., 2002). Experimental studies in rodents further support the relationship between HFD, obesity, and cognitive dysfunction. Experimental studies in rodents further support the relationship between HFD, obesity, and cognitive dysfunction. HFD is a broad term that encompasses different dietary regimens characterized by a higher fat content than standard laboratory chow. Among these, the Western diet (WD), characterized not only by a high fat content but also by elevated levels of refined sugars and salt, is one of the most widely used experimental models to reproduce metabolic alterations associated with obesity and sporadic AD (Tan & Norhaizan, 2019; Więckowska-Gacek et al., 2021). Diet-induced obesity has been associated with memory impairment (Alzoubi et al., 2018), while mice exposed to long-term HFD and WD exhibit reduced learning and memory abilities (Cordner & Tamashiro, 2015), together with anxiety- and depressive-like behaviors (Tan & Norhaizan, 2019). Moreover, several rodent studies have shown that obesity induced during midlife is associated with increased levels of APP and p-tau (Więckowska-Gacek et al., 2021). HFD-fed mice also display increased body weight accompanied by elevated hippocampal Aβ levels, reduced hippocampal neurogenesis, and impairments in cognitive performance (Więckowska-Gacek et al., 2021). An excessive influx of free fatty acids into the liver promotes the production of very low-density lipoprotein (VLDL) triglycerides, thereby contributing to the development of dyslipidemia. These metabolic alterations may subsequently lead to the onset of metabolic syndrome.

Elevated circulating free fatty acid levels and the lipotoxic effects associated with dyslipidemia can also negatively affect the CNS (O'Brien et al., 2017). Over time, these dysfunctions may contribute to the development of neurological disorders, including MCI and AD (O'Brien et al., 2017).

1.6.2 Social Isolation and Loneliness as Risk Factors for Alzheimer's Disease

Social interaction plays a fundamental role in maintaining both physical and mental health, whereas social isolation and loneliness have consistently been identified as important risk factors for adverse health outcomes and increased mortality (Ren et al., 2023). Social isolation refers to an objective condition characterized by insufficient social support, communication, and engagement with others (McPherson et al., 2006). In contrast, loneliness describes the subjective perception that existing social relationships are inadequate (Livingston et al., 2024). Unlike loneliness, social isolation can be objectively assessed through indicators such as social network size, marital status, and living arrangements (Cornwell & Waite, 2009; Hawthorne, 2008). Growing evidence indicates that both social isolation and loneliness are associated with an increased risk of cognitive decline and dementia (Ren et al., 2023). Several epidemiological studies support this association. A systematic review including eight studies and 15,762 participants reported that individuals with less frequent social interactions had a significantly greater risk of dementia (RR 1.57, 95% CI 1.32–1.85) than those with more frequent social contact, although the definition of social contact varied among studies (Kuiper et al., 2015). Similarly, dementia incidence was higher among individuals considered socially isolated at baseline, defined by the presence of at least two of the following criteria: living alone, interacting with family or friends less than once per month, and not participating in weekly group activities (Livingston et al., 2024). Conversely, socially integrated individuals appear to exhibit greater resilience against processes that negatively affect neurobiological function (Ren et al., 2023). Although this association is now well established, the biological mechanisms linking social isolation and loneliness to cognitive decline remain only partially understood. Current evidence suggests that prolonged social isolation acts as a chronic psychosocial stressor capable of promoting persistent inflammation and accelerating neurodegenerative processes that affect brain regions involved in cognition (Ren et al., 2023). In addition, socially isolated individuals frequently exhibit other lifestyle characteristics associated with an increased risk of dementia, including reduced physical activity, impaired sleep quality, smoking, and excessive alcohol consumption (J. T. Cacioppo & Hawkley,

2009). Among the proposed biological mechanisms, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis has received considerable attention. Chronic activation of the HPA axis has been observed in lonely individuals and has been associated with abnormal diurnal cortisol secretion patterns (J. T. Cacioppo & Hawkley, 2009; Conrad & Bimonte-Nelson, 2010; Miller et al., 2007). Moreover, elevated bedtime cortisol concentrations have been linked to poorer cognitive performance in individuals experiencing loneliness (Montoliu et al., 2019). Several biological mechanisms have been proposed to explain how social isolation and loneliness may contribute to dementia. Chronic loneliness can act as a persistent psychosocial stressor, activating the sympathetic nervous system, increasing glucocorticoid secretion, and promoting inflammation (Sapolsky, 1999; Sundström et al., 2020). Over time, these alterations may damage brain regions particularly vulnerable to AD, including the hippocampus, orbital prefrontal cortex, and medial prefrontal cortex (S. Cacioppo et al., 2014; Sutin et al., 2020). In addition, prolonged loneliness may reduce cognitive reserve, thereby decreasing the brain's resilience to age-related cognitive decline (Wilson et al., 2007). Reduced social engagement may limit cognitive and neural stimulation, whereas participation in social activities may exert neuroprotective effects and preserve neural circuits involved in social behavior, potentially through mechanisms related to neurogenesis (Holmes, 2016; Lieberwirth & Wang, 2012).

1.6.3 Physical Exercise as a Protective Factor Against Alzheimer's Disease

Physical inactivity is considered one of the major potentially modifiable risk factors associated with dementia, accounting for nearly 40% of dementia cases occurring in older age (Livingston et al., 2020). Increasing evidence suggests that regular physical activity (PA) may exert protective effects against AD and other dementias through multiple interconnected mechanisms. These include reduced Aβ production, enhanced Aβ clearance, improvements in cerebral blood flow and vascular integrity, as well as antioxidant and anti-inflammatory effects within the brain (Iso-Markku et al., 2022). In addition, PA may indirectly contribute to cognitive health by improving sleep quality, mood, and cardiovascular risk factors (Iso-Markku et al., 2022). Consequently, maintaining an active lifestyle appears to be important both in reducing the risk of AD and in slowing disease progression in older individuals (De La Rosa et al., 2020). Current evidence also suggests that the association between exercise and dementia may be bidirectional, as cognitive decline itself can reduce an individual’s ability or motivation to remain physically active (Livingston et al., 2020). Nevertheless, several

epidemiological studies consistently support the protective role of PA. A systematic review and meta-analysis including 58 studies and 257,983 participants demonstrated that physically active individuals had a lower risk of both all-cause dementia (RR 0.80, 95% CI 0.77–0.84) and AD (RR 0.86, 95% CI 0.80–0.93), independently of baseline age and even during follow-up periods exceeding 20 years (Iso-Markku et al., 2022). Similarly, a prospective study involving 29,826 participants followed for a median of 24.5 years reported that individuals who maintained optimal levels of PA showed a lower dementia risk compared with persistently inactive subjects (HR 0.75, 95% CI 0.58–0.97). A reduced risk was also observed in individuals who increased their PA over time (HR 0.83, 95% CI 0.72–0.96) (Tari et al., 2022). The beneficial effects of exercise on cognition appear to occur across all ages and are likely mediated by several physiological adaptations. PA can improve cerebral perfusion and oxygenation through reductions in hypertension and increased nitric oxide availability, ultimately enhancing brain plasticity and reducing neuroinflammation (Huuha et al., 2022). Consistently, individuals engaging more frequently in moderate-to-vigorous exercise exhibit larger brain volumes compared with sedentary individuals (Casaletto et al., 2020; Raji et al., 2024). Exercise has also been associated with improvements in endothelial function, lipid metabolism, and reductions in obesity and pro-inflammatory activity, mechanisms that may collectively contribute to the approximately 45% reduction in AD risk reported by Hamer and Chida (2009) (Hamer & Chida, 2009). Several molecular mechanisms have been proposed to explain the neuroprotective effects of PA, including the stimulation of neurotrophic and growth factors involved in neuronal survival and synaptic plasticity. In particular, exercise has been associated with increased levels of brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), all of which support neurogenesis and synaptic remodeling (Silva et al., 2019). Experimental evidence further suggests that irisin, a myokine released during exercise, may exert additional neuroprotective effects (De Freitas et al., 2020). Moreover, PA has been linked to reduced free radical accumulation in the hippocampus and increased levels of antioxidant enzymes, including superoxide dismutase and endothelial nitric oxide synthase (eNOS) (Mendiola-Precoma et al., 2016). Studies conducted in healthy older adults have shown that regular exercise is associated with increased hippocampal volume and elevated plasma BDNF concentrations, while in patients with AD, PA positively correlates with BDNF levels (Paillard et al., 2015). Since BDNF plays a crucial role in neuronal and synaptic development and survival, these findings further support the neuroprotective potential of physical exercise (Silva et al., 2019).

1.7 Animal Models in Alzheimer's Disease Research

Experimental animal models have become indispensable tools for investigating the molecular mechanisms underlying AD and for evaluating potential therapeutic strategies. Among the available experimental systems, mouse models are the most widely used because they allow the reproduction of several pathological and behavioral features of the disease under controlled conditions (Zhong et al., 2024). Although some species, including non-human primates and canines, may naturally develop age-related cognitive decline and neuropathological features that resemble those observed in AD, no animal species fully recapitulates the human disease spontaneously. Consequently, a wide range of vertebrate and invertebrate models has been generated to reproduce specific aspects of AD pathology and to investigate the mechanisms responsible for disease onset and progression (Z.-Y. Chen et al., 2022). The primary objective of AD animal models is to reproduce the major neuropathological hallmarks of the disease, including Aβ plaque deposition, NFTs, synaptic dysfunction, neurodegeneration, and cognitive impairment (Z.-Y. Chen et al., 2022). Many of these models have been generated by introducing AD-associated mutations involving the APP, PSEN1, PSEN2, or MAPT genes, thereby reproducing pathological features that closely resemble those observed in FAD (Z.-Y. Chen et al., 2022; Epis et al., 2010; Zhong et al., 2024). However, the development of representative models of SAD, which accounts for the vast majority of AD cases, remains considerably more challenging. Unlike FAD, SAD is not caused by a single genetic mutation but results from the complex interaction of multiple genetic, environmental, and lifestyle-related factors (Epis et al., 2010). Consequently, a substantial gap still exists between the pathological processes occurring in human SAD and those reproduced in currently available animal models. Ideally, an experimental model should reproduce not only the characteristic neuropathological features and behavioral deficits of AD, but also the temporal sequence of pathological events observed in humans (Duyckaerts et al., 2008). According to the neuroinflammatory hypothesis of AD, this progression should include the early development of chronic neuroinflammation, preceding Aβ deposition and tau hyperphosphorylation. Nevertheless, most currently available models exhibit a relatively rapid onset of pathology and cognitive impairment, primarily reflecting intermediate or advanced stages of the disease rather than the prolonged preclinical phase that characterizes human AD (Z.-Y. Chen et al., 2022). Since patients typically experience a gradual progression from subjective cognitive decline (SCD) to MCI before the onset of dementia, the development of experimental models capable of reproducing these early stages remains a major challenge (Z.-Y. Chen et al., 2022). Animal models

remain essential for investigating the mechanisms underlying AD and for the preclinical assessment of potential therapeutic strategies. However, as simplified experimental systems, they cannot fully reproduce the complexity of the human disease, and their findings should therefore be interpreted together with evidence from human studies (Ameen-Ali et al., 2017).

1.7.1 Transgenic Mouse Models of Alzheimer's Disease

A substantial part of AD research has relied on transgenic mouse models generated through the introduction or overexpression of human mutations associated with FAD, mainly involving the APP, PSEN1/PSEN2, and MAPT genes (Götz et al., 2018; Holcomb et al., 1998; Zhong et al., 2024). These models were developed to reproduce specific pathological components of AD, including Aβ deposition, tau-related alterations, neuroinflammation, synaptic dysfunction, and cognitive impairment, although no single model is able to fully recapitulate the complexity of the human disease (Ameen-Ali et al., 2017; Zhong et al., 2024). Depending on the genetic alterations introduced, transgenic models can be broadly classified into single-transgenic, double-transgenic, and triple-transgenic models, each reflecting different aspects and stages of AD-like pathology (Eriksen & Janus, 2007; Holcomb et al., 1998; Oddo et al., 2003). Among the earliest single-transgenic models, the PDAPP mouse represented an important milestone, as it was one of the first models to develop robust amyloid plaque deposition following the expression of human APP carrying the V717F mutation (Games et al., 1995). In this model, amyloid deposition is associated with dystrophic neurites, astrocytic and microglial activation around plaques, and reduced synaptic density (Games et al., 1995; Masliah et al., 2001). However, PDAPP mice do not develop NFTs or marked neuronal loss, highlighting one of the main limitations of APP-based models (Ameen-Ali et al., 2017). To reproduce a broader spectrum of pathological features, double-transgenic models have been generated by combining mutations involved in different mechanisms of AD pathology. APP/PSEN1 mice, obtained by crossing APP-overexpressing mice with animals carrying PSEN1 mutations, show an accelerated amyloid phenotype compared with single APP models, with earlier accumulation of insoluble Aβ and increased amyloid burden (Holcomb et al., 1998). Other double-transgenic models, such as TAPP mice, combine APP pathology with mutant MAPT expression, allowing the investigation of the interaction between amyloid accumulation and tau pathology (Lewis et al., 2001). In these models, amyloid pathology appears to exacerbate tau aggregation and NFT formation,

suggesting a functional link between these two major pathological pathways (Lewis et al., 2001). Triple-transgenic models were developed to reproduce both amyloid and tau pathology within the same animal. The 3xTg-AD model combines APP, PSEN1, and MAPT mutations and develops both Aβ plaques and NFTs, together with progressive synaptic alterations (Oddo et al., 2003). However, although this model represents a more complete approximation of AD pathology than single- or double-transgenic models, Aβ and tau abnormalities appear to develop with partially independent temporal patterns, and therefore do not fully reproduce the sequence of pathological events observed in human AD (Ameen-Ali et al., 2017; Oddo et al., 2003). More recently, knock-in (KI), knock-out (KO), and CRISPR gene-editing technologies models have been developed to improve the representation of both familial and sporadic AD-related genetic risk factors while reducing the artefacts associated with excessive overexpression of human proteins in the mouse brain (Zhong et al., 2024). Overall, transgenic models have provided essential insights into AD mechanisms and remain valuable tools for preclinical research. Their inability to fully reproduce the multifactorial nature of human AD, largely because they model selected aspects of FAD-related pathology and often exhibit limited neuronal loss, has supported the development of complementary approaches such as ICV Aβ administration (Ameen-Ali et al., 2017).

1.7.2 Intracerebroventricular Amyloid-β Injection Model

Aβ is widely recognized as a central pathological factor in both familial and SAD. Progressive accumulation of Aβ oligomers and extracellular plaques is a hallmark of the AD brain and has been associated with synaptic dysfunction, impairment of long-term potentiation, and the development of cognitive deficits (H. Y. Kim et al., 2016). Experimental and clinical evidence suggests that acute elevations of Aβ levels within the brain are sufficient to induce several pathological and behavioral features resembling those observed in AD patients (H. Y. Kim et al., 2016). Unlike transgenic mouse models, which develop AD-like pathology as a consequence of disease-associated genetic mutations, wild-type (WT) mice do not spontaneously exhibit progressive neurodegeneration or cognitive impairment. This characteristic makes WT animals particularly suitable for investigating the contribution of environmental and lifestyle-related risk factors, such as diet, social isolation, and physical inactivity, to the development of AD-like pathology (Kelliny et al., 2021). Because WT mice do not carry mutations that inevitably lead to neurodegeneration, they provide a more appropriate

experimental background for modelling the gradual development of sporadic AD and for evaluating the effects of modifiable risk factors before the induction of pathology (Kelliny et al., 2021; H. Y. Kim et al., 2016). To overcome the limitations of transgenic models, non-transgenic approaches based on the direct administration of Aβ into the brain have been developed. Among these, intracerebroventricular (ICV) injection of Aβ peptides into WT mice represents a rapid and reproducible approach for inducing AD-like alterations (Kelliny et al., 2021; H. Y. Kim et al., 2016). This strategy makes it possible to investigate the interaction between pre-existing environmental risk factors and Aβ-induced neurotoxicity while avoiding the confounding effects associated with transgene expression. One of the main advantages of this model is the possibility of controlling the concentration of Aβ administered, the timing of pathology onset, and the simultaneous induction of disease-related phenotypes in a large number of animals (H. Y. Kim et al., 2016). Following ICV Aβ administration, animals develop several neuropathological and behavioral abnormalities that resemble key features of AD. Studies have reported significant impairments in learning and memory, as demonstrated by behavioral paradigms such as the Y-maze, Morris water maze, object recognition, passive avoidance, and fear-conditioning tests (H. Y. Kim et al., 2016). In addition to cognitive deficits, Aβ injection induces a series of neurotoxic events, including neuronal loss, disruption of calcium homeostasis, increased neuronal apoptosis, and excessive production of ROS (Perl, 2010). Evidence from rat models further indicates that Aβ administration can reproduce important pathological hallmarks of AD, including intracellular Aβ accumulation, NFT formation, structural neuronal alterations, and spatial memory impairment (Xiaoguang et al., 2018). Since Aβ burden and NFT aggregation are considered the principal histopathological characteristics of AD, their presence in experimental models strengthens their relevance for investigating disease mechanisms. These pathological changes disrupt neuronal architecture and signaling pathways, ultimately leading to neuronal dysfunction and cognitive decline similar to those observed in AD patients (Xiaoguang et al., 2018). Nevertheless, some variability has been reported among Aβ-based models. For example, a single ICV injection of Aβ may consistently induce memory deficits, neuronal loss, and gliosis, while the extent of Aβ deposition and NFT formation can vary between studies (Xiaoguang et al., 2018). Despite these limitations, Aβ-injected animal models remain valuable experimental tools for investigating AD-related neurodegeneration and for evaluating potential therapeutic strategies targeting disease pathology (H. Y. Kim et al., 2016; Xiaoguang et al., 2018).

1.7.3 Behavioral Tests for Cognitive Dysfunction in AD Mouse Models

The hippocampus is one of the earliest brain regions affected in AD, and impairments in declarative and working memory are among the first symptoms reported by patients (Nestor et al., 2006). Consequently, hippocampal-dependent behavioral tasks are extensively used to investigate cognitive deficits in AD mouse models (Ameen-Ali et al., 2017). Commonly employed paradigms include the Morris water maze, radial arm maze (RAM), T-maze and Y-maze alternation tasks, , the Novel Object Recognition (NOR) test (Antunes & Biala, 2012) and the open field test (OFT) (Pentkowski et al., 2021). The Morris water maze evaluates hippocampal-dependent spatial memory by measuring the ability of rodents to locate a hidden platform using spatial cues (R. Morris, 1984). Spatial working memory is frequently assessed using the RAM, in which animals must remember previously visited baited arms (Olton & Samuelson, 1976). T-maze and Y-maze paradigms rely on rodents' natural exploratory behavior and tendency to enter less recently visited arms, allowing the assessment of spatial alternation with minimal training requirements (Rawlins & Olton, 1982). The NOR test assesses recognition memory by exploiting the natural tendency of rodents to preferentially explore a novel object over a familiar one. Unlike many other behavioral paradigms, the NOR test does not require external motivation, reward, or punishment, requires only minimal habituation and training, and can be completed within a relatively short period, making it one of the most widely used tests for evaluating recognition memory in rodent models of neurodegenerative diseases (Antunes & Biala, 2012). The OFT is a widely used behavioral paradigm for assessing anxiety-like behavior and has frequently been applied in transgenic rodent models of AD. The apparatus typically consists of either a circular or square arena, whose floor is divided into sectors by concentric circles or square grids, while high surrounding walls prevent the animals from escaping. Although the OFT can be used to evaluate anxiety-related responses, it is more commonly employed to assess locomotor activity and patterns of spatial exploration (Pentkowski et al., 2021). Since these behavioral paradigms are believed to rely on neural mechanisms comparable to those involved in human cognition, they provide valuable tools for assessing cognitive dysfunction in neurodegenerative disease models.

CHAPTER 2: AIM OF THE WORK

The work described in this thesis was carried out at the Department of Neuroscience, Istituto Superiore di Sanità (ISS), Rome, Italy. The present study aims to investigate the early neurodegenerative mechanisms associated with SAD through the development of a multifactorial experimental animal model based on the combined exposure to modifiable lifestyle-related risk factors. Unlike familial forms of AD, which are linked to rare genetic mutations, SAD arises from the complex interaction between aging, environmental influences, metabolic alterations, and lifestyle-associated factors. In this context, the project focuses on reproducing experimental conditions that more closely resemble the multifactorial nature of human SAD by combining different chronic risk factors, including hypercaloric diet exposure, physical inactivity, and social isolation. To investigate the contribution of these factors, WT mice were exposed to two different environmental conditions: a protective-factor (PF) condition, consisting of a standard diet, group housing, and voluntary physical exercise, and a risk-factor (RF) condition, characterized by a Western diet, social isolation, and physical inactivity. To further investigate their interaction with an AD-related insult, animals within each environmental condition received an ICV injection of either $A\beta_{1-42}$ or vehicle. This experimental design allowed us to assess the contribution of prolonged lifestyle-related risk exposure and its interaction with Aβ administration. Behavioral performance was evaluated through the OFT and NOR test, followed by molecular and histological analyses of the hippocampus and cerebral cortex. Molecular analyses included the evaluation of neuroinflammation through Real-Time PCR analysis of pro-inflammatory cytokines, oxidative stress through Western blot analysis of antioxidant enzymes, and mitochondrial function through Western blot analysis of OXPHOS proteins. Histological analyses were performed to assess glial activation through Iba1 and GFAP immunohistochemistry and to evaluate Aβ deposition. Particular attention was devoted to neuroinflammation, oxidative stress, mitochondrial function, and other markers associated with AD-related pathology, as these processes are widely recognized as key contributors to the early pathophysiological changes associated with AD. Overall, the study aimed to determine whether the combined exposure to modifiable lifestyle-related risk factors could promote early cognitive and neurobiological alterations relevant to the initial stages of SAD.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental Design

To investigate the effects of the interaction among multiple risk factors associated with dementia and to identify potential protective mechanisms against neurodegeneration, a mouse model was employed. Mice were exposed to a combination of environmental and behavioral risk factors commonly associated with sporadic dementia, including a hypercaloric diet rich in fat, refined carbohydrates, and salt (Western Diet, WD), social isolation, and physical inactivity. These experimental conditions were designed to induce a phenotype resembling the main features of sporadic human dementia. The impact of these risk factors was assessed through a multidimensional approach that included behavioral, histological, molecular, metabolic, and morphological analyses. In addition, to further investigate the interaction between environmental risk factors and neurodegenerative mechanisms associated with AD, a subset of animals received an ICV injection of Aβ42, generating an AD-like neurodegenerative model.

3.2 Animal Model and Experimental Groups

Six-month-old male C57BL/6 WT mice were used in this study. Animals were maintained for a total period of nine months on either a standard diet (Normal Diet, ND) or a hypercaloric diet enriched in fat, refined sugars, and salt (Western Diet, WD). Two experimental cohorts were established, each consisting of at least 10 animals (*Figure 1*):

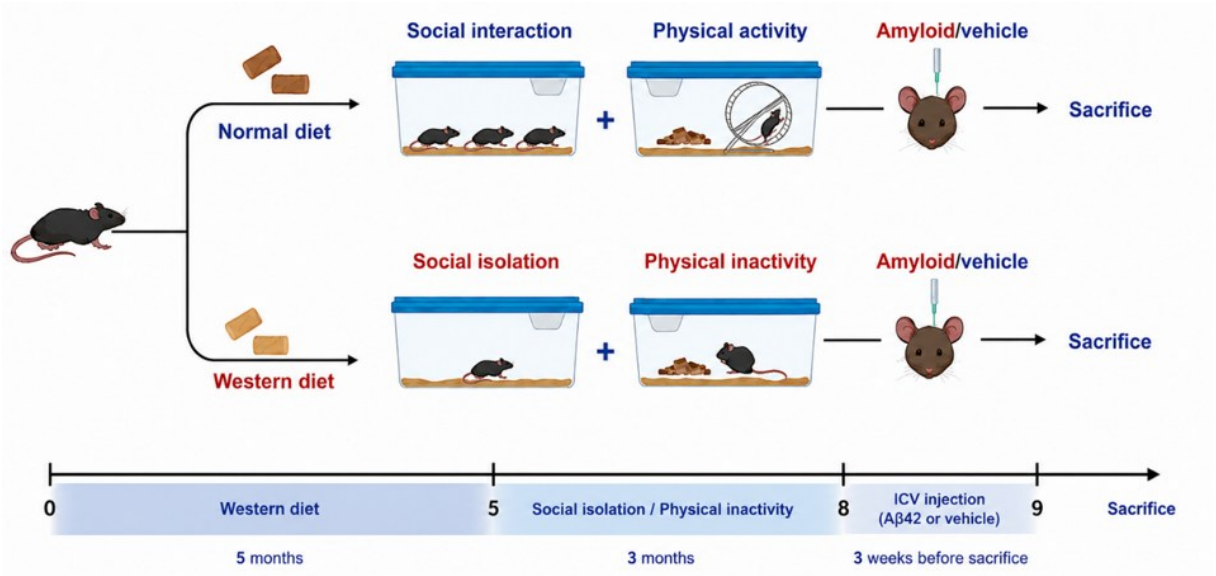

Figure 1. Experimental design of MRI/MRS studies in animal models. WD + Social Isolation + Standard Cage Activity. ND + Physical Exercise + Group Housing

Social isolation and physical exercise were introduced after five months of dietary exposure and maintained for an additional three months. Voluntary PA was provided through running wheels equipped with activity counters and available 24 h per day within the home cages. Three weeks before sacrifice, animals from each experimental group were further divided into two subgroups and subjected to ICV injection of either Aβ42 peptide or saline solution (sham control). Amyloid-β peptide (Aβ1–42; Abcam, AB120301; molecular weight = 4514 Da) was prepared before ICV administration according to a standardized protocol, as outlined below:

1. Dissolve 1 mg of lyophilized Aβ1–42 peptide in 1 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a final concentration of 1 mg/mL. HFIP was used to disrupt pre-existing aggregates and stabilize the peptide in its monomeric form.

2. Calculate the molar concentration according to the following equation:

$$[C] = \frac{\text{gr}}{\text{PM} \times \text{L}}$$

$$\frac{1 \times 10^{-3}}{4514 \times 10^{-3}} = 2.21 \times 10^{-4}\ \text{M} = 0.221\ \text{mM} = 221\ \mu\text{M}$$

3. Aliquot the solution into ten Eppendorf tubes (100 µL each).

4. Evaporate the HFIP completely and store the dried aliquots at −80°C until use.

5. Immediately before administration, resuspend one aliquot containing 0.1 mg of Aβ1–42 in 5.54 µL DMSO to obtain a 4 mM stock solution.
6. Dilute the stock solution 1:10 by mixing 5.54 µL of stock solution with 49.86 µL of sterile PBS, obtaining a final concentration of 0.4 mM (400 pmol/µL).
7. Incubate the diluted solution at 37°C, under continuous agitation (400 rpm) for 72 h to promote the formation of Aβ oligomers.
8. Immediately before injection, mix 1 µL of the 0.4 mM Aβ1–42 solution with 4 µL of sterile PBS to obtain a final injection volume of 5 µL, containing 400 pmol of Aβ1–42.
9. Prepare the sham solution by diluting 1 µL DMSO in 49 µL of sterile PBS (1:50 dilution).
10. Anesthetize the animals and perform the intracerebroventricular injection using a stereotaxic apparatus according to the following coordinates relative to bregma:
    - AP: −0.58 mm
    - ML: +1.0 mm
    - DV: −2.2 mm
11. Monitor the animals until complete recovery from anesthesia and return them to their home cages until the end of the experimental protocol.

For molecular and biochemical analyses, whole-blood samples were collected at three experimental time points:

- T0: before exposure to the risk factors;
- T1: after five months of dietary exposure;
- T2: at the end of the experimental period, including Aβ42 ICV administration.

At the end of the study, animals were sacrificed and the cortex and hippocampus were dissected for subsequent histological, molecular, and biochemical analyses. To accommodate the different experimental procedures, including molecular biology analyses, magnetic resonance spectroscopy, and behavioral testing, each cohort was further divided into subgroups exposed to the experimental conditions at different time points.

3.3 Behavioral Evaluation

Open Field Test

The OFT was used to evaluate spontaneous locomotor activity and exploratory behavior. Animals were individually placed in a standard open-field arena and their behavior was recorded for a predetermined period of time. Parameters analyzed included the total distance traveled and the time spent in the central area of the arena, which are indicative of locomotor activity and alterations in exploratory or emotional behavior, respectively.

Novel Object Recognition Test

The NOR test was used to assess recognition memory. The experimental protocol consisted of a familiarization phase with two identical objects, followed by a testing phase in which one familiar object was replaced with a novel object. Exploration times were recorded and used to evaluate the animal's ability to discriminate between familiar and novel objects, serving as an indicator of cognitive performance.

Both behavioral tests were performed in all experimental groups to allow systematic comparisons among the different experimental conditions.

3.4 Analysis of Inflammatory Cytokines

RNA Extraction from Brain Tissue

RNA isolation was carried out using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol, as outlined below:

1. Disrupt and homogenize ≤100 mg fatty tissue (≤50 mg other tissue) in 1 ml QIAzol Lysis Reagent using the TissueRuptor®, TissueLyser LT or TissueLyser II.
2. Incubate the homogenate at room temperature for 5 min.
3. Add 200 µl chloroform, and shake vigorously for 15 s.
4. Incubate sample at room temperature for 2–3 min.
5. Centrifuge at 12,000 x g for 15 min at 4°C.

6. Transfer upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 1 volume of 70% ethanol, and vortex. Do not centrifuge. Proceed at once to step 7.

7. Transfer up to 700 µl of the sample to RNeasy Mini spin column in 2 ml collection tube (supplied). Close the lid, centrifuge at room temperature for 15 s at ≥8000 x g and discard flow-through.

8. Using the same collection tube, repeat step 7 using the remainder of the sample. Discard the flow-through.

   Optional DNase digest: Follow steps in "Optional On-Column DNase Digestion with the RNase-Free DNase Set" in Appendix C of the RNeasy Lipid Tissue Mini Handbook.

9. Add 700 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g and discard flow-through. (Skip this step if performing optional DNase digestion.)

10. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g and discard flow-through.

11. Add 500 µl Buffer RPE to RNeasy column. Close lid and centrifuge for 2 min at ≥8000 x g.

    Optional: To further dry membrane, place RNeasy column in new 2 ml tube, close lid and centrifuge at full speed for 1 min.

12. Place RNeasy column in a new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid and centrifuge for 1 min at ≥8000 x g.

Quantization

Following RNA extraction, total RNA concentration was quantified using a NanoDrop spectrophotometer. This instrument is based on the principle of spectrophotometry and measures the absorbance of a 1 µL sample by directing a beam of light through it.

Sample concentration is subsequently calculated according to the Beer–Lambert law:

$$c = A / (\varepsilon \times b)$$

where c is the concentration (mol/L), A is the absorbance, ε is the molar extinction coefficient (which depends on the wavelength), and b is the optical path length (cm).

Nucleic acid absorbance is measured at a wavelength of 260 nm. The purity of RNA samples was evaluated using the A260/A280 and A260/A230 absorbance ratios, which are considered acceptable when ranging between 1.8 and 2.2.

cDNA Synthesis

Following RNA extraction and quantification, complementary DNA (cDNA) was synthesized from total RNA using the SensiFAST™ cDNA Synthesis Kit (Bioline), according to the manufacturer's instructions. Reverse transcription was performed to generate cDNA suitable for subsequent quantitative real-time PCR (qRT-PCR) analysis (*Figure 2*).

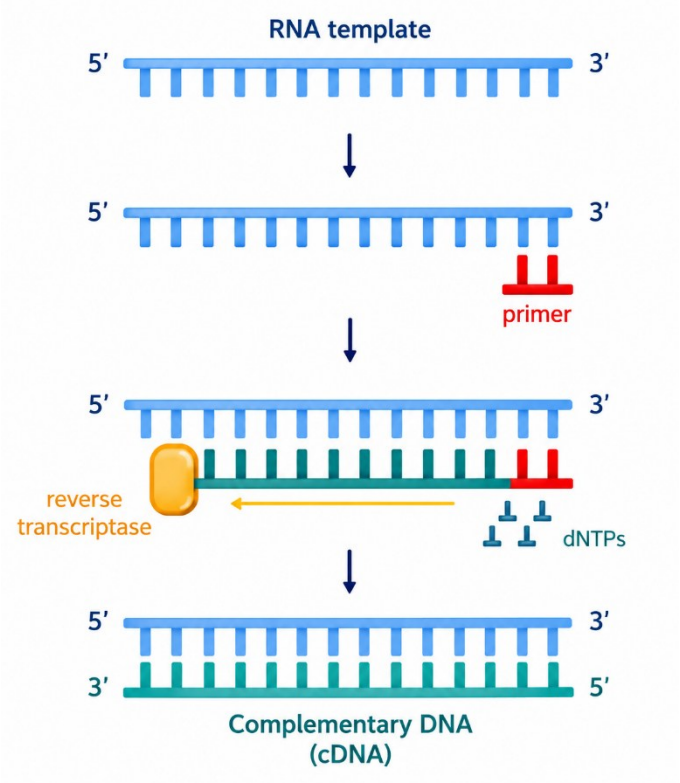

Figure 2. Reverse transcribe from RNA to cDNA.

The reverse transcription protocol consisted of the following steps:

1. Prepare the master mix on ice.
2. Vortex solutions and centrifuge briefly before use.

Total RNA or mRNA (up to 1000 ng/µl)	n µL
5x TransAmp Buffer	4 µL
Reverse Transcriptase	1 µL
DNase/RNase free-water*	Up to 20 µL

* Not supplied

3. Mix gently by pipetting.
4. Set up the following program in a thermal cycler:
    - 25 °C for 10 min (primer annealing)
    - 42 °C for 15 min (reverse transcription)
    - Optional Step: 48 °C for 15 min (for highly-structured RNA)
    - 85 °C for 5 min (inactivation)
    - 4 °C hold (or chill on ice)
5. Use up to 4 µL (1/5th volume) of cDNA synthesis reaction product in a 20 µL volume qPCR. If desired, reaction product can be diluted in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA prior to use.
6. Alternatively, store reaction product or diluted cDNA at 4 °C for 1 week or -20 °C for long term storage.

Quantitative Real-Time PCR (qRT-PCR)

Gene expression levels of IL-6, IL-1β, TNF-α, and TGF-β transcripts were quantified by qRT-PCR using the SensiFAST SYBR kit and a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). All reactions were performed in duplicate, and HPRT was used as the endogenous reference gene.

The qRT-PCR protocol consisted of the following steps:

1. A reaction master mix was prepared for each target gene containing:

Reaction Master Mix (per reaction)	n µL
SensiFAST SYBR Master Mix	5 µL

Specific primer*	0.5 µL
Nuclease-free water	3.5 µL

*Primers specific for IL-6, IL-1β, TNF-α, TGF-β, or HPRT.

2. A volume of 9 µL of the prepared master mix was dispensed into each well of the PCR plate.
3. Subsequently, 1 µL of cDNA sample was added to each well, resulting in a final reaction volume of 10 µL.
4. All samples were analyzed in duplicate.
5. Real-time PCR amplification was performed using the following cycling conditions: an initial hold stage consisting of 50°C for 2 min followed by 95°C for 10 min, and a subsequent amplification stage of 40 cycles comprising denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.
6. Amplification data were collected and analyzed using GraphPad Prism software.

Polymerase chain reaction (PCR) is a molecular technique used to amplify a specific DNA fragment whose flanking sequences are known. Specific primers are designed to anneal to these target regions, enabling selective amplification of the sequence of interest.

Each PCR cycle consists of three main steps (*Figure 3*):

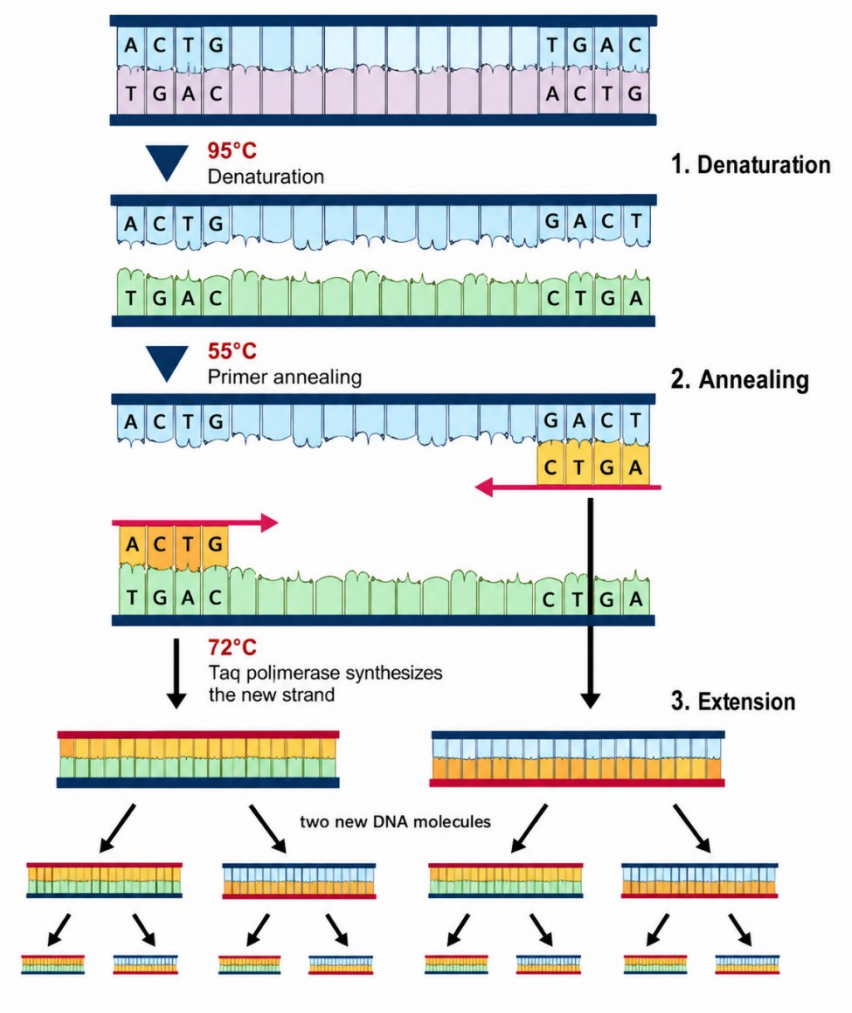

Figure 3. Schematic representation of the PCR amplification process.

- Denaturation: during which double-stranded DNA is separated into single strands by exposure to high temperatures.
- Annealing: in which primers bind to their complementary target sequences at a temperature that depends on the characteristics of the oligonucleotides used. In this study, Locked Nucleic Acid (LNA) primers were employed. These modified oligonucleotides contain chemically constrained nucleotides that enhance duplex stability, allowing the use of shorter primers with increased specificity.
- Extension: during which DNA polymerase synthesizes the cDNA strand using the target sequence as a template.

qRT-PCR not only amplifies DNA but also provides information about the amount of amplified product through the use of a fluorescent reporter molecule. In the present study, SYBR Green was used as the fluorescent dye. SYBR Green is a DNA-intercalating agent that does not interfere with DNA replication or enzymatic activity. When free in solution, it exhibits minimal fluorescence; however, upon binding to double-stranded DNA, its fluorescence increases markedly. As PCR amplification proceeds, the accumulation of fluorescence parallels the accumulation of PCR products and is therefore proportional to the amount of amplified DNA. During each cycle, fluorescence decreases during the denaturation step, when DNA strands separate, and increases again during extension as new double-stranded DNA molecules are synthesized (*Figure 4*).

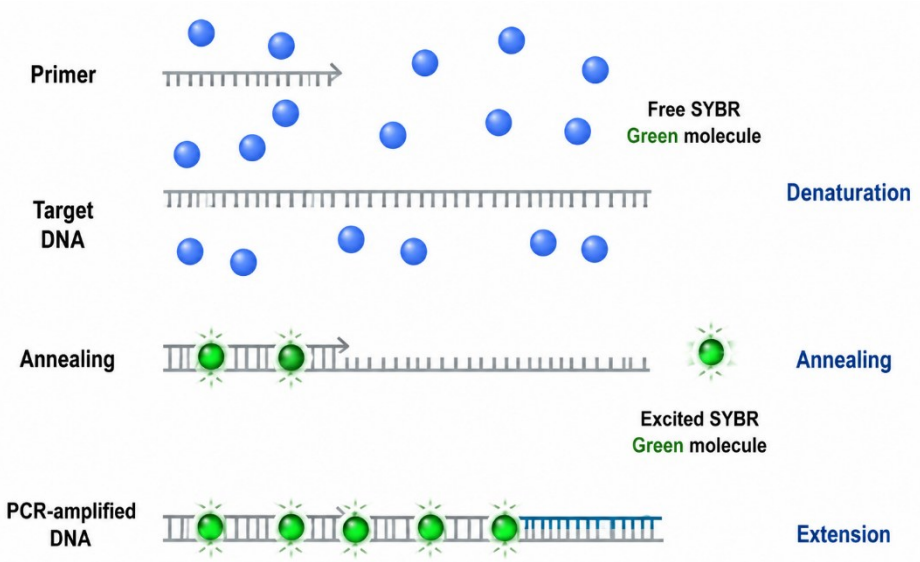

Figure 4. SYBR Green binding during the three phases of PCR.

Data Analysis and Quantification

qRT-PCR generates an amplification curve that relates fluorescence intensity to the number of PCR cycles. Three main phases can be distinguished (*Figure 5*):

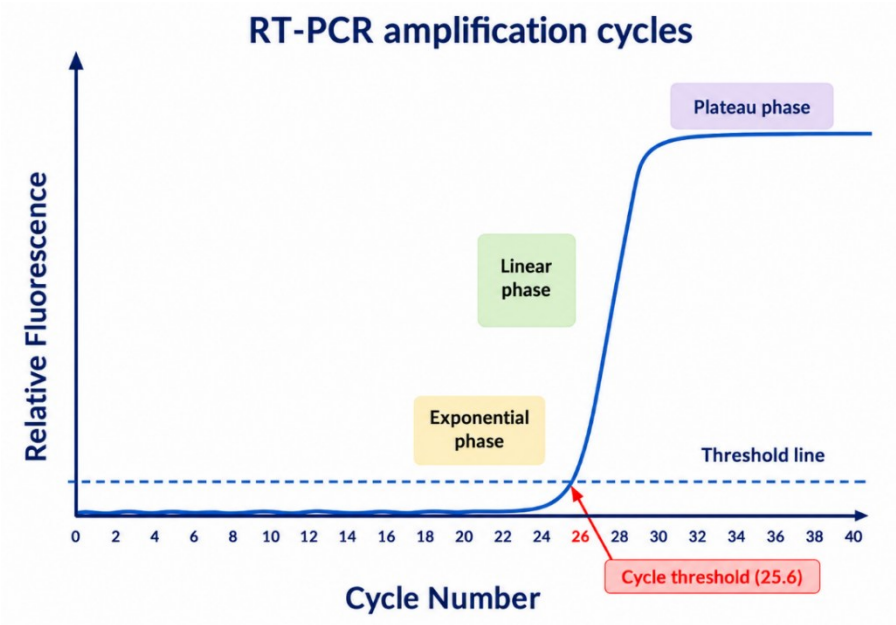

Figure 5. Representative qRT-PCR amplification curve showing the baseline, exponential, and plateau phases

The baseline phase, in which the amount of amplified product is too low to generate a detectable fluorescent signal.

The exponential phase, during which amplification proceeds at maximum efficiency and the amount of product increases exponentially with each cycle. This phase can be described by the equation:

$$Cn = Ci\,(1 + E)^n$$

where C_n is the number of copies at cycle n, C_i is the initial number of copies, E is the amplification efficiency, and n is the number of cycles.

Since amplification efficiency is often close to its theoretical maximum ($E = 1$), the equation can be approximated as:

$$Cn = Ci\,2^n$$

The plateau phase, during which amplification slows and eventually stops because reaction components become limiting.

A fluorescence threshold is established at the beginning of the exponential phase. The PCR cycle at which the fluorescence signal exceeds this threshold is defined as the cycle threshold (Ct).

Gene expression levels were quantified by performing qRT-PCR using both the target-specific assay and the endogenous reference gene assay. Because each target reaches the fluorescence threshold at a different cycle number, relative expression levels were calculated using the following equation:

$$2^{-\Delta Ct}$$

where $\Delta Ct = Ct(target) - Ct(reference)$.

3.5 Immunohistochemistry

To evaluate neuroinflammation, glial activation, and amyloid deposition, histological and immunohistochemical analyses were performed on brain tissue samples, according to the protocol outlined below.

Tissue Processing

1. Collect brains and fix them in 4% buffered formaldehyde.
2. Section the fixed brains into 2-mm-thick coronal slices.
3. Wash tissue slices in running water.
4. Dehydrate samples through graded alcohols.
5. Clear the tissue in xylene.
6. Embed the tissue in paraffin wax.
7. Cut 5-µm-thick sections using a microtome and mount them onto glass slides.

Histological Analysis

1. Deparaffinize the tissue sections.
2. Rehydrate the sections through graded alcohols.
3. Stain sections with hematoxylin and eosin (H&E).

4. Examine tissue morphology to identify possible anatomical alterations, including neuronal loss.

Immunohistochemical Analysis

1. Mount 5-µm-thick sections onto Super Frost Plus slides were processed according to the avidin–biotin complex (ABC) method.
2. Deparaffinize and rehydrate the tissue sections.
3. Perform antigen retrieval by microwave treatment in citrate buffer.
4. Block endogenous peroxidase activity by incubating the sections in 3% hydrogen peroxide in methanol.
5. Rinse the sections with phosphate-buffered saline (PBS).
6. Block non-specific binding by incubating the sections for 1 h in PBS containing 3% normal goat serum.
7. Incubate the sections overnight at 4°C with one of the following primary antibodies:
    - anti-GFAP (rabbit, 1:200 dilution) for astrocytes;
    - anti-IBA1 (rabbit, 1:200 dilution) for microglia;
    - anti-TMEM119 (rabbit, 1:200 dilution) for microglia;
    - anti-amyloid-β (6E10, mouse, 1:200 dilution) for amyloid deposition.
8. Prepare negative controls by omitting the primary antibody.
9. Wash the sections with PBS.
10. Incubate the sections for 1 h at room temperature with the appropriate biotinylated secondary antibodies (1:200 dilution; Vector Laboratories).
11. Incubate the sections with the avidin–biotin–peroxidase complex (Vectastain® ABC Elite Kit, Vector Laboratories) according to the manufacturer's instructions.
12. Visualize immunoreactivity using 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) as the chromogenic substrate.

13. Counterstain the sections with hematoxylin.

14. Examine and acquire images using a Zeiss Axiophot microscope.

3.6 Neurodegeneration Analysis

To evaluate β-secretase activity in the brain, a subgroup of samples was analyzed using the Elabscience β-Secretase 1 (BACE1) Activity Fluorimetric Assay Kit according to the manufacturer's instructions (Elabscience).

BACE1 Activity Assay

BACE1 activity was evaluated in cortical and hippocampal samples using the β-Secretase 1 (BACE1) Activity Fluorometric Assay Kit (Elabscience), according to the manufacturer's protocol, as outlined below:

1. Weigh 20 mg of brain tissue sample.
2. Homogenize the tissue in 180 µL of extraction solution at 4°C.
3. Centrifuge the homogenate at 10,000 × g for 10 min at 4°C to remove insoluble material.
4. Collect the supernatant and keep it on ice for subsequent analysis.
5. Determine the protein concentration of each supernatant in parallel using the Pierce™ BCA Protein Assay (Thermo Fisher Scientific), as described below.
6. No additional dilution of mouse brain homogenates was required before analysis, according to the manufacturer's instructions.
7. Add 10 µL of each standard or sample into the appropriate wells of a black 96-well microplate.
8. Add 120 µL of buffer to the standard wells and 120 µL of working solution to the sample wells.
9. Mix the plate briefly and measure the fluorescence intensity using a fluorescence microplate reader at an excitation wavelength of 325 nm and an emission wavelength of 393 nm. This first reading was recorded as F_1.
10. Incubate the plate at 37°C for 20 min protected from light.

11. Measure the fluorescence intensity again under the same conditions. This second reading was recorded as F_2.
12. Calculate BACE1 activity according to the manufacturer's instructions and express the results relative to protein concentration.

Standard Curve Construction

1. Average the duplicate readings for each standard.
2. Subtract the mean fluorescence value of the blank from all standard readings to obtain the absolute fluorescence value.
3. Plot the standard curve using the absolute fluorescence value of each standard on the y-axis and the corresponding concentration on the x-axis.
4. Generate the standard curve equation (y = ax + b) using graphing software or Excel.

BACE1 activity is defined as the amount of substrate hydrolyzed by 1 g of tissue or cell protein to produce 1 μmol of product in 1 min at 37°C.

BACE1 activity was calculated using the following formula:

$$\text{BACE1 activity (U/gprot)} = (\Delta F - b) \div a \div T \times f \div C_{pr}$$

Where:

ΔF: The change fluorescence value of sample, F2 – F1.

T: The incubation time, 20 min.

f: Dilution factor of sample before tested.

C_{pr}: Concentration of protein in tissue sample, gprot/L.

Amyloid Deposition

The presence of amyloid aggregates was assessed by immunohistochemical staining using the anti-amyloid-β (6E10) antibody described above.

BDNF Gene Expression

BDNF transcript expression levels in cortical and hippocampal samples were evaluated by qRT-PCR using cDNA generated by reverse transcription and specific Qiagen primers, as described above.

3.7 Oxidative Damage and Mitochondrial Dysfunction Analysis

To evaluate oxidative damage and mitochondrial dysfunction associated with exposure to the selected risk factors, a panel of markers involved in oxidative stress pathways and mitochondrial activity was analyzed by Western blot protein expression analysis.

Protein Extraction

1. Total proteins were extracted from cortical and hippocampal tissue samples using radioimmunoprecipitation assay (RIPA) buffer (pH 8.0) supplemented immediately before use with protease inhibitor cocktail (Sigma-Aldrich, P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, P5726).
2. Brain tissue samples were maintained on ice throughout the procedure.
3. Tissue samples were weighed and homogenized in RIPA buffer at a ratio of 10 µL of buffer per milligram of tissue.
4. Homogenization was performed by sonication on ice using three pulses of 10 s at 50% power, with 10-s intervals between pulses to prevent sample overheating.
5. Homogenates were centrifuged at 14,000 rpm for 15–20 min at 4°C.
6. The resulting supernatants containing the soluble protein fraction were collected and stored on ice until protein quantification.

Protein Quantification

1. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.
2. Protein samples and bovine serum albumin (BSA) standards were incubated with the BCA working reagent.

3. Absorbance was measured at 562 nm using a microplate reader.
4. Protein concentrations were calculated from the standard calibration curve.
5. Protein concentrations were used to normalize protein loading for Western blot analysis.

Western Blot Analysis

1. Equal amounts of total protein were mixed with Bolt™ LDS Sample Buffer (4×) and Bolt™ Reducing Agent (10×).
2. Samples were brought to the desired final volume using deionized water.
3. Protein samples were denatured at 70°C for 10 min.
4. Protein samples and molecular weight markers were loaded onto Bolt™ Bis-Tris Plus 4–12% precast gels (Thermo Fisher Scientific).
5. Electrophoresis was performed in 1× MOPS running buffer supplemented with antioxidant at a constant voltage of 200 V for approximately 32 min at 4°C.
6. Following electrophoretic separation, proteins were transferred onto nitrocellulose membranes using a wet-transfer system.
7. Transfer sandwiches were assembled using sponges and filter papers soaked in 1× transfer buffer, ensuring complete removal of air bubbles.
8. Protein transfer was carried out at 10 V for 60 min.
9. Membranes were briefly rinsed with distilled water and stained with Ponceau S solution to verify transfer efficiency and equal protein loading.
10. Membranes were washed with distilled water and equilibrated in Tris-buffered saline (TBS).
11. Non-specific binding sites were blocked by incubation in 5% non-fat dry milk prepared in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature under gentle agitation.
12. Membranes were incubated with the following primary antibodies diluted in 2% milk/TBST for 1 h at room temperature or overnight at 4°C.

Primary antibody	Dilution	Target
Anti-Catalase (H9) (Santa Cruz, sc-271803)	1:1000	Catalase
Anti-SOD1 (Santa Cruz, sc-271014)	1:500	SOD1
Total OXPHOS Cocktail (Abcam, ab110413)	1:3000	Complexes I–V

13. Membranes were washed three times in TBST.
14. Membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature.
15. Membranes were washed twice in TBST followed by one wash in TBS.
16. Chemiluminescent substrate was prepared by mixing Substrate A and Substrate B in a 1:1 ratio.
17. Membranes were incubated with the substrate solution for 5 min at room temperature in the dark.
18. Chemiluminescent signals were acquired using a ChemiDoc Imaging System (Bio-Rad Laboratories).
19. Immunoreactive bands were quantified by densitometric analysis using Image Lab software (Bio-Rad Laboratories).
20. Statistical analyses were performed using GraphPad Prism software.

3.8 Statistical Analysis

Gene expression data were expressed as mean (Δ) ± standard error of the mean (SEM) for each experimental group. Statistical comparisons between two groups were performed using Student's t-test. This analysis allowed estimation of the p-value, representing the probability that observed differences occurred by chance. Statistical significance was set at $p < 0.05$. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) was performed, followed by Bonferroni's post hoc test.

CHAPTER 4: RESULTS

4.1 Experimental Cohorts

At the end of the experimental protocol, all surviving animals included in the study were allocated to the experimental groups summarized in the *table 1* below. The PF groups (protective factor) consisted of mice maintained under a normal diet, physical exercise, and group housing, whereas the RF groups (risk factor) consisted of mice exposed to a WD and social isolation. Within each condition, animals received either ICV injection of PBS (Sham) or $A\beta_{1-42}$ peptide (Aβ).

Experimental group	Total number animals	Aβ ICV	PBS (Sham)
PF	11	6	5
RF	14	7	7

Table 1. Experimental groups and number of animals included in each group at the end of the study.

4.2 Behavioral Evaluation

Voluntary Physical Activity

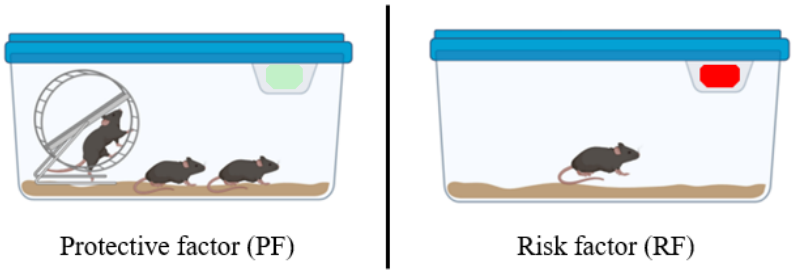

Figure 6. Experimental housing conditions. Group sizes were PF: $n = 11$ (Aβ, $n = 6$; Sham, $n = 5$) and RF: $n = 14$ (Aβ, $n = 7$; Sham, $n = 7$).

Voluntary physical activity (*Figure 6*) was monitored throughout the experimental period in mice maintained under the protective condition, whereas mice assigned to the risk-factor condition did not have access to running wheels. As expected, mice maintained under the PF condition performed a significantly higher number of wheel rotations during the dark phase than during the light phase, consistent with their physiological circadian rhythm. No significant differences were observed in either the number of wheel rotations or the total time spent using the running wheels between the PF-Sham and PF-Aβ groups. Throughout the three-month intervention period, all mice maintained under

the PF condition regularly used the running wheels, indicating good adherence to the voluntary exercise protocol.

Open Field Test

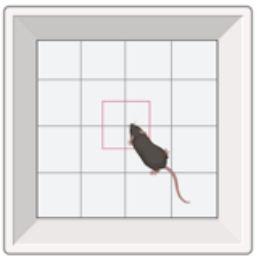

Figure 7. Schematic representation of the OFT. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7).

Locomotor activity was assessed using the OFT (*Figure 7*). No significant differences in spontaneous locomotor activity were observed between mice maintained under the PF condition and those exposed to the RF condition. Similarly, no significant differences were detected in the time spent in the central area of the arena or in overall locomotor performance among the experimental groups. Furthermore, intracerebroventricular Aβ administration did not markedly affect spontaneous locomotor activity or exploration of the central area of the arena under either the PF or RF condition.

Novel Object Recognition Test

Recognition memory was evaluated using the NOR test (*Figure 8*) by comparing the time spent exploring the novel (N) and familiar (F) objects. Under the PF condition, both sham- and Aβ-treated mice spent more time exploring the novel object than the familiar one, indicating preserved recognition memory. In contrast, mice exposed to the RF condition showed no clear preference for the novel object, regardless of ICV Aβ administration, suggesting impaired object recognition and reduced discrimination between familiar and novel objects. Overall, exposure to the combined environmental risk factors was associated with a marked reduction in novel object preference compared with the protective condition, indicating impaired recognition memory.

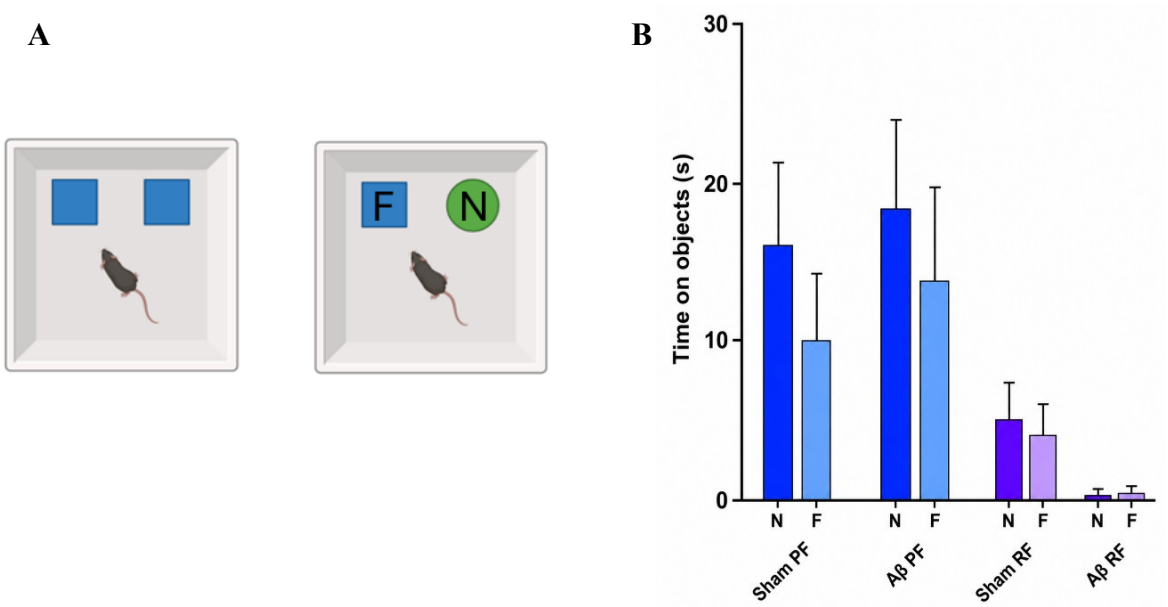

Figure 8. NOR test performed at the end of the experimental protocol (T2). (**A**) Schematic representation of the NOR test. (**B**) The graph shows the time spent exploring the N and F objects by mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. Data are expressed as time spent exploring each object (s) and presented as mean ± SEM. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7).

4.3 Analysis of Inflammatory Cytokines

The expression of pro-inflammatory cytokines was evaluated in cortical and hippocampal tissue collected at the end of the experimental protocol (T2). Transcript levels were quantified by qRT- PCR to assess the effects of the experimental conditions on neuroinflammatory responses. Cytokine mRNA expression was compared between mice maintained under the PF condition and those exposed to the RF condition, in both sham-treated and ICV Aβ-treated animals. In the cerebral cortex (*Figure 9*), IL-6 transcript expression tended to be higher in RF-Sham mice than in PF-Sham mice (0.4673 vs. 0.1089; $P = 0.0639$), although the difference did not reach statistical significance. Furthermore, IL-1β transcript expression was significantly increased in RF-Sham mice compared with PF-Sham mice (0.2875 vs. 0.0842; $P = 0.04$). A significant increase was also observed in RF-Aβ mice compared with PF-Sham mice (0.3504 vs. 0.0842; $P = 0.04$). Overall, cortical IL-6 and IL-1β transcripts expression was approximately fourfold higher in mice exposed to the RF condition than in mice maintained under the PF condition. In addition, IL-1β transcript expression remained significantly elevated in mice receiving ICV Aβ under the RF condition. Consistent with the findings observed in the cortex, the hippocampus (*Figure 10*) also exhibited a marked increase in pro-

inflammatory cytokine expression following exposure to the RF condition. Specifically, IL-6 transcript expression was significantly increased in RF-Sham mice compared with PF-Sham mice (0.1679 vs. 0.03754; $P = 0.01$). A significant increase was also observed in RF-Aβ mice compared with PF-Sham mice (0.1541 vs. 0.03754; $P = 0.02$). Similarly, IL-1β transcript expression was significantly higher in RF-Sham mice than in PF-Sham mice (0.3160 vs. 0.1070; $P = 0.0038$).

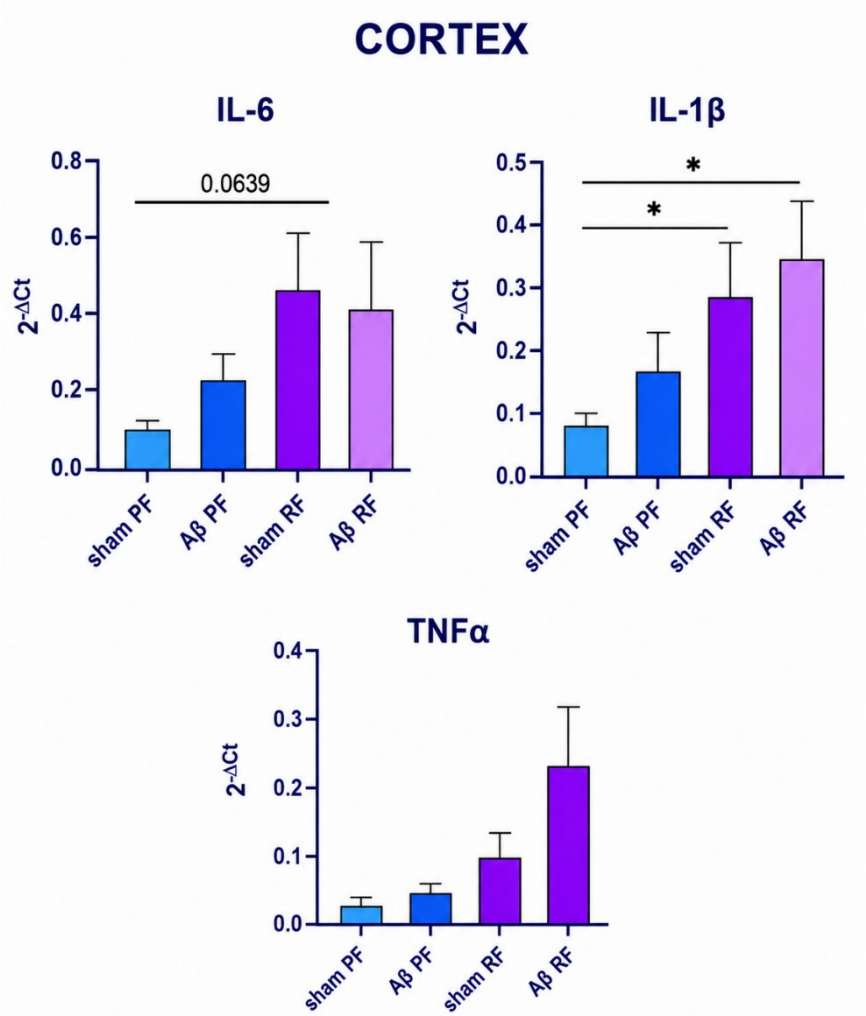

Figure 9. Expression of IL-6, IL-1β, and TNFα transcripts in the cerebral cortex of mice. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7). Cytokine expression is reported as $2^{-\Delta Ct}$ and presented as mean ± SEM. Statistical significance is indicated by (*): $p \leq 0{,}05$; (**): $p \leq 0{,}01$; (***): $p \leq 0{,}0001$.

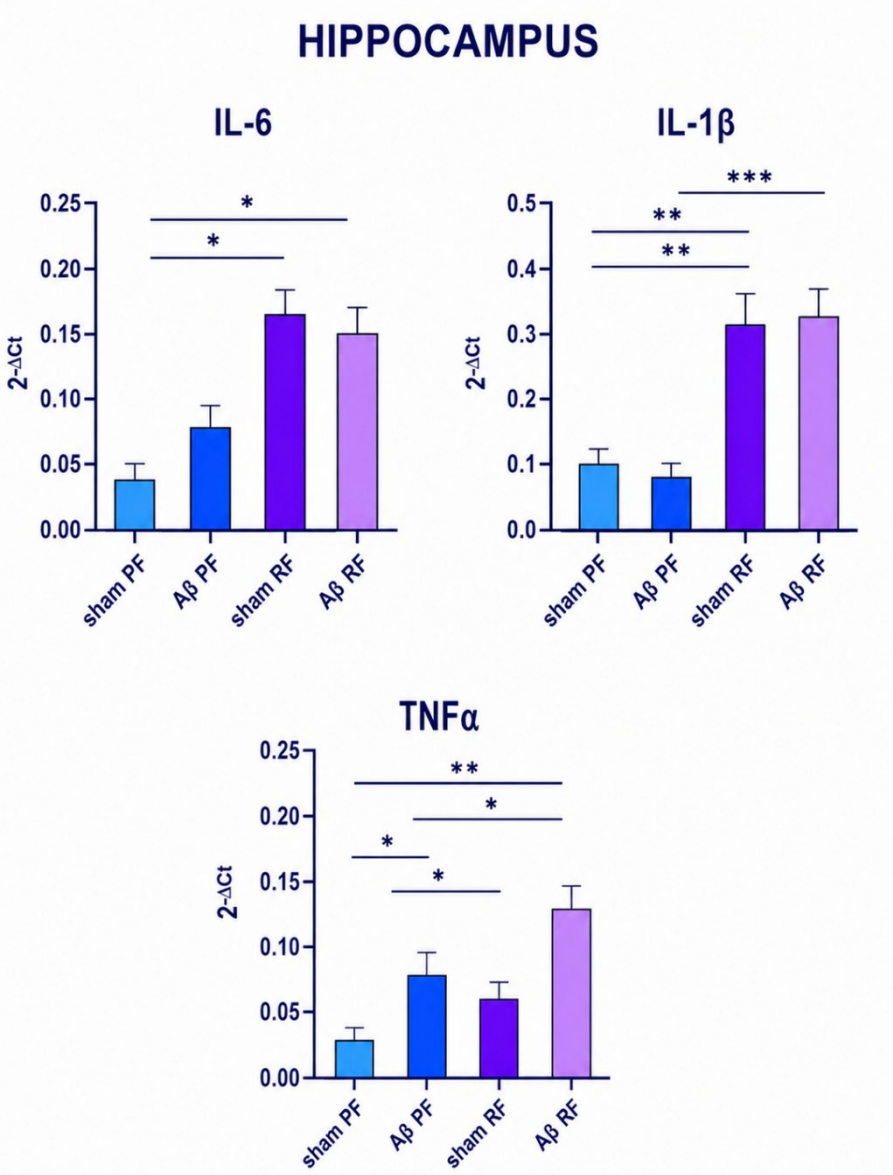

Figure 10. Expression of IL-6, IL-1β, and TNFα transcripts in the hippocampus of mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7). Cytokine expression is reported as $2^{-\Delta Ct}$ and presented as mean ± SEM. Statistical significance is indicated by (*): p ≤ 0,05; (**): p ≤ 0,01; (***): p ≤ 0,0001.

IL-1β transcript expression was also significantly increased in RF-Aβ mice compared with both PF-Sham mice (0.3301 vs. 0.1070; $P = 0.0063$) and PF-Aβ mice (0.3301 vs. 0.08468; $P = 0.0002$). Regarding TNFα transcript, significantly higher expression levels were observed in RF-Sham mice than in PF-Sham mice (0.06297 vs. 0.02717; $P = 0.03$), in RF-Aβ mice than in PF-Aβ mice (0.1320 vs. 0.07652; $P = 0.04$), and in RF-Aβ mice than in PF-Sham mice (0.1320 vs. 0.02717; $P = 0.005$). In addition, PF-Aβ mice exhibited significantly higher TNFα transcript expression than PF-Sham

mice (0.07652 vs. 0.03130; $P = 0.02$). Overall, exposure to the combined environmental risk factors was associated with an approximately fourfold increase in hippocampal IL-6 transcript expression, a three- to fourfold increase in IL-1β transcript expression, and a two- to fivefold increase in TNFα transcript expression compared with the protective condition, depending on the experimental comparison performed.

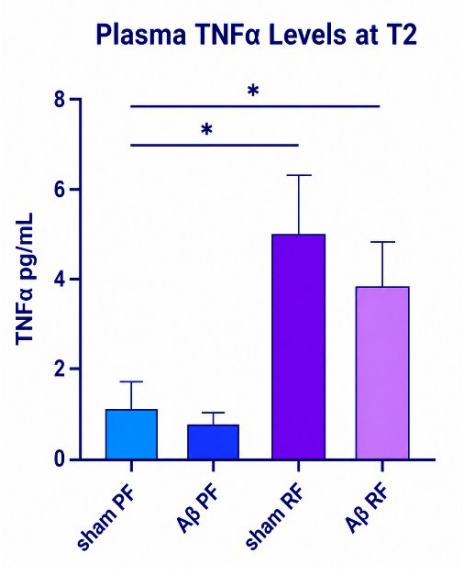

Figure 11. Plasma TNFα levels measured at the end of the experimental protocol (T2) in mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7). Data are expressed as pg/mL and presented as mean ± SEM. Statistical significance is indicated by (*): $p \leq 0,05$; (**): $p \leq 0,01$; (***): $p \leq 0,0001$.

Plasma cytokine analysis (*Figure 11*) revealed detectable levels of TNFα, whereas the concentrations of IL-6 and IL-1β were below the detection limit of the assay. At T2, plasma TNFα levels were significantly higher in RF-Sham mice than in PF-Sham mice (4.710 vs. 1.043 pg/mL; $P = 0.01$). Similarly, RF-Aβ mice exhibited significantly higher TNFα concentrations than PF-Sham mice (3.932 vs. 1.043 pg/mL; $P = 0.03$). Overall, exposure to the combined environmental risk factors was associated with an approximately fourfold increase in circulating TNFα levels compared with the protective condition.

4.4 Immunohistochemical Evaluation

Representative hippocampal sections stained for GFAP and Iba1 are shown in *Figures 12*. Qualitative immunohistochemical evaluation did not reveal evident differences in either astrocytic or microglial staining among the experimental groups. Similarly, ICV Aβ administration did not produce appreciable changes in the overall staining patterns of either marker.

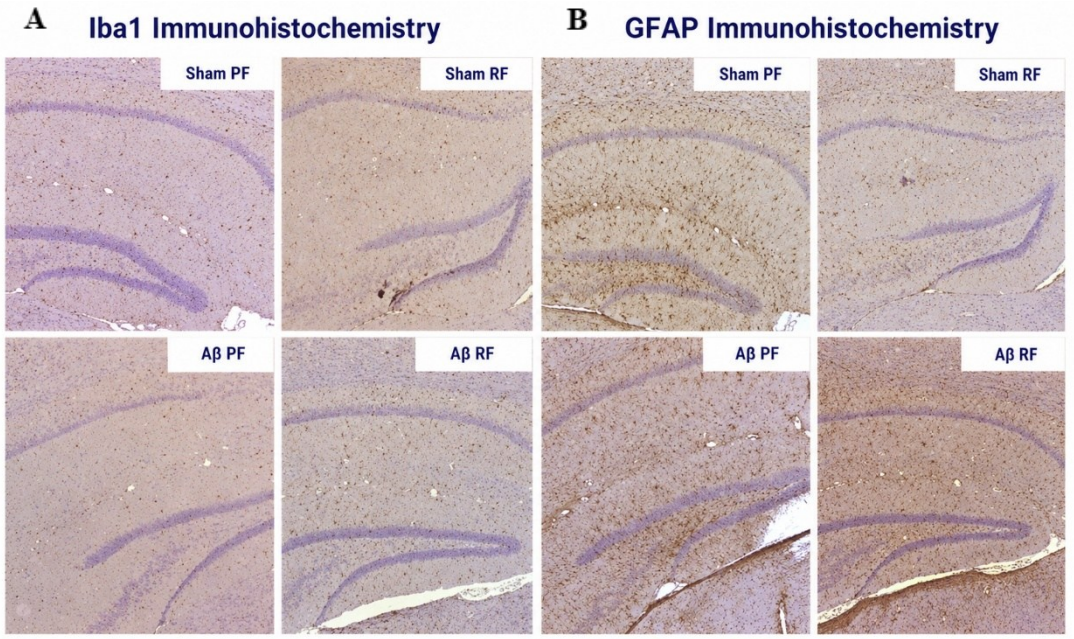

Figure 12. (**A**) Representative Iba1 immunohistochemical staining of hippocampal sections from mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. Images are representative of microglial distribution in the hippocampus. (**B**) Representative GFAP immunohistochemical staining of hippocampal sections from mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. Images are representative of astrocyte distribution in the hippocampus (Aβ, n = 2; Sham, n = 2), (Aβ, n = 2; Sham, n = 2).

GFAP immunostaining showed a relatively homogeneous distribution of astrocytes throughout the hippocampal regions examined, with no obvious alterations associated with either the PF or RF condition. Likewise, Iba1 immunostaining did not reveal evident differences in microglial distribution or morphology among the experimental groups. Overall, qualitative immunohistochemical analysis did not reveal overt astroglial or microglial activation in response to the experimental conditions.

4.5 Neurodegeneration Analysis

β-Secretase (BACE1) Activity

BACE1 activity was evaluated in a subset of brain samples using a fluorometric assay. The measured enzymatic activity values were below the linear range of the standard curve provided by the assay kit, preventing accurate quantification and reliable statistical analysis. Therefore, no conclusions could be drawn regarding the effects of the PF and RF conditions or ICV Aβ administration on BACE1 activity.

Aβ42 Deposition

The presence of Aβ42 aggregates was assessed by immunohistochemical analysis of brain sections. No macroscopically detectable amyloid deposits or histopathological patterns clearly indicative of amyloid plaque formation were observed in any of the experimental groups. Therefore, no evidence of detectable amyloid deposition was observed following exposure to either the PF or RF condition or after ICV Aβ administration within the timeframe of the present study (data not shown).

BDNF Expression

BDNF transcript expression was evaluated by qRT-PCR in brain tissue collected at the end of the experimental protocol (T2). As shown in *Figure 13*, comparable BDNF transcript expression levels were observed among the experimental groups. No statistically significant differences were identified between mice maintained under the PF or RF conditions or following ICV Aβ administration. Overall, these findings indicate that neither exposure to the PF or RF condition nor ICV Aβ administration significantly affected BDNF transcript expression under the experimental conditions examined.

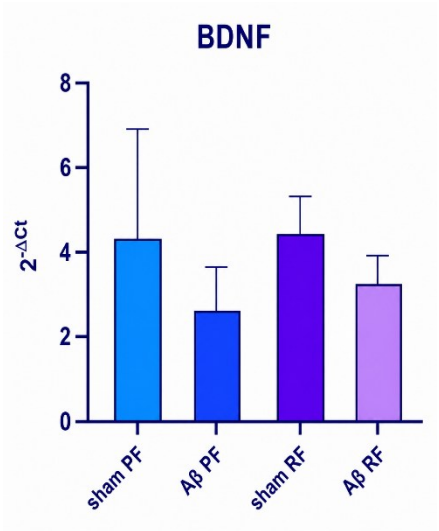

Figure 13. BDNF transcript expression in brain tissue collected at the end of the experimental protocol (T2). Relative BDNF transcript expression was determined by qRT-PCR in mice maintained under the PF or RF conditions, following sham or ICV Aβ administration. The PF group included 11 mice (Aβ, n = 6; Sham, n = 5), whereas the RF group included 14 mice (Aβ, n = 7; Sham, n = 7). Gene expression is reported as $2^{-\Delta Ct}$ and presented as mean ± SEM. Statistical significance is indicated by (*): $p \leq 0,05$; (**): $p \leq 0,01$; (***): $p \leq 0,0001$.

4.6 Oxidative Damage and Mitochondrial Dysfunction Analysis

Protein expression analyses were performed by Western blot to evaluate oxidative stress markers and mitochondrial respiratory chain components in cortical and hippocampal tissue collected at the end of the experimental protocol (T2).

4.6.1 Oxidative Stress Markers

The expression of the antioxidant enzymes Catalase and Superoxide Dismutase 1 (SOD1) was evaluated to investigate changes in the antioxidant defense system. Representative immunoblots and corresponding densitometric analyses are shown in *Figure 14*. Preliminary analyses revealed no significant differences between sham-treated and ICV Aβ-treated animals for the parameters examined. Therefore, to increase statistical power and sample size, data from the two treatment groups were pooled, and subsequent analyses were performed by comparing PF and RF conditions irrespective of Aβ treatment. In the cortex, Catalase expression was significantly higher in mice exposed to the RF condition than in those maintained under the PF condition (142% relative to the PF group, PF = 100%; P = 0.03), corresponding to an approximately a twofold increase compared

with the PF condition. In contrast, SOD1 expression showed a tendency to decrease under the RF condition, although this difference did not reach statistical significance. A similar trend was observed in the hippocampus, where Catalase expression tended to increase in mice exposed to the RF condition compared with the PF condition, whereas SOD1 expression showed a tendency to increase. However, neither difference reached statistical significance. Overall, these findings suggest that exposure to the RF condition was associated with alterations in the antioxidant defense system, with a significant increase in cortical Catalase expression and similar, although non-significant, trends in the hippocampus.

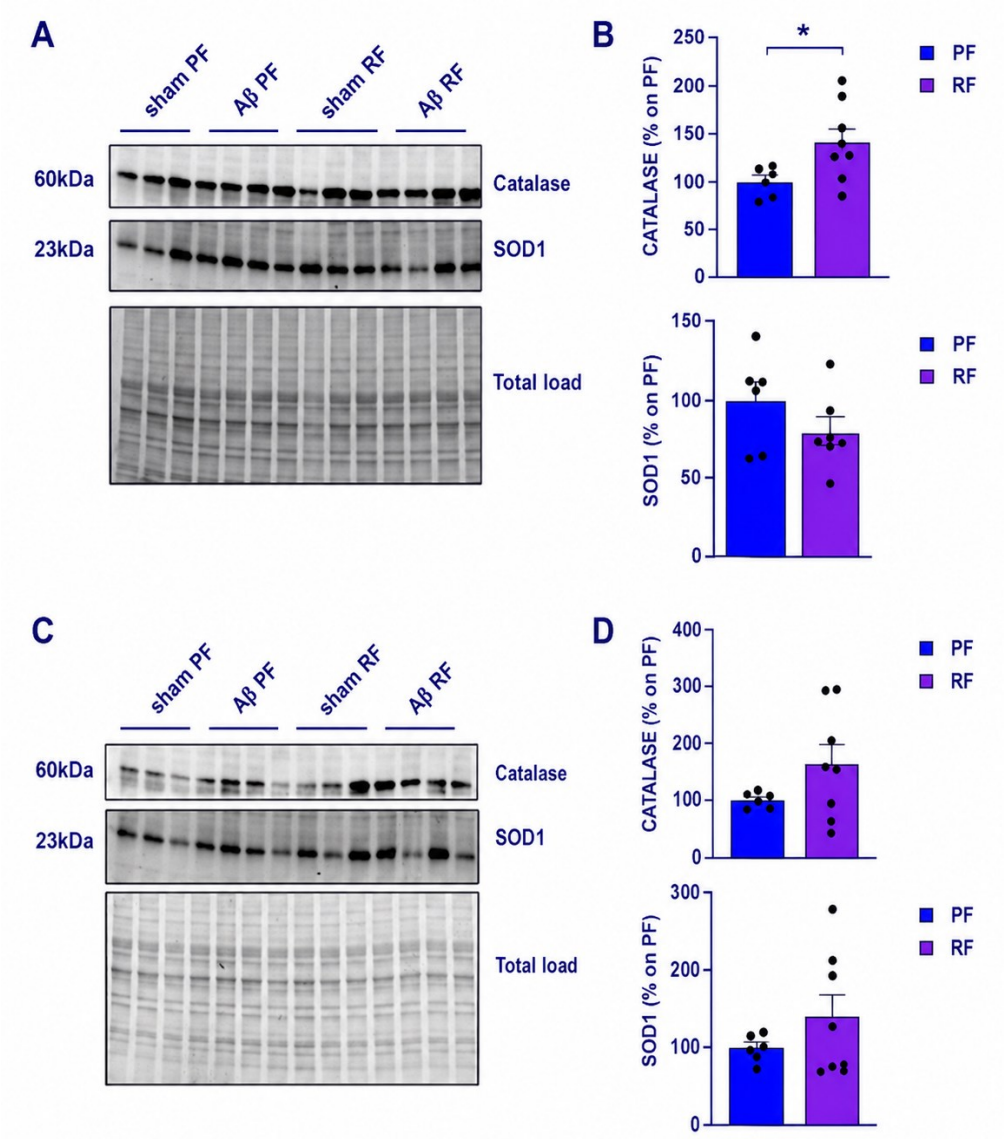

Figure 14. Expression of the antioxidant enzymes Catalase and SOD1 in the cerebral cortex (**A–B**) and hippocampus (**C–D**). Representative Western blots (**A, C**) and corresponding densitometric analyses (**B, D**) are shown. Protein expression was normalized to total protein load and expressed as percentage of the PF group (PF = 100%). Data are presented as mean ± SEM. For both Catalase and SOD1, the number of samples analyzed was PF, n

= 6 and RF, n = 8 in the cortex, and PF, n = 5 and RF, n = 8 in the hippocampus. Statistical significance is indicated by (*): $p \leq 0,05$; (**): $p \leq 0,01$; (***): $p \leq 0,0001$.

4.6.2 Mitochondrial Respiratory Chain Complexes

The expression of mitochondrial oxidative phosphorylation (OXPHOS) complexes was subsequently evaluated to investigate the impact of the experimental conditions on mitochondrial function. Representative immunoblots and corresponding densitometric analyses are shown in *Figure 15*. In the cortex, mice exposed to the RF condition showed significant alterations in the expression of several mitochondrial respiratory chain complexes compared with mice maintained under the PF condition. Specifically, Complex III (UQCRC2) expression was significantly reduced to 75.8% of the PF group (PF = 100%; P = 0.001), while Complex II (SDHB) expression decreased to 70% of the PF group (P = 0.0003). Furthermore, Complex I (NDUFB8) expression increased to 257% of the PF group (P = 0.03), corresponding to an approximately 2.5-fold increase, whereas Complex IV (MTCO1) expression was approximately fivefold higher than the PF group (485% relative to PF; P = 0.006). No significant differences were observed in the expression of Complex V (ATP5A). In the hippocampus, significant alterations were detected only for Complexes I and IV. Specifically, Complex I (NDUFB8) expression increased to 390% of the PF group (P = 0.0004), corresponding to an approximately 3.9-fold increase, whereas Complex IV (MTCO1) expression was approximately 24-fold higher than the PF group (2401% relative to PF; P = 0.001). No significant differences were observed in the expression of Complex II (SDHB), Complex III (UQCRC2), or Complex V (ATP5A). Overall, exposure to the RF condition was associated with region-specific alterations in mitochondrial respiratory chain complex expression, characterized by increased expression of Complexes I and IV in both brain regions and reduced expression of Complexes II and III selectively in the cortex. Overall, exposure to the RF condition was associated with alterations in the expression of selected mitochondrial respiratory chain complexes in both the cortex and hippocampus, suggesting that the combined environmental risk factors affected mitochondrial homeostasis in both brain regions.

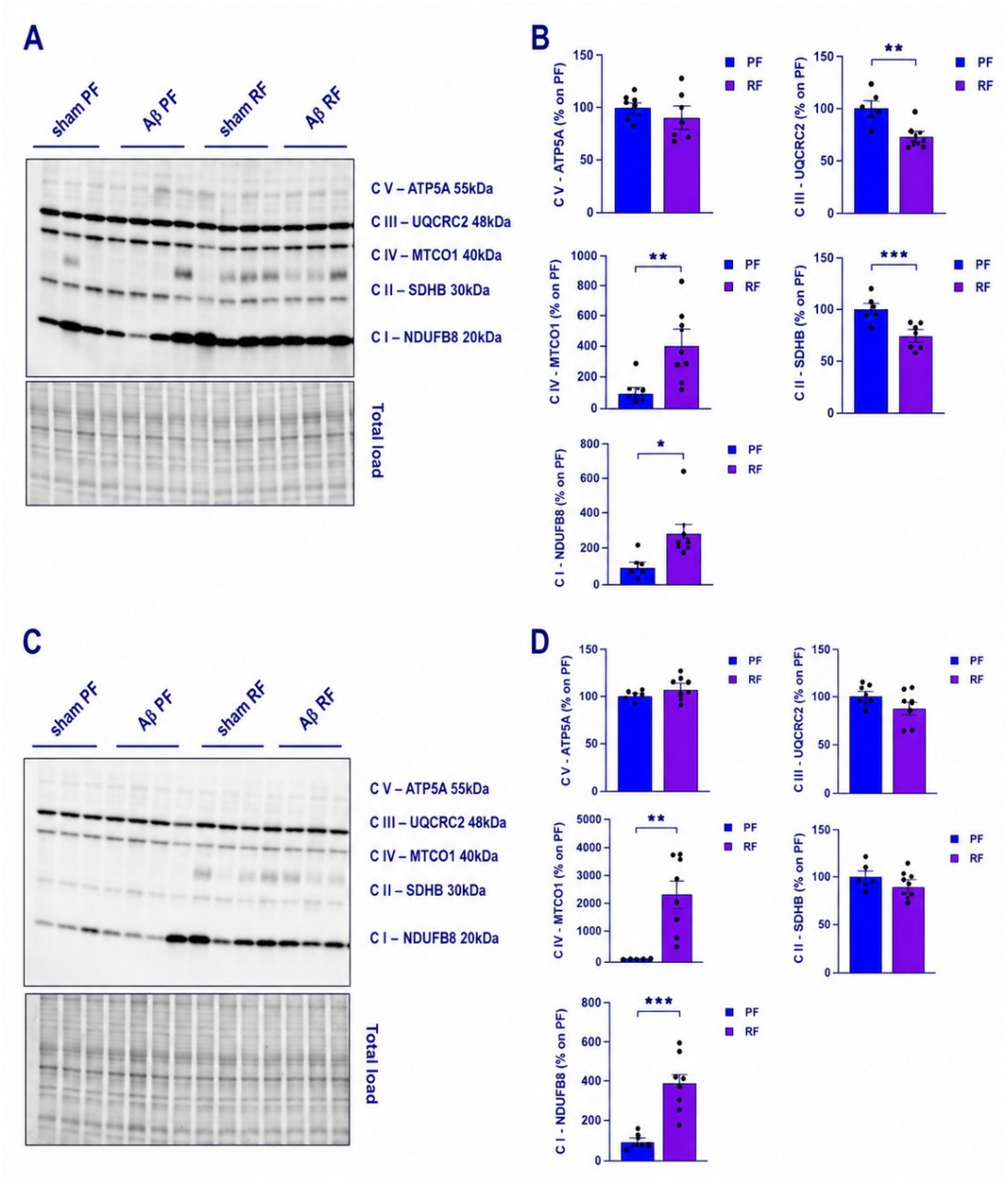

Figure 15. Expression of mitochondrial OXPHOS complexes in the cerebral cortex (**A–B**) and hippocampus (**C–D**). Representative Western blots (**A, C**) and corresponding densitometric analyses (**B, D**) are shown. Protein expression was normalized to total protein load and expressed as percentage of the PF group (PF = 100%). Data are presented as mean ± SEM. PF: n = 6; RF: n = 8 for both cortical and hippocampal analyses. Statistical significance is indicated by (*): $p \leq 0,05$; (**): $p \leq 0,01$; (***): $p \leq 0,0001$.

CHAPTER 5: DISCUSSION

AD is a complex and multifactorial neurodegenerative disorder in which aging, genetic susceptibility, and environmental factors interact over several years before the onset of clinically evident symptoms. Although the pathological hallmarks of the disease are represented by extracellular Aβ plaques and intracellular NFTs composed of hyperphosphorylated tau, increasing evidence suggests that these alterations are preceded by a series of molecular and cellular events involving chronic neuroinflammation, oxidative stress, mitochondrial dysfunction, and metabolic impairment. Most experimental studies have traditionally relied on transgenic mouse models carrying mutations in genes such as APP, PSEN1, or PSEN2. Although these models have substantially contributed to the understanding of amyloid pathology and FAD, they reproduce only a small proportion of human cases and do not fully reflect the multifactorial nature of SAD. In particular, it is thought to arise from the cumulative effects of multiple risk factors rather than from a single pathogenic event, highlighting the importance of investigating how modifiable lifestyle-related factors contribute to disease initiation and progression (Di Crescenzo et al., 2026; Livingston et al., 2024; Safiri et al., 2024). Environmental factors including unhealthy dietary habits, physical inactivity, and social isolation have emerged as important contributors to the development of neurodegenerative disorders, acting through mechanisms that extend beyond amyloid accumulation alone. Consequently, increasing attention has been directed toward the development of experimental models that more closely reproduce the complex interaction between lifestyle-related risk factors and the biological processes underlying SAD. Based on these considerations, the present study was designed to investigate the combined effects of WD and social isolation in a WT mouse model, using normal diet, voluntary physical exercise and group housing as a protective condition. In addition, ICV administration of $A\beta_{1-42}$ was included to evaluate whether acute amyloid exposure could further influence the molecular and behavioral alterations induced by environmental risk factors. To provide a comprehensive characterization of the experimental model, behavioral performance was assessed together with multiple biological processes known to be involved in AD pathogenesis, including neuroinflammation, oxidative stress, mitochondrial function, glial activation, and neurodegeneration. The first objective of the present study was to determine whether prolonged exposure to combined environmental risk factors induce early behavioral alterations associated with AD. Cognitive impairment represents an important clinical manifestation of AD. While molecular and cellular

alterations generally precede cognitive decline, assessing cognitive function remains essential to determine whether these changes translate into functional deficits. To this end, locomotor activity and recognition memory were assessed, with locomotor activity serving as a control for potential differences in spontaneous locomotion. Indeed, the OFT revealed no significant differences in spontaneous locomotor activity between mice maintained under the PF condition and those exposed to the RF condition. Similarly, ICV administration of $A\beta_{1–42}$ did not significantly affect exploratory behavior or the time spent in the central area of the arena. These findings indicate that neither the environmental conditions nor the acute amyloid challenge produced detectable alterations in general locomotion or exploratory drive under the experimental conditions adopted. This observation is particularly relevant because it excludes the possibility that subsequent differences observed in cognitive performance were secondary to impaired motor activity or reduced exploration, thus strengthening the interpretation of the behavioral findings obtained in the memory task. In contrast, a different pattern emerged from the NOR test, which specifically evaluates recognition memory, a cognitive domain that is highly dependent on the integrity of the hippocampus and associated cortical regions. Mice maintained under the PF condition consistently displayed a clear preference for the novel object, indicating preserved recognition memory. Conversely, animals exposed to the RF condition failed to discriminate between the familiar and novel objects, demonstrating an impairment in recognition memory. Interestingly, this RF-induced behavioral phenotype was observed irrespective of ICV Aβ administration, suggesting that prolonged exposure to WD and social isolation was sufficient to compromise cognitive performance independently of an acute amyloid insult. These findings are consistent with an increasing body of evidence indicating that environmental and lifestyle-related risk factors contribute to cognitive decline through mechanisms that extend beyond amyloid deposition alone. Several experimental studies have shown that prolonged consumption of a WD or HFT promotes deficits in learning and memory by inducing metabolic dysfunction, neuroinflammation, and oxidative stress within brain regions critically involved in cognition, particularly the hippocampus (Tan & Norhaizan, 2019). Likewise, chronic social isolation has been associated with impaired synaptic plasticity, reduced cognitive performance, and increased vulnerability to neurodegeneration, supporting the concept that the social environment plays an important role in maintaining normal brain function during aging (Ren et al., 2023). Taken together, these observations suggest that the behavioral alterations identified in the present study are more likely to reflect the cumulative effects of prolonged exposure to adverse environmental conditions

than the direct consequences of acute Aβ administration. This interpretation is further supported by the absence of overt neuropathological changes in the experimental model, indicating that cognitive dysfunction can develop before the appearance of classical histopathological hallmarks of AD. Since cognitive impairment is generally considered the functional consequence of underlying molecular alterations, these behavioral findings prompted further investigation into the biological mechanisms potentially responsible for the observed phenotype. Among the molecular pathways investigated, neuroinflammation emerged as one of the earliest and most prominent alterations associated with the RF condition. The molecular analyses performed in the present study demonstrated that the cognitive impairment observed under the RF condition was accompanied by a robust inflammatory response involving both the CNS and the peripheral circulation. Neuroinflammation is now recognized as one of the earliest events in AD pathogenesis and is increasingly viewed as a key contributor to disease progression rather than a mere consequence of neuronal degeneration (Long et al., 2025). Indeed, chronic activation of inflammatory pathways has been shown to disrupt synaptic function, impair neuronal communication, promote oxidative stress, and ultimately facilitate neurodegenerative processes long before the appearance of the classical neuropathological hallmarks of AD (Almikhlafi, 2026; Di Crescenzo et al., 2026; Long et al., 2025). Within this context, mice exposed to the RF condition exhibited a marked increase in the expression of the pro-inflammatory cytokines IL-6 and IL-1β in both the cerebral cortex and hippocampus. These findings suggest that the combination of WD and social isolation was sufficient to establish a persistent inflammatory environment affecting two brain regions that are critically involved in cognitive function. A similar inflammatory profile was observed in RF mice following ICV administration of $A\beta_{1-42}$, indicating that acute Aβ exposure did not substantially modify the inflammatory response already induced by the RF condition. Taken together, these findings suggest that the neuroinflammatory changes observed in the present study were primarily driven by prolonged exposure to lifestyle-related risk factors rather than by the acute amyloid challenge. Among the cytokines analyzed, IL-1β deserves particular attention because it is considered one of the principal mediators linking chronic inflammation to neurodegeneration. Activated microglia represent the major source of IL-1β within the CNS, and sustained production of this cytokine has been associated with impaired synaptic plasticity, altered hippocampal-dependent memory, and increased neuronal vulnerability. Likewise, IL-6 plays a pleiotropic role in the regulation of immune responses and, although transient IL-6 signaling may exert protective functions, prolonged overexpression has consistently been associated with chronic neuroinflammation, glial

activation, and cognitive decline (Almikhlafi, 2026). The approximately fourfold increase observed for both IL-6 and IL-1β in mice exposed to the RF condition therefore suggests that prolonged exposure to environmental stressors promotes a sustained inflammatory response that may contribute to the behavioral impairment observed in the NOR test. The inflammatory profile identified in the present study was not restricted to the brain. Plasma analysis revealed a significant increase in circulating TNFα levels in mice maintained under the RF condition, supporting the presence of a concomitant systemic inflammatory response. This observation is particularly relevant because growing evidence indicates that peripheral inflammation and neuroinflammation are closely interconnected. Circulating pro-inflammatory mediators can influence CNS homeostasis through multiple mechanisms, including alterations of BBB permeability, activation of endothelial cells, and amplification of microglial responses, thereby establishing a bidirectional communication between the peripheral immune system and the brain (S. Zhang et al., 2025). Consequently, the elevated plasma TNFα levels observed in the present study further support the hypothesis that chronic exposure to environmental risk factors promotes a systemic pro-inflammatory state capable of contributing to early brain dysfunction. Overall, the inflammatory findings presented here are consistent with the growing view that chronic low-grade inflammation represents a key mechanism through which modifiable lifestyle-related risk factors increase the risk of SAD. Rather than acting as isolated events, WD, social isolation and reduced physical activity are likely to converge on common inflammatory pathways that progressively alter brain homeostasis, thereby creating conditions that favor the subsequent development of oxidative stress, mitochondrial dysfunction, and neuronal damage. Based on these considerations, it was of particular interest to determine whether the inflammatory alterations observed in the present study were accompanied by evidence of oxidative imbalance, as oxidative stress represents one of the principal downstream consequences of chronic neuroinflammation and a critical contributor to neuronal dysfunction during the early stages of AD. Consistent with this hypothesis, mice exposed to the RF condition exhibited molecular changes indicative of an altered antioxidant response, particularly within the cerebral cortex. Oxidative stress is considered an early contributor to AD pathogenesis and is closely linked to chronic neuroinflammation (Lovell & Markesbery, 2007). Among the antioxidant enzymes analyzed, catalase exhibited a significant increase in cortical tissue from mice maintained under the RF condition. Since catalase is a key enzyme involved in the detoxification of hydrogen peroxide, this increase may reflect a compensatory response aimed at limiting oxidative stress rather than enhanced antioxidant capacity

itself. However, this interpretation remains speculative and cannot be directly demonstrated by the present findings because, although its antioxidant function supports this hypothesis, the present data only demonstrate increased catalase expression and do not establish whether this increase actually represents a compensatory response to oxidative stress. In contrast, SOD1 expression showed a tendency to decrease in the cortex of RF mice. SOD1 constitutes the first enzymatic line of defense against oxidative stress by catalyzing the conversion of superoxide anions into hydrogen peroxide, which is subsequently detoxified by catalase and other antioxidant systems. Although the absence of statistical significance prevents definitive conclusions, the opposite trends observed for SOD1 and catalase may nevertheless reflect an early imbalance in the regulation of antioxidant defenses. Reduced superoxide detoxification combined with increased catalase expression could indicate that oxidative stress had already begun to challenge redox homeostasis, triggering selective activation of downstream antioxidant pathways. Interestingly, these alterations were considerably less evident in the hippocampus, where neither catalase nor SOD1 expression differed significantly between experimental groups. Although the reasons underlying this regional difference remain unclear, several studies have shown that the temporal progression of oxidative damage is not necessarily uniform throughout the brain. Different brain regions may exhibit distinct metabolic demands, antioxidant capacities, and susceptibilities to environmental insults, resulting in region-specific patterns of oxidative imbalance during the early phases of neurodegeneration (Buccellato et al., 2021; Elahi et al., 2016). Therefore, with regard specifically to oxidative stress, the more pronounced alterations observed in the cerebral cortex suggest a greater cortical susceptibility to oxidative imbalance under the RF condition. This regional pattern differed from that observed for neuroinflammation, which was more pronounced in the hippocampus, suggesting that the effects of the RF condition may involve distinct region-specific molecular responses. Taken together, these findings support the hypothesis that prolonged exposure to WD and social isolation promotes the establishment of an early pro-oxidant environment. Rather than representing irreversible oxidative damage, the observed changes appear to reflect an active adaptive response aimed at preserving redox homeostasis under conditions of persistent metabolic and inflammatory stress. However, chronic activation of antioxidant defenses is unlikely to remain effective indefinitely. Sustained oxidative stress may progressively impair mitochondrial function, leading to reduced ATP production and further ROS generation, thereby establishing a vicious cycle that contributes to neuronal dysfunction and disease progression (Swerdlow et al., 2014). Based on this close relationship between oxidative

stress and mitochondrial dysfunction, mitochondrial respiratory chain proteins were investigated to determine whether mice model showed early alterations in cellular bioenergetics. Actually, exposure to the RF condition was associated with significant alterations in the expression of several OXPHOS complexes, indicating an early disruption of mitochondrial bioenergetics. Interestingly, the pattern of mitochondrial alterations did not suggest a generalized loss of respiratory chain components. Instead, distinct complexes exhibited different expression profiles, indicating the activation of adaptive mechanisms rather than irreversible mitochondrial failure. Among the proteins analyzed, Complex II and Complex III were significantly reduced in the cerebral cortex of RF mice. Both complexes play a crucial role in electron transfer within the ETC, and impairment of their activity has been associated with reduced respiratory efficiency, increased electron leakage, and enhanced ROS production. In particular, Complex III is considered one of the principal mitochondrial sites of superoxide generation, and its dysfunction may therefore further amplify oxidative stress, reinforcing the molecular alterations already suggested by the antioxidant profile observed in the present study. The concomitant reduction of Complex II may further compromise mitochondrial respiration by limiting electron flux into the ETC, thereby contributing to decreased bioenergetic efficiency. Conversely, Complex I and Complex IV exhibited a marked increase in expression, particularly within the hippocampus, where both complexes were substantially upregulated. At first glance, these findings may appear counterintuitive, as mitochondrial dysfunction is often associated with reduced expression of respiratory chain proteins. However, increasing evidence indicates that during the earliest stages of neurodegeneration mitochondria frequently undergo adaptive remodeling aimed at maintaining ATP production despite persistent cellular stress. Increased expression of specific OXPHOS complexes has therefore been interpreted as a compensatory response designed to preserve mitochondrial function under conditions of increased energetic demand or oxidative challenge rather than as evidence of improved mitochondrial performance. This interpretation is consistent with the overall biological profile observed in the present study. Although mice exposed to the RF condition displayed clear evidence of neuroinflammation, oxidative imbalance, and cognitive impairment, no overt neurodegenerative changes were detected. Under these circumstances, mitochondrial adaptation may represent an attempt to sustain neuronal metabolism and preserve cellular homeostasis before irreversible structural damage develops. The simultaneous decrease in some respiratory complexes together with the upregulation of others therefore suggests that mitochondrial function was undergoing dynamic remodeling rather than uniform deterioration. Such compensatory responses

have been described in several experimental models of early AD and are thought to reflect the remarkable metabolic plasticity of neurons during the initial phases of disease progression (Andersen et al., 2021; Comyn et al., 2024). Taken together, these findings indicate that chronic exposure to WD and social isolation induces early disturbances in mitochondrial homeostasis that are closely associated with the inflammatory and oxidative alterations described above. Rather than representing isolated molecular events, neuroinflammation, oxidative stress, and mitochondrial dysfunction appear to constitute interconnected components of the same pathogenic cascade, each amplifying the effects of the others. Nevertheless, despite these molecular alterations, the absence of detectable glial activation and overt neurodegeneration suggests that the experimental model reproduces an early preclinical stage of the disease, in which compensatory mechanisms are still active and neuronal integrity is largely preserved. This aspect is further supported by the histological analyses performed in the present study, which provide additional insight into the temporal sequence of pathological events. The histological findings are fully consistent with the molecular profile described above and provide further evidence that the experimental model reproduces an early stage of disease progression. Although the molecular findings suggest the presence of early inflammatory, oxidative, and mitochondrial alterations accompanied by cognitive impairment, the analyses did not reveal detectable Aβ plaque deposition, increased GFAP immunoreactivity, or significant alterations in the expression of BACE1 or BDNF. Rather than contradicting the molecular findings, these observations strengthen the hypothesis that the pathological alterations induced by prolonged exposure to environmental risk factors precede the appearance of the classical neuropathological hallmarks of AD. The absence of detectable Aβ deposition was not entirely unexpected. Unlike FAD, in which genetic mutations directly promote excessive Aβ production and plaque formation, SAD is generally considered a slowly evolving disorder in which molecular dysfunction progressively accumulates over many years before the appearance of overt neuropathological lesions. In this context, chronic exposure to environmental risk factors is thought to initiate a cascade of pathogenic events that initially affects cellular homeostasis, synaptic function, and neuronal metabolism, whereas extracellular amyloid deposition represents a relatively late manifestation of the disease process. Therefore, the lack of detectable plaques in the present study is consistent with the hypothesis that the experimental paradigm reproduced an early preclinical stage of SAD, preceding the development of classical amyloid pathology. A similar interpretation can be applied to the absence of significant changes in BACE1 expression. BACE1 is the rate-limiting enzyme responsible for the amyloidogenic

processing of APP and is frequently found to be upregulated in advanced stages of AD, where increased Aβ production contributes to progressive plaque accumulation. However, alterations in BACE1 expression are not necessarily among the earliest molecular events occurring during disease initiation. The lack of significant differences observed in the present study therefore suggests that the pathological mechanisms induced by prolonged exposure to WD and social isolation had not yet reached the stage at which substantial activation of the amyloidogenic pathway becomes detectable. This observation further supports the concept that inflammatory and metabolic disturbances may precede overt amyloid pathology during the evolution of sporadic AD. Likewise, the absence of significant differences in GFAP immunoreactivity indicates that, despite the marked increase in pro-inflammatory cytokines, widespread astrocytic activation had not yet developed. This finding does not necessarily imply the absence of neuroinflammation. Instead, it highlights the dynamic nature of glial responses during disease progression. Functional activation of inflammatory pathways may occur before the establishment of the morphological changes typically detected by GFAP immunostaining. In other words, cytokine dysregulation may represent an earlier event than overt astrogliosis, reflecting the progressive transition from molecular immune activation to the structural remodeling of glial cells. Similarly, the lack of detectable changes in BDNF expression suggests that neuronal trophic support remained largely preserved despite the presence of cognitive impairment and multiple molecular alterations. Although reduced BDNF levels have frequently been associated with synaptic dysfunction and cognitive decline in AD, their decrease is generally more evident during later stages of disease, when neuronal damage becomes more pronounced. Although no detectable Aβ plaque deposition was observed, the molecular alterations observed in the present study may have been sufficient to impair neuronal and synaptic function, thereby contributing to the cognitive deficits. This interpretation is consistent with current evidence indicating that functional alterations within vulnerable neuronal circuits precede widespread neurodegeneration and the appearance of the classical neuropathological hallmarks of AD. Moreover, this temporal sequence resembles the earliest Braak stages (I–II), in which tau pathology is initially confined to the transentorhinal and entorhinal cortex, while widespread neuronal loss and extensive neuropathological changes have not yet developed (Braak et al., 2011; S. Kim et al., 2022). Consequently, the present model provides a valuable experimental platform for investigating the mechanisms responsible for disease initiation and for identifying therapeutic strategies aimed at preventing or delaying the transition from early molecular dysfunction to irreversible

neurodegeneration. Although the present study provides a comprehensive characterization of the early molecular alterations induced by environmental risk factors, several limitations should be considered when interpreting the findings. First, the experimental paradigm was specifically designed to reproduce the initial stages of SAD rather than the advanced phases of the disease. The absence of overt amyloid pathology marked glial activation, and extensive neurodegeneration should not be interpreted as a limitation of the model itself, but rather as a reflection of the relatively early time point investigated. Extending the duration of exposure to the RF condition may therefore provide valuable information regarding whether the molecular alterations identified here eventually evolve into the classical neuropathological features of AD. Another important consideration concerns the acute ICV administration of $A\beta_{1-42}$. Although this approach allows the investigation of early amyloid-related mechanisms without relying on genetic manipulation, it does not fully reproduce the gradual accumulation of endogenous Aβ that characterizes SAD. Similarly, while the combination of WD and social isolation successfully mimics relevant modifiable risk factors, human disease results from the interaction of numerous environmental, metabolic, vascular, and genetic variables that cannot be entirely replicated within a single experimental model. Therefore, although the present paradigm captures important aspects of the multifactorial nature of SAD, it inevitably represents only part of the complexity of the human condition. Despite these limitations, the model offers several important advantages. Unlike traditional transgenic models, which primarily reproduce FAD, the present experimental approach focuses on mechanisms that are directly relevant to the sporadic disease, emphasizing the contribution of modifiable lifestyle-related risk factors to disease initiation. This feature makes the model particularly suitable for investigating the earliest pathogenic events that occur before irreversible neurodegeneration becomes established, a phase during which preventive interventions are expected to be most effective. Future studies should further explore the temporal evolution of the molecular alterations identified in the present work by extending the duration of environmental exposure and evaluating whether the inflammatory, oxidative, and mitochondrial changes observed here ultimately progress toward overt amyloid pathology, glial activation, synaptic degeneration, and neuronal loss. In parallel, the development of a complementary model characterized by enhanced protection against oxidative stress could help determine whether increased cellular resilience is able to prevent or attenuate the cognitive and molecular alterations induced by prolonged exposure to lifestyle-related risk factors. In addition, integrating transcriptomic, proteomic, and metabolomic approaches may provide a more comprehensive understanding of the biological

networks linking lifestyle-related risk factors to neurodegeneration and may facilitate the identification of early biomarkers and potential therapeutic targets for early intervention.

CHAPTER 6: CONCLUSIONS

The findings of the present study indicate that prolonged exposure to combined lifestyle-related risk factors promotes molecular and functional alterations consistent with an early stage of SAD. The molecular changes observed, together with cognitive impairment and the absence of overt neuropathological hallmarks, support the hypothesis that environmental risk factors contribute to the earliest phases of disease development. These results further highlight the value of this experimental model for investigating the mechanisms underlying disease initiation and for evaluating preventive approaches before irreversible neurodegeneration becomes established.

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