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Crosstalk between Depression, Anxiety, Dementia, and Chronic Pain

Comorbidity in Behavioral Neurology and Neuropsychiatry Volume III

Edited by
Masaru Tanaka and Simone Battaglia

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Guest Editors

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About the Editors

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Masaru Tanaka, M.D., Ph.D., is Senior Research Fellow at the Danube Neuroscience Research Laboratory, HUN-REN-SZTE Neuroscience Research Group, Hungarian Research Network, University of Szeged (HUN-REN-SZTE). His research spans neuroimmune mechanisms, tryptophan and kynurenine pathway dynamics, and network-level biomarkers to explain how affective, cognitive, and nociceptive circuits destabilize across depression, dementia, and chronic pain and their comorbidity. Current projects integrate plasma proteomics, multimodal imaging, and digital phenotyping in longitudinal cohorts and preclinical models to build predictive tools and inform adaptive trials for precision neuropsychiatry. He has authored more than 140 peer-reviewed papers, is ranked in the D1 percentile of global publication impact, and several works are in the top 99.9 citation percentile in neuroscience and psychiatry; he appears on the 2025 Stanford Elsevier World's Top 2 Percent Scientists list. His scholarship has received Best Paper, Hot Paper, and Most Cited Paper awards, along with recognition as Best Reviewer of the Month, and he serves on editorial boards including Biomedicines, Biomolecules and Biomedicine, and Frontiers in Neuroscience. He earned an MD and a PhD in Medicine from the University of Szeged and a Bachelor's degree in Biophysics from the University of Illinois Urbana-Champaign.

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Preface

This Reprint examines the intertwined pathophysiology and clinical expression of depression, anxiety, dementia, and chronic pain within a neuroimmune and biomarker-anchored framework. Its scope spans cellular mechanisms involving microglia and stress signaling, multimodal measurement from imaging to proteomics, and clinical investigations of comorbidity, progression, and treatment response. The aim is to integrate heterogeneous evidence into an analytic synthesis that links molecules to circuits and symptoms while identifying validated targets and endpoints for precision intervention. The motivation arises from the substantial patient and system burden created by overlapping syndromes and from the fragmentation of current evidence that impedes translation. This Reprint is addressed to clinicians, neuroscientists, psychiatrists, pain specialists, methodologists, and trainees seeking rigorous orientation and practical guidance. It is intended as a compact compass for designing adaptive trials and for implementing biomarker-informed care across the lifespan.

Masaru Tanaka and Simone Battaglia
Guest Editors

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Editorial

From Biomarkers to Behavior: Mapping the Neuroimmune Web of Pain, Mood, and Memory

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1. Setting the Stage: Mood, Memory and Pain in Clinical Convergence

Mounting evidence situates mood disturbance, memory decline, and chronic pain within a single neuro-immune conversation [1,2]. Cytokines, glial activation, and hypothalamic-pituitary-adrenal signals braid nociceptive and limbic circuits, so a flare in one domain can echo as affective dysregulation or cognitive drift [3–5]. Yet biology tells only half the story; social support, fear-avoidance beliefs, and lifestyle patterns modulate this signaling milieu, amplifying or dampening vulnerability [6,7]. Multimodal neuroimaging and plasma proteomics now reveal convergent fingerprints across depression, anxiety, dementia, and pain, hinting at transdiagnostic biomarkers and inviting predictive models that respect context [1,3,8–10]. Appreciating this intricate biopsychosocial weave is the first step toward interventions that target both the molecule and the milieu [11–13].

Despite rapid expansion of multimodal datasets across depression, anxiety, dementia, and chronic pain, robust transdiagnostic biomarkers remain elusive [14,15]. Meta-analytic neuroimaging reveals both shared and disorder-specific network alterations, while inflammatory and self-referential markers fragment across symptom clusters, underscoring heterogeneity that defeats single-signature diagnostics [8,16–19]. Clinical translation falters further when biomarker readouts shift with body mass, activity, sleep, medication exposure, and broader lifestyle ecologies; in some cohorts, sickness behavior outperforms cytokines for explaining psychological comorbidity [14,15,20,21]. These inconsistencies demand predictive modeling pipelines that integrate dimensional symptoms, multimodal biology, and environmental context to support individualized risk stratification and adaptive care pathways [15,16,22–24]. Without such integrative frameworks, precision interventions will continue to lag behind need in practice [14,15,25–27].

This editorial pursues four interconnected goals. First, we integrate preclinical and clinical evidence that maps convergent neuro-immune circuitry linking affective, cognitive, and nociceptive phenotypes. Second, we appraise the most promising, yet still provisional, transdiagnostic biomarkers—from network-level signatures to inflammatory proteomes—assessing their reproducibility and translational readiness. Third, we interrogate how lifestyle and environmental exposures, such as diet, physical activity, and psychosocial enrichment, recalibrate these pathways and modulate symptom trajectories. Finally, we outline robust predictive-modeling architectures that fuse multimodal biology with contextual data, ultimately offering a roadmap toward individually tailored prevention and treatment strategies for co-occurring mood, memory, and pain disorders.

2. Where the Gaps Were

Despite prolific datasets, the biological choreography linking affective dysregulation, mnemonic decline, and nociception remains speculative [28–32]. Neuro-immune signals correlate with symptom clusters, yet causal arrows are blurred by cross-sectional snapshots that average out temporal dynamics [28,33,34]. Most human studies still lean on single magnetic resonance imaging (MRI) scans and one-off cytokine panels, analytical conveniences that miss diurnal swings in network excitability or immune tone [28,33–36]. Consequently, conflicting associations proliferate and mechanistic narratives stall [28,30,33,37,38]. Progress will require multi-scale frameworks in which cell-type gene expression, circuit oscillations, and whole-brain connectomes are integrated within longitudinal designs, permitting inference on how perturbations ripple upward and downward across the neuro-immune hierarchy [28,30,34,39,40].

Longitudinal knowledge is perilously thin. Outside a handful of decade-long registries, few cohorts follow patients as mood symptoms, cognitive drift, and pain wax and wane across treatment cycles; without these trajectories, models cannot learn temporal signatures of risk or recovery [41–43]. At the same time, granular information that wearables, smartphones, and in-home sensors could supply remains largely untapped, leaving laboratory snapshots to stand in for lived experience [44–46]. When candidate biomarkers do emerge, translation stalls within siloed pipelines: discovery groups rarely collaborate with device engineers, regulators, frontline clinicians, and health-system decision-makers, so results languish in preclinical vaults. These fissures collectively stifle predictive algorithms, thwart stratified trials, and delay the timely delivery of precision care [44,47,48].

Five deficits frame the road ahead: first, a lack of mechanistic alignment linking molecular signals to whole-brain networks [49–51]; second, the absence of large, harmonized longitudinal cohorts to chart symptom trajectories [50,52,53]; third, biomarker panels that ignore contextual nuance and remain unstandardized [50,53,54]; fourth, predictive algorithms seldom stress-tested across diverse populations [49,50,52]; and fifth, fragile pipelines that fail to convert discoveries into patient-centered tools [49,52,54] (Table 1).

Table 1. Key research deficits in translational neuropsychiatry. Five major gaps hinder progress in precision neuropsychiatric care, spanning mechanistic integration, longitudinal tracking, biomarker standardization, algorithmic robustness, and translational pipelines.

	Deficit	References
1	Lack of mechanistic alignment linking molecular signals to whole-brain networks	[49–51]
2	Absence of large, harmonized longitudinal cohorts to chart symptom trajectories	[50,52,53]
3	Biomarker panels that ignore contextual nuance and remain unstandardized	[50,53,54]
4	Predictive algorithms seldom stress-tested across diverse populations	[49,50,52]
5	Fragile pipelines that fail to convert discoveries into patient-centered tools	[49,52,54]

3. Snapshot of the Ten Papers

3.1. Chronic Pain and Mechanisms

Zhang and colleagues uncover a unidirectional molecular cascade where spinal neurotrophic tyrosine kinase receptor type 1 (NTRK1) drives insulin-like growth factor II (IGF2) expression, fueling endoplasmic reticulum (ER) stress and sustaining chronic postsurgical pain [55]. Using the skin/muscle incision–retraction (SMIR) rat model, they show that blocking tropomyosin receptor kinase A (TrkA) or silencing IGF2 breaks this pathogenic loop. Their findings highlight a novel, non-opioid target for precision pain therapy. In this prospective real-world study, Della Vecchia and colleagues reveal that anti-calcitonin gene-related peptide/receptor (CGRP/R) monoclonal antibodies do more than just ease migraine pain [56]. They also alleviate depression, anxiety, fatigue, and allodynia in

treatment-resistant patients. Interestingly, even non-responders showed some psychiatric improvement, hinting at deeper neuroaffective effects worth exploring. (Table 2).

Table 2. Thematic clustering of ten recent papers relevant to mood, memory, and pain convergence. CGRP/R, calcitonin gene-related peptide/receptor; ER, endoplasmic reticulum; IGF2, insulin-like growth factor II; NTRK1, neurotrophic tyrosine kinase receptor type 1.

Thematic Group	Paper Title (Shortened)	References
Chronic Pain and Molecular Mechanism	Unidirectional Crosstalk Between NTRK1 and IGF2 Drives ER Stress in Chronic Pain	[55]
	Impact of Anti-CGRP/R mAbs on Comorbid Symptoms in Resistant Migraine	[56]
Comorbidities and Cognitive Decline	Periodontal Indices as Predictors of Cognitive Decline (PerioMind Cohort)	[57]
	Impact of Comorbid Conditions on Conversion from Mild Cognitive Impairment to Dementia	[58]
	Predictive Models for Progression to Major Neurocognitive Disorder	[59]
Neuroinflammation, Stress, and Depression	Blood Plasma Markers in Mice under Chronic Social Defeat Stress	[60]
	Neuroinflammation and Natural Antidepressants	[61]
Behavioral and Lifestyle Factors in Pain, Depression, and Anxiety	Physical Inactivity Amplifies Anxiety, Depression, and Neck Pain in Breast-Cancer Survivors	[62]
	Advances in Classifying Chronic Orofacial Pain via Biopsychosocial Models	[63]
Neurodevelopmental Disorders and Novel Therapeutic Targets	Yin/Yang Angiotensin System Components in Autism Spectrum Disorder; Astaxanthin as Therapy	[64]

3.2. Comorbidity and Cognitive Decline

This study brings fresh light to an urgent issue: how gum health might reflect brain health. Using data from the PerioMind Colombia Cohort, the authors show that specific periodontal indices—like gingival redness and pocket depth—closely align with mild cognitive impairment in older adults [57]. Their models suggest dentists could become frontline detectors of cognitive decline. This compelling study explores how comorbidities influence the transition from mild cognitive impairment to dementia. By clustering patients based on their comorbidity profiles, the authors uncover striking risk patterns—diabetes and obesity sharply accelerate decline, while traumatic brain injury shows surprisingly low progression [58]. It is a sharp call to personalize dementia risk management. This study offers a fresh lens on predicting dementia. Tracking 132 individuals with mild neurocognitive disorder over two years, the authors crafted a model that nails an 83.7% accuracy rate [59]. As for risk, it rises with alcohol and a higher body mass index (BMI). In terms of protection, female sex, strong praxis, and surprisingly, higher scores on the geriatric depression scale (GDS) prevail. This study uses simple inputs to obtain powerful foresight.

3.3. Neuro-Immune Stress Circuitry

Smagin and colleagues dive deep into the biological aftermath of social stress [60]. Using a robust mouse model, they reveal striking shifts in blood plasma proteins linked to depression and neurodegeneration. What stands out? A telltale signature of amyloid and apolipoproteins—mirroring patterns seen in Alzheimer's. A compelling read. This elegant review explores how neuroinflammation, oxidative stress, and mitochondrial dysfunction

converge in major depression—and how phytochemicals might disrupt that vicious cycle [61]. Drawing from both bench and bedside, the authors highlight natural compounds like curcumin and flavonoids as promising multitarget tools for mood modulation, with fewer side effects and broader neuroprotective impact than traditional drugs [65].

3.4. Lifestyle and Biopsychosocial Angles

In this timely study, the authors reveal how being inactive physically can turn up the volume on the connection between anxiety, depression, and neck pain in breast cancer survivors. Surprisingly, this link does not hold for low back pain [62]. Their message is clear: movement matters—and not just for the body, but the mind too. This timely and thorough review dives deep into the tangled web of chronic orofacial pain [63]. It challenges outdated diagnostic norms and champions a biopsychosocial lens—merging the International Classification of Diseases, Eleventh Revision (ICD-11); the International Classification of Headache Disorders, Third Edition (ICHD-3); the International Classification of Orofacial Pain (ICOP); and the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria. This way, the authors spark a necessary rethinking of classification and care, urging a unified, patient-centered approach.

3.5. Neurodevelopment and Novel Targets

Samra and colleagues thrust the carotenoid astaxanthin into the autism spotlight [60]. In valproic acid (VPA)-exposed rats, the pigment steered the renegade renin–angiotensin axis toward its protective arm, muted Notch signaling and nuclear factor kappa B (NF-κB) activation, untangled microtubule-associated protein tau, softened gliosis, and, crucially, coaxed the pups back into sociability. Biology and behavior clicked in one tidy preclinical arc.

4. Bridges Built: How These Studies Advance the Field

By deploying the SMIR rat paradigm, investigators traced a spinal NTRK1-triggered cascade that amplifies IGF2 expression and culminates in maladaptive ER stress, a trio now validated as driver of postsurgical pain [55]. Parallel real-world trials with anti-CGRP/R monoclonals show that targeting sensory peptides simultaneously softens migraine, depression, anxiety, and mechanical allodynia [56]. Together these studies fuse molecular neurobiology with bedside pain management, sketching an immediate roadmap for non-opioid interventions and broader psychiatric benefit in chronic headache populations [66–68].

Periodontal indices now function as surrogate biomarkers of neural integrity, with gingival redness, pocket depth, and alveolar loss tracking early cognitive slippage [57]. Clustering comorbidities such as diabetes, obesity, and cardiovascular burden sharpens dementia risk stratification by exposing synergistic rather than additive trajectories [58]. This cross-pollination between dentistry, neurology, and geriatric psychiatry recasts the dental clinic as a cognitive sentinel, enabling nuanced risk profiles that drive personalized interventions and feed next-generation prediction algorithms [57–59].

Plasma proteomic profiling after controlled social stress uncovers a signature that bridges cytokine flux with neurodegenerative cascades [60,69]. Parallel animal and cell studies show curcumin, quercetin, and related phytochemicals dampening microglial activation and improving mitochondrial function, positioning natural compounds as innovative neuroimmune modulators [61,70]. Integrating immunologic analytics with psychiatric phenotyping clarifies how inflammatory and mitochondrial crosstalk drives depressive symptoms [71]. These converging insights nominate biomarkers for depression and prodromal Alzheimer's disease while advancing low-toxicity phytochemical therapeutics over conventional antidepressants [72–74].

By isolating physical inactivity within post-cancer cohorts, targeted analyses compute novel multivariate paths, exposing sedentarism as a catalyst of the anxiety-depression-pain triad in breast cancer survivors [62,75,76]. Psychology, oncology, and rehabilitation converge, refreshing the biopsychosocial paradigm [77–79]. Such transdisciplinary clarity reorients clinical priorities. Evidence thus advocates early, personalized activity prescriptions to blunt cascading affective and nociceptive burdens and to integrate mental-health prevention into routine oncology pain care [75,77,78,80].

By fusing ICD-11, ICHD-3, ICOP, and DSM-5 within a biopsychosocial scaffold, these studies recalibrate neurodevelopmental disorder nosology, exposing phenotypes that transcend categorical silos [63,81,82]. Such conceptual innovation invites neurologists, psychiatrists, dentists, and developmental scientists into a shared space, aligning biomarkers with behavioral heuristics and unveiling novel therapeutic targets [81,83]. Clinically, patient-centered algorithms and interdisciplinary guidelines emerge, accelerating therapeutics from bench to bedside [81,82,84].

5. Still Unpaved Roads: Future Research Directions

5.1. Multi-Omics Mega-Cohorts

Big data only matters if it stays in step with biology [85–87]. The next wave should launch mega-cohorts that blend single-cell transcriptomics, proteomics, metabolomics, and connectomics with repeated neuroimaging and biosamples every three to six months [85,88–90]. Such "rolling deep phenotypes" will let us watch how a flare of neuropathic pain, a dip in mood, or a slip in recall leaves footprints from microglia to default-mode hubs [91–93]. Harmonized pipelines and open cloud workspaces will finally allow algorithms to trace directional signals, validating the mechanistic hypotheses flagged in the gaps above and building a living atlas that evolves with each new participant—and across diverse ancestries, socioeconomic contexts, and life stages, capturing latent resilience alongside vulnerability [94,95].

5.2. Longitudinal Pain-Mood Tracking and Lifestyle-Targeted Randomized Controlled Trials (RCTs)

Pair those omics streams with real-world longitudinal tracking. Smartphone diaries, passive gait sensors, and cross-platform ecological momentary assessment (EMA) can capture minute-to-minute swings in pain intensity, affect, sleep, and cognitive fog [96–99]. By feeding those trajectories into adaptive, lifestyle-targeted randomized controlled trials (RCTs)—think exercise-timing, personalized nutrition, light therapy, or mindfulness-dose algorithms—we can test causal levers in near real time [98,100]. Each participant becomes a micro-trial, and aggregated n-of-1 readouts will reveal who benefits, when, and why [100]. This approach does more than shrink sample sizes; it closes the discovery-translation loop, letting mechanistic insights instantly inform behavioral prescriptions delivered at the wrist [98,100].

5.3. Cross-Talking Neuro-Immune Combination Therapies

The final gap is therapeutic: neuro-immune dialog rarely respects disciplinary borders. Hybrid interventions that co-modulate cytokine tone and neural circuit excitability—such as low-dose interleukin (IL)-1 antagonists paired with transcranial alternating current, vagus-paced breathing, or gut-microbiota modulators—could rewrite maladaptive set points [101–103]. We need factorial trials that cross these modalities, mapped against the multi-omics atlas, to pinpoint synergistic nodes [104–106]. A regulatory sandbox shared by neuroscientists, immunologists, and digital-health engineers would speed iteration, while federated analytics guard privacy. Success would move us from symptom silos to network rewiring, turning the still-unpaved road into an integrated highway toward precision recovery [107].

6. Take-Home Message

Together, these ten papers narrow down the critical mechanistic and translational gaps—further developing our understanding of how mood, memory, and pain braid through neuro-immune circuitry—while simultaneously prying open fresh investigative frontiers. From spinal NTRK1-IGF2 loops that propel postsurgical hyperalgesia to gingival biomarkers that foreshadow cognitive drift, this collection delivers concrete answers yet sparks new questions about bidirectional brain–body crosstalk. Proteomic signatures, connectomic shifts, and wearable-captured behaviors now coalesce into a reproducible risk compass, steering clinicians toward earlier, kinder interventions. Lifestyle, comorbidity, and social context emerge not as confounders but as tunable levers, suggesting that a step counter or food log can be as informative as a positron emission tomography (PET) scan. The horizon beckons with multi-omics cohorts tethered to smartphone phenotyping, adaptive n-of-1 trials that turn each patient into their own laboratory, and combination neuro-immune therapies poised to retune both cytokine tone and cortical rhythm. With the stage now set, the next wave of precision relief is already cresting.

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Abbreviations

The following abbreviations are used in this manuscript:

CGRP/R	Calcitonin gene-related peptide/receptor
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
ER	Endoplasmic reticulum
IGF2	insulin-like growth factor II
ICD-11	International Classification of Diseases, Eleventh Revision
ICHD-3	International Classification of Headache Disorders, Third Edition
ICOP	International Classification of Orofacial Pain
NTRK1	Neurotrophic tyrosine kinase receptor type 1

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Preclinical Evidence for the Role of the Yin/Yang Angiotensin System Components in Autism Spectrum Disorder: A Therapeutic Target of Astaxanthin

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Abstract: Autism spectrum disorder (ASD) prevalence is emerging with an unclear etiology, hindering effective therapeutic interventions. Recent studies suggest potential renin–angiotensin system (RAS) alterations in different neurological pathologies. However, its implications in ASD are unexplored. This research fulfills the critical gap by investigating dual arms of RAS and their interplay with Notch signaling in ASD, using a valproic acid (VPA) model and assessing astaxanthin's (AST) modulatory impacts. Experimentally, male pups from pregnant rats receiving either saline or VPA on gestation day 12.5 were divided into control and VPA groups, with subsequent AST treatment in a subset (postnatal days 34–58). Behavioral analyses, histopathological investigations, and electron microscopy provided insights into the neurobehavioral and structural changes induced by AST. Molecular investigations of male pups' cortices revealed that AST outweighs the protective RAS elements with the inhibition of the detrimental arm. This established the neuroprotective and anti-inflammatory axes of RAS (ACE2/Ang1-7/MasR) in the ASD context. The results showed that AST's normalization of RAS components and Notch signaling underscore a novel therapeutic avenue in ASD, impacting neuronal integrity and behavioral outcomes. These findings affirm the integral role of RAS in ASD and highlight AST's potential as a promising treatment intervention, inviting further neurological research implications.

Keywords: astaxanthin; Notch1 receptor; renin–angiotensin system; autism spectrum disorder; gliosis; tauopathies; Mas receptor; carotenoids; valproic acid; rats

1. Introduction

Autism, a neurodevelopmental disorder, is classified as a "spectrum" disorder (ASD) since it displays a range of symptoms during early childhood [1], including motor challenges [2], abnormal social communication, and restricted/repetitive movements [3,4] due to alterations in different brain areas [2]. Indeed, cortical/subcortical overgrowth and dysfunction were detected in post-mortem samples [5].

ASD originates from genetic, environmental, and mother-and-child-associated risk factors [4,6]. The prenatal environment includes exposure to harmful substances, infections, and medication use [7], such as in the case of valproic acid (VPA), which is used in neurological and psychiatric disorders. VPA in pregnancy can cause ASD [8], initially known as fetal valproate syndrome [9,10], partly by increasing oxidative stress, which is detected in ASD patients [11,12].

ASD is not a standalone disorder but is associated with other neurological conditions such as anxiety disorders [13], depression [14], and epilepsy [15,16]. In addition, ASD is highly engaged with Alzheimer's disease (AD), sharing common characteristics despite impacting individuals at different stages of life [6]. Similar symptoms as dementia, cognitive impairment, speech difficulties, and intellectual disability are common in both diseases, in addition to the presence of different signaling pathways such as the Notch signal [17] and the processing mechanism of amyloid-β precursor protein (APP) [18], highlighting their overlapping pathophysiology. Recently, the renin–angiotensin system (RAS) has participated in the pathophysiology of AD and other neurodegenerative diseases [19], but not in ASD so far. Moreover, the aberrant phosphoinositide 3-kinase (PI3K) signaling pathway contributes to ASD pathogenesis since the PI3K/protein kinase B (Akt) axis has a crucial role in neuronal morphogenesis and survival [20,21]. The cardiogenic effect of the Ang 1-7/Mas receptor was linked to the PI3K/Akt survival signal; however, Mas receptor, PI3K, or Akt inhibitors unequivocally abolish the beneficial effects [22].

Furthermore, accumulated amyloid-β (Aβ) plaques and the presence of hyperphosphorylated tau (p-tau) protein characterize AD [23], as well as autism [24,25]. Therefore, it is noteworthy to understand the tau pathomechanisms in autism and to delve deeper into the effects of reducing tau levels on neurogenesis since its reduction prevents excessive brain growth (megalencephaly) and alleviates behavioral abnormalities, which are facts that nominate it as a targeted therapy against ASD [26].

To date, p-tau is known to be augmented by different kinases, including activated glycogen synthase kinase (GSK)-3β and cyclin-dependent kinase 5 (CDK5) [27]. GSK-3β is activated when the PI3K/Akt axis is turned off [21,28], whereas the effect of CDK5 is mediated by calpain-dependent cleavage of p35 to give the active p25 moiety [21,25]. However, CDK5 enhances learning and memory by playing a role in synaptic plasticity when bound to p35 [29]. Apart from these kinases, our comprehension of the mechanisms underlying tau hyperactivation remains incomplete.

For example, Notch signaling is implicated in AD and ASD [17]. Moreover, neurofibrillary tangles in AD brains contain Notch1, which overlaps with p-tau in plaque-like structures [30]. A previous study suggested a potential association between tau aggregates and Notch-1 expression in neurodegenerative diseases [31].

Furthermore, limited research has been conducted on the involvement of the renin-angiotensin system (RAS) in this process. In one study, the activation of the angiotensin II (Ang II)-converting enzyme (ACE) enhanced p-tau, while its inhibition decreased p-tau [32]. A subsequent study clarified that Ang II activates tau by stimulating CDK5, which is reversed by losartan [33]. Nonetheless, the activated Yang arm of RAS (ACE2/Ang1-7/Mas receptor hub) reduced tau pathology in both AD and Parkinson's disease (PD) models [34, 35]. In addition, AD, with its hallmark p-tau, is linked to decreased ACE2 [36]. Although not extensively studied in autism, one study linked ASD to ACE genetic variations [37].

Although the search for a specific pathology underlying ASD is still challenging, recent research suggests that glial cell pathology may be a defining feature of ASD. Astrocytes, the most abundant glial cells in the brain, play a crucial role in neuronal function during development and adulthood [12]. An altered astrocyte number and function have been linked to impaired connectivity, highlighting the potential role of activated astrocytes in neuronal disorders, including ASD [38,39]. Though limited, the available data indicates that while the number of astrocytes is reduced in ASD, their activation level and hallmark glial fibrillary acidic protein (GFAP) expression are increased [12]. Similarly, GFAP is elevated in the cerebrospinal fluid of ASD patients [40]. However, further research is necessary to better understand the role of astrocytes in ASD. Autistic patients with increased levels of secreted APP-α show increased GFAP expression and Notch1 alteration [41]. Furthermore, the activated Notch1/Hes axis promotes the phosphorylation of signal transducer and activator of transcription 3 (STAT3), which is responsible for the expression of GFAP [42,43]. While no correlation was found between RAS and GFAP in neuronal disorders, a previous

study reported that the renin inhibitor (aliskiren) reduced LPS-induced astrogliosis in a depression model [44].

Although recent studies highlighted crosstalk between RAS and Notch1, few data are available on their link to neuronal disorders, autism, tauopathy, or astrogliosis. In an in vitro study, Ang II upregulated Notch1 to mediate its harmful effect on renal podocytes [45], while the activation of angiotensin receptor 1 (AT1R) activated the Notch pathway in human vascular smooth muscle cells [46].

ASD lacks reliable treatments due to its diverse pathomechanisms [47]. Hence, preclinical studies are necessary to find new therapies and evaluate their potential mechanisms. Astaxanthin (AST), a natural xanthophyll carotenoid, may hold promise as an adjunctive treatment for autism and neurodegenerative diseases. AST has potent antioxidant capabilities [48] and can cross the blood–brain barrier [49], characteristics that make it a potential option, as proven in a VPA-induced ASD model [50]. However, more data are needed to unveil additional mechanisms beyond its antioxidant role in autism.

We investigated the potential of AST in treating tauopathy and astrogliosis associated with ASD. We hypothesize that AST's modulation of the Yin/Yang arms of RAS and correction of the inflammatory signal may prove effective. We used a prenatal VPA-induced ASD model in rats to explore this hypothesis. Indeed, animal models are essential in understanding ASD since they mimic certain traits observed in ASD patients.

2. Materials and Methods

Animal models play a pivotal role in elucidating the intricacies of ASD. These models are reliable in translational research to replicate clinical behavioral and neurobiological traits of ASD. Moreover, as highlighted previously, they are instrumental in exploring potential therapeutic avenues such as mTOR, GABAergic, and glutamatergic systems [51,52]. These insights have spurred efficient interventions in reversing behavioral anomalies in these models. A comparative analysis drew intriguing parallels between clinical observations in ASD patients and findings in animal models [52]. In that review, various ASD animal models, including environmental-induced (e.g., VPA), immune-induced (e.g., maternal immune activation), and genetic (e.g., Shank3 deletion) models, were examined. Among these, the VPA model produced the most pronounced and severe phenotypes across behavioral and cellular aspects with gender relevance, especially to males. This model effectively simulated the consequences of prenatal exposure to certain environmental factors, such as medications, on neurodevelopmental outcomes. However, the review acknowledged the limitations of animal models, emphasizing that not all ASD symptoms, such as cognitive stereotypy, intellectual disability, and speech deficits, are reproducible in rodents.

Moreover, animal models prove to be a critical aspect in understanding the comorbidities often accompanying autism. These comorbidities, like epilepsy [53], mood disturbance [54], and other psychiatric disorders [55], have been noted in animals and have been attributed to the lens of disturbances in the tryptophan–kynurenine metabolic pathway [55].

In our quest to adopt a reliable, reproducible, ethically sound, and methodologically valuable approach to investigating the complex mechanisms underlying ASD, we utilized the in utero VPA model. Specifically, the use of VPA during pregnancy, a prevalent strategy for managing central disorders, often leads to fetal valproate syndrome, reinforcing the ecological validity of this model's findings. In this study, a methodological decision was made to utilize male offspring to parallel the gender ratio discrepancy observed in ASD prevalence, where males are significantly more affected than females. This is compatible with [55], who documented the behavioral traits of ASD in male offspring more than in female offspring.

2.1. Animals and Induction of the ASD Model

After one week of acclimatization at the institutional animal facility (Faculty of Pharmacy, Cairo University), adult 8-week-old male and female Wistar rats (200–250 g; El-Salam

Breeding Center, Cairo, Egypt) were allowed to mate. Nine females were examined every morning for the presence of vaginal plugs or spermatozoa in vaginal smears to confirm pregnancy. Once confirmed, the date was designated as gestational day 0.5 (G 0.5) [56]. A well-established, reliable, and integral autism model, namely, the in utero-VPA paradigm, was used to induce neurotypical autistic signs in offspring, following previous studies [57–60], in which 6 pregnant female rats received a single intraperitoneal injection of 500 mg/kg sodium VPA (Depakine; SANOFI, Paris PA, France) [57–60] at the critical period of exposure to VPA [61]. Similarly, the other 3 normal control pregnant female rats received an equal volume of distilled water. The experimental protocol was followed with minor modifications regarding the housing of the animals. Instead of single caging, dams were maintained with their partner till the weaning of the offspring to avoid cannibalism or maternal death noted in our pilot study, resulting from pre-gestational stress, which increases vulnerability to depression [62]. Pups were weaned on postnatal day (PND) 23, and the male offspring enrolled in our study were separated into new cages until the end of the experimentation. Notably, all rats were kept under controlled environmental conditions of constant temperature (23 ± 2 °C), humidity (60 ± 10%), and light/dark cycle (12/12 h; lights on at 6:00 a.m.) and were allowed free access to standard chow pellets and water ad libitum. The handling of animals strictly adhered to the Guide for the Care and Use of Laboratory Animals protocol (NIH publication No.85-23, 2011) adopted by the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (Cairo, Egypt; PT 2613). All efforts were exerted to minimize animal suffering during the experimental period.

2.2. Experimental Design

On PND 34 (early adolescence), animals were allocated into 3 groups containing 9 rats each. The in utero saline-injected male offspring (group 1) served as the control group (CONT), whereas VPA-exposed male offspring were randomly allocated into two groups (2 and 3). Animals in group 2 were left untreated and designated as the model group (ASD), whereas those in group 3, were administered AST daily (25 mg/kg; Nutrex Hawaii Inc., Kailua-Kona, HI, USA) [63] until PND 58 (early adulthood). Animals in the first two groups (CONT and ASD) received distilled water p.o daily for 25 days (PND 58). Starting from PND 56, behavioral tests were performed. The marble burying test (MBT) was conducted to assess repetitive, stereotyped behavior, whereas the three-chamber test (3CT) was carried out on PND 57 to assess sociability and social novelty preference. Finally, general locomotor activity was assessed in open field apparatus on PND 58 (Figure 1).

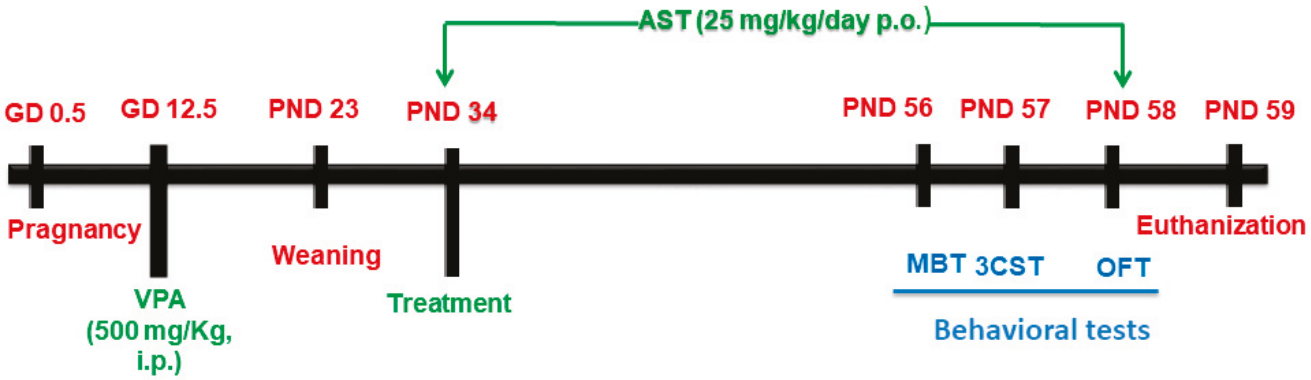


Figure 1. Schematic timeline for prenatal VPA model induction, AST treatment schedule, behavioral experiments, and euthanization. AST: astaxanthin; 3-CST: three-chamber sociability test; GD: gestational day; MBT: marble burying test; OFT: open field test; PND: postnatal day.

2.3. Assessment of Autistic-Like Behaviors

2.3.1. Marble-Burying Test (MBT)

In this test, rats were placed individually in a standard Plexiglas cage containing a 5 cm depth of clean bedding. Fifteen glass marbles (15 mm diameter) were evenly

placed in 3 rows on the bedding, and animals were tested for 20 min for burying/hiding behavior. The number of marbles buried with bedding up to two-thirds of their depth was counted [64,65].

2.3.2. Three-Chamber Sociability Test

The test was conducted as mentioned before [60,61,66]. The three-chamber apparatus (60 × 45 × 45 cm) is divided into three connected chambers with openings between the compartments, allowing the animals to freely explore the three chambers, and a wire cage is placed in each of the lateral compartments. Notably, the chambers were cleaned with 70% alcohol after each tested animal. The 3-CST consisted of three phases: the habituation phase, in which the tested rat was placed in the central chamber before the sociability phase and was allowed to explore for 5 min. The second is the sociability phase, which started directly after the habituation phase, where a novel rat (novel rat 1) of the same age, sex, and strain was placed into a wire cage in one of the lateral chambers, and the other wire cage was left empty, representing the novel object. The tested rat was left for 10 min, and the time spent exploring novel rat 1 or the novel object was recorded. The sociability index (*SI*) was calculated as follows:

$$SI = \frac{(\text{time exploring novel rat 1} - \text{time exploring novel object})}{(\text{time exploring novel rat 1} + \text{time exploring novel object})} \quad (1)$$

The third phase is the social novelty preference phase, which starts directly after the sociability phase for an additional 10 min. A new, unfamiliar rat was placed into the opposite chamber's empty wire cage and considered "novel rat 2". This session evaluates the preference for the social novelty of the tested rat to choose whether to interact with the familiar animal (novel rat 1) or the novel one (novel rat 2). The social novelty preference index (*SNI*) was calculated using the following equation:

$$SNI = \frac{(\text{time exploring novel rat 2} - \text{time exploring familiar rat})}{(\text{time exploring novel rat 2} + \text{time exploring familiar rat})} \quad (2)$$

2.3.3. Open Field Test (OFT)

A 60 × 60 × 45 cm quadrangular arena divided into 16 equal squares was used. Rats were allowed to acclimatize to the test room for 30 min before starting the experiment, and then each rat was individually placed in the center of the dimly illuminated arena for 5 min. The number of crossed squares visited with all 4 feet, the entries to the central zone compromising the central 4 squares, the time spent in this zone, and the latency to leave it were recorded. In addition, the duration (s) of rearing and grooming was documented. The test arena was cleaned with 70% alcohol after each rat [67,68].

2.4. Cortical Processing

After behavioral testing, all animals were euthanized under deep anesthesia (Phenobarbital 100 mg/kg) followed by transcardiac perfusion with phosphate-buffered saline (PBS; pH 7.4), and the cortices were rapidly dissected and divided into 3 sets (each containing 3 rats). The left hemispheres of animals in the first set were fixed in 10% neutral buffered formalin for histopathological/immunochemical examinations, whereas those of animals in the second set were kept in glutaraldehyde for electron microscopy. From 3 animals in set 3, the left cortices were submerged in a RIPA buffer with protease and phosphatase inhibitors for measurements with Western blots. After that, the right cortex of the first 3 rats (set 1) was placed in lysis buffer for qRT-PCR analysis, and the remaining right cortices from rats in sets 2 and 3 were flash frozen in liquid nitrogen and then homogenized in PBS and aliquoted for the assessment of ELISA parameters and stored at −80 °C.

2.4.1. Assessments of $p^{(S396)}$-tau, $p^{(Y458/199)}$-PI3K p85/p55, Notch1, NICD, p25, and p35 Using the Western Blot Technique

Following cortical protein quantification (Bio-Rad Protein Assay Kit, Hercules, CA, USA), 10 μg protein of each sample was separated using SDS polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and then blocked with 5% bovine serum albumin (BSA). Afterward, the membrane was incubated with anti-$p^{(S396)}$-tau (cat#: 35-5300), anti- $p^{(Y458/199)}$-PI3K p85/p55 (cat#: PA5-17387), anti-Notch1 (cat#: PA5-23181), anti-NICD-1 (cat#: PA5-99448), anti-P35 (cat#: MA5-14834), or β-actin (cat#: PA1-183) polyclonal antibody (ThermoFisher Scientific, Waltham, MA, USA) and anti-P25 (ABClonal Technology, Woburn, MA, USA; cat#: A4637) overnight at 4 °C on a roller shaker. Next, the membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dianova, Hamburg, Germany). Finally, the blots were developed with an enhanced chemiluminescence detection reagent (Amersham Biosciences, Chicago, IL, USA), and the corresponding expressed protein was quantified with densitometric analysis using a scanning laser densitometer (GS-800 system, Bio-Rad, Hercules, CA, USA). The results were expressed as arbitrary units (AUs) after normalization to β-actin.

2.4.2. Assessments of Cortical Gene Expressions of Mas Receptor with qRT-PCR

Total RNA was extracted from cortical tissue using an RNeasy Mini kit (Qiagen, Venlo, Netherlands), and the obtained RNA was verified for pureness spectrophotometrically at OD 260/280 nm. According to the manufacturer's procedure, equal amounts of RNA (1 μg) were reverse transcribed into cDNA using a reverse transcription system (Promega, Leiden, Netherlands). The qRT-PCR of the Mas receptor was performed using SYBR Green Master Mix (Applied Biosystems, Waltham, CA, USA). In a 25 μL reaction volume, 5 μL of cDNA was combined with 12.5 μL SYBR Green mixture, 5.5 μL RNase-free water, and 2 μL of each primer (Table 1). The PCR amplifications were accomplished with 40 cycles for 15 s at 95 °C (denaturation), 60 s at 60 °C (annealing), and 60 s at 72 °C (extension). The relative expression of the target gene was obtained using the $2^{-\Delta\Delta CT}$ formula using β-actin as a housekeeping gene.

Table 1. Primer sequences used for qPCR.

Gene Name	Primer Sequence (5′–3′)	Accession Number
MAS receptor	F: TGACCATTGAACAGATTGCCA R: TGTAGTTTGTGACGGCTGGTG	NM_153722
β-Actin	F: CCCATCTATGAGGGTTACGC R: TTTAATGTCACGCACGATTTC	NM_031144.3

2.4.3. Quantification of Cortical Contents of Ang II, ACE2, and Ang1-7 Using the ELISA Technique

MyBioSource ELISA kits (San Diego, CA, USA) were used for the determination of Ang II (cat#: MBS705139), ACE2 (cat#: MBS014209), and Ang1-7 (cat#: MBS2604372). All procedures were performed according to the manufacturer's instructions.

2.5. Microscopic Investigation

For both histopathological and immunohistochemistry inspections, brain specimens were embedded in paraffin, and sections of 5 μm thickness were blindly inspected by a pathologist under a light microscope (BX43, Olympus, Tokyo, Japan) and photographed using Cellsens dimension software version 4.1 CS-EN-V4.1 (Olympus) connected to an Olympus DP27 camera. The Nissl staining and immune reactivity of the corresponding antigens were assessed using image analysis software (Image J, version 1.46a, NIH, MD, USA). Measurements were prepared from five non-overlapping randomly chosen fields in each section at 200× magnification and averaged.

2.5.1. Hematoxylin and Eosin Staining

Brain sections were stained with hematoxylin and eosin (H & E), and the cortical neuropathologic damage was graded from 0 to 4 as follows: (0) indicated no changes; (1) indicated <10% area affected; (2) indicated 20–30% area affected; (3) indicated 40–60% area affected; and (4) indicated >60% area affected [69].

2.5.2. Nissl Staining

Brain sections were stained with cresyl violet to analyze the intensity of the Nissl proteins in cortical neurons. Brain sections mounted on gelatinized slides were air-dried and stained with 0.1% aqueous cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 60 °C. The slides were then washed in distilled water, differentiated in 70% ethyl alcohol, dehydrated in ascending grades of ethyl alcohol, and cleared in xylene to be mounted with DPX (Sigma-Aldrich). The number of both intact and degenerated neurons was counted.

2.5.3. Cortical GFAP and NF-κB p65 Immunoreactivity

The deparaffinized and rehydrated sections were incubated with primary rabbit monoclonal GFAP antibody (Cat#: SAB4501162; Merck KGaA, ON, Canada) or primary mouse monoclonal nuclear factor-kappa B (NF-κB) p65 (cat #: sc-8008; Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibody. Subsequently, they were washed with PBS and incubated with a secondary antibody using the HRP EnVision kit (DAKO, Santa Clara, CA, USA). Sections were developed and visualized using diaminobenzidine tetrachloride. The substrate system produced a crisp brown end-product at the site of the target antigen. Sections were counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and covered and slipped for microscopic examination. GFAP and NF-κB p65 quantification was estimated by measuring the area% immune expression.

2.5.4. Electron Microscopy

For transmission electron microscopy (TEM) preparation, the samples were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.0) for 2 h at room temperature, rinsed in the same buffer, and post-fixed in 1% osmium tetroxide for 2 h at room temperature. The samples were dehydrated in an ethanol series ranging from 10% to 90% for 15 min in each alcohol dilution and finally with absolute ethanol for 30 min. The samples were infiltrated with epoxy resin and acetone through a graded series until they were finally in pure resin. Ultrathin sections were collected on formvar-coated copper grids. The sections were then double stained in uranyl acetate followed by lead citrate. The stained sections were observed with a JEOL JEM 1010 transmission electron microscope at 70 kV at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University (Cairo, Egypt).

2.6. Statistical Analysis

For parametric data, values were expressed as mean ± SD, and statistical differences between groups were computed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests. For non-parametric data, values were expressed as median (minimum-maximum) and analyzed using a Kruskal–Wallis test followed by a Dunnett's post hoc or Mann–Whitney test to compare every 2 groups. The level of significance was set at $p < 0.05$. GraphPad Prism version 9.4.1 (GraphPad Software Inc., San Diego, CA, USA) software was used to carry out all the statistical tests and to draw the corresponding figures.

3. Results

3.1. AST Increases Sociability While Reducing Repetitive/Compulsive Behaviors in Autistic Rats

Both impaired social and repetitive/compulsive behaviors are diagnostic hallmarks of ASD. During the 3-CST, male autistic rats (Figure 2A) displayed significant reductions in the SI (A) and SNI (B) by 59% ($F_{(2,24)}$ = 196.7, $p < 0.0001$) and 120% ($F_{(2,24)}$ = 818.5,

$p < 0.0001$), respectively compared with the CONT group. However, socialization improvement was noted following AST treatment, where the carotenoid abrogated social withdrawal and normalized both SI ($F_{(2,24)} = 196.7$, $p < 0.0001$) and SNI ($F_{(2,24)} = 818.5$, $p < 0.0001$) in VPA in utero exposed offspring. Additionally, VPA exposure enhanced repetitive stereotyping in the marble burying test, as indicated by the 4-fold ($F_{(2,24)} = 77.85$, $p < 0.0001$) increment in the number of hidden marbles (C) compared with the CONT group. This repetitive compulsive-like disorder was, however, normalized in the AST-treated group, indicating reduced disruptive behavior and social discourse difficulties ($F_{(2,24)} = 77.85$, $p < 0.0001$). A defective exploration of the surroundings was evaluated with the OFT (Figure 3) and manifested by the significant decrease in the total number of crossed squares (A) by 55% ($F_{(2,24)} = 51.56$, $p < 0.0001$), along with the entries to the central zone (B) by 67% ($F_{(2,24)} = 44.80$, $p < 0.0001$). These decrements were associated with a 2-fold ($F_{(2,24)} = 107.7$, $p < 0.0001$) prolongation of time spent in the central zone (C) and latency (D) (2 folds; $F_{(2,24)} = 26.87$, $p < 0.0001$) along with a reduction in rearing behavior (E) by 59% ($F_{(2,24)} = 42.92$, $p < 0.0001$). In addition, animals experienced a longer duration of grooming (F) (4.7 folds; $F_{(2,24)} = 100.4$, $p < 0.0001$), denoting higher repetitive stereotyped behaviors compared with the CONT group. In contrast, AST-enhanced locomotion was indicated by a large number of crossed squares (1.8 folds; $F_{(2,24)} = 51.56$, $p < 0.0001$) and entries to the central zone (2.2 folds; $F_{(2,24)} = 44.80$, $p < 0.0001$) with a shorter time spent in the central zone (30%; $F_{(2,24)} = 107.7$, $p < 0.0001$) compared with the VPA untreated group. Additionally, AST normalized the latency to leave the central zone (45%; $F_{(2,24)} = 26.87$, $p < 0.0001$), increased rearing to 2 folds ($F_{(2,24)} = 42.92$, $p < 0.0001$), and abrogated grooming (41%; $F_{(2,24)} = 100.4$, $p < 0.0001$), as compared with the ASD group. The amendments mentioned above in AST-treated rats signify the ability of AST to improve sociability and reduce repetitive/compulsive core signs of autism.

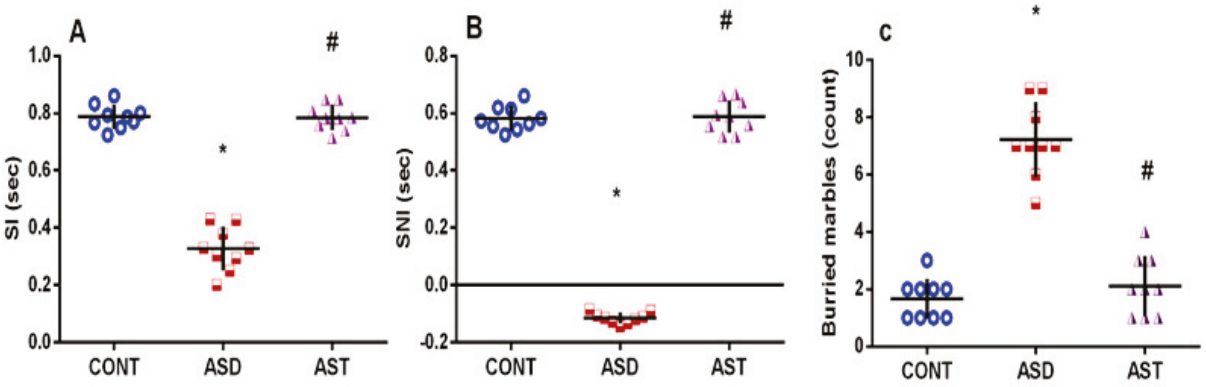


Figure 2. Effect of AST on (**A**) SI and (**B**) SNI in 3-CST, and (**C**) MBT in autistic rats. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) on PND 34 (early adolescence) until PND 58 (early adulthood). Values are expressed as mean ± SD ($n = 9$ rats/group). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, as compared with the CONT (*) and ASD (#) groups. ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; 3-CST: three-chamber sociability test; GD: gestational day; MBT: marble burying test; p.o: per os; PND: postnatal day; SI: sociability index; SNI: social novelty preference index; VPA: valproic acid. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

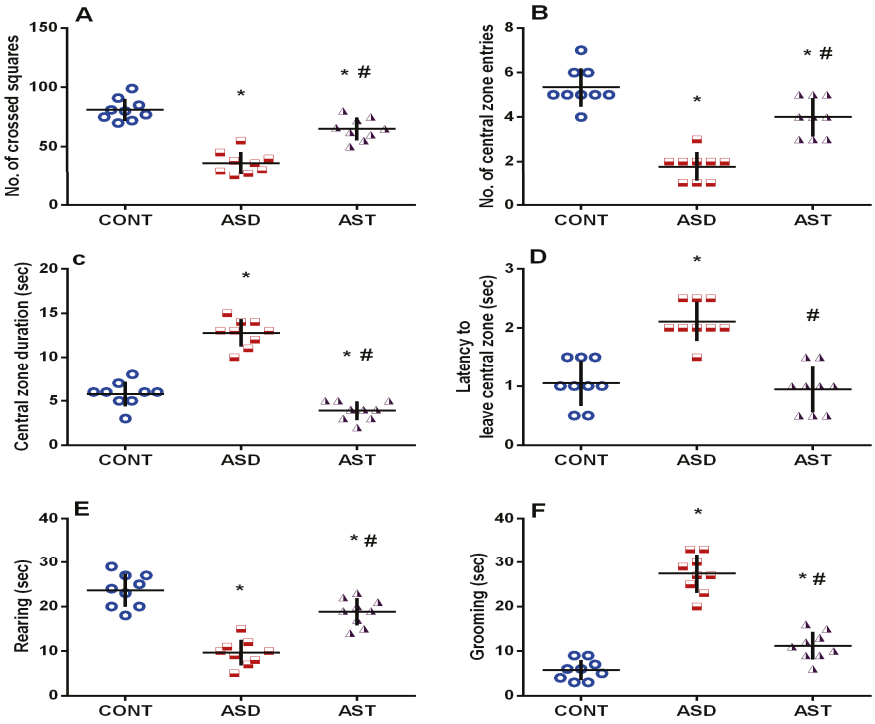


Figure 3. Effect of AST on locomotor behavior and exploration of surroundings using the OFT test in autistic rats: (**A**) the total number of crossed squares; (**B**) frequency of entering the central zone; (**C**) stay duration in the central zone; (**D**) latency to leave the central zone; (**E**) duration of rearing; and (**F**) grooming. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) on PND 34 (early adolescence) until PND 58 (early adulthood). Values are expressed as mean ± SD (n = 9 rats/group). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, as compared with the CONT (*) and ASD (#) groups. ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; GD: gestational day; OFT: open field test; p.o: per os; PND: postnatal day; VPA: valproic acid. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

3.2. AST Modulates Cortical AngII/ACE2/Ang1-7/Mas Receptor/Trajectory and Augments PI3K p85/p55 in Autistic Rats

As depicted in Figure 4, the ASD model resulted in a significant increase of 3.4-fold ($F_{(2,15)}$ = 1887, $p < 0.0001$) in the cortical content of Ang II (A), a vital activator of the canonical RAS. However, ASD reduced ACE2 (B), an enzyme that catalyzes the hydrolysis of Ang II into Ang1-7 for homeostatic regulation, as well as Ang1-7 (C), a heptapeptide that counterbalances the Ang II harmful effects, by 72% ($F_{(2,15)}$ = 264.8, $p < 0.0001$) and 74% ($F_{(2,15)}$ = 892.1, $p < 0.0001$), respectively. Additionally, the mRNA expression of Mas receptor (D), the Ang1-7 receptor that activates the alternative protective RAS arm, was downregulated by 71% ($F_{(2,6)}$ = 20.87, p = 0.0019). Furthermore, the insult led to a depletion of 73% in the protein expression of (E) $p^{(Y458/199)}$-PI3K p85/p55 ($F_{(2,6)}$ = 53.63, p = 0.0001), a downstream transducer for the Mas receptor signaling. However, treatment with AST effectively corrected the altered molecules of RAS and increased PI3K protein expression to nearly normal levels across most parameters. Hence, modulation of the Yin/Yang RAS axis can be a novel player in the pathomechanism of ASD and can be one of the anti-autistic mechanisms of AST by reducing Ang II, activating the ACE2 enzyme to trigger the Ang1-7/Mas receptor axis, and stimulating its neuroprotective downstream target PI3K p85/p55.

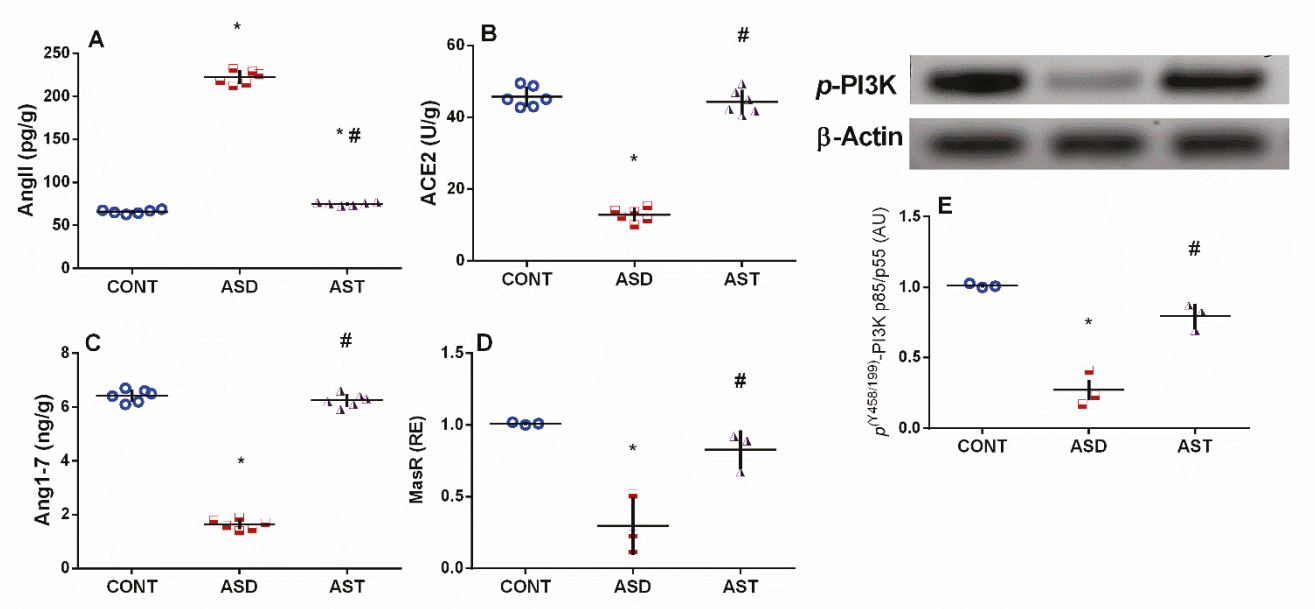


Figure 4. Effect of AST on cortical contents/gene expression of (**A**) Ang II, (**B**) ACE2, (**C**) Ang1-7, (**D**) MasR, and (**E**) p(Y458/199)-PI3K p85/p55 of autistic rats. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) on PND 34 (early adolescence) until PND 58 (early adulthood). Values are expressed as mean ± SD (n = 3 rats/group for PCR and 6 rats/group for ELISA). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, as compared with the CONT (*) and ASD (#) groups. ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; ACE2: angiotensin-converting enzyme 2; Ang1–7: angiotensin 1–7; Ang II: angiotensin 2; MasR: Mas receptor; p.o: per os; p(Y458/199)-PI3K: phosphorylated phosphoinositide 3-kinase at tyrosine 458 and 199; PND: postnatal day; VPA: valproic acid; GD: gestational day. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

3.3. AST Deactivates the Cortical Notch1/NICD-1/NF-κB Trajectory and Increases the Cortical p25/p35 Ratio in Autistic Rats

Figure 5 shows that the autistic rats displayed an elevated protein expression of Notch1 (A), a single-pass transmembrane receptor activated by Ang II and CDK5/p25 kinase, and its downstream intracellular signaling molecule NICD-1 (B). These parameters were increased by approximately 6.7 fold ($F_{(2,6)} = 74.80$, $p < 0.0001$) and 6 fold ($F_{(2,6)} = 52.58$, $p = 0.0002$), respectively. Additionally, the insult led to a downregulation in the protein expression of the neuron-specific CDK5 activator (C), p35, by 61%, while upregulating p25 (D) generated by Ca^{2+}-dependent calpain cleavage of p35, to approximately 4.7 fold, resulting in a significant increase in the p25/p35 ratio (E) by 13.4-fold. However, treatment with AST effectively inhibited the protein expression of the Notch1/NICD-1 axis and reversed the impact of VPA on the p25/p35 ratio by increasing p35 (2.3-fold) while reducing p25 and the p25/p35 ratio by 62% and 84%, respectively, compared with the insulted group.

ASD-induced activation of the Notch1/NICD-1 axis extended to impact its downstream target NF-κB, a transcription factor with pleiotropic effects, as presented in Figure 6. The ASD insult resulted in an increased immunoreactivity of the transcription factor NF-κB, reaching a level approximately 12.7-fold higher ($F_{(2,12)} = 232.0$, $p < 0.0001$) compared with the CONT group (A, D), whereas treatment with AST (C, D) blunted this expression by 75% ($F_{(2,12)} = 232.0$, $p < 0.0001$). These results confirmed the involvement of the active inflammatory signal Notch1/NICD-1/NF-κB in the ASD model and the inhibited CDK5, which plays a role in synaptic plasticity and the formation of learning and memory. Oppositely, AST acted by reverting the ASD effect to enhance the neurogenic CDK5 indicated by stimulation of p35 in addition to abating the inflammatory trajectory.

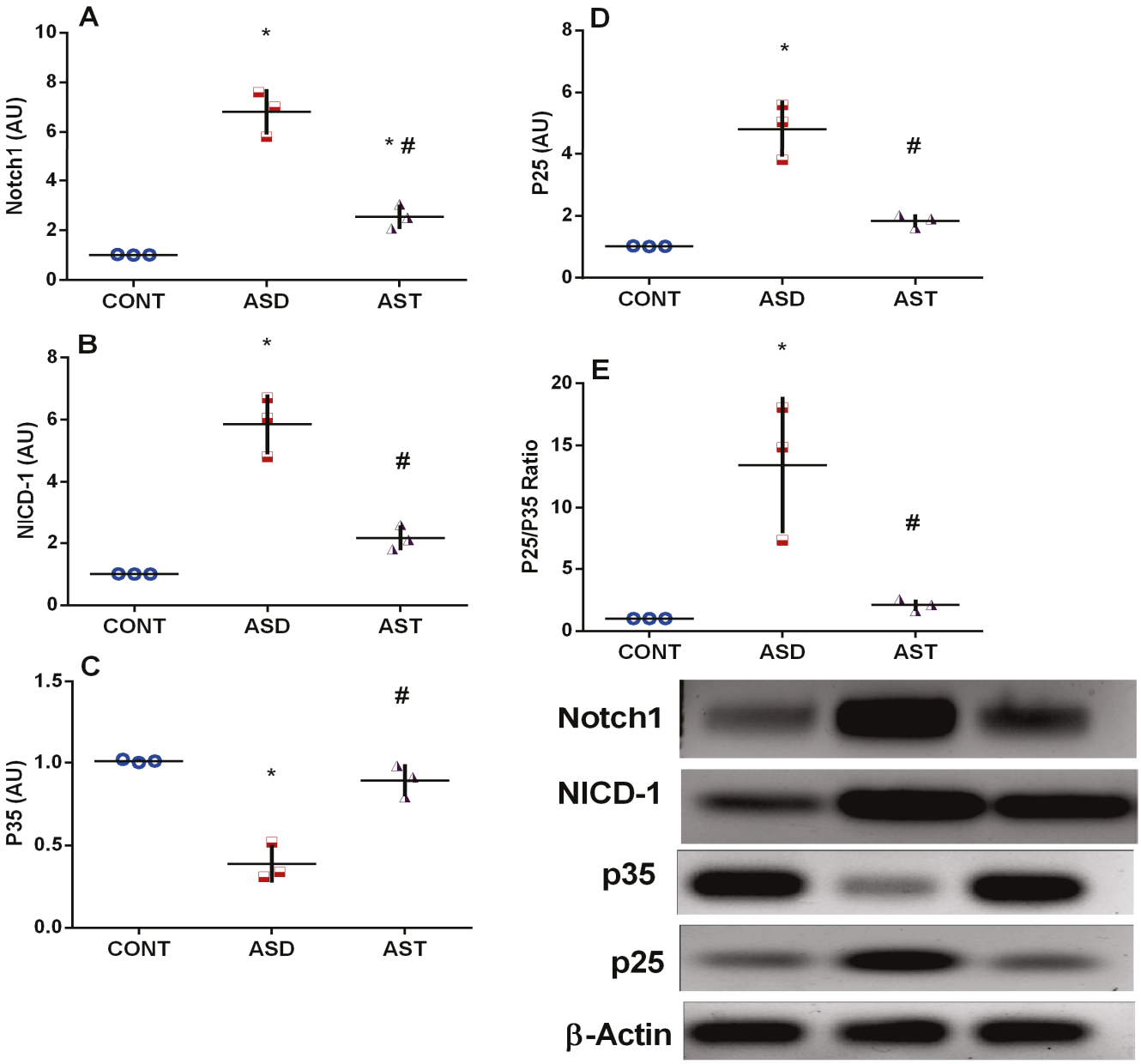


Figure 5. Effect of AST on the protein expression of (**A**) Notch1, (**B**) NICD, (**C**) p35, (**D**) p25, and their ratio (**E**) p25/35 in the cerebral cortex of autistic rats. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) on PND 34 (early adolescence) until PND 58 (early adulthood). Values are expressed as mean ± SD (n = 3 rats/group). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$, as compared with the CONT (*) and ASD (#) groups. ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; GD: gestational day; Notch1: neurogenic locus notch homolog protein 1; NICD: Notch intracellular domain; p.o: per os; PND: postnatal day; VPA: valproic acid. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

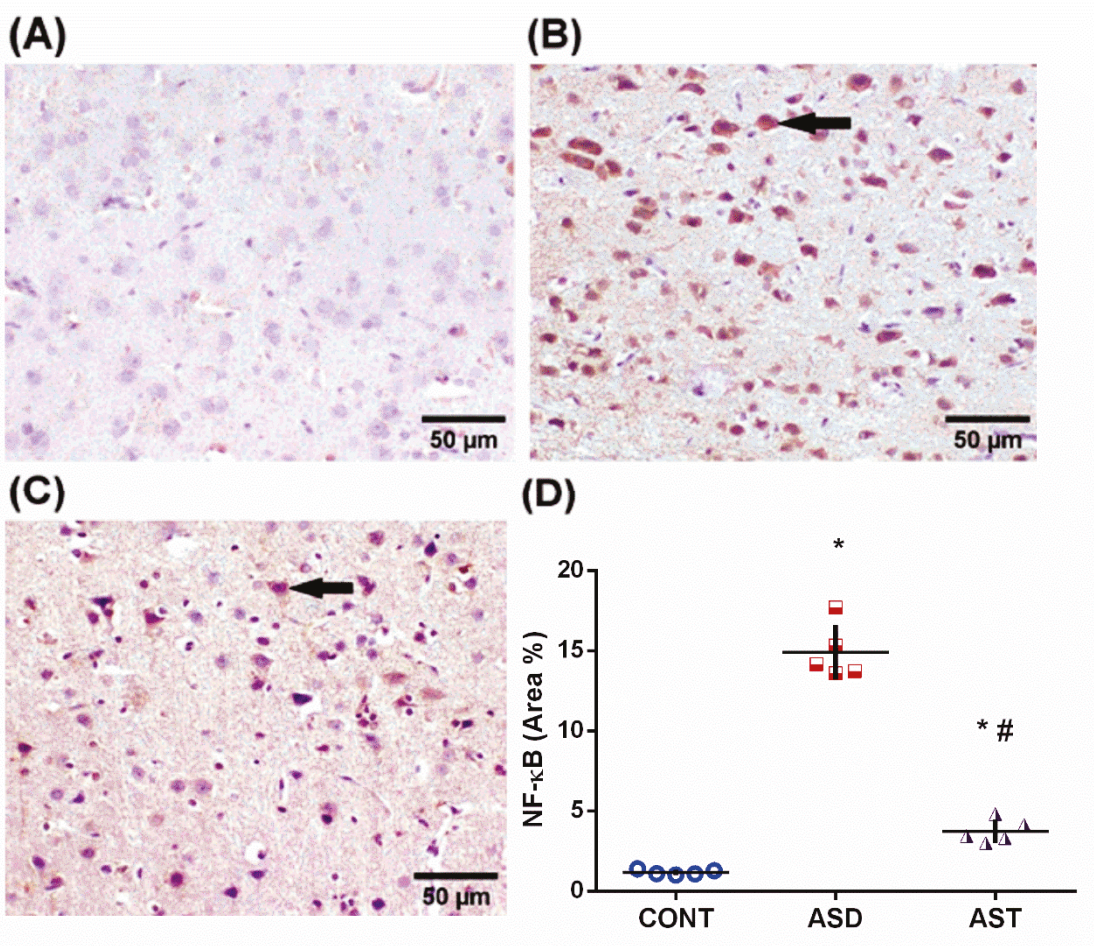


Figure 6. Representative photomicrographs of NF-κB immuno-expression in the cerebral cortex illustrating the differences between the groups. Section of the CONT (**A**) group shows negative immune expression, while that from the ASD (**B**) rats depicts strong immunoreactivity (arrow). Section from the AST-treated group (**C**) represents a weak NF-κB immune reaction (arrow; scale bar 50 µm). The data in panel (**D**) represent the mean ± SD, with 5 fields analyzed from 3 rats per group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. (*) and (#) indicate comparisons with the CONT and ASD groups, respectively. ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; NF-κB: nuclear factor kappa B. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

3.4. AST Reduces ASD Hallmarks Astrogliosis/p-tau, Amends Cortical Structure, and Rescues Neurons in Autistic Rats

Figure 7 shows strong immunoreactivity of the astrocyte marker GFAP in the section of (B) autistic rats that shows deep brown-stained hypertrophied astrocytes in the cerebral cortex compared with the lightly stained section of the CONT rats (A) that have normal small-sized astrocytes. Moreover, AST (C) treatment weakened astrogliosis, as evidenced by the light GFAP immunoreactivity, compared with the ASD section. The data are summarized in panel D ($F_{(2,12)} = 453.8$, $p < 0.0001$). Moreover, the ASD insult caused a 6.6-fold increase in the protein expression of $p^{(S396)}$-tau (E), which disables neural network function in ASD to be significantly downregulated upon treatment with AST.

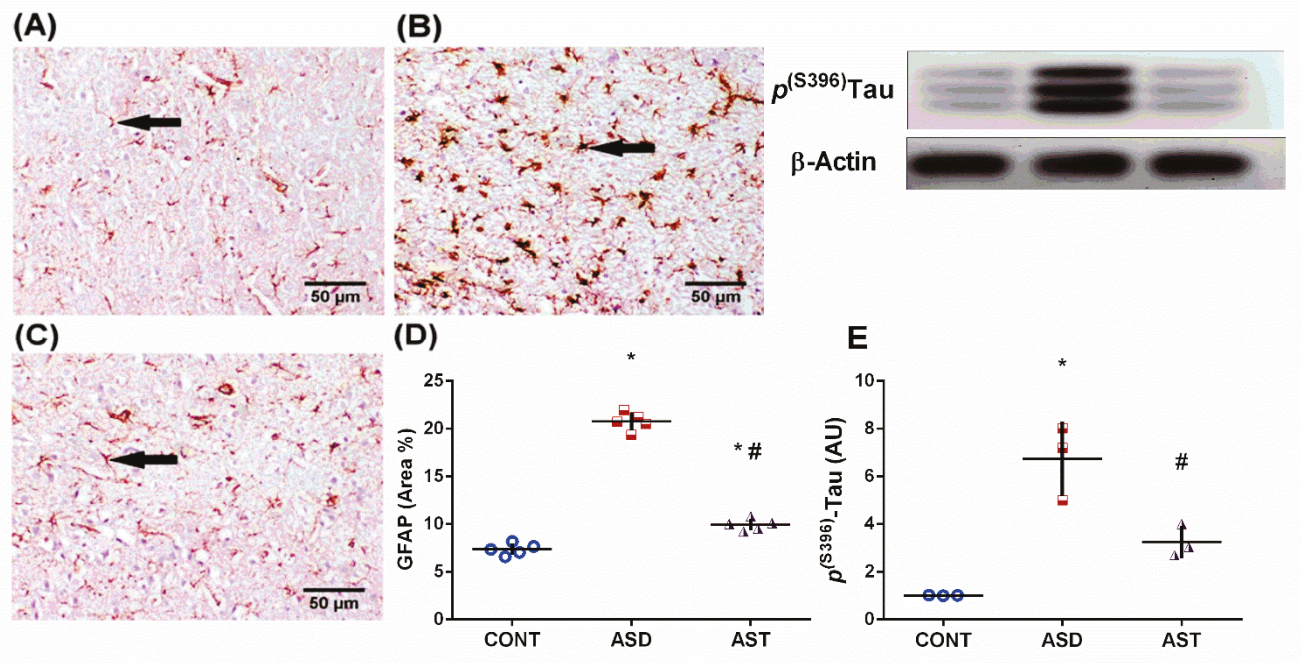


Figure 7. Effect of AST on GFAP and p(ser396)-tau protein expression. Representative photomicrographs of cortical GFAP immuno-expression in the CONT (**A**) rats showing normal small-sized astrocytes with lightly stained GFAP positive-short processes. In contrast, the section from the ASD rats (**B**) displays astrogliosis, indicated by strongly immunoreactive hypertrophied astrocytes with deeply stained GFAP-positive brown processes. However, the section from the AST-treated rats (**C**) demonstrates a soothing effect on this reaction, with mild GFAP-positive expression (scale bar = 50 µm). The data are summarized in panel (**D**). Panel (**E**) presents the protein expression of p(ser396)-tau. The data are presented as mean ± SD, with 5 fields analyzed from 3 rats per group for GFAP and 3 rats per group for tau. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. (*) and (#) indicate comparisons with the CONT and ASD groups, respectively ANOVA: analysis of variance; AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; GFAP: glial fibrillary acidic protein; p(ser396)-tau: phosphorylated tau protein at serine 396; p.o: per os; PND: postnatal day; VPA: valproic acid. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

The photomicrographs (Figure 8) of ASD rats (C, D) showed severe neuropathic lesions surrounded by empty areas and were characterized by increased cell-packing density, pyknosis, and reduced neuronal size. In addition, vacuolation of neuropil and proliferation of glial cells were noted compared with CONT rats (A, B), which exhibited normal histological architecture with intact neurons. On the contrary, sections of the AST-treated rats (E, F) depicted a marked upturn with some shrunken necrotic neurons associated with mild proliferation of glial cells. As shown in panel G, ASD scores reached 11 ($p = 0.0010$), whereas AST abated it to 2 ($p = 0.0079$); furthermore, it improved the individual lesions, as presented in panel H.

Sections stained with Nissl stain (Figure 9) revealed that ASD rats (C, D) possess shrunken and darkly stained neurons, as compared with sections of the CONT rats (A, B) that depict intact neurons with a visible nucleus and blue-violet stained perinuclear Nissl proteins. Furthermore, the AST-treated rat (E, F) sections revealed decreased degenerated neuronal cells. Panels G ($F_{(2,12)} = 139.1$, $p < 0.0001$) and H ($F_{(2,12)} = 229.5$, $p < 0.0001$) represent the number of intact and degenerated neurons, respectively.

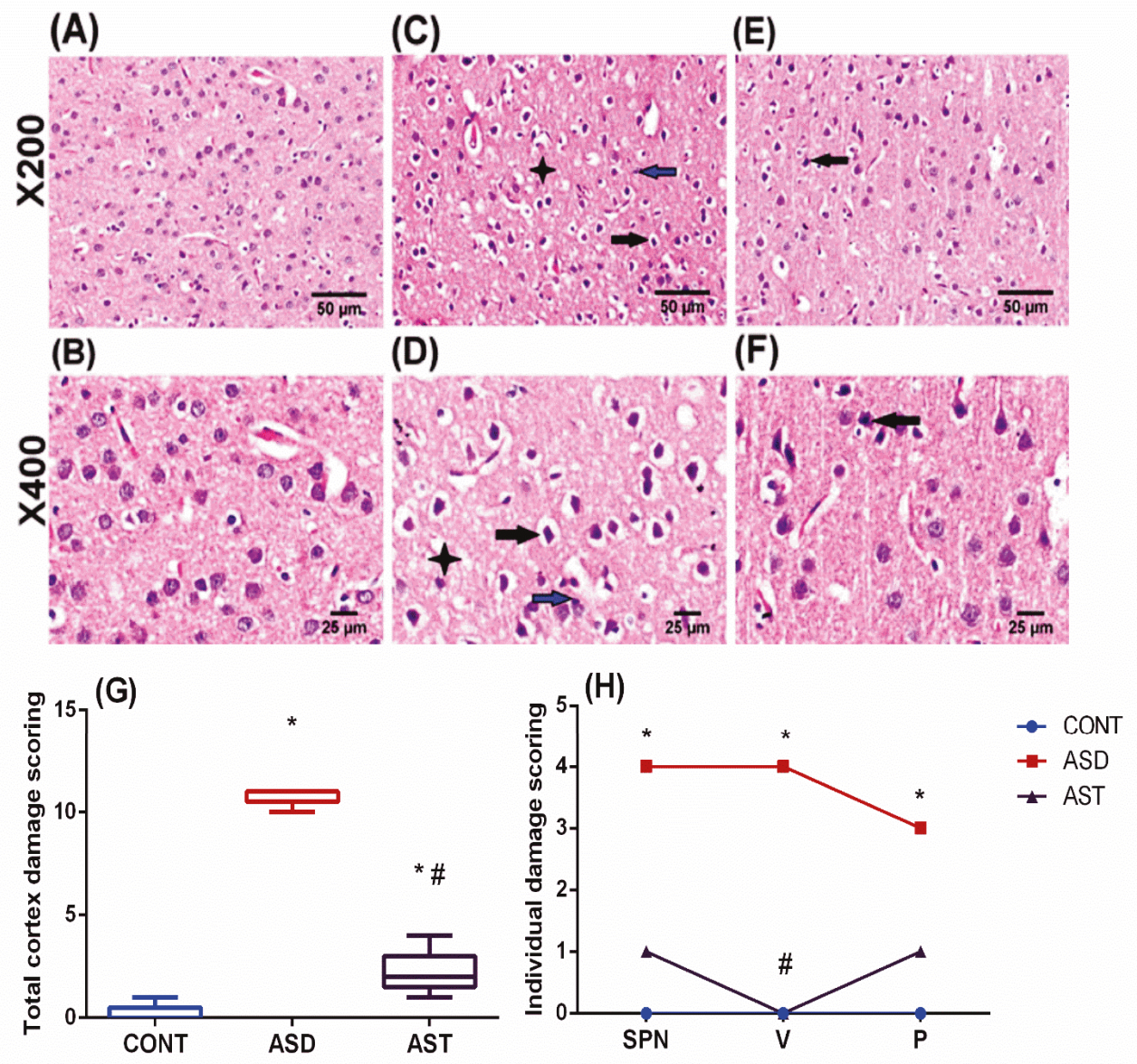


Figure 8. Representative photomicrographs of H & E-stained cerebral cortexes of rats in the CONT group (**A**,**B**) show normal histological architecture with intact neurons, whereas sections of the ASD rats (**C**,**D**) show pyknosis with shrunken and reduced neuronal size (black arrow), vacuolation of neuropil (asterisk), and proliferation of glial cells (blue arrow). Sections from the AST-treated rats (**E**,**F**) depict a few shrunken necrotic neurons (black arrow) with a mild proliferation of glial cells (scale bar, 50 and 25µm). Panel (**G**) illustrates the collective cortical damage scores presented as box and whiskers; median (minimum–maximum) data were analyzed using the Mann–Whitney test to compare between every 2 groups. Panel (**H**) depicts individual cortical alteration scores expressed as median (max–min) and analyzed using the Kruskal–Wallis test followed by Dunnett's posthoc test. Values were calculated from 5 fields of 3 rats/group ($p < 0.05$), as compared with the CONT (*) and ASD (#) groups. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) on PND 34 (early adolescence) until PND 58 (early adulthood). AST: astaxanthin; ASD: autism spectrum disorder; CONT: control; GD: gestational day; P: proliferation of glial cells; p.o: per os; PND: postnatal; SPN: shrunken, pyknosis, and necrosis of neurons; V: vacuolation of neuropil; VPA: valproic acid; Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

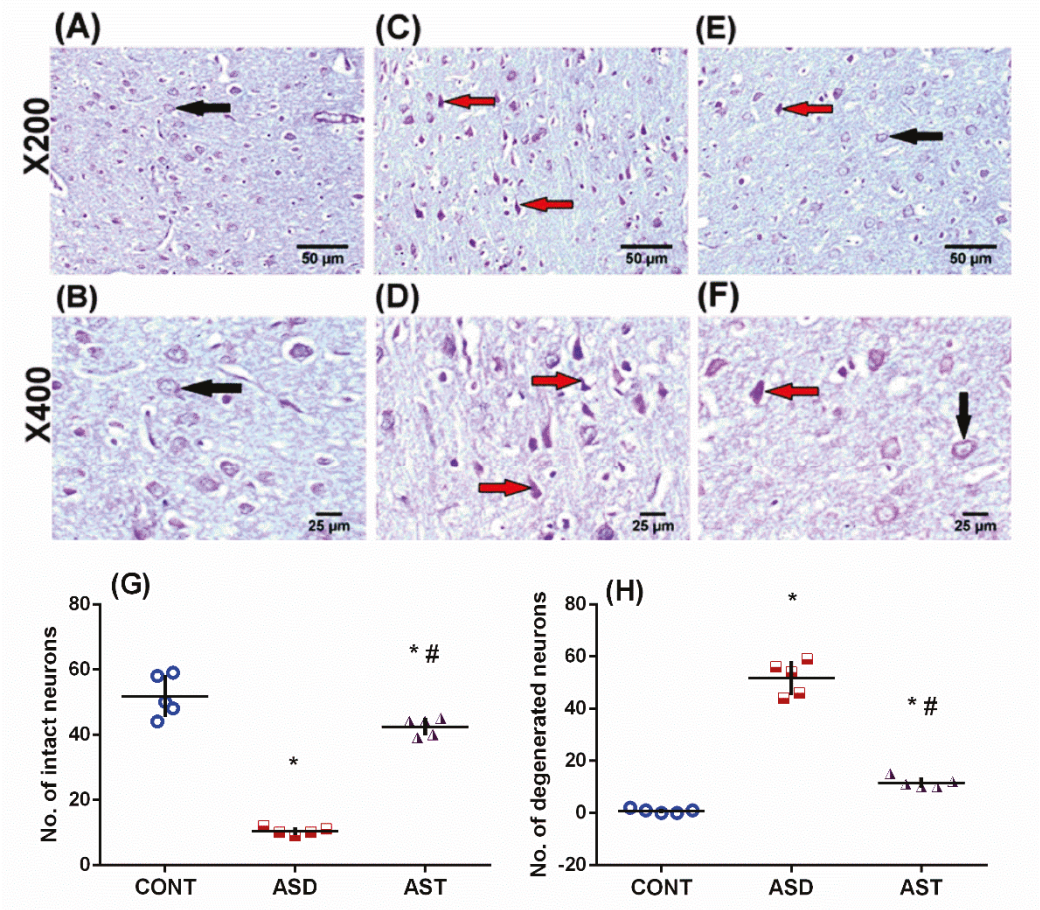


Figure 9. Representative photomicrographs of cresyl violet-stained sections of the cerebral cortex illustrate the differences between the groups. In the CONT group (**A**,**B**), normal intact neurons with visible nuclei are observed, and Nissl proteins around the nucleus are stained blue-violet (black arrow). Sections from the ASD rats (**C**,**D**) show shrunken, darkly stained neurons (red arrow), while sections from the AST-treated group (**E**,**F**) display a decreased number of degenerated, darkly stained neurons (red arrow) and an increased number of intact neurons (black arrow) (scale bar, 50 and 25 µm). The panels (**G**,**H**) provide quantitative data on the number of intact and degenerated neurons, respectively. The values are expressed as mean ± SD, with 5 fields of view analyzed from 3 rats per group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons tests, with $p < 0.05$ considered significant. (*) and (#) indicate comparisons with the CONT and ASD groups, respectively. Male offspring rats of dams were injected with VPA on GD 12.5 and were treated with AST (25 mg/kg/day, p.o) from PND 34 (early adolescence) until PND 58 (early adulthood). ANOVA: analysis of variance; ASD: autism spectrum disorder; AST: astaxanthin; CONT: control, GD: gestational day; p.o: to per os; PND: postnatal day; VPA: valproic acid. Blue circles for control group; red squares for ASD group; purple triangles for AST treated group are indicative of individual data.

In Figure 10, electron micrographs of cortical sections of CONT rats (A) show normal nuclei with regular nuclear membranes and prominent nucleoli. In contrast, the sections of the ASD model (B, C) depict a shrunken irregular nucleus with an irregular nuclear membrane, empty lysed cytoplasm, and degenerated mitochondria. However, the section of the AST-treated rats (D) shows a large normal nucleus surrounded by apparently normal cytoplasm containing slightly swollen mitochondria. Accordingly, the enhanced cortical structure and neuronal count by AST is a consequence of its ability to deter astrogliosis and p-tau, two decisive pathological events related to autism.

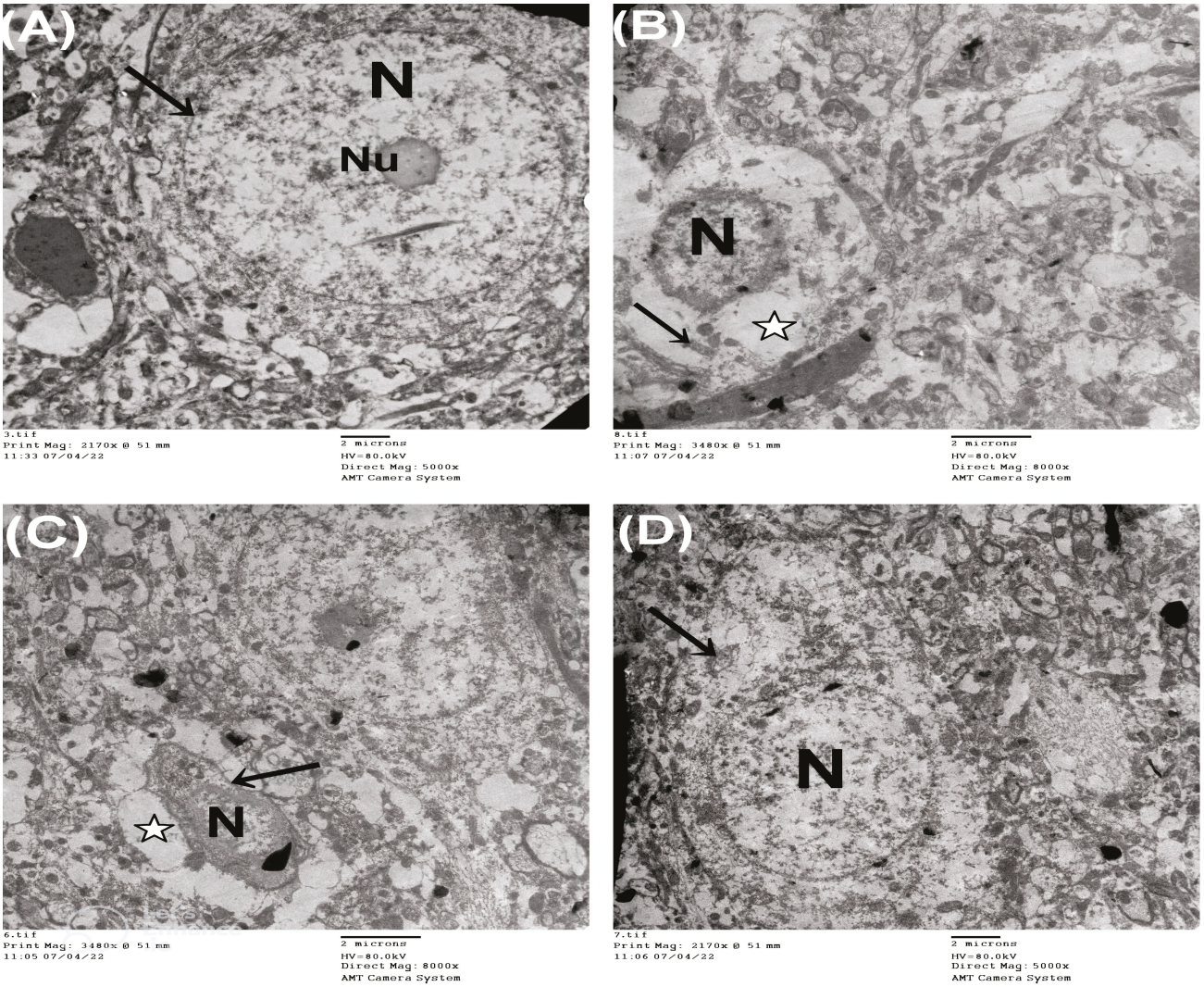


Figure 10. Electron micrographs of cerebral cortex sections from different groups provide insights into cellular changes. In the CONT group (**A**), the images show normal nuclei (N) with regular nuclear membranes (arrow) and prominent nucleoli (Nu). In contrast, the ASD group (**B**) exhibits a shrunken nucleus (N), empty lysed cytoplasm (white star), and degenerated mitochondria (arrow). Similarly, section (**C**) displays an uneven nucleus (N) surrounded by an irregular nuclear membrane (arrow) and lysed cytoplasm (white star). However, in the AST-treated group (**D**), the section reveals a large, normal nucleus (N) surrounded by apparently normal cytoplasm containing slightly swollen mitochondria (arrow).

To sum up, AST, by activating the Mas receptor and stimulating ACE2-induced Ang1-7 production, halted Ang II, Notch1/NICD1/p25, and NF-κB hubs. Additionally, AST enhanced two survival molecules, namely, PI3K and p35. The crosstalk between these proteins amended structural alterations and behavioral deficits induced by the VPA-induced autistic model. Moreover, the interplay between these signals limits both tauopathy and astrogliosis, which participate in the pathomechanism of ASD.

4. Discussion

ASD is a complex neurodevelopmental disorder characterized by difficulties in social interaction, communication, and repetitive behaviors. Despite its prevalence, the pathophysiology of ASD is not well understood, making it challenging to develop effective treatments. The underlying causes of ASD are believed to be a combination of genetic and

environmental factors, but specific mechanisms are still unclear. This lack of understanding hampers the progress in finding targeted therapies for individuals with ASD, leaving many struggling to find suitable treatments and interventions. As research continues to uncover the underlying mechanisms and causes of ASD, it is hoped that more effective treatments will be developed to improve the lives of those affected by this disorder.

Our study revealed, for the first time, the involvement of the imbalanced RAS in the pathomechanisms of ASD, and the correction of this imbalance with AST serves as an in vivo "proof of concept". By redirecting the RAS toward the beneficial arm, as evidenced by the strengthening of the ACE2/Ang1-7/Mas receptor axis, AST effectively diminished the unwanted effects of Ang II and the Notch1/NICD/NF-κB inflammatory cascade. Simultaneously, AST increased the expression of the neurogenesis marker PI3K and improved the p35/p25 ratio. The interplay between these pathways reduced astrogliosis and p-tau, which play a critical role in ASD pathogenesis. Furthermore, on a behavioral level, these events led to an improvement in sociability/social preference and a decrease in stereotypies/perseverative behavior, which are cardinal features of ASD. Additionally, AST facilitated the repair of the histological structure and enhanced neuronal survival, as indicated with H & E stain, Nissel stain, and electron microscopy (Figure 11).

While previous research has linked genetic variations in ACE to autism [37], this study is the first to examine the role of the Yin/Yang arms of RAS in in utero VPA-exposed animals. The results showed that ASD is associated with a surge in Ang II, the surrogate marker of the classical arm of RAS, and a significant suppression of the good RAS arm ACE2/Ang1-7/MasR. It is worth noting that although there are no existing data on the effect of AST on central cortical RAS, our findings on the inhibitory effect of AST on Ang II can be explained by the work of Gao et al. [70]. They reported that chronic infusion of AST in the hypothalamic paraventricular nucleus in spontaneously hypertensive rats reduced the expression of ACE and AT1R and pointed to the ability of AST to hinder both the synthesis of Ang II and its effect.

Moreover, the effect of AST on the neuroprotective arm of the RAS was investigated to provide a comprehensive understanding. The results revealed that the Yang arm of the RAS was significantly activated by treatment with AST to provide one possible explanation for its neuroprotection in ASD pups. Interestingly, the central role of AST on the beneficial axis of the RAS aligns with its central effect observed in spontaneously hypertensive rats [70], where in addition to inhibiting Ang II, the chronic infusion of AST enhanced the expression of ACE2 and the Mas receptor, reinforcing our findings. The modulatory effect of AST on the RAS may partly contribute to the improved behavior observed in our study, as the disturbed Yang arm of the RAS has previously been implicated in impaired cognition [71]. ACE2 knockout rodents have shown impaired cognitive functions, while upregulation of the Mas receptor has been found to improve social interaction and emotions [72]. These findings highlight the ACE2/Ang1-7/Mas receptor pathway as a potential target for managing disturbed behavior in ASD.

The corrective effect of AST on the beneficial aspect of the RAS can be partially attributed to the activation of PI3K, as observed in our study. This is noteworthy because the neuroprotective role of the PI3K/Akt pathway relies, in part, on the inactivation of Ang II, as demonstrated in a model of hypertension-induced cardiac hypertrophy [73] and the stimulation of the Ang1-7/Mas receptor axis. The latter has been reported to activate PI3K [74], which subsequently counteracts the harmful effects of the Ang II/AT1R axis [75]. Indeed, a previous study strengthened our finding since weakening this protein and its downstream trail, Akt-Bcl-xL, enhanced cell death [18]. Furthermore, in a VPA-induced autistic rat model, Nicolini and coauthors [76] found that the PI3K/AKT trajectory was downregulated. Similarly, in BTBR mice, Luo et al. [77] found that the PI3K/Akt axis was inhibited but reactivated when moesin was overexpressed to mitigate autism-associated behaviors.

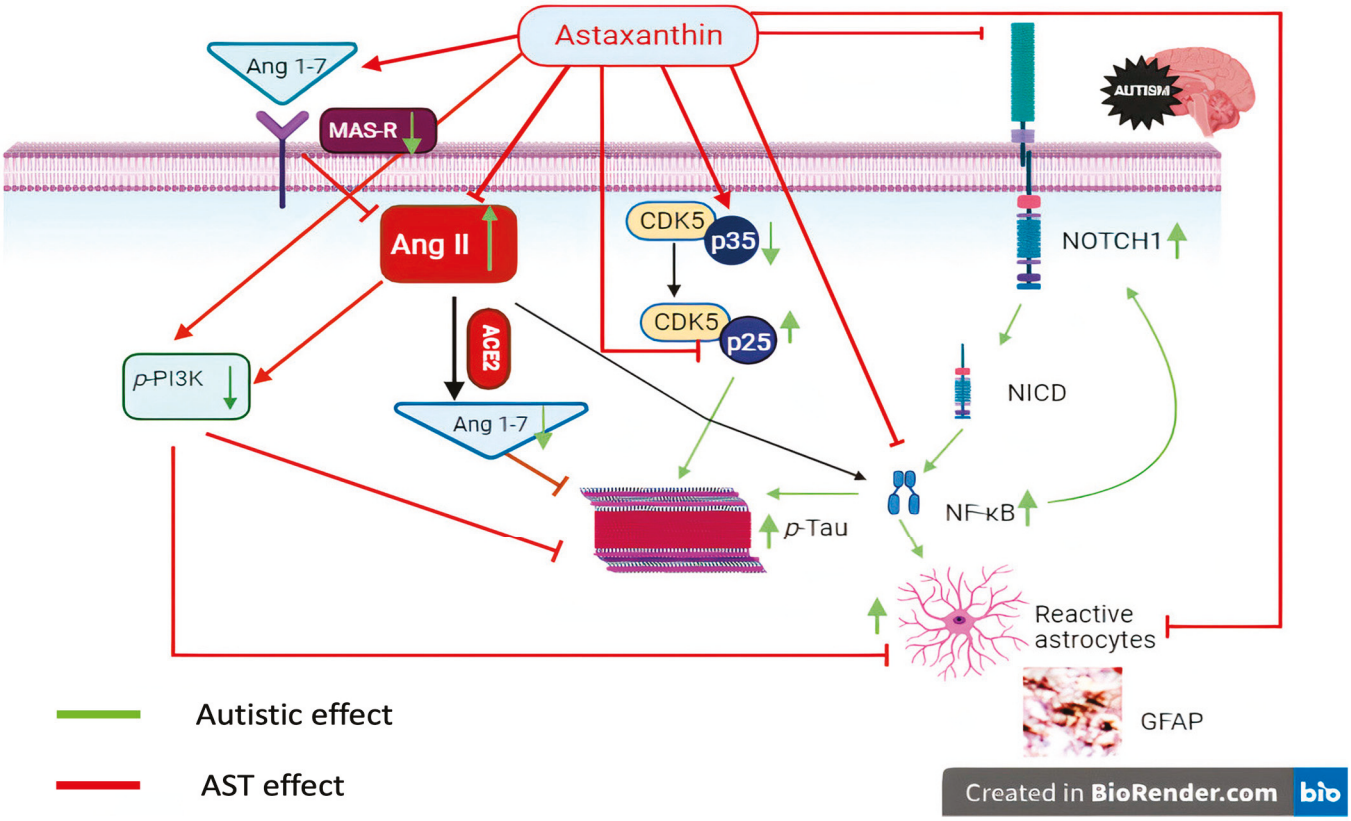


Figure 11. Illustrating diagram representing the effect of AST through interaction with different signaling proteins to mitigate ASD. AST increased Ang 1-7 by binding to its receptor Mas, inhibited Ang II, and activated ACE2 to increase the formation of Ang 1-7 to abate p-tau. In addition, AST and activated Ang 1-7/Mas receptor activated the survival proteins PI3K and p35 to decrease p-tau and GFAP. AST inhibited the inflammatory cascade Notch1/NICD-1/NF-κB to lessen GFAP and p-tau. Ang: angiotensin; ACE2: angiotensin-converting enzyme 2; GFAP: glial fibrillary acidic protein; Notch 1: neurogenic locus notch homolog protein 1; NICD-1: Notch intracellular domain; NF-κB: nuclear factor kappa B p65; PI3K: phosphoinositide 3-kinase; p-tau: phosphorylated tau protein. The green arrows are indication of autistic effect while red inhibitory lines are indication of AST effect.

In addition to rebalancing the RAS, AST treatment has been shown to curb the inflammatory Notch1/NICD-1/NF-κB cascade, shedding light on another neuroprotective mechanism against the ASD model. It is noteworthy that the dysfunctional Notch pathway has a potential correlation with autism. Consistent with our findings, it has been reported that VPA-induced ASD activates the Notch1 signaling, and its inhibition alleviates autistic-like behavior [78]. In addition, activated Notch1 cascade stimulates its downstream target NF-κB, which has been linked to autism, as documented from post-mortem tissue analysis of autistic patients showing increased NF-κB expression [79]. An interplay between Notch1 and NF-κB has been recognized since NF-κB, in addition to being activated by Notch1, enhances the expression of Jagged-1 to activate the Notch signaling in recipient cells [80]. Our findings demonstrated that AST inhibits the Notch1/NICD-1/NF-κB pathway, explaining the increased survival of neurons in the ASD model.

Moreover, the inhibition of NF-κB has been reported to alleviate synaptic deficits associated with ASD [81]. A previous study explained the inhibitory effect of AST on NICD-1 by enhancing the protein numb homolog (Numb), which degrades NICD-1 proteolytically [82]. Additionally, the ability of AST to downregulate NF-κB protein expression aligns with its effects in a model of subarachnoid hemorrhage [83], supporting its potential as an anti-autistic agent by reducing one of the inflammatory markers associated with autism [79].

The current study highlighted the cross-communication and integration between the RAS and the Notch signaling pathways. In a previous report, Ang II, which is activated by ACE [84], was reported to stimulate both the Notch system [45] and its downstream target NF-κB [85]. Mutually, increased levels of Ang II have been shown to cause Notch1 cleavage and subsequent release of NICD-1 in podocytes [45]. Thus, the reduction in the cortical Ang II by AST is considered a novel culprit for deactivating the Notch1/NICD-1/NF-κB axis, which together intermingles to mitigate autism.

Since p-tau is a characteristic trait of ASD and its reduction mitigates autism key features [86], the AST modulatory effect on the previous markers explains the reduced p-tau. One contributing factor to the observed decrease in tau levels is the AST-mediated inhibition of Ang II since activation of its synthesizing enzyme ACE is associated with an increase in p-tau, an effect that can be reversed by inhibiting the converting enzyme [32]. Additionally, the aptitude of AST to augment the p35/p25 ratio, as observed in our study, partakes in the activation of CDK5 to promote neurogenesis [87]. Similarly, the activation of PI3K plays a role in tau inhibition by inactivating GSK3β [19,26], which is another reason for the inhibited p-tau. Notably, inflammatory Notch1 signaling was reported to increase p-tau in an AD model, and turning off this cascade was shown to reduce this marker [88]. Therefore, the ability of AST to modulate these mechanisms offers a potential explanation for the inhibited p-tau levels observed in our study.

Increased astrogliosis associated with an increase in GFAP is another participant in ASD [89] that is partly linked to the activated inflammatory Notch1/NICD-1/NF-κB axis. Notably, the dysfunctional Notch pathway has a potential correlation with autism [78], and via its pleiotropic actions, the activated Notch/NICD axis has been reported to promote the formation of GFAP [41] by activating NF-κB p65 [90]. Therefore, AST extends the anti-autistic mechanisms via reducing astrogliosis by repressing Notch1 signaling and NF-κB, as presented in our findings. Moreover, the inhibitory effect of AST on NF-κB was recounted earlier in a model of subarachnoid hemorrhage [91], supporting our results. The modulation of RAS may play a role in the reduced GFAP immunoreactivity, as documented earlier, where blocking AT1R with losartan [92] or inhibiting ACE with perindopril [93] in models of AD have inhibited this astrogliosis marker.

In conclusion, our study unveiled novel molecular mechanisms through which AST can partially improve the core behavioral abnormalities associated with ASD. Our results demonstrated that AST effectively mitigates tauopathy by activating the neuroprotective ACE2/Ang1-7/Mas receptor pathway and the neuroprotective proteins PI3K p85/p55 and p35. Moreover, AST, by inhibiting AngII and the Notch1/NICD/NF-κB pathway, leads to reduced levels of p-tau and astrogliosis, as evidenced by decreased GFAP expression. Therefore, by modulating these intersecting pathways, AST plays a crucial role in ameliorating behavioral disabilities and improving the compromised cortical structure observed in VPA-induced ASD. Consequently, our findings suggest that targeting both arms of the RAS, specifically the Ang1-7/Mas receptor and AngII, as well as the inflammatory Notch1/NICD/NF-κB signaling pathway, could serve as potential therapeutic approaches for treating ASD.

It is important to acknowledge the challenges and limitations of the present study. While this study highlighted the role of the two arms of RAS in the pathophysiology of ASD, it did not encompass the contribution of other angiotensin metabolites to ASD. Therefore, further validations may be needed to establish the role of the examined angiotensin arms and metabolites in the pathophysiology of ASD and its associated abnormal behaviors. However, these limitations should not overshadow the merits of this study. It is pioneering in investigating the involvement of dysregulated RAS in the pathological environment of ASD.

Furthermore, it sheds light on the interaction between the RAS and Notch signaling pathways, providing a deeper understanding of the complex mechanisms underlying ASD. For future clinical applications and drug development, the findings emphasize the need to consider RAS modulators as potential treatments for ASD. Additionally, the effectiveness of

anti-inflammatory drugs that inhibit the inflammatory Notch1/NICD-1/NF-κB signaling pathway suggests they could be promising therapeutic approaches for ASD treatment.

5. Conclusions

Preclinical studies are essential to gain insights into the disease mechanism and to test novel interventions. Despite its cardiovascular role, the RAS attracted attention as a target in neuronal disorders. Recent reviews highlighted the RAS's crucial role in AD by hindering Aβ plaque and p-tau accumulation, improving symptoms, and slowing disease progression. The promising preclinical results extended to clinical trials, where blocking AT1R reduced stroke risk, improved cognition, and offered neuroprotection in AD patients.

Considering the similarities between AD and ASD, we evaluated the role of RAS in an ASD VPA acid model and studied the possible role of AST in correcting imbalanced arms of the RAS. Our results uncovered a new pathomechanism of ASD and a neuroprotective role for AST to improve behavior. AST was found to mitigate tauopathy and astrogliosis by activating the neuroprotective arm ACE2/Ang1-7/Mas receptor pathway, as well as the neuroprotective proteins PI3K p85/p55 and p35 while inhibiting AngII and the Notch1/NICD/NF-κB pathway. Although our preclinical study supported our hypothesis, additional experiments involving the modulation of several RAS molecules and their targeted administration to the brain are necessary in the future to validate this strategy. Furthermore, clinical trials are still required to substantiate the approach.

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Abbreviations

3-CT	Three chamber test
ACE2	Angiotensin-converting enzyme 2
AD	Alzheimer disease
Akt/PKB	Protein kinase B
Ang1-7	Angiotensin 1–7
Ang II	Angiotensin 2
ASD	Autism spectrum disorder
AST	Astaxanthin
CDK5	Cyclin-dependent kinase 5
GD	Gestational day
GFAP	Glial fibrillary acidic protein
MasR	Mas receptor
MBT	Marble burying test
NF-κB p65	Nuclear factor Kappa B p65

NICD	Notch intracellular domain
Notch1	Neurogenic locus notch homolog protein 1
OFT	Open field test
PI3K p85/p55	Phosphoinositide 3-kinase p85/p55
PND	Postnatal day
p-Tau	Phosphorylated tau protein
RAS	Renin angiotensin system
SI	Sociability index
SNI	Social novelty preference index
VPA	Valproic acid

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Review

Advancements in Understanding and Classifying Chronic Orofacial Pain: Key Insights from Biopsychosocial Models and International Classifications (ICHD-3, ICD-11, ICOP)

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Abstract: In exploring chronic orofacial pain (COFP), this review highlights its global impact on life quality and critiques current diagnostic systems, including the ICD-11, ICOP, and ICHD-3, for their limitations in addressing COFP's complexity. Firstly, this study outlines the global burden of chronic pain and the importance of distinguishing between different pain types for effective treatment. It then delves into the specific challenges of diagnosing COFP, emphasizing the need for a more nuanced approach that incorporates the biopsychosocial model. This review critically examines existing classification systems, highlighting their limitations in fully capturing COFP's multifaceted nature. It advocates for the integration of these systems with the DSM-5's Somatic Symptom Disorder code, proposing a unified, multidisciplinary diagnostic approach. This recommendation aims to improve chronic pain coding standardization and acknowledge the complex interplay of biological, psychological, and social factors in COFP. In conclusion, here, we highlight the need for a comprehensive, universally applicable classification system for COFP. Such a system would enable accurate diagnosis, streamline treatment strategies, and enhance communication among healthcare professionals. This advancement holds potential for significant contributions to research and patient care in this challenging field, offering a broader perspective for scientists across disciplines.

Keywords: orofacial pain; burning mouth syndrome; chronic pain; models; biopsychosocial; toothache/diagnosis; trigeminal neuralgia; International Classification of Diseases (ICD-11); International Classification of Orofacial Pain (ICOP); International Classification of Headache Disorders (ICHD-3); Diagnostic and Statistical Manual of Mental Disorders (DSM-5)

1. Introduction

Chronic pain, affecting an estimated 30% of the global population, ranks as one of the predominant reasons for seeking medical care [1,2]. Despite the higher mortality associated with conditions such as heart attacks, strokes, infectious diseases, cancer, and diabetes, chronic pain remains a leading cause of human suffering and disability, surpassing many other health challenges in its impact on quality of life [1,3,4].

The mismanagement of pain not only leads to severe physical, psychological, and social repercussions but also incurs substantial economic costs, both in terms of healthcare expenditure and lost productivity [5–7].

Patients enduring chronic pain often experience a protracted journey through the healthcare system, spanning months or years, without adequate recognition of their condition [8,9]. This lack of validation can contribute to feelings of alienation and exacerbate their

suffering [10]. The inability of healthcare systems to effectively acknowledge and address the multifaceted impact of chronic pain has emerged as a critical issue, necessitating urgent attention in medical and dental practices as well as at the policy level [11–13].

Understanding and effectively treating chronic pain is not just a clinical challenge but a societal imperative, in medicine, dentistry, and for the national healthcare system [14,15].

In response to the complexities presented by chronic pain, particularly in the orofacial region, this review seeks to illuminate the various dimensions of chronic orofacial pain (COFP) [16,17]. The face and mouth hold special significance in human physiology and social interaction, making pain in this region particularly complex [18,19]. The prevalence of COFP is notable, with approximately 10% of chronic pain cases falling within this category [20–22]. It encompasses a range of conditions, from trigeminal neuralgia to burning mouth syndrome, each presenting unique challenges in terms of diagnosis and management [23–25]. The review will explore these conditions in depth, focusing on their epidemiology, clinical presentation, and the intricacies of their classification.

A central theme of this review is the exploration of the biopsychosocial model of chronic pain [26,27]. This model emphasizes that pain is not solely a physical symptom but is significantly influenced by psychological and social factors [28,29].

This understanding is crucial for healthcare professionals as it necessitates a highly individualized approach to pain management [30–32].

We will specifically examine the classification systems currently in use for COFP, including the International Classification of Orofacial Pain (ICOP 2020), the International Classification of Headache Disorders (ICHD-3), and the International Classification of Diseases (ICD-11). This review will critique these systems, highlighting their strengths and limitations, and discuss the future directions for a more effective and comprehensive classification of COFP. This exploration is vital for enhancing diagnostic accuracy, optimizing treatment modalities, and ultimately improving patient outcomes.

Finally, this review aims to provide a thorough understanding of COFP, its classification, and its management, with a focus on the integration of the biopsychosocial model and the latest diagnostic frameworks, in order to contribute to the broader understanding of orofacial pain within the healthcare community, facilitating better patient care and outcomes.

According to the International Association for the Study of Pain (IASP), "pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" [33–35].

The IASP task force expanded this definition in 2020 by adding six key notes [35]:

Pain is always a personal experience that is influenced by biological, psychological, and social factors;

Pain and nociception are different phenomena. Pain cannot be solely deduced from the activity of sensory neurons;

Individuals learn the concept of pain through their life experiences;

The individual's personal history of pain should be respected;

Although pain usually plays an adaptive role, it can have negative effects on social, psychological, and functional well-being;

Verbal description is just one of many behaviors used to express pain. The inability to communicate does not negate the possibility that a human or non-human being experiences pain [35].

Chronic pain is defined as pain that persists beyond the normal time of healing that is associated with a particular type of damage or disease [36]. According to the IASP task force, chronic pain is defined as persistent or recurrent pain lasting more than 3 months [37].

Pain, a complex and multifaceted experience, can be categorized into three distinct types based on mechanistic differences: nociceptive, neuropathic, and nociplastic pain [38].

Nociceptive pain is defined as pain that arises from actual or potential damage to non-neural tissue due to the stimulation of nociceptors [35]. Therefore, neural tissue is

generally healthy, and signal transmission is normal; what creates pain is the stimulation of "nociceptors" present both somatically and viscerally [39].

Nociceptors are primary afferent neurons with a high activation threshold. Acute pain is due to the activation of myelinated nociceptors (mainly Aδ fibers), while chronic pain is generally due to the activation of unmyelinated nociceptors [40,41]. This pain type is distinguished from neuropathic pain by its association with a normally functioning somatosensory nervous system [39,42].

Neuropathic pain is defined as pain caused by a lesion or disease affecting the somatosensory nervous system [43]. It is generally perceived within the territory of innervation of the damaged nerve [37].

Depending on the affected system, it is categorized as peripheral or central: it is peripheral when the lesion and disease affect the peripheral system, and it is central when the central nervous system is affected [36,44].

The somatosensory system includes peripheral receptors and neural pathways through which the central nervous system can detect and process information about the body, such as touch, pressure, vibration, pain, temperature, position, and movement of its parts [45,46]. Therefore, by definition, within the concept of pain, a lesion or disease of the somatosensory system is related to altered pain processing [47,48].

Neuropathic pain can be either spontaneous or evoked, marked by either negative (hypoalgesia, hypoesthesia) or positive sensory signs (hyperalgesia, allodynia) that must be perceived in the territory of innervation of the injured nervous structure [49,50] (Appendix **??**

Confirming neuropathic pain often requires demonstrating a nervous system lesion or disease via diagnostic methods such as imaging, biopsies, or neurophysiological tests, with questionnaires serving as supplementary screening tools [51].

The concept of nociplastic pain, a term introduced by the pain research community [37,52], represents the third category [53].

Nociplastic pain is characterized by altered nociception in the absence of clear evidence of either actual or potential tissue damage that would activate peripheral nociceptors, or of any injury to the somatosensory system [54]. Typically, this type of pain presents as multifocal, widespread, and intense, and is often accompanied by a constellation of symptoms including fatigue, sleep disturbances, memory impairment, and mood alterations [38].

Baliki et al. showed changes in brain connectivity in patients with nociplastic pain (chronic back pain and osteoarthritis), demonstrating reduced connectivity of the medial prefrontal cortex and increased connectivity of the insular cortex in proportion to the intensity of pain [55].

This type of pain can manifest itself in isolation, as often occurs in conditions such as fibromyalgia or tension-type headache, or as part of a mixed pain state in combination with nociceptive or neuropathic pain in progress, as might occur in chronic low back pain or chronic orofacial pain [56].

It responds differently to treatments, often showing limited response to anti-inflammatory drugs and opioids [38].

It is crucial to recognize that "neuropathic", "nociceptive", and "nociplastic" pain are not specific pathologies in themselves but are used to categorize pain types with potentially shared pathophysiological features [57].

The perception and experience of pain are complex processes that have a multidimensional character, influenced by various factors that do not only involve sensory-discriminative biological processes, but also the interaction of affective, motivational, cognitive, and social factors in a biopsychosocial model, as emphasized by the expansion of the definition by the IASP [33,58]. The model considers the interaction of these factors in the experience of pain [58]. This convergence of diverse elements renders everyone's pain experience uniquely personal, necessitating a highly individualized approach by healthcare professionals [59,60] (Figure 1).

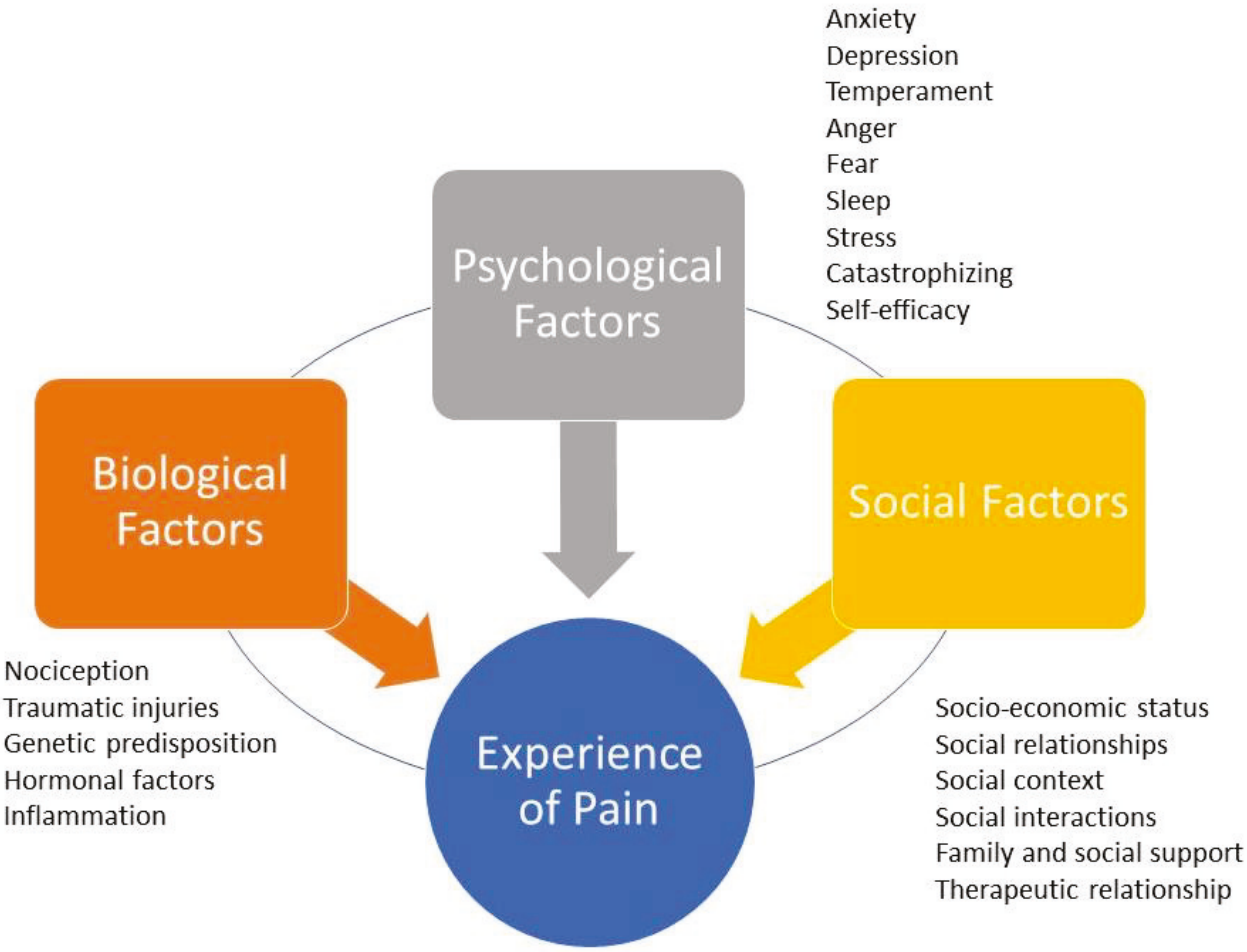


Figure 1. The biopsychosocial model of pain. The experience of pain is personal because it is mediated by the dynamic, interdependent, and synergistic integration of three factors/domains: biological factors, psychological factors, and social factors. Each domain may have characteristics shared with the others. Biological factors that can negatively affect the perception of pain include traumatic injury, the severity of overall health conditions, and genetic/hormonal predisposition. Psychological factors that can amplify pain include anxiety, depression, sleep disorders, excessive catastrophizing, and low self-efficacy. Social factors that can negatively influence the perception of pain include gender roles, ethnic identity, discrimination, poor family support, and healthcare provider prejudice.

The face and mouth hold a special significance in human physiology and social interaction, serving not just in essential functions like taste, smell, chewing, swallowing, and sensory–motor activities, but also playing a crucial role in interpersonal communication [58,61–63]. Consequently, pain in this region gains a multidimensional aspect, often more complex than pain in other body areas [64–66]. Facial pain (FP) is commonly defined as pain predominantly occurring below the orbito-meatal line, anterior to the earlobes, and above the neck, with some definitions extending to the forehead, linking certain headaches to orofacial structures [67–70].

Orofacial pain (OFP) encompasses all structures of the oral cavity [71]. In dental practice, such pain is often associated with dental issues or temporomandibular joint disorders; however, in 30% of cases, dentists may be faced with a type of pain that cannot be attributed to any of the above situations [72–74].

Approximately 20% of acute craniofacial pains have the potential to evolve into COFP if not addressed effectively and in good time [16,75]. Dentists play a pivotal role in identifying and managing these conditions, especially when the pain source is not immediately apparent, thus avoiding unnecessary procedures [18,76]. The complexity and often elusive

etiology of COFP make diagnosis and treatment challenging, with patients frequently facing delayed diagnosis and intervention, typically 2–3 years post onset, regardless of the pain's nature [77–79].

COFP can be categorized into those with a clear etiology, such as post-herpetic trigeminal neuralgia or post-traumatic neuropathic trigeminal pain [80,81], those associated with chronic diseases like arthritis or diabetes, and a significant number of idiopathic cases where the cause remains unidentified, including conditions like burning mouth syndrome (BMS), persistent idiopathic facial pain (PIFP), or persistent idiopathic dentoalveolar pain (PIDP) [82]. Consequently, COFP is classified as primary when idiopathic and secondary when linked to an identifiable pathological process [16,83]. The impact of COFP extends beyond physical discomfort, often correlating with mood disorders, sleep disturbances, cognitive impairments, and a substantial reduction in quality of life [3,84,85].

Symptomatically, COFP shares common features with other pain types, such as neuropathic characteristics, including spontaneous, intermittent, lancinating, or burning sensations, alongside both positive and negative sensory symptoms [35,86] (Appendix A).

The prevalence of OFP varies, with estimates ranging from 16.1% to 33.2%, and a more realistic figure seems to hover around 25% [87]. Within this spectrum, COFP accounts for about 10%, positioning it among the most common types of chronic pain, after low back, neck, and knee pain [1]. Recent studies indicate an incidence rate of COFP at approximately 38.7 per 100,000 person-years. It is more prevalent in women and tends to increase with age [88].

The majority of COFP cases are linked to musculoskeletal disorders, particularly temporomandibular disorders (TMDs), which affect an average of 4.6% of the general population, with a higher incidence in women (6.3%) compared to men (2.8%) [89].

Another highly prevalent form of COFP is BMS [90].

The worldwide prevalence of BMS is estimated at 1.73% in the general population, which notably increases to 7.72% among dental patients, as detailed in a 2021 meta-analysis conducted by Wu et al. [91]. This extensive study included both clinical and population-based research, offering a comprehensive view. However, the exact prevalence rates vary due to differences in population demographics, geographic regions, and diagnostic criteria used. In terms of geographic distribution, BMS prevalence is higher in Europe (5.58%) and North America (1.10%), compared to Asia (1.05%), within the general population. This trend reverses in clinical settings, with Asia reporting a higher prevalence (8.96%) than South America (6.05%) and Europe (6.46%) [91]. Such variations underscore the significant impact of geographic factors on BMS occurrence. A gender-specific analysis of the data reveals a more pronounced prevalence of BMS in females (1.15%) than in males (0.38%) within the general population, indicating a ratio of approximately 3:1 [92]. This notable gender disparity in BMS prevalence may be linked to physiological and behavioral factors specific to each gender [93].

Trigeminal neuralgia (TN) is relatively rare, with a prevalence ranging from 0.03% to 0.3% in the general population, and an incidence of approximately 1 case per 70,000 to 100,000 individuals annually [94,95]. The likelihood of developing TN increases with age and the presence of certain medical conditions, such as migraines [96]. Notably, TN is 15–20 times more prevalent in individuals with multiple sclerosis, where its prevalence ranges between 1.1% and 6.3% [97].

PIFP is also rare, occurring in about 0.03% of the population, with an incidence rate of 4.4 per 100,000 person-years. PIFP predominantly affects females, typically manifesting around the age of 40 [98].

An ideal classification system in medicine is pivotal, serving as a comprehensive, biologically plausible, clinically useful, and reliable framework. In the realm of OFP, such a system is indispensable for accurate disease identification, effective treatment planning, and enhanced patient understanding and acceptance of their condition.

Classification in medicine organizes pathologies into categories based on specific criteria [99]. For OFP, these criteria often stem from etiology, pathophysiology, diagnosis, and management [72]. Clinicians rely on this classification for precise disease identifi-

cation and treatment strategies. Patients benefit from a clear diagnosis framed within a globally recognized system, aiding in their understanding and acceptance of the disease. Moreover, for research purposes, classification is crucial as it ensures consistent diagnostic criteria are applied across studies, whether in pharmacological trials or pathophysiological investigations.

According to Fillingim et al. [100], an ideal classification system should be:

1. exhaustive (including all clinical diseases or disorders within the field of interest);
2. biologically plausible (symptoms and signs should correspond to known biological processes) and mutually exclusive (there should be no overlap between disease entities due to common symptoms);
3. clinically useful (so that it can be used to aid in treatment and prognosis);
4. reliable (applicable consistently and reproducibly among clinicians);
5. simple for practical use.

However, most current OFP classification systems suffer from deficits in at least one of these qualities.

The variability in definitions across different classifications, drafted by independent clinician groups, further complicates this issue.

Although there is no consensus yet regarding a universal and unique OFP classification, the latest ICOP 2020 classification [71] seems to encompass most of Fillingim et al.'s requirements [100].

Other notable systems include:

1. The International Classification of Headache Disorders, 3rd edition (ICHD-3) beta version [101];
2. The International Association for the Study of Pain for the International Classification of Diseases (ICD-11) [14].

The ongoing updates to these classifications, readily available online, reflect the scientific community's commitment to enhancing the understanding of these pathologies. This process exemplifies the dynamic and evolving nature of medical knowledge, driven by continual research and clinical findings. As new insights emerge, classification systems are systematically refined and expanded, ensuring they remain current and applicable. This iterative approach is integral to advancing medical science and improving diagnostic accuracy, ultimately leading to better patient outcomes and more effective treatments.

The International Classification of Headache Disorders, 3rd edition (ICHD-3) beta version.

Since its inaugural publication in 1988 by the International Headache Society (IHS), the ICHD has undergone significant revisions, with major updates in 2004, 2013 (ICHD-3), in 2018 (ICHD-3 beta version), and in 2020 [67,101]. This taxonomic classification system, primarily focused on headache-related disorders and painful craniofacial conditions, is organized into a three-part system. Parts I and II categorize primary and secondary headaches across 12 main groups. Part III addresses painful cranial neuropathies and other facial pain conditions, particularly those related to the trigeminal nerve [102] (Table 1).

This part has two distinct sections, one for primary painful craniofacial neuralgias and facial pain and the other for unclassified or unspecified headaches. A reference to OFP conditions also appears in Part II of the ICHD-3, dedicated to secondary headaches, which occur when a primary headache worsens in close temporal relationship with a causative OFP condition. Therefore, the ICHD-3 recognizes that some disorders arising from dental, maxillary, maxillary–sinus, eye, ear, and nose elements can cause headaches. However, despite its comprehensive structure, ICHD-3 falls short in encompassing all clinical phenotypes of OFP and does not adequately consider biopsychosocial and behavioral factors [102,103].

Table 1. Classification system from the International Headache Society (ICHD-3 beta version).

Part 1: Primary Headache	
1. Migraine	
2. Tension-type headache	
3. Trigeminal autonomic cephalalgias	
4. Other primary headache disorders	
PART 2: SECONDARY HEADACHE	
5. Headache attributed to trauma or injury to the head and/or neck	
6. Headache attributed to cranial or cervical vascular disorder	
7. Headache attributed to non-vascular intracranial disorder	
8. Headache attributed to a substance or its withdrawal	
9. Headache attributed to infection	
10. Headache attributed to homeostasis disorders	
11. Headache or facial pain attributed to disorders of the skull, neck, eyes, ears, nose, paranasal sinuses, teeth, mouth, or other facial or cervical structures	
12. Headache attributed to psychiatric disorder	
PART 3: CRANIAL NEUROPATHIES, AND OTHER FACIAL PAINS	
13. Painful cranial neuropathies and other facial pains	
14. Other headache disorders	
13. Painful cranial neuropathies and other facial pains	
ICHD code	Types of Pain
13.1	Trigeminal neuralgia
13.1.1	Classical trigeminal neuralgia
13.1.1.1	Purely paroxysmal classical trigeminal neuralgia
13.1.1.2	Classical trigeminal neuralgia with concurrent persistent facial pain
13.1.2	Painful trigeminal neuropathy
13.1.2.1	Painful trigeminal neuropathy attributed to acute herpes zoster
13.1.2.2	Post-herpetic trigeminal neuropathy
13.1.2.3	Painful post-traumatic trigeminal neuropathy
13.1.2.4	Painful trigeminal neuropathy attributed to multiple sclerosis (MS) plaques
13.1.2.5	Painful trigeminal neuropathy attributed to space-occupying lesion
13.1.2.6	Painful trigeminal neuropathy attributed to other disorders
13.11	Burning mouth syndrome (BMS)
13.12	Persistent idiopathic facial pain (PIFP)

Abbreviations: OFP: orofacial pain; ICOP: International Classification of Orofacial Pain; IASP: International Association for the Study of Pain; ICD: International Classification of Diseases. Trigeminal neuralgia attributed to multiple sclerosis and trigeminal neuralgia attributed to space-occupying lesion are only coded within the context of central chronic neuropathic pain (MG 30.50).

The hierarchical nature of the ICHD-3 allows for varying levels of diagnostic detail, adaptable to different clinical settings in which clinicians may decide the necessary level of diagnosis based on the type of practice. Therefore, coding can range from one digit up to five depending on the detail of the criteria used in the diagnosis. This flexibility is crucial for accurately diagnosing and coding over 200 headache-related disorders categorized in 12 main headache categories. The ultimate goal is to align this coding system with the WHO's International Classification of Diseases (ICD-11) [14,33].

In oral medicine, the most frequently referenced category within Part III is Category 13, dealing specifically with OFP conditions [104]. (Table 1)

Parallel to the ICHD's development, the IASP has collaborated with the WHO to integrate a systematic classification of chronic pain into the ICD-11.

Every update or recent development related to the ICHD or ICD-11 since 2018 is available on-line.

This integration represents a significant step towards a more comprehensive understanding of chronic pain conditions, including OFP, and emphasizes the need for a multidimensional approach to diagnosis and treatment.

The International Classification of Diseases 11th Revision (ICD-11).

The 11th Revision of the ICD-11 [12], published by the WHO in June 2018, represents a significant evolution in the global standard for health data classification. Its international and multilingual taxonomy is used for collecting and reporting data on health services (e.g., statistics on morbidity and mortality, quality and safety, health costs, and clinical research).

This latest revision, which has been the international standard for coding since 2022, offers enhanced features for research applicability, clinical detail encoding, and modernized scientific content. One of the pivotal advancements in ICD-11, as highlighted by Scholz et al. [37], is its refined approach to chronic pain, now recognized both as a symptom and as a distinct disease entity, complete with a supportive coding framework.

A groundbreaking feature of ICD-11 is the concept of "multiple parenting", which allows diagnostic entities to be classified under more than one category. This flexibility overcomes previous limitations where diseases could only be classified singularly by etiology or site [14]. For instance, "Chemotherapy-induced chronic pain" can be categorized both under "Chronic cancer pain" (etiology) and "Chronic neuropathic pain" (mechanism). This multifaceted approach aligns more closely with the interdisciplinary nature of medical practice, bridging gaps between specialties such as oncology and neurology.

The ICD-11 pain classification introduces nine main diagnostic categories [14] (Table 2).

Table 2. International Association for the Study of Pain's (IASP) classification system with the International Classification of Diseases (ICD-11), categorizing different types of chronic pain.

Types of Pain	ICD-11 Code
Primary chronic pain	MG 30.0
Chronic cancer pain	MG 30.1
Chronic post-surgical and post-traumatic pain	MG 30.2
Chronic musculoskeletal pain	MG 30.3
Secondary chronic visceral pain	MG 30.4
Chronic neuropathic pain	MG 30.5
Secondary chronic headache or orofacial pain	MG 30.6
Other specified chronic neuropathic pain	MG 30.Y
Chronic neuropathic pain, unspecified	MG 30.Z

Specifically, neuropathic pain conditions are divided into two major categories:

1. Chronic central neuropathic pain (MG 30.50);
2. Chronic peripheral neuropathic pain (MG 30.51).

Orofacial pain in the ICD-11 is recognized as a significant medical entity, reflecting advancements in understanding pain as a complex, multidimensional experience [105].

Notably, OFP is categorized under secondary chronic headaches (code MG 30.62) with specific codes assigned to different conditions such as NT, PIFP, and BMS [14,106].

Specific codes for orofacial pain conditions:

1. NT: 8B82.0 under trigeminal disorders (Foundation ID in the ICD-11 browser: 1803581281);
2. PIFP: 8B82.1 under trigeminal disorders (Foundation ID in the ICD-11 browser: 248232693);
3. BMS: DA0F.0: sensory disturbances involving the orofacial region (Foundation ID in the ICD-11 browser: 618998878) (Figure 2).

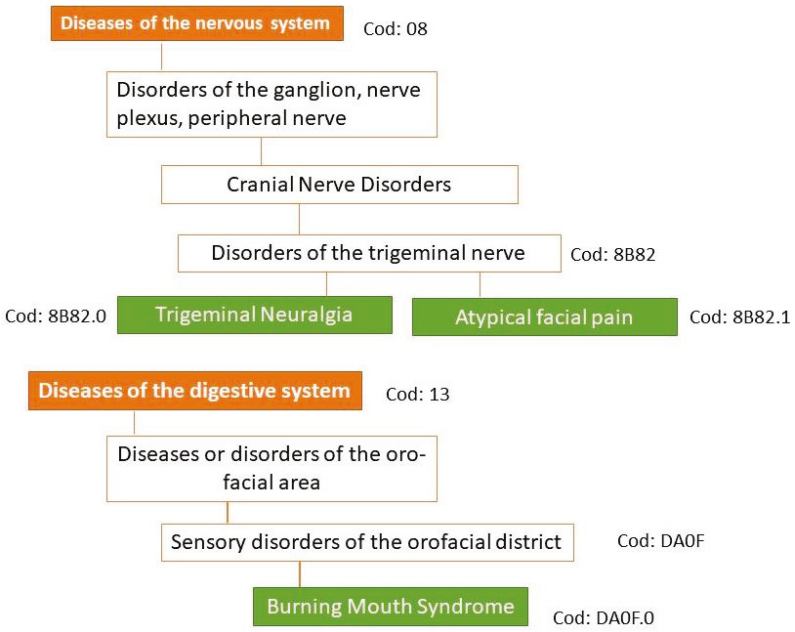


Figure 2. Classification of the three main types of orofacial pain according to ICD-11. Under the new concept of multiple parenting in ICD-11, each entity can be assigned to other divisions of the classification of chronic pain. In ICD-11, persistent idiopathic facial pain is referred to as atypical facial pain.

See Appendix B for extension codes.

See website https://icd.who.int/en accessed on 5 November 2023 for the complete classification of ICD-11.

The codes for the most common forms of OFP in different classifications are indicated in Table 3.

Table 3. Codes for common forms of orofacial pain according to different classification systems.

Types of OFP	ICOP 2020	ICHD-3 v. Beta	IASP for ICD-11
Trigeminal neuralgia	4.1.1	13.1	8B82.0
Purely paroxysmal classical trigeminal neuralgia	4.1.1.1.1	13.1.1.1	N.D
Classical trigeminal neuralgia with concomitant continuous pain	4.1.1.1.2	13.1.1.2	N.D
Trigeminal neuralgia attributed to multiple sclerosis	4.1.1.2.1	13.1.2.4	MG 30.50
Trigeminal neuralgia attributed to space-occupying lesion	4.1.1.2.2	13.1.2.5	MG 30.50
Trigeminal neuropathic pain attributed to herpes zoster	4.1.2.1	13.1.2.1	1E91.4
Post-herpetic trigeminal neuralgia	4.1.2.2	13.1.2.2	1E91.5
Post-traumatic trigeminal neuropathic pain	4.1.2.3	13.1.2.3	N.D
Trigeminal neuralgia attributed to another cause	4.1.1.2.3	13.1.2.6	8B82.Z
Burning mouth syndrome	6.1	13.11	DA0F.0
Persistent idiopathic facial pain	6.2	13.12	8B82.1
Persistent idiopathic dento-alveolar pain	6.3	N.D	N.D

The ICOP 2020 marks a significant evolution in the understanding and classification of OFP [71]. This system, modeled after the ICHD-3, offers a nuanced approach to diagnosing OFP based on symptomatology and suspected pathophysiology, prioritizing the disorder's

characteristics over its location. This novel approach broadens the diagnostic criteria and fosters a more comprehensive understanding of various OFP forms improving a proper diagnosis and management.

The hierarchical classification system of the ICOP 2020 enables a granular diagnosis, ranging from a broad categorization (first digit) to highly specific classifications (up to the seventh digit) [71]. This level of detail adapts to different clinical contexts, with general medicine often utilizing first or second digit diagnoses, while specialist practices may require more detailed coding.

Unlike ICHD-3, primary and secondary OFP are included in the same group in separate subgroups because, according to experts, the strict structure of the ICHD criteria, using Criterion A to describe the pain, Criterion B to identify the presumed cause, and Criterion C to establish evidence of causality, is not easily applicable to OFP.

In cases where patients present with multiple types of pain, diagnoses should be prioritized based on the patient's experience of suffering and disability, also including the longitudinal history of pain (how and when did the pain start?), family history, medication effect, relationship with menstrual cycle, age, sex, etc.

For a definitive OFP diagnosis, patients must experience a minimum number of attacks or days with pain, as specified in the detailed criteria for each type or subtype of OFP. Different OFPs must meet a series of other requirements described in the criteria under separate letter headings, i.e., A, B, C, etc. Some letter headings are monothematic, expressing a single requirement. Other letter headings are polythematic and require, for example, two of the four listed characteristics. This structure is common in the ICHD-3 [67].

The diagnostic criteria in this classification do not include the evaluation of the severity and frequency of painful attacks, which should be evaluated and specified [107,108].

Another innovative feature of this classification is the introduction of the "psychosocial assessment of patients with OFP". This approach integrates psychometric testing for a holistic evaluation of the patient, acknowledging the significant role of psychological factors in pain perception and management [71]. The recommended tests are summarized in Table 4. The ICOP classification considers six groups. However, in this text, we will focus on pain types 4 and 6 (Table 5). (See cross-references for the complete classification of ICOP 2020).

Table 4. Recommended psychometric tests for the assessment of pain, psychological profile, and coping strategies of the patient.

Abbreviations	Questionnaires	
Pain		
Pain drawing	/	Evaluate the extent of the pain
GCPS	Graded Chronic Pain Scale	Scale of pain intensity and disability
JFLS	Jaw Functional Limitation Scale	Scale of functional limitation (specific to TMD)
OBC	Oral Behaviors Checklist	Checklist of dysfunctional behaviors (specific to TMD)
Depression and anxiety		
GAD7	General Anxiety Disorders	Scale for the evaluation of generalized anxiety disorder
PHQ-9	Patient Health Questionnaire-9	Depression evaluation
PHQ-15	Patient Health Questionnaire-15	Scale for the evaluation of the severity of somatic symptoms
PCS	Pain Catastrophizing Scale	Scale for the evaluation of catastrophizing
Coping strategies		
CSQ	Coping Strategies Questionnaires	Scale for the evaluation of coping strategies
TSK	Tampa Scale for Kinesiophobia	Scale of pain avoidance

Table 5. Classification system for COFP by the International Classification of Orofacial Pain (ICOP 2020).

Orofacial Pain	
1. Orofacial pain attributed to disorders of the dento-alveolar and anatomically related structures 2. Myofascial orofacial pain 3. Temporomandibular joint (TMJ) pain 4. Orofacial pain attributed to lesion or disease of cranial nerves 5. Orofacial pains with presentations similar to primary headaches 6. Idiopathic orofacial pain	
ICOP Code	**Types of Pain**
4.1	Pain attributed to lesion or disease of the trigeminal nerve
4.1.1	Trigeminal neuralgia
4.1.1.1	Classical trigeminal neuralgia
4.1.1.1.1	Purely paroxysmal classical trigeminal neuralgia
4.1.1.1.2	Classical trigeminal neuralgia with concomitant continuous pain
4.1.1.2	Secondary trigeminal neuralgia
4.1.1.2.1	Trigeminal neuralgia attributed to multiple sclerosis
4.1.1.2.2	Trigeminal neuralgia attributed to space-occupying lesion
4.1.1.2.3	Trigeminal neuralgia attributed to another cause
4.1.1.3	Idiopathic trigeminal neuralgia
4.1.1.3.1	Idiopathic trigeminal neuralgia, purely paroxysmal
4.1.1.3.2	Idiopathic trigeminal neuralgia with concomitant continuous pain
4.1.2	Other trigeminal neuropathic pain
4.1.2.1	Trigeminal neuropathic pain attributed to herpes zoster
4.1.2.2	Post-herpetic trigeminal neuralgia
4.1.2.3	Post-traumatic trigeminal neuropathic pain
4.1.2.3.1	Probable post-traumatic trigeminal neuropathic pain
4.1.2.4	Trigeminal neuropathic pain attributed to another disorder
4.1.2.4.1	Probable trigeminal neuropathic pain attributed to another disorder
4.1.2.5	Idiopathic trigeminal neuropathic pain
ICOP Code	**Types of Pain**
6.1	Burning mouth syndrome (BMS)
6.1.1	Burning mouth syndrome without somatosensory alterations
6.1.2	Burning mouth syndrome with somatosensory alterations
6.1.3	Probable burning mouth syndrome
6.2	Persistent idiopathic facial pain (PIFP)
6.2.1	Persistent idiopathic facial pain without somatosensory alterations
6.2.2	Persistent idiopathic facial pain with somatosensory alterations
6.2.3	Probable persistent idiopathic facial pain
6.3	Persistent idiopathic dento-alveolar pain (PIDP)
6.3.1	Persistent idiopathic dento-alveolar pain without somatosensory alterations
6.3.2	Persistent idiopathic dento-alveolar pain with somatosensory alterations
6.3.3	Probable persistent idiopathic dento-alveolar pain

2. Discussion and Future Directions

The modern approach to understanding COFP emphasizes the importance of a comprehensive biopsychosocial perspective. This model holistically considers the intertwined nature of biological (physiological), psychological, and social factors in pain perception and management. By advocating for the integration of diagnostic codes from various contemporary classification systems—the ICOP 2020, the ICHD-3, and the ICD-11—a more nuanced understanding of COFP is fostered. Additionally, incorporating the code for somatic symptom disorder (SSD) with predominant pain from the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), by the American Psychiatric Association, underscores the crucial role of psychological factors in the experience of chronic pain. This integration acknowledges that COFP is not solely a physical ailment but a complex interplay of various aspects of human health, necessitating a multifaceted approach to diagnosis and treatment [14,71,103,109]. This integration, aligned with the ICD coding system, could enhance the standardized coding of chronic pain, including in mental health contexts, underscoring the vital interplay of biological, psychological, and social factors in chronic pain syndromes [12,110].

The primary DSM-5 code for somatic symptom disorder is [109]—300.82 (F45.1)—somatic symptom disorder.

In the realm of COFP, and other chronic pain types, this disorder can be conceptualized within the SSD framework when patients exhibit heightened attention to physical symptoms such as pain, accompanied by intense health-related anxiety, disproportionate concerns regarding their symptoms, and significant investment of time and energy in these concerns, leading to considerable distress or functional impairment [111,112].

These persistent reactions often continue even after extensive medical evaluations and reassurances. The DSM-5's transition from the DSM-4 has shifted the focus from the presence of physical complaints to the subjective experience of distress [113,114]. The DSM-5 criteria for SSD with predominant pain emphasize the excessive thoughts, feelings, and behaviors related to somatic symptoms, irrespective of whether the pain has a known medical cause [115–117].

In instances where COFP patients display characteristics indicative of SSD, this diagnostic code might be considered supplementary. This alignment with modern pain classification systems underscores the importance of a multidisciplinary and holistic approach, centering patient-focused care and treatment strategies [118–120] (Figure 3).

This review, comprising an in-depth examination of COFP and its classification systems, sets the stage for a comprehensive understanding of this complex condition [121,122]. Beginning with a summary of the previous sections, we have explored the prevalence and impact of chronic pain, the intricacies of COFP, and existing classification frameworks such as the ICOP 2020, ICHD-3, and ICD-11. In this study, we delve into the potential for extension of previous works on COFP, highlighting its implications and paving the way for future research. By providing a holistic view of COFP classification and management, this study significantly extends the current understanding of the condition. It emphasizes the integration of the biopsychosocial model, underlining the importance of considering not just the biological aspects of pain, but also incorporating psychological and social factors into our perception and treatment of pain. This comprehensive approach marks a pivotal advancement in the field, offering new directions for subsequent investigations [123–125]. This approach offers a significant extension of traditional perspectives, which predominantly focused on the physiological aspects of pain [126].

The implications of this review are far-reaching. It challenges the existing paradigms in COFP diagnosis and management, advocating for a more integrated approach [16,127,128]. The review highlights the limitations of current classification systems and suggests that a more nuanced system is essential for better patient outcomes. This could significantly influence how healthcare providers approach COFP, leading to more personalized and effective treatment plans [129,130].

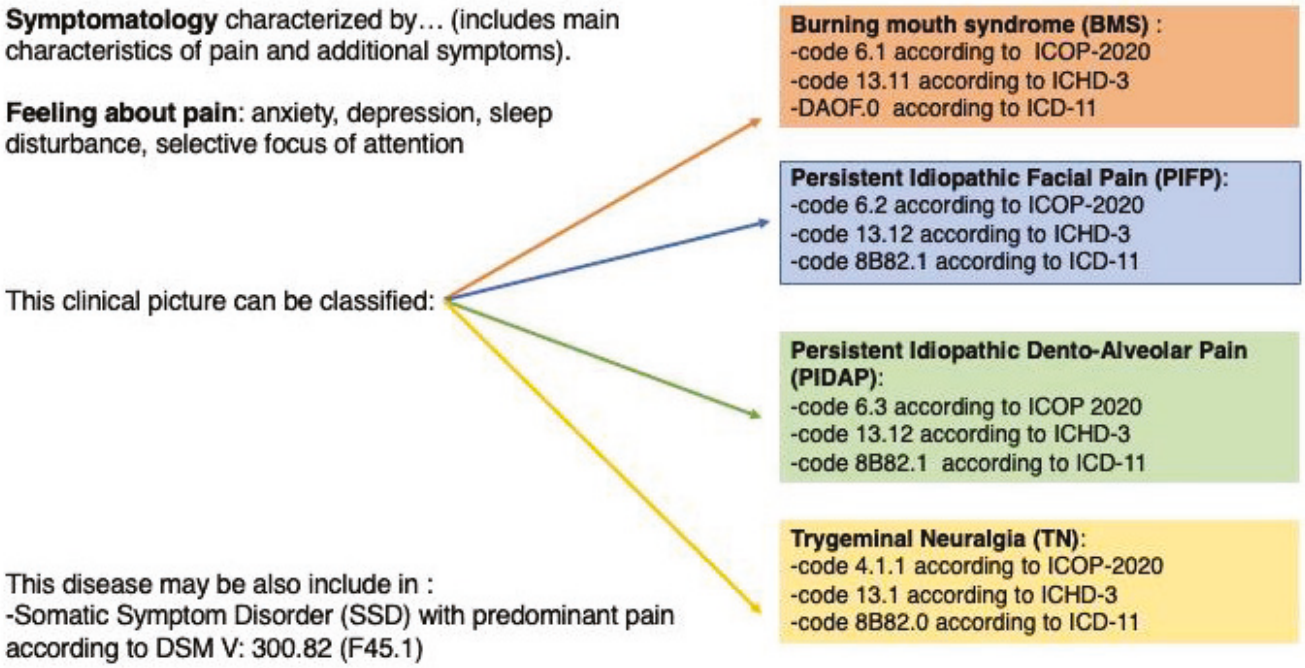


Figure 3. Classification and coding of chronic orofacial pain disorders. Abbreviations—BMS: burning mouth syndrome; PIFP: persistent idiopathic facial Pain; PIDP: persistent idiopathic dento-alveolar pain; TN: trigeminal neuralgia; SSD: somatic symptom disorder; ICOP: International Classification of Orofacial Pain (2020 Edition); ICHD-3: International Classification of Headache Disorders (3rd Edition); ICD-11: International Classification of Diseases (11th Revision); DSM-5: Diagnostic and Statistical Manual of Mental Disorders (5th Edition).

This review lays the groundwork for future research in several ways. Firstly, by identifying gaps in current classification systems, it calls for the development of a unified framework that can adapt to evolving understandings of COFP [131,132]. Secondly, it opens avenues for research into the integration of psychological and social factors in pain management, which is relatively unexplored in COFP. This could lead to new treatment modalities that address the patient's overall well-being [133–135].

The ultimate goal is to establish a comprehensive, universally applicable classification system for COFP that effectively integrates the biopsychosocial model. The challenge lies in balancing the need for a system that is detailed enough to be clinically useful yet simple enough for widespread adoption. Additionally, overcoming the traditional siloed approach to pain management and fostering a more holistic, interdisciplinary methodology remains a significant hurdle [136,137].

Achieving this goal requires an amalgamation of knowledge from various fields, including neurology, psychology, and sociology, coupled with advancements in technology [138]. Machine learning and data analytics could play a crucial role in analyzing patient data to derive more precise classifications [139]. Additionally, developing technologies for better pain measurement and monitoring could significantly enhance diagnosis and treatment [140,141].

The field of research focusing on COFP holds significant importance, extending far beyond the boundaries of a singular medical condition. This line of inquiry is instrumental in enhancing the overall understanding of COFP, a complex and often debilitating condition that impacts a substantial portion of the population. By delving into the multifaceted aspects of COFP, including its origins, progression, and effective management strategies, this research contributes profoundly to the broader discipline of pain management. It offers invaluable insights into the nuanced interplay between physiological, psychological, and social factors in pain perception and response. Furthermore, advancements in COFP research have the potential to inform and improve therapeutic approaches, leading to

more effective and personalized treatment plans for various types of pain conditions. This not only benefits patients suffering from COFP but also enriches the entire field of pain management with new knowledge and perspectives, ultimately enhancing the quality of life for individuals affected by chronic pain worldwide [142]. It challenges the existing frameworks and methodologies, pushing for innovations that can have a transformative impact on healthcare practices [143,144].

Despite its comprehensive approach, this review has limitations. The primary limitation is the reliance on existing literature, which may not fully capture the latest developments in the field.

However, the strengths of this study include a comprehensive examination of the various types of COFP, their underlying mechanisms, and their impact on patients' quality of life through an in-depth analysis of current classification systems [145,146]. However, the manuscript also emphasizes the significance of using the biopsychosocial model to understand pain perception and integrating biological, psychological, and social dimensions in pain management, which provide valuable insights for both clinicians and researchers [147].

Clinically, this review can aid in the development of more effective treatment strategies for COFP, leading to better patient outcomes and quality of life, taking into account the importance of psychometric evaluation in the treatment of patients with COFP as well as the importance of evaluating psychiatric comorbidities when making treatment decisions [148,149].

Finally, this review marks a significant step forward in the understanding of COFP. By highlighting the limitations of the current systems and advocating for a more holistic approach, it sets the stage for future advancements in this field. The integration of diverse knowledge domains and the application of new technologies are key to realizing the potential of this research, ultimately contributing to the evolution of pain management practices [144,150].

3. Conclusions

This review advocates for an integrated, multidisciplinary approach to COFP, emphasizing the necessity of considering physical, psychological, and social factors in pain management. The adoption of the biopsychosocial model is essential in developing more effective treatments that focus on a patient's overall well-being. Our analysis of classification systems like the ICOP 2020, ICHD-3, and ICD-11 underscores the need for a refined, universally applicable diagnostic framework that aligns with the evolving understanding of COFP. We highlight the challenges in accurate diagnosis and classification, indicating the need for continuous methodological and theoretical advancement. Future research should aim at creating a unified classification system capturing COFP's complexity and investigating how psychological and social factors can be integrated into individualized treatment plans. This work signals a shift towards a more holistic, patient-centered approach to COFP in both clinical and research settings.

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Appendix A. Terminologies, Definitions, and Classifications Related to COFP

Terminologies	Definitions
Allodynia	Pain evoked by a stimulus that is normally not harmful (mechanical, tactile) and does not cause tissue damage. Example: light brushing, light pressure, mild cold or heat. Note: the stimulus leads to an unexpectedly painful response. Lowered threshold—stimulus and mode of response differ.
Hyperalgesia	Increased sensitivity to pain, often disproportionate, produced by a harmful stimulus. Note: hyperalgesia reflects an increase in pain to above-threshold stimulation. Increased response: stimulus and mode of response are the same.
Hyperesthesia	Increased sensitivity to stimulation. The word is used to indicate both a decrease in the threshold to any stimulus and an increased response to normally recognized stimuli. It includes both allodynia and hyperalgesia, but more specific terms should be used wherever applicable.
Hyperapathia	Pain syndrome characterized by an abnormal painful reaction to a stimulus, especially a repetitive stimulus, as well as by an increased threshold. Note: it can occur with allodynia, hyperesthesia, hyperalgesia, or dysesthesia. There may be a delay in identifying the stimulus or incorrect localization with pain irradiation that can take on an explosive character. Increased threshold, increased response—stimulus and mode of response may be the same or different.
Hypoalgesia	Reduced sensitivity to pain produced by a normally painful stimulus. Relatively low pain in response to a stimulation that produces pain. Increased threshold, decreased response—stimulus and mode of response are the same.
Analgesia	Absence of pain in response to a harmful stimulus.
Hypoesthesia	Reduced sensitivity to stimulation that is normally painful.
Paresthesia	Spontaneous or provoked abnormal sensation that is not unpleasant.
Dysesthesia	Spontaneous or provoked abnormal sensation, unpleasant.
Trigeminal first neuron	First neuron of the somatosensory pathway that detects mechanical, thermal, or chemical stimuli at the peripheral end and transmits an action potential to the Gasser ganglion that synapses with the second-order neuron or projection neuron.
Nociceptor	Primary afferent (sensory) neuron that is exclusively activated by a painful stimulus. They are free nerve endings that may be thin myelinated Aδ fibers or unmyelinated C fibers.
Nociception	Process through which the nociceptor detects the painful stimulus and generates an action potential that propagates towards the higher centers.
Second-order neuron or projection neuron	Neuron that from the Gasser ganglion and/or the dorsal horn of the spinal cord that receives input from the primary afferent neuron and/or interneurons and projects it to the higher pain processing centers.
Interneuron	Neuron equipped with a cell body, axon, and dendrites present at the level of the Gasser ganglion and the spinal cord. It can be excitatory (glutamatergic) or inhibitory (GABAergic).
Spinothalamic tract	Tract of neurons that project from the Gasser ganglion and/or the spinal cord to the thalamus.
Central sensitization	Exaggerated or amplified response of the central nervous system to peripheral nociceptive and sensory stimuli. This amplified response and neuronal hyperexcitability contribute to the development of persistent pain characterized by hyperalgesia, allodynia, spontaneous pain, and referred pain.

Adaptation of terminology and definitions from the IASP.

Appendix B. ICD-11 Extension Codes

The "optional specifiers" or "extension codes" present in the ICD-11 allow for the assessment of pain severity, its temporal course, and the presence of psychological and social factors. The "severity" of pain is not only related to intensity, but also to the discomfort and interference of pain in the patient's life.

Each determinant of severity (intensity, pain-related discomfort, and interference) is evaluated by the patient on a numerical or visual rating scale from 0 to 10 (visual analog

scale (VAS) and numerical rating scale (NRS)) and then transformed into severity stages of "mild", "moderate", and "severe". Temporal characteristics can be coded as continuous pain (pain is always present), episodic recurrent pain (there are recurrent pain attacks with intervals without pain), and continuous pain with pain attacks (there are recurrent pain attacks as exacerbations of underlying continuous pain).

There is also an extension code that codes for cognitive, emotional, behavioral, and/or social factors that accompany chronic pain. This extension code should be used when it is believed that psychological and social factors contribute to the onset, maintenance, or exacerbation of pain, or are considered relevant consequences of pain.

Additional extension codes for each of these diagnoses are:

- XS7G: Psychosocial factors present;
- XS8B: No psychosocial factors present;
- Determinants of severity:
- Intensity:
- XS5B: No pain;
- XS5D: Mild pain (NRS: 1–3; VAS: <31 mm);
- XS9Q: Moderate pain (NRS: 4–6; VAS: 31–54 mm);
- XS2E: Severe pain (NRS: 7–10; VAS: 55–100 mm);
- Discomfort:
- XS1J: No discomfort;
- XS3R: Mild discomfort (NRS: 1–3; VAS: <31 mm);
- XS7C: Moderate distress (NRS: 4–6; VAS: 31–54 mm);
- XS7N: Severe distress (NRS: 7–10; VAS: 55–100 mm);
- Interference:
- XS71: No pain-related interference;
- XS5R: Mild pain-related interference (NRS: 1–3; VAS: <31 mm);
- XS2L: Moderate pain-related interference (NRS: 4–6; VAS: 31–54 mm);
- XS2U: Severe pain-related interference (NRS: 7–10; VAS: 55–100 mm).

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Article

Beyond Pain Relief: Unveiling the Multifaceted Impact of Anti-CGRP/R mAbs on Comorbid Symptoms in Resistant Migraine Patients

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Abstract: The study aimed to evaluate the effects of monoclonal antibodies (mAbs) acting on the calcitonin gene-related peptide (CGRP) or its receptor (anti-CGRP/R mAbs) on migraine comorbidities of depression, anxiety, and fatigue in patients resistant to traditional therapies. The issue addressed in this study is pivotal to unveiling the role of this neurotransmitter beyond pain processing. We conducted an open-label prospective study assessing comorbidities in patients with high frequency (HFEM) and chronic migraine (CM), medication overuse headache (MOH), and resistance to traditional prophylaxis. All patients were treated with anti-CGRP/R mAbs for 3 months. Seventy-seven patients were enrolled with either HFEM (21%) or CM (79%) with or without MOH (56% and 44%, respectively). We identified 21 non-responders (27%) and 56 responders (73%), defined on the reduction $\geq$50% of headache frequency. The two groups were highly homogeneous for the investigated comorbidities. Disease severity in terms of headache frequency, migraine-related disability, and affective comorbid symptoms was reduced in both groups with different thresholds; allodynia and fatigue were ameliorated only in responders. We found that anti-CGRP/R antibodies improved pain together with affection, fatigue, and sensory sensitization in a cohort of migraine patients resistant to traditional prophylaxis. Our results offer novel perspectives on the early efficacy of anti-CGRP/R mAbs in difficult-to-treat patients focusing on clinical features other than pain relief.

Keywords: migraine disorders; comorbidity; depression; anxiety; fatigue; allodynia; calcitonin gene-related peptide

1. Introduction

Migraine ranks as the most prevalent, disabling, and long-term neurological disease [1,2]. Migraine is an evolutive disease, in which the clinical features can vary over a long time course, probably according to different pathophysiological mechanisms [3].

The prevalence of migraine in the general population is around 15%, and 8% of migraineurs suffer from chronic migraine (CM) [4,5]. CM is more burdensome than episodic migraine (EM) in terms of disability, quality of life, use of health resources, involvement of comorbidities, and drug resistance [5,6]. If both EM and CM are considered, the disease has a paramount financial burden on Western economies, with an estimate of USD 19.6 billion in the United States [7] and EUR 27 billion in the European Union [8] per year. However, the true socioeconomic burden comprises the intangible costs and the reduced productivity of

the affected population [9]. Migraine affects females and males differently with a 3:1 ratio, and impairs their quality of life during the most productive period of their lives [10]. The clinical features of migraine attacks are prevalently unilateral, throbbing head pain, with sensitivity to visual, auditory, and other input (e.g., olfactory) [2,11,12]. Prodromic and postdromic symptoms such as fatigue, food craving, irritability, yawning, and reduced concentration can impair the so-called interictal period, between two pain phases [13]. Furthermore, a variable percentage of migraine patients have their attacks associated with transitory neurological symptoms (prevalently visual), collectively named migraine aura [14,15].

In other words, recognizing the multifaced aspects of the clinical presentation of migraine can be challenging. Pain might not be the pivotal clinical symptom and can evolve with time, sometimes paradoxically less troublesome than other features. The background literature clearly imposes to go beyond the headache, and considering migraine exclusively as a pain-processing disorder is extremely simplistic misleading, affecting its management [16].

The comorbidity between migraine and psychiatric disorders has been extensively explored in the literature [17–24]. The strongest association was described with depression and anxiety, which seem to have a bidirectional relationship with migraine. Subjects with a combination of major depression and anxiety disorders are more likely to have migraine compared with those with depression or anxiety only and without both [2,22].

Fatigue has also been recognized as a dominant feature of migraine [25]. It is estimated that approximately 60% of migraineurs report pathologic fatigue [26]. Furthermore, fatigue seems to be correlated with symptoms of depression and headache intensity in migraineurs [26].

Depression, anxiety, and fatigue are pivotal elements of migraine-related disability and disease progression, regarded as risk factors for transforming EM into CM [3,27].

Prophylactic treatments for migraine can be also effective on psychiatric comorbidities (e.g., antidepressants) that are considered when choosing the proper drug for headache frequency control [27,28]. The role of novel targeted anti-migraine drugs in this fearful triad (depression, anxiety, and fatigue) is still unclear and unpredictable based on the putative pathophysiological mechanism.

We are still largely ignorant about migraine causative molecules and signaling pathways, although findings on both clinical and basic neuroscience have identified the central role of the activation of the trigeminovascular system and the antidromic release of molecules, such as the calcitonin gene-related peptide (CGRP) [28–30]. CGRP is released by small unmyelinated sensory (C-)fibers whose hyperactivation could result in what is called neurogenic inflammation [28].

Other neuropeptides and molecules contribute to the migraine pathophysiology and can be found in the innervation of cranial vessels (intra- and extracerebral), such as vasoactive intestinal polypeptide (VIP), nitric oxide (NO), adenylate cyclase-activating peptides (PACAP), amylin, and ions, although their role needs to be further verified [31]. Clinical studies have supported the importance of PACAP the pathophysiology of migraine but the clinical trials are still inconclusive with both a receptor monoclonal antibody (AMG 301) that showed no effects in preventing migraine, and a PACAP ligand monoclonal antibody (Lu AG09222) that showed promising results on headache frequency bit is still in Phase 2 clinical trial [32].

Compared to other neuropeptides, CGRP levels were consistently elevated in external jugular vein blood samples during the pain phase of migraine [33] and 5-hydroxytryptamine (5-HT, serotonin) receptors (5-HT1B and 5-HT1D) agonists (e.g., triptans) are effective suppressors of CGRP release and significantly abort migraine attacks [34].

Serotonin reuptake is also one of the main targets of the pharmacological treatment of psychiatric syndromes [35]. CGRP and serotonin signaling are tightly intertwined in the migraine pathophysiology, still little is known about the effects of CGRP blockage and

migraine comorbidities [36,37] that could shed light on the common pathophysiology of the diseases and translate the results of CGRP-interference on mood disorders.

According to most clinical trials and real-world studies, the efficacy of novel treatments targeting the CGRP is not significantly different between EM and CM [38–40], including patients experiencing previous preventive treatment failures that were considered most difficult to treat [41,42]. However, real-world trials showed non-responders more numerous in CM [43,44], probably because CM patients with continuous pain and medication overuse headache (MOH) have been excluded from most clinical trials [45]. It has been reported in real-world studies that high frequency is not a negative predictor, but daily headache could prognosticate the failure of novel treatments [27]. In this context, we evaluated comorbid symptoms of anxiety, depression, and fatigue at baseline and 3 months after starting a treatment with monoclonal antibodies (mAbs) acting on the CGRP (Fremanezumab and Galcanezumab) or its receptor (CGRPR) (Erenumab) in a cohort of 77 subjects with a diagnosis of migraine resistant to traditional drug prophylaxis either high-frequency EM (HFEM > 8 headache days/month) or CM with or without MOH. Additionally, we decided to consider anxiety–depressive symptoms and fatigue as potential prognostic factors for drug efficacy. Hence, we sorted the patients into two groups according to whether the treatment was clinically effective or not, to observe if any differences in these symptoms were present at baseline.

2. Methods

2.1. Study Design

The study was an open-label prospective study evaluating symptoms of anxiety, depression, and fatigue in 77 HFEM and CM patients resistant to traditional prophylaxis and, therefore, undergoing treatment with anti-CGRP/R mAbs (Erenumab 70 or 140 mg/month, Galcanezumab 120 mg/month, and Fremanezumab 225 mg/month, administered by subcutaneous injection once a month). The study consisted of a preliminary evaluation (V0) to verify the eligibility of the patient, a baseline assessment (V1) with the first administration of anti-CGRP/R mAbs, and a 3-month follow-up visit (V2). The selected study period was conceived to observe the rapid effects of the novel molecules on migraine comorbidities. No acute medication was used during the visits. Information on acute medication used by the participants during the study period was recorded.

2.2. Study Participants

We enrolled adult outpatients, consecutively enrolled from November 2021 to December 2022 in the Neurology Unit, Center for Diagnosis and Treatment of Headaches and Craniofacial Pain at the University of Pisa, suffering from HFEM or CM according to the International Classification of Headache Disorders—3rd edition (ICHD-3) [46], and resistant to the common prophylaxis therapies according to the European Headache Federation (EHF) Consensus [47].

All patients received and failed at least three preventive medication classes (beta-blockers, calcium-channel blockers, anticonvulsants, antidepressants, onabotulinumtoxinA) due to a lack of efficacy or intolerable side effects.

Patients were discontinued from other preventive treatments at least 3 months before the baseline or were treated with stable oral migraine prophylaxis (defined as stable dosage of the medication for at least 6 months before the inclusion visit and for the duration of the study). No specific medication for psychiatric comorbidities was allowed or used by the participants. The exclusion criteria were: (1) age under 18; (2) diagnosis of schizophrenia, chronic psychosis, acute psychosis; (3) diagnosis of somatic and related symptom disorders; (4) diagnosis of ongoing substance use disorders; (5) patients with impaired speech; (6) patients with mental retardation; (7) diagnosis of other neurological diseases; (8) patients unable to provide valid written informed consent; (9) pregnant or breastfeeding patients; (10) patients with a desire to become pregnant during the study period.

This study was performed in accordance with the Declaration of Helsinki, and it was approved by the local ethics committee (Comitato Etico Area Vasta Nord Ovest—Sezione Autonoma del Comitato Etico Regionale per la Sperimentazione Clinica—Via Roma 67, 56126, Pisa, Italy) with approval code ID-14.518. All subjects involved provided written, informed consent before their inclusion.

2.3. Clinical Assessment

The study visits were performed before (V1) and after (V2) the administration of the treatment. Clinical characteristics of migraine were collected through an interview and based on patients' self-reported diaries. Moreover, the following questionaries were administered at each visit: Migraine Disability Assessment (MIDAS) [48,49] for the evaluation of migraine-related disability, Fatigue Severity Scale (FSS) [50,51] to assess migraine-associated fatigue, the Generalized Anxiety disorder (GAD-7) [52], and Patient Health Questionnaire (PHQ-9) [53], to monitor anxiety and depressive symptoms, and Allodynia Symptoms Checklist 12 (ASC-12) to report ictal allodynia [54]. The questionnaires were administered by trained neurologists and neurology residents of a tertiary care outpatient clinic, specialized in the diagnosis and treatment of headaches and craniofacial pain. The neurologists administered the questionnaires properly translated into Italian.

2.4. Statistical Analysis

All demographic and clinical data were presented for continuous variables in terms of medians and interquartile ranges.

The quantitative variables of the sample, evaluated with the Shapiro–Wilk test, did not have a normal distribution. For this reason, to test the possible differences before and after the treatment with anti-CGRP/R mAbs, the Wilcoxon rank test was used, whereas to compare the two subgroups identified (responders and not-responders to anti-CGRP/R mAbs) the Mann–Whitney test was used.

Categorical variables were expressed as percentages and the comparison was performed by the Chi-square test with continuity correction (Yates test). Binary logistic regression analysis was performed to predict the likelihood of the patients responding to the anti-CGRP/R mAbs, according to the measured variables.

The differences were considered statistically significant for values of probability $p < 0.05$ (two tails). IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA: IBM Corp was used for statistical analyses.

3. Results

The study population was composed of 77 patients, of which 59 (77%) were females, with a median age of 49.0 years old (IQR 15.0).

All patients were diagnosed with migraine without aura; 4 subjects (5%) had a concomitant diagnosis of migraine with aura and, according to the ICHD-3, were coded as both migraine with aura and migraine without aura. At baseline, 16 patients (21%) self-reported a frequency compatible with HFEM, and 61 (79%) with CM of which 43 were also diagnosed with medication overuse headache (MOH). MOH diagnosis was not reassessed at V2, as the duration of the symptoms required for the diagnosis was longer than the study period. All patients were resistant to traditional drug prophylaxis.

All patients were treated with mAbs therapy: 44 patients (57%) received monoclonal antibodies acting on the CGRP (12 were treated with Fremanezumab and 32 with Galganezumab) and 33 patients (43%) received monoclonal antibodies acting on the CGRPR (Erenumab).

For all patients, we evaluated comorbidity symptoms of anxiety, depression, fatigue, and allodynia at baseline and 3 months after starting the treatment. All patients completed the study. No adverse events, tolerability, or safety issues were reported.

The overall analysis showed that the treatments were highly effective in reducing migraine frequency which dropped from a median of 23 days/month to 6 days/month (p

< 0.001). The same highly significant impact was registered for migraine-related disability, anxiety and depressive symptoms, allodynia, and fatigue, as summarized in Table 1.

Table 1. Clinical assessment at baseline and after 3 months.

	V1 Raw Data		Clinical Score (75–25 Percentile)	V2 Raw Data		Clinical Score (75–25 Percentile)	
	Median	**75–25 Percentiles**		**Median**	**75–25 Percentiles**		p [1]
Age (years)	47.00	57–41					
Headaches frequency (days/month)	23.00	30–15		6.00	13–4		**<0.001** *
MIDAS	93.00	133–69		24.50	49–4		**<0.001** *
ASC-12	6.00	10–4	Moderate (3.00–1.00)	3.00	7–0	Mild (2.00–0.00)	**<0.001** *#§
GAD7	9.00	13–6	Mild (2.00–1.00)	6.50	9–4	Mild (1.75–0.00)	**<0.001** *#§
PHQ9	8.00	13–6	Mild (2.00–1.00)	6.00	9.00–3.00	Mild (1.00–0.00)	**<0.001** *#§
FSS	46.00	57–34		36.00	44–20		**<0.001** *

[1] Test Wilcoxon; MIDAS: Migraine Disability Assessment Scale; FSS: Fatigue Severity Scale; ASC-12: Allodynia Symptom Checklist 12; GAD7: Generalized Anxiety Disorder 7; PHQ9: Patient Health Questionnaire 9; Clinical score:. Severe = 3; Moderate = 2; Mild = 1; Absent = 0; # raw data; § clinical score (* $p < 0.001$).

We decided to run a subgroup analysis dividing the population at baseline into two sets (responders and non-responders), based on the clinical effectiveness of the drugs measured at V2. According to the EHF treatment guidelines, non-responders were defined as subjects that did not have a reduction of at least 50% in the frequency of migraine after the administration of drugs for at least three months. We identified 21 non-responders (27%) and 56 responders (73%).

The two groups were homogeneous, without distinctive features among the analyzed variables. The burden of disease, the distribution of mild psychiatric symptoms (anxiety and depression), fatigue, and ictal allodynia did not show significant differences (Table 2). The same result was obtained considering crude scores of the tests or clustering raw data according to validated clinical significance as absent, mild, moderate, and severe (from 0 to 3 in crescent order).

The responders' group exhibited a reduction of disease severity, non-exclusively in terms of headache frequency but also associated disability, allodynia ($p < 0.001$), and psychiatric comorbidities (Table 3).

Non-responders showed a significant reduction of the headache frequency although below the 50% threshold ($p = 0.003$), and reduced migraine-related disability ($p < 0.001$). No significant improvement was registered for ictal allodynia, depressive symptoms, and fatigue. Although the raw scores for anxiety did not significantly change for this group, the clinical classification varied from a median value of moderate to mild symptoms during the 3 months of observation ($p = 0.034$).

However, the homogeneity of the groups did not allow for the prediction of treatment outcomes based on the investigated characteristics (Table 4).

Table 2. Clinical features of responders and non-responders at baseline.

	Responder (% of All Patients)	**Non-Responder (% of All Patients)**	
	N (%)		p [1]
EM	13 (17%)	3 (4%)	0.586
CM	43 (56%)	18 (23%)	
Patients with MOH	34 (44%)	9 (12%)	0.981
Patientes without MOH	22 (28%)	12 (16%)	
	Median (75–25 percentiles)		p [2]
Age (years)	50.00 (57–44)	41.00 (49–36)	0.082
Headaches frequency (days/month)	22.00 (30–14)	30.00 (30–15)	0.237
MIDAS	90.00 (129–67)	99.00 (152–78)	0.171
ASC-12 (raw data)	6.00 (10–4)	5.00 (12–3)	0.812
ASC-12 (clinical score)	Moderate (3–1)	Mild (3–1)	0.628
GAD7 (raw data)	8.00 (13–6)	10.00 (14–6)	0.491
GAD7 (clinical score)	Mild (2–1)	Moderate (3–1)	0.250
PHQ9 (raw data)	7.50 (13–5)	10.00 (14–6)	0.132
PHQ9 (clinical score)	Mild (2–1)	Moderate-Mild (2–1)	0.259
FSS	45.00 (57–33)	54.00 (59–39)	0.151

[1] Chi-Squared test; [2] U Mann–Whitney test; MIDAS: Migraine Disability Assessment Scale; FSS: Fatigue Severity Scale; ASC-12: Allodynia Symptom Checklist 12; GAD7: Generalized Anxiety Disorder 7; PHQ9: Patient Health Questionnaire 9; EM: episodic migraine; CM: chronic migraine; MOH: medication overuse headache; Clinical score:. Severe = 3; Moderate = 2; Mild = 1; Moderate-Mild = 1.5; Absent = 0.

Table 3. Differences between parameters at baseline and after three months in responders and non-responders.

	Responder Median (75–25 Percentiles)			**Non-Responder Median (75–25 Percentiles)**		
	V1	**V2**	p [1]	**V1**	**V2**	p [1]
Headaches frequency (days/month)	22.00 (30–14)	5.00 (6–3)	**<0.001** ***	30.00 (30–15)	20.00 (30–12)	**0.003** **
MIDAS	96.00 (135–70)	8.00 (39–2)	**<0.001** ***	99.00 (15278)	38.00 (60–26)	**<0.001** ***
ASC-12 (raw data)	6.00 (9–3)	1.50 (5–0)	**<0.001** ***	5.00 (12–2)	6.00 (9–1)	0.241
ASC-12 (clinical score)	Moderate (3–1)	Absent (1–0)	**<0.001** ***	Mild (3–0)	Mild(3–0)	0.476
GAD7 (raw data)	8.00 (12–6)	6.00 (7–3)	**0.001** **	10.00 (13–5)	7.00 (10–6)	0.130

Table 3. *Cont.*

	Responder Median (75–25 Percentiles)			Non-Responder Median (75–25 Percentiles)		
	V1	**V2**	p [1]	**V1**	**V2**	p [1]
GAD7 (clinical score)	Mild (2–1)	Mild (1–0)	**0.002** **	Moderate (2–1)	Mild (2–1)	**0.034** *
PHQ9 (raw data)	7.00 (12–5)	4.00 (7–2)	**<0.001** ***	10.00 (14–6)	8.00 (13–6)	0.184
PHQ9 (clinical score)	Mild (2–1)	Absent (1–0)	**<0.001** ***	Mild-Moderate (2–1)	Mild (2–1)	0.414
FSS	43.50 (57–26)	29.00 (40–17)	**0.001** **	54.00 (61–44)	42.00 (55–39)	0.109

[1] Wilcoxon test; MIDAS: Migraine Disability Assessment Scale; FSS: Fatigue Severity Scale; ASC-12: Allodynia Symptom Checklist 12; GAD7: Generalized Anxiety Disorder 7; PHQ9: Patient Health Questionnaire 9; Clinical score:. Severe = 3; Moderate = 2; Mild = 1; Moderate-Mild = 1.5; Absent = 0 (* $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$).

Table 4. Logistic regression predicting the likelihood of responding to therapy with anti-CGRP/R mAbs at baseline (responders vs non-responders).

	p	OR	95% CI	
			Lower	**Upper**
Sex	0.223	0.406	0.095	1.731
Age	0.225	1.030	0.982	1.081
ASC12	0.619	1.036	0.901	1.191
FSS	0.374	0.978	0.932	1.027
GAD7	0.485	1.070	0.885	1.292
PHQ9	0.158	0.886	0.748	1.048

FSS: Fatigue Severity Scale; ASC-12: Allodynia Symptom Checklist 12; GAD7: Generalized Anxiety Disorder 7; PHQ9: Patient Health Questionnaire 9; OR: odds ratio.

4. Discussion

The overall analysis of the study indicated that the efficacy of mAbs targeting the CGRP in a real-life study for migraine prevention was combined with a significant improvement in psychiatric symptoms. Of note, the studied population was exclusively composed of HFEM or CM, resistant to the common drug prophylaxis prophylaxes (with or without the concomitant diagnosis of MOH).

A subgroup analysis was undertaken in patients who experienced $\geq$ 50% reductions in headache frequency (defined as responders) [41] after anti-CGRP/R mAbs treatment to detect clinical predictive factors. The relationship between anxiety, depression, fatigue, and migraine has been reported in several investigations. However, it is not clear whether the CGRP neurotransmission pathway may be directly involved in tuning affective symptoms [28]. The pain reduction due to anti-CGRP treatment could induce modification of "pain matrix" activity in the central nervous system and indirectly restore a neurotransmitter imbalance (e.g., serotonin and dopamine), pivotal for mood disorders [17]. CGRP could be related to chronic pain sensitization and cortical hyperexcitability, combined with other factors such as oxidation/reduction (redox) state [18,55,56]. In this framework, a chronic pain condition reporting several therapeutical failures represents, per se, a risk factor for psychiatric disease onset mining personal resilience. Furthermore, a link between migraine and psychiatric symptoms has been associated to genetic [57] and neurotransmitter modifications [58]. Altered endocannabinoid levels and decreased cerebrospinal fluid levels of GABA in CM patients with comorbid depression [59,60] may be part of the common pathophysiological signaling underlying affective comorbidity within migraine.

CGRP receptors, on the other hand, are usually relegated to the trigeminovascular system for the description of their causative role in migraine [28,29]. However, CGRP signaling is also found in the superior and inferior colliculi (phonophobia and photophobia), stria terminalis (anxiety-like behavior), hypothalamus (appetite regulation), thalamus (allodynia), amygdala, cerebellum, and neocortex (anxiety and depression), pointing towards multiple anatomic and functional interactions between migraine and its comorbidities [61–64].

Headache disorders, according to the global burden of disease (GBD) are the third most prevalent cause of global disability, expressed as years lived with disability (YLDs), just below depressive disorders if considering all genders and ages. The selection of young adults (age 15–49) of both genders makes headaches the most impacting condition in this stage of life, overcoming mood disorders [1]. We did not assess the pediatric (children and adolescents) population of migraineurs. The opportunity to treat these young patients with anti-CGRP/R drugs is one of the unsolved questions for the novel treatments [65]. One of the main issues is the physiological role of CGRP in bone formation. However, the rapid efficacy of these molecules, corroborated by our data, could allow treatment for short-term periods (e.g., 3 months), reducing the risk of metabolic interference.

Considering that the anti-CGRP/R antibodies are ineffective in a significant proportion of patients and are costly, it would be useful for the headache specialist, the patient, and public healthcare to be able to predict who will probably benefit from the molecule. This has always been a problem in migraine therapy and a tailored approach was based on comorbid conditions or contraindications more than on presumed efficacy. Predictors of effect have been retrospectively identified in clinical trials or real-world studies, but their clinical usefulness is scarce or there is none on the single patient, considering the measured predictive values [37,39,43,66].

Previous failures of preventive treatments, according to most trials, do not represent a negative predictor of anti-CGRP/R antibodies success, and our study confirms the evidence [41,65]. It should be noted that an inverse relationship between the responder rate and number of prior treatments was reported in other real-world studies [43,67,68]. It should be mentioned that Erenumab was tested in the LIBERTY trial with a lower responder rate (30% compared to 50%) after two to four previous treatment failures [69,70].

CM patients with daily headache, classified in the ICHD-3 as A1.3.2, may be poor responders to anti-CGRP/R antibodies as published after a compassionate use of Erenumab [43]; very low responder rates were found in this cohort of patients. These data are paralleled by higher rates of response in CM patients with pain-free periods (A1.3.1). Other real-world studies have found negative response rates in such patients [66], even when switching the molecules that were used [71]. We did not check for the A1.3.2 vs. A1.3.1 subgroups of CM in our cohort; however, the headache frequency was not a predictor of poor outcome in our population. More studies need to be conducted to understand if CM patients with continuous pain or MOH are less prone to responding to neuromodulation of CGRP signaling [72]. Psychiatric comorbidities were considered possible culprits and concomitant depression was more frequent in non-responders than in super-responders [44], but our data are not in line with this hypothesis. The baseline migraine frequency and interictal allodynia were also found as poor outcome predictors, however, our data do not support these results [66,73].

Other features that were identified as good predictors were not found in our real-world study, in particular headache unilaterality, less severe disability at baseline, good response to triptans, typical migraine features and vomiting, and young age [44,65,66].

Interestingly the responders and non-responders in this study had a similar age, burden of disease, and associated comorbidities. This evidence, on the one hand, further supports the homogeneity of the selected population and reduces the prognostic values of these characteristics but, on the other hand, suggest a common pathogenetic pathway between migraine and psychiatric comorbidities. This study provided additional evidence about mAbs anti-CGRP/R efficacy. In particular, the treatment consistently reduced allodynia, from moderate to absent in responders, whilst the non-responder group was

unchanged. This observation may imply a modification of the central circuitry for the conscious perception of pain and cortical excitability [55,74–76], probably through the reduction of peripheral sensitization in responders [56,77,78]. Indeed, it was recently described that distinct thalamocortical circuits underlie allodynia induced by depression-like state rather than tissue damage [74]. Our group and others described a reduction in the allodynic symptoms in CM using onabotulinumtoxinA. The mechanisms of these preventive treatments could be convergent in reducing the peripheral and central excitability of the trigeminovascular/thalamic relays [78,79].

Our study has some caveats. First, a precise psychiatric diagnosis was not extensively investigated. Then, the sample number was relatively small, although quite homogenous, including only individuals reporting disabling migraine with a serious negative impact on daily life. The main strength of the study is the evaluation of the impact of monoclonal antibodies targeting the CGRP/R on comorbid symptoms of depression, anxiety, and fatigue in migraine patients who are resistant to conventional prophylaxis. This was an open-label prospective study, with a valuable contribution to the real-world data on the early efficacy on a specific and homogeneous population of patients.

The subgroup analysis responders versus non-responders offered insights into potential clinical predictive factors. There was a remarkable reduction of MIDAS in responders (more than 10-fold from V1 values, see Table 3) with the median score classified as mild disability at V2. The non-responders also experienced a significant reduction in the MIDAS score (2.6-fold), being still over the threshold of severe disability ($\geq$21). Both groups of patients had baseline scores of disabilities (median above 85), substantially out-of-scale compared to the typical migraine patients. The cutoff levels were indeed difficult to apply, and the perceived amelioration of the personal disability was still of great impact also in non-responders.

The opposite can be said for anxiety and depressive symptoms. The responders' group showed, after three months of treatment, a significant reduction in the median values of the relative questionnaires; however, the scores of the symptoms can still be categorized as mild in GAD7 while passing from mild to absent for PHQ9 [52,53]. Notably, the anxiety symptoms for non-responders improved from moderate to mild during the period of the study ($p = 0.034$), even if crude scores showed a non-significant reduction ($p = 0.130$). This observation needs to be confirmed and considered with caution, as the responder and non-responder groups did not significantly differ for both clinical grading and scores of GAD7. Longer follow-up might detect if migraine modifications affect the onset or reduction of psychiatric symptoms or vice versa. However, the strict relationship between migraine, anxiety, and depressive disorders is confirmed in our study [19,80].

Fatigue that can be also subtended by thalamocortical dysfunctional mechanisms in migraine, was notably reduced only in the responders' group with a decrease of 33% from the baseline values [18,81,82]. The FSS scale has no clinically validated cutoffs. As for allodynia, it seems that the responders' group had a highly significant reduction compared to non-responders. The FSS score reduction in migraine patients responding to anti-CGRP antibodies could be due to the improvement of the dysfunctional migraine-related mechanisms but could also suggest the potential role of CGRP neurotransmission in fatigue and dysfunctional pain-perception syndromes if these findings are replicated in further studies. This study increases the understanding of the potential advantages of the anti-CGRP/R mAbs in the treatment of both migraine and related affective, fatigue, and hyperalgesic comorbidities.

5. Conclusions

The role of the novel prophylactic agents for migraine targeting the CGRP system is not limited to the improvement of disease severity but also affects anxiety, depressive symptoms, and fatigue. Among those conditions, allodynia and fatigue seem to be responsive to these treatments in those patients who experienced the highest clinical impact. The clinical benefits are remarkably precocious, and the non-responders still manifest high

clinical impacts on their quality of life. There are still open questions about the therapeutical opportunities of these new molecules, and we assessed a relatively unexplored field of study. Our results are hastening the interlocked bidirectional link between affective disorders and migraine. The future direction of CGRP studies should try to unravel a common pathophysiological mechanism for interictal and ictal manifestations of migraine and its comorbidities. These common features could speculatively open a new field of investigation for the CGRP neurotransmission that goes beyond pain modulation and directly affects the resilience of the CNS.

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Predictive Models for the Transition from Mild Neurocognitive Disorder to Major Neurocognitive Disorder: Insights from Clinical, Demographic, and Neuropsychological Data

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Abstract: Neurocognitive disorders (NCDs) are progressive conditions that severely impact cognitive function and daily living. Understanding the transition from mild to major NCD is crucial for personalized early intervention and effective management. Predictive models incorporating demographic variables, clinical data, and scores on neuropsychological and emotional tests can significantly enhance early detection and intervention strategies in primary healthcare settings. We aimed to develop and validate predictive models for the progression from mild NCD to major NCD using demographic, clinical, and neuropsychological data from 132 participants over a two-year period. Generalized Estimating Equations were employed for data analysis. Our final model achieved an accuracy of 83.7%. A higher body mass index and alcohol drinking increased the risk of progression from mild NCD to major NCD, while female sex, higher praxis abilities, and a higher score on the Geriatric Depression Scale reduced the risk. Here, we show that integrating multiple factors—ones that can be easily examined in clinical settings—into predictive models can improve early diagnosis of major NCD. This approach could facilitate timely interventions, potentially mitigating the progression of cognitive decline and improving patient outcomes in primary healthcare settings. Further research should focus on validating these models across diverse populations and exploring their implementation in various clinical contexts.

Keywords: mild cognitive impairment; neurocognitive disorders; prediction; neuropsychological tests; risk factors; body mass index; alcohol drinking; depression; demographic factors; longitudinal studies

1. Introduction

Neurocognitive disorders (NCDs) encompass a wide range of conditions characterized by a decline in cognitive functioning, with Alzheimer's disease being the most well-known. According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), mild neurocognitive disorder (mild NCD) is defined as a noticeable decline in cognitive functioning that does not significantly interfere with daily activities [1, 2]. This includes conditions like mild cognitive impairment (MCI), which serves as a precursor to major neurocognitive disorder (major NCD) [3–5]. Major NCD is characterized by a significant decline in cognitive abilities that impairs daily life, affecting memory, language, and other cognitive functions [6]. Major NCD, previously known as dementia, is defined by the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5),

as a significant cognitive decline from a previous level of performance in one or more cognitive domains (complex attention, executive function, learning and memory, language, perceptual–motor function, and social cognition). This decline must be substantial enough to interfere with independence in daily activities [7]. Major NCD can arise from various etiologies, including Alzheimer's disease, vascular disease, traumatic brain injury, and other conditions. Symptoms vary depending on the cause but generally include memory loss, impaired judgment, language difficulties, and changes in personality and behavior [8]. The diagnostic process for major NCD involves a thorough clinical assessment, including medical history, cognitive testing, and possibly neuroimaging or laboratory tests to identify the underlying cause and rule out other conditions. Clinicians use standardized tools like the Mini-Mental State Examination (MMSE) or the Montreal Cognitive Assessment (MoCA) to evaluate cognitive decline [9].

Neurocognitive disorders represent a significant and growing public health concern as populations age. Alzheimer's disease, the most prevalent form of major NCD, affects millions worldwide and incurs substantial emotional and economic costs [10]. These disorders not only affect the individuals diagnosed but also have profound impacts on families and caregivers, often necessitating long-term care and support. Understanding the distinctions between mild NCD and major NCD is crucial for early intervention and management [11]. While mild NCD might not severely disrupt daily life, it signals a higher risk of progressing to more severe forms, highlighting the need for early detection and therapeutic strategies.

The intermediate phase between minor NCD and major NCD is characterized by mild cognitive impairments that, while noticeable, do not severely impact daily activities. Common symptoms include memory loss, difficulty with problem solving, challenges with planning and organizing, and language difficulties, as well as decreased attention span and concentration [12]. Patients in this stage often retain independence but may need help with complex tasks and may experience frustration or anxiety due to their cognitive challenges, with changes sometimes first noticed by friends and family [1]. Studies on minor NCD often include patients in this intermediate phase to track progression to major NCD, highlighting the importance of longitudinal research to monitor cognitive, structural, and biomarker changes over time [2]. Progression from minor to major NCD varies widely, typically occurring within 3 to 5 years, and is influenced by factors such as age, genetics, and comorbid conditions [13].

Early detection and intervention in neurocognitive disorders are paramount for improving patient outcomes. Predictive models that can accurately identify individuals at risk for progressing from mild NCD to major NCD are crucial for implementing timely interventions that can slow cognitive decline [14]. Recent advancements have demonstrated the transformative impact of artificial intelligence (AI)-based techniques in early detection and diagnosis, emphasizing the potential of these tools in clinical settings [15,16]. AI approaches have been applied to detect early signs of cognitive impairment, allowing for more timely and precise diagnoses. This is essential as the aging population grows and the prevalence of dementia increases globally.

Timely intervention can significantly alter the trajectory of neurocognitive disorders. For instance, lifestyle modifications, cognitive training, and pharmacological treatments can be more effective if initiated during the early stages of cognitive decline. AI and machine learning models are particularly promising in this regard. By analyzing vast datasets, these technologies can identify subtle patterns and predictors of disease progression that might be missed by traditional diagnostic methods. Moreover, AI can enhance the accuracy and efficiency of neuropsychological assessments, reducing the burden on healthcare systems and providing more accessible diagnostic options for patients.

Several studies have focused on identifying predictive markers for the transition from mild NCD to major NCD. Cognitive performance, particularly episodic memory deficits, has consistently been highlighted as a robust predictor of progression. Delayed recall in episodic memory tests is one of the most significant indicators of future decline [17–19].

Additionally, biomarkers, such as cerebrospinal fluid (CSF) tau and amyloid levels, and neuroimaging findings, like hippocampal atrophy, have been integrated with cognitive assessments to enhance predictive accuracy [20–22].

Biomarkers play a critical role in the early detection of neurocognitive disorders. For example, AI-driven approaches have shown potential in improving predictive accuracy by analyzing complex patterns of brain connectivity. Another study highlighted the importance of transdiagnostic biomarkers, which can provide insights across different neurocognitive and psychiatric disorders, thereby improving the overall understanding of disease mechanisms [23]. Integration of multiomics profiles and multimodal electroencephalographic (EEG) data has contributed significantly to personalized diagnostic strategies, enhancing the precision of early detection methods [24].

The field of biomarker research is rapidly evolving, with significant advancements in both molecular and imaging techniques. CSF biomarkers, such as tau protein and amyloid-beta, have been extensively studied for their roles in the pathophysiology of Alzheimer's disease [21]. Elevated levels of these proteins are associated with neuronal damage and plaque formation, key features of Alzheimer's pathology. Additionally, neuroimaging techniques, including magnetic resonance imaging (MRI) and positron emission tomography (PET) scans, provide critical insights into brain structure and function, enabling the identification of atrophy patterns and metabolic changes associated with cognitive decline [25].

Functional biomarkers, such as changes in brain connectivity observed through resting-state functional MRI, have also shown promise in early detection. These biomarkers can reveal disruptions in neural networks that precede clinical symptoms, offering a window for early intervention [26]. Moreover, advancements in genomics and proteomics are paving the way for the discovery of novel biomarkers that could further refine diagnostic accuracy and prognostic assessments [27].

Genetic factors also play a crucial role in predicting cognitive decline. The presence of the apolipoprotein E (APOE) ε4 allele has been strongly associated with an increased risk of Alzheimer's disease and faster cognitive decline [18]. Demographic factors, including age, education, and gender, further influence the risk of progression from mild NCD to major NCD. For instance, older age and lower educational attainment are associated with higher risks [21,22]. The interplay between genetics and environmental factors is complex and multifaceted [28–30]. While the APOE ε4 allele is the most well-established genetic risk factor for Alzheimer's disease, other genes are also being investigated for their roles in neurocognitive disorders [31]. Genome-wide association studies (GWASs) have identified numerous genetic variants that contribute to disease susceptibility, highlighting the polygenic nature of these conditions [32]. Understanding the genetic architecture of neurocognitive disorders can inform personalized medicine approaches, where interventions are tailored based on an individual's genetic profile [33].

Demographic factors also offer valuable insights into disease risk and progression. Studies have shown that individuals with higher educational attainment or greater cognitive reserve tend to exhibit a slower rate of cognitive decline [34]. This suggests that lifelong cognitive engagement may confer protective effects against neurocognitive disorders. Additionally, sex differences in disease prevalence and progression rates have been observed, with women generally exhibiting a higher risk of developing Alzheimer's disease [35]. Hormonal factors, lifestyle differences, and genetic variations may all contribute to these disparities [36].

Despite advancements in identifying individual risk factors, comprehensive models that integrate multiple data sources to predict progression to major NCD are still lacking. Most studies have examined cognitive markers, genetic factors, and biomarkers separately, but there is a need for a unified approach that combines these elements to enhance predictive capabilities. Furthermore, longitudinal studies are needed to better understand the trajectory of cognitive decline and biomarker changes in individuals with mild NCD [37].

Recent findings underscore the urgent need for innovative and cost-effective early-stage intervention strategies to address the growing global challenge of dementia [38].

The development of comprehensive predictive models involves integrating diverse datasets, including clinical, genetic, biomarker, and neuroimaging data [39]. Machine learning and artificial intelligence are instrumental in this endeavor, offering powerful tools to analyze complex datasets and generate predictive models with high accuracy [40]. These models can identify individuals at high risk for progression to major NCD, facilitating early intervention and personalized treatment plans. Longitudinal studies are particularly valuable in understanding the natural history of neurocognitive disorders. By following individuals over time, researchers can observe the progression of cognitive decline and identify early markers of disease. Such studies also allow for the assessment of intervention efficacy, providing critical insights into which treatments are most effective at different stages of the disease [41].

In conclusion, the integration of AI-based techniques, biomarkers, genetic factors, and comprehensive predictive models holds significant promise for the early detection and intervention of neurocognitive disorders. As the prevalence of dementia continues to rise globally, these advancements are crucial for enhancing diagnostic accuracy, understanding disease mechanisms, and developing personalized treatment strategies. Ongoing research and longitudinal studies are essential to refine these approaches and ensure their effective implementation in clinical settings. By leveraging the power of technology and multidisciplinary research, we can make significant strides in combating the challenges posed by neurocognitive disorders and improving the quality of life of affected individuals.

The current study aims to develop and validate predictive tools that enhance the screening and risk assessment of major NCD in primary healthcare settings by integrating clinical, demographic, and neuropsychological data.

2. Materials and Methods

A brief description of the study's methodological approach towards the inclusion of predictive factors and the implementation of specific models for data analysis follows. The methodology of this study was carefully designed to ensure robust and reliable data collection, analysis, and interpretation. The study aimed to develop and validate predictive models for the transition from minor neurocognitive disorder (minor NCD) to major neurocognitive disorder (major NCD). We employed a comprehensive approach that integrates demographic, clinical, and neuropsychological assessments to identify significant predictors of cognitive decline. The study design and methodology are grounded in established research practices and supported by the relevant literature to enhance the validity of our findings. Prior studies have demonstrated the importance of longitudinal data in understanding the progression of neurocognitive disorders [12,13]. Additionally, the use of neuropsychological tests, such as the CAMCOG and the GDS, has been validated in various settings to predict cognitive decline [42,43]. Our approach ensures a comprehensive evaluation of factors contributing to the transition from minor to major NCD, providing a robust framework for early intervention strategies.

Furthermore, the international literature emphasizes the importance of focusing on the structure of the data and correlations in Generalized Estimating Equations (GEE) analysis rather than a fixed minimum number of observations per variable [44–46]. GEE analysis can be applied even to small sample sizes [47]. However, it is worth noting that, in our research, we adhered to the general assumption for regression models that requires a minimum threshold of 10 observations per independent variable [48–50]. In our case, there were 394 observations/24 variables = 16.4 observations per variable.

2.1. Subjects

Data regarding 132 participants in an ongoing registry of the Neurology Department of the University Hospital of Alexandroupolis with minor NCD and with available diagnostic follow-up assessments for at least 2 years were included in the study. The final sample

consisted of participants who visited the outpatient dementia clinic and underwent the examination as a part of their routine neuropsychological assessment (Figure 1).

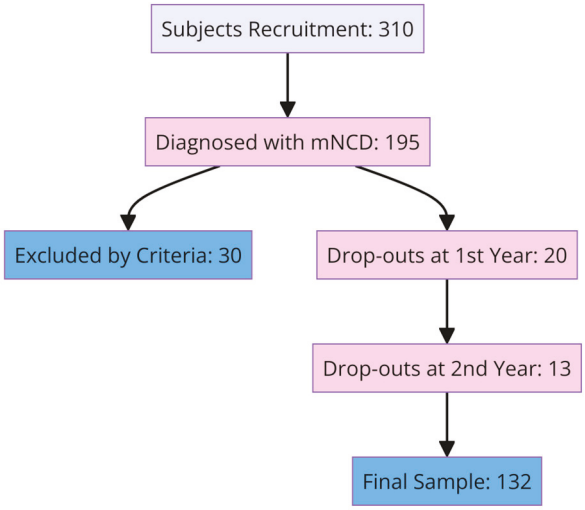


Figure 1. Subjects' recruitment process.

All participants signed an informed consent form prior to their participation. Approval was also required for the patients with dementia by their caregiver and/or by a legal representative. Approval for the study was granted by the Ethics Committee of the University Hospital of Alexandroupolis (ΔΣ1/Θ68/06-04-2020). The data were analyzed anonymously.

2.2. Inclusion/Exclusion Criteria

The interview collected biographical information and medical data, including information regarding any medical diagnosis; history of cardiovascular, metabolic, and neurological syndromes; and history of affective diseases. Individuals with NCDs underwent a neurological examination, neuropsychological assessment, neuroimaging, and specific biochemical and hematological testing.

A total of 132 individuals at baseline met the diagnostic criteria for minor NCD as defined in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) [51]. These criteria include the following: (a) self-reported or observed decline in cognitive functioning by the patient, family member, or clinician; (b) cognitive impairment for the individual's age demonstrated by formal neuropsychological testing; (c) evidence of gradual cognitive decline in objective tasks beyond normal aging but not meeting criteria for dementia; (d) preserved general cognitive and daily function; and (e) no prior diagnosis of dementia or other conditions (e.g., depression, delirium, intoxication, or psychosis) that could explain the impairment. Additional inclusion criteria were as follows: age over 40 years; mild cognitive decline based on Mini-Mental State Examination (MMSE) [52,53] and Montreal Cognitive Assessment (MoCA) score [54,55], defined as 1 to 1.5 standard deviations (SDs) below the mean for age- and education-adjusted norms based on normative data; absence of other neurological diseases; not currently taking cholinesterase inhibitors, antipsychotics, and/or anticholinergic drugs. The inclusion criterion of age over 40 years in studies of minor NCD is significant for capturing early markers of cognitive decline and understanding the progression of the disorder. While cognitive decline is more common in older adults, including patients starting at age 40 helps identify early symptoms of minor NCD, as this age group may begin to show subtle signs of cognitive impairment [12]. This inclusion is associated with several critical parameters: genetic factors like the presence of the APOE ε4 allele may start influencing cognitive decline in individuals in their 40s [2]; lifestyle factors, such as diet, exercise, and smoking, as well as comorbidities like hypertension, diabetes, and cardiovascular diseases, begin to impact

cognitive health in midlife, increasing the risk of minor NCD [13]; and neuropsychological changes in cognitive performance and brain health can show measurable changes in this age group, making it a critical period for early detection and intervention [1]. Therefore, including patients over 40 years old provides valuable insights into the early onset and progression of cognitive decline.

The exclusion criteria were as follows: secondary causes of cognitive deficits confirmed with laboratory tests, including vitamin B12/folate determination and thyroid functioning tests; structural lesions on conventional brain MRI, such as territorial infarction, intracranial hemorrhage, brain tumor, hydrocephalus, and traumatic brain injury. The subjects were followed annually with two repeated clinical visits after baseline.

2.3. Conversion to Major NCD

Our primary study outcome was progression to major NCD. In order to avoid circularity, the major NCD diagnostic criteria did not include any of our predictive neuropsychological testing markers. We used the criteria based on modes of assessment that were independent of our neuropsychological decline measure. Participants were diagnosed with major NCD [56] based on DSM-V and decline in activities of daily living as well as cognitive test scores for MMSE and MoCa (total scores) falling 2 or more standard deviations (SDs) below the mean based on available normative data.

2.4. Neuropsychological Predictive Factors

All participants underwent a battery of neuropsychological tests comprising the Greek version of the Cambridge Cognitive Examination Scale (CAMCOG) as part of the Cambridge Mental Disorders of the Elderly (CAMDEX) [42,57]. The cognitive domains assessed through the subtasks were abilities of praxis, orientation, understanding, language, memory, and perception. Confrontation naming was evaluated by the Boston Naming Test (BNT) [58] and executive functioning by the Functional Cognitive Assessment Scale (FUCAS) [59]. The Functional Rating Scale of Symptoms of Dementia (FRSSD) was administered to assess the patient's functionality in daily activities based on the caregiver's perspective. Emotional status was evaluated by the Geriatric Depression Scale (GDS) for the detection of depressive symptoms [43,60]. The Hamilton Depression Scale (HAM-D) [61] was administered to assess patients' emotional states through 17 questions from the caregiver's perspective. Patients' neuropsychiatric disturbances were assessed through the Neuropsychiatric Inventory (NPI) administered to caregivers [62]. The neuropsychological examinations took place in a quiet room, and every participant was tested individually by the same neuropsychologist of the Neurology Department.

2.5. Demographic and Clinical Predictive Factors

Age (years), sex (male/female), and education (years) were the demographic factors studied, while the clinical variables included duration of minor NCD (years), body mass index (BMI) $\geq$ 25 [63] (yes/no), smoking (yes/no), alcohol consumption (yes/no), history of cardiovascular disease (yes/no), presence of white matter lesions (yes/no), and cerebrovascular burden (at least one of the following risk factors: atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia).

2.6. Statistical Analysis

Data were analyzed using a Generalized Estimating Equations (GEE) framework to account for the longitudinal nature of the data with repeated measures over time (baseline, first year, and second year) [64,65]. The dependent variable was diagnosis, coded as 0 for minor NCD and 1 for major NCD. The independent variables included in the model are shown in Tables 1 and 2. The model was specified with a binomial distribution and a logit link function. The working correlation structure was set as unstructured, allowing for a general form of the covariance matrix for the repeated measures [66,67]. Employing an unstructured correlation matrix is beneficial when the intervals between measurements are

uniform across subjects [44], which aligns with the methodology of our study. Wald χ^2 tests were used to determine the significance of the predictors, with a 95% confidence interval. All analyses and graphical representations were performed using SPSS (version 25.00) software, and the significance level was set at $p < 0.05$.

Table 1. Descriptive statistics for baseline demographic, clinical, and cognitive data.

	N	Percent	Mean	Std.	Min.	Max.
Age (years)	132	-	70.6	7.5	44	83
Sex (M/F)	39/93	29.5/70.5				
Education (years)	132	-	8.3	4.8	0	18
Duration (years)	132	-	1.7	1.3	0	11
BMI $\geq$ 25 (Y/N)	20/112	15.2/84.8	-	-	-	-
WML (Y/N)	41/91	31.1/68.9	-	-	-	-
Cardiovascular diseases (Y/N)	34/98	25.8/74.2	-	-	-	-
Smoking (Y/N)	16/116	12.1/87.9	-	-	-	-
Alcohol (Y/N)	2/130	1.5/98.5	-	-	-	-
CB (3/2/1/0)	7/30/40/55	5.3/22.7/30.3/41.7	-	-	-	-
CAMCOG—praxis	132	-	25.7	3.0	13	30
CAMCOG—orientation	132	-	9.8	0.6	6	10
CAMCOG—understanding	132	-	6.3	0.8	4	7
CAMCOG—language	132	-	23.3	2.8	15	29
CAMCOG—memory	132	-	18.9	3.8	6	25
CAMCOG—perception	132	-	17.2	3.8	1	24
CAMCOG—time perception	132	-	1.9	1.2	0	14
BNT	132	-	47.0	7.3	29	60
BNT—time completion	132	-	489.5	159.6	170	875
FUCAS	132	-	45.9	8.8	42	88
GDS	132	-	2.8	2.3	0	9
HAM-D	132	-	4.6	3.3	0	21
FRSSD	132	-	4.3	2.9	0	14
NPI	132	-	2.1	2.7	0	14

Notes. M/F: Male/female; BMI: Body mass index; WML: White matter lesions; Y/N: Yes/no; CB: Cerebrovascular burden; CB-0: No cerebrovascular factor among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-1: One cerebrovascular factor among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-2: Two cerebrovascular factors among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-3: Three cerebrovascular factors among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CAMCOG: Cambridge Cognitive Examination Scale; BNT: Boston Naming Test; FUCAS: Functional Cognitive Assessment Scale; GDS: Geriatric Depression Scale; HAM-D: Hamilton Depression Scale; FRSSD: Functional Rating Scale of Symptoms of Dementia; NPI: Neuropsychiatric Inventory.

Table 2. Parameter estimates for predictors of major NCD diagnosis.

Parameter	B	Std. Error	Wald Chi-Square	df	Sig.	Exp(B)
(Intercept)	6.45	2.76	5.45	1	0.02	633.70
[Sex = F]	−0.66	0.23	8.33	1	0.00	0.51
[Sex = M]	0					1.00
[Cardiovascular diseases = Y]	0.15	0.28	0.28	1	0.60	1.16
[Cardiovascular diseases = N]	0					1.00
[BMI $\geq$ 25 = Y]	0.82	0.27	9.07	1	0.00	2.27
[BMI $\geq$ 25 = N]	0					1.00
[WML = Y]	−0.24	0.27	0.82	1	0.37	0.79
[WML = N]	0					1.00
[Smoking = Y]	−0.29	0.36	0.65	1	0.42	0.75
[Smoking = N]	0					1.00
[Alcohol = Y]	0.86	0.29	9.04	1	0.00	2.36
[Alcohol = N]	0					1.00
[CB = 3.00]	−0.31	0.59	0.28	1	0.59	0.73

Table 2. *Cont.*

Parameter	B	Std. Error	Wald Chi-Square	df	Sig.	Exp(B)
[CB = 2.00]	−0.28	0.31	0.82	1	0.36	0.75
[CB = 1.00]	−0.16	0.28	0.33	1	0.57	0.85
[CB = 0.00]	0					1.00
CAMCOG—praxis	−0.12	0.05	6.40	1	0.01	0.89
GDS	−0.14	0.06	4.80	1	0.03	0.87
Age	−0.01	0.02	0.47	1	0.49	0.99
Education	−0.04	0.03	1.45	1	0.23	0.96
Duration	0.00	0.11	0.00	1	1.00	1.00
CAMCOG—orientation	0.04	0.17	0.04	1	0.83	1.04
CAMCOG—understanding	0.12	0.17	0.49	1	0.49	1.12
CAMCOG—language	−0.09	0.05	2.67	1	0.10	0.92
CAMCOG—memory	−0.09	0.06	2.20	1	0.14	0.92
CAMCOG—perception	0.00	0.05	0.00	1	0.97	1.00
CAMCOG—time perception	0.12	0.08	1.91	1	0.17	1.12
FUCAS	−0.02	0.02	2.27	1	0.13	0.98
BNT	0.01	0.02	0.16	1	0.69	1.01
BNT—time completion	0.00	0.00	0.01	1	0.94	1.00
NPI	−0.06	0.06	0.82	1	0.36	0.95
FRSSD	0.07	0.05	2.16	1	0.14	1.07
HAM-D	0.08	0.05	2.47	1	0.12	1.08

Notes. M: Male; F: Female; Y: Yes; N: No; BMI: Body mass index; WML: White matter lesions; CB: Cerebrovascular burden; CB-0: No cerebrovascular factor among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-1: One cerebrovascular factor among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-2: Two cerebrovascular factors among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CB-3: Three cerebrovascular factors among atrial fibrillation, cerebrovascular diseases, hypertension, diabetes, and hypercholesterolemia; CAMCOG: Cambridge Cognitive Examination Scale; GDS: Geriatric Depression Scale; FUCAS: Functional Cognitive Assessment Scale; BNT: Boston Naming Test; NPI: Neuropsychiatric Inventory; FRSSD: Functional Rating Scale of Symptoms of Dementia; HAM-D: Hamilton Depression Scale.

3. Results

The Quasi-Likelihood under Independence Model Criterion (QIC) was 428.865, and the Corrected Quasi-Likelihood under Independence Model Criterion (QICC) was 454.655, indicating the model's adequacy in fitting the data in comparison to alternative models that were tested. The model performed well, with an accuracy of 83.7%. Table 1 shows the descriptive statistics for the categorical (nominal/ordinal) and continuous (scale) variables of the model.

The GEE analysis revealed significant effects of sex, BMI $\geq$ 25, alcohol, CAMCOG-praxis, and GDS on the likelihood of major NCD (Table 2). Specifically, females were less likely to be diagnosed with major NCD than males (B = −0.664, OR = 0.515, Wald $\chi^2(1) = 8.329$, $p = 0.004$). Participants with increased BMI (BMI $\geq$ 25) had higher odds of receiving a major NCD diagnosis (B = 0.820, OR = 2.272, Wald $\chi^2(1) = 9.069$, $p = 0.003$). Similarly, alcohol consumption was associated with a higher likelihood of major NCD diagnosis (B = 0.857, OR = 2.356, Wald $\chi^2(1) = 9.035$, $p = 0.003$). Additionally, the results showed that higher scores on the CAMCOG—praxis were significantly associated with lower odds of being diagnosed with major NCD (B = −1.115, OR = 0.891, Wald $\chi^2(1) = 6.396$, $p = 0.011$). Furthermore, higher scores on the GDS were significantly associated with decreased odds of major NCD diagnosis (B = −1.137, OR = 0.872, Wald $\chi^2(1) = 4.801$, $p = 0.028$).

The interaction effects of time with sex, BMI $\geq$ 25, and alcohol consumption were visually explored through estimated marginal means plots. The charts in Figure 2 show the estimated probabilities of major NCD diagnosis for various subgroups based on sex, BMI $\geq$ 25, and alcohol consumption. The diagrams present the mean values and the 95% confidence intervals for each subgroup at three time points. The charts reveal the following: (A) males have a higher likelihood of major NCD diagnosis compared to females at all time points, with the differences being more pronounced in the second

year; (B) individuals with excessive BMI (BMI $\geq$ 25) appear to have a higher likelihood of major NCD diagnosis in the second year compared to those without excessive weight; (C) individuals who consume alcohol have a greater likelihood of major NCD diagnosis in the second year compared to those who do not consume alcohol.

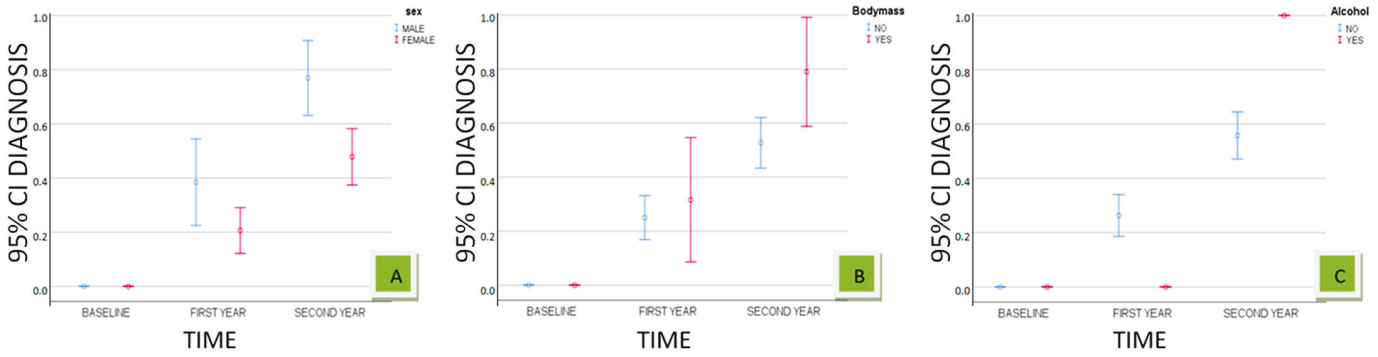


Figure 2. Estimated probabilities of major NCD diagnosis over time with 95% confidence intervals of means by sex, body mass, and alcohol consumption.

Figure 3 includes two charts with the 95% confidence intervals for the variables CAMCOG—praxis and GDS among patients with minor NCD and patients with major NCD over the time periods. Both in the first and second year, individuals displaying major NCD showed lower values in CAMCOG—praxis and GDS. Indeed, even visually, it appears that for CAMCOG—praxis the differences are statistically significant.

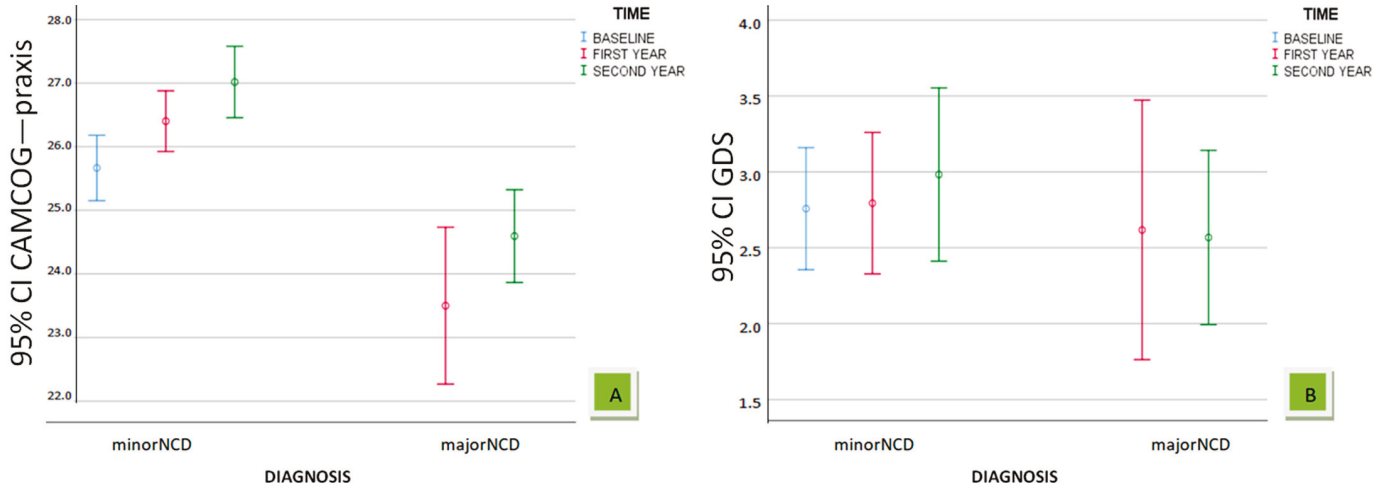


Figure 3. Changes in mean values of CAMCOG—praxis (**A**) and GDS (**B**) with 95% confidence intervals by diagnosis and time.

In summary, the results from the GEE show the influence of categorical variables like sex, BMI, and alcohol consumption on the likelihood of being diagnosed with major NCD. For instance, females showed a reduced likelihood of a major NCD diagnosis compared to males, those with a BMI over 25 were more likely to receive a major NCD diagnosis, and alcohol consumers also showed a higher probability of a major NCD diagnosis. Additionally, higher scores on the CAMCOG—praxis and higher scores on the GDS were associated with a decreased likelihood of a major NCD diagnosis.

4. Discussion

This study aimed to address the critical need for early detection and effective management of neurocognitive disorders by developing a validated predictive model for the

transition from minor neurocognitive disorder (minor NCD) to major neurocognitive disorder (major NCD). By focusing on demographic, clinical, and neuropsychological factors, we aim to enhance early intervention strategies within primary healthcare settings, ultimately improving patient outcomes and quality of life. The ultimate goal of this research is to facilitate early identification of individuals at high risk for major NCD, enabling timely interventions that can delay or prevent severe cognitive decline. The primary challenge lies in integrating diverse data sources—demographic information, clinical history, and neuropsychological test results—into a cohesive and accurate predictive model. Overcoming this challenge requires extensive knowledge in neuropsychology, geriatrics, and advanced statistical modeling techniques.

Our study highlights a crucial predictive model for identifying individuals at risk of major NCD among those with minor NCD. Baseline clinical profiles, particularly emphasizing increased BMI (BMI $\geq$ 25) and alcohol consumption, emerged as significant factors associated with heightened major NCD risk. Conversely, certain predictors appeared to lower the likelihood of major NCD, including being female, exhibiting higher scores on the GDS, and achieving higher scores in praxis assessments. Of note, to achieve the study's goals, a deep understanding of neurocognitive disorders and the factors influencing their progression is essential. This includes expertise in neuropsychological assessment, familiarity with demographic and lifestyle risk factors, and proficiency in statistical methods for analyzing longitudinal data.

Alcohol consumption, defined as consuming more than one glass of alcohol per day based on self-reported questionnaires, emerged as a predictive factor for progression to major NCD. Considering previous research, Kuang et al. [68] highlighted alcohol consumption as a significant predictive factor in various models for dementia, emphasizing its importance in assessing cognitive health alongside other variables, such as age, activities of daily living questionnaire score, and smoking status. Conversely, Koch et al. [69] emphasized caution in alcohol consumption among individuals with minor NCD, consistent with Xu et al. [70] who identified a J-shaped relationship between alcohol intake and cognitive decline, suggesting that light to moderate drinking might reduce dementia risk in this vulnerable population. While moderate alcohol intake has been linked to potential cognitive benefits in certain contexts, such as reduced progression to major NCD in individuals with minor NCD [71], caution is warranted. Xu et al. [72] demonstrated a nonlinear association between alcohol consumption and dementia risk, suggesting that excessive drinking may actually elevate the risk.

The present study identified BMI (BMI $\geq$ 25) as a predictive factor for conversion to major NCD. This finding aligns with previous research exploring the complex relationship between BMI and cognitive decline. Hessler et al. [73] investigated cardiovascular health metrics and dementia risk in older adults. While their study did not find a direct association between BMI and dementia risk, it emphasized the importance of other cardiovascular risk factors, such as smoking, physical activity levels, and glucose levels, which could indirectly impact cognitive health. A more recent study [74] examined the effects of the MIND diet on cognition in older adults with a higher BMI and suggested that dietary interventions alone may not significantly alter cognitive outcomes, highlighting the complexity of factors influencing dementia risk beyond BMI. Mirza et al. [75] explored trajectories of depressive symptoms and their association with dementia risk. Although not directly related to BMI, their findings underscored the importance of mental health factors in cognitive decline, which could interact with BMI-related mechanisms. Another study [76] provided insights into the obesity paradox, where late-life lower BMI was associated with increased cortical amyloid burden and dementia risk, especially in APOE4 carriers.

In our comprehensive investigation into predictive factors associated with the conversion to major NCD, a notable finding emerged regarding the impact of female sex on dementia risk. Our analysis indicated that female sex is linked to a lower risk for major NCD progression, a conclusion that draws upon a body of research exploring sex-specific influences on cognitive health. Studies [77,78] have consistently observed a higher preva-

lence and elevated risk of AD among women compared to men. These findings suggest potential sex-specific vulnerabilities to certain forms of dementia, underscoring the need to explore biological and environmental factors that may contribute to these disparities. Conversely, other researchers [68,79] have highlighted educational disparities and hormonal fluctuations, particularly estrogen changes, as potential contributors to variations in dementia risk between the sexes. Moreover, recent studies [80,81] have reported differential associations of risk factors with dementia among men and women, indicating that certain risk factors may have distinct impacts based on sex.

Our research finding that increased scores on the GDS predict a slower progression to major NCD adds a significant dimension to the discourse on the potential of depressive symptoms as early indicators of cognitive impairment. This result is particularly insightful in the context of our study methodology, where participants identified with elevated GDS scores at baseline were subsequently enrolled in a comprehensive monitoring program. This intervention included both pharmacological treatment and counseling services for the participants and their family systems, aimed specifically at mitigating the impact of depressive factors on cognitive health. Supporting our approach, the study by Mirza et al. [75] aligns well with our findings, suggesting that increasing trajectories of depressive symptoms over time are associated with a higher risk of dementia, thereby reinforcing the notion that actively managing these symptoms could significantly alter the progression trajectory of cognitive decline. In contrast, another study [82] found that mild depressive symptoms did not predict AD, highlighting the importance of the severity and management of symptoms in their role as potential predictors. Moreover, while Nation et al. [83] emphasize the utility of neuropsychological markers in predicting dementia, the integration of therapeutic interventions in response to depressive symptomatology in our study provides a model for combining psychological and neuropsychological assessments to enhance predictive accuracy.

Our investigation aligns with previous studies, showing that improved praxis abilities can have a protective effect against cognitive decline. These abilities are integral to our daily cognitive functions and may play a role in mitigating the symptoms or delaying the progression of neurocognitive disorders. One study highlights the positive impact of literacy and education on visuo-constructional abilities, suggesting that educational activities enhancing these abilities could support cognitive health in the elderly [84]. This is particularly evident in individuals who engage in complex cognitive activities, showing better performance in cognitive tests compared to those with lower engagement levels. However, the relationship between praxis abilities and cognitive health is not straightforward. Martins-Rodrigues et al. found that certain visuo-constructional tasks did not effectively differentiate between individuals with minor NCD and healthy controls, suggesting that the protective effects of enhanced praxis abilities may be limited to specific cognitive functions or early stages of cognitive decline [85].

In summary, our results highlight the importance of specific demographic and lifestyle factors in predicting the transition from minor NCD to major NCD. Increased BMI and alcohol consumption were associated with a higher risk, while female sex, higher GDS scores, and better praxis abilities were linked to a lower likelihood of progression. These findings underscore the need for comprehensive risk assessments in primary care settings to identify and support at-risk individuals effectively.

This research is significant as it addresses a growing public health concern: the rising prevalence of neurocognitive disorders among aging populations. Early detection of individuals at high risk for major NCD can lead to timely interventions, potentially delaying the onset of severe cognitive decline and reducing the overall burden on healthcare systems.

4.1. Strenghts and Limitations of the Study

One of the study's primary strengths is the integration of multiple types of predictive data, including clinical risk factors and neuropsychological tests, which make it a useful

tool for general practitioners in primary healthcare settings. Additionally, the study focused on developing and validating predictive tools that can be effectively implemented within community-based healthcare frameworks. These tools are designed to facilitate early intervention strategies and improve patient outcomes, making them particularly valuable in remote areas where they can aid general practitioners in referring patients to specialized centers. Moreover, the longitudinal study design, which included follow-ups, allowed for a more accurate assessment of disease progression from minor NCD to major NCD.

The study has certain limitations. The sample size of 132 participants from a single institution may limit the generalizability of the findings to broader populations or different geographic locations, potentially affecting its utility in diverse or remote settings. There is also a need for further validation studies across diverse populations to ensure the model's generalizability and clinical applicability. Additionally, the study acknowledges gaps in the literature where comprehensive models integrating various data sources (cognitive, genetic, and biomarker data) are still lacking.

4.2. Potential Clinical Implications and Future Research

The clinical applications of our findings are substantial. The predictive model can aid in the early identification of patients at risk of major NCD, facilitating timely referrals to specialized care and the implementation of personalized intervention strategies. The findings from this study pave the way for several avenues of future research. First, further refinement of predictive models is necessary to enhance their accuracy and reliability. This can be achieved by incorporating additional biomarkers and genetic information alongside the current demographic, clinical, and neuropsychological factors. Advanced machine learning techniques could also be employed to analyze these complex datasets more effectively. Secondly, the impact of targeted interventions based on the predictive model should be explored. Moreover, it is crucial to validate the predictive model across diverse populations. Finally, the integration of the predictive model into primary healthcare systems requires careful consideration of its implementation and usability by general practitioners. Research should focus on developing user-friendly tools and training programs that facilitate the adoption of these predictive models in everyday clinical practice.

5. Conclusions

The study successfully developed and validated predictive tools that integrate clinical risk factors and neuropsychological tests tailored for implementation in primary healthcare settings. These tools enable effective screening and risk assessment for neurocognitive disorders (NCDs), thereby facilitating early intervention strategies which can significantly improve patient outcomes. The findings underscore the influence of demographic factors, such as gender, with females exhibiting a lower risk of major neurocognitive disorder (MNCD) progression compared to males, suggesting potential sex-specific interventions. Additionally, lifestyle factors like BMI and alcohol consumption were significant in MNCD progression, presenting opportunities for targeted interventions to delay or prevent severe cognitive impairments. Moreover, certain neuropsychological predictors appeared to lower the likelihood of major NCD, including exhibiting higher scores on the GDS and achieving higher scores in praxis assessments. The proposed comprehensive and validated model can be adaptable to various healthcare settings and is poised to significantly influence clinical practice, especially in primary care environments, by providing tools that support early diagnosis and tailored intervention strategies.

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Blood Plasma Markers in Depressed Mice under Chronic Social Defeat Stress

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Abstract: It has previously been shown that, in mice, chronic social defeat stress in daily agonistic interactions leads to a depression-like state similar to that in depressive patients. With this model, it has become obvious that it is possible to study peripheral markers of the depression-like state in an experiment. This paper was aimed at searching for protein markers in the blood plasma of depressed mice in the chronic social conflict model, which allows for us to obtain male mice with repeated experiences of defeat. Proteomic analysis of blood plasma samples was conducted to identify proteins differentially expressed in this state. There were changes in the expression levels of the amyloid proteins SAA1, SAA4, and SAMP and apolipoproteins APOC3, APOD, and ADIPO in the blood plasma of depressed mice compared with controls (unstressed mice). Changes in the expression of serine protease inhibitors and/or proteins associated with lipid metabolism, inflammation, or immune function [ITIH4, SPA3, A1AT5, HTP (HP), CO9, and A2MG] were also found. Here, we showed that chronic social stress is accompanied by increased levels of amyloid proteins and apolipoproteins in blood plasma. A similarity was noted between the marker protein expression changes in the depressed mice and those in patients with Alzheimer's disease. These data indicate a psychopathogenic role of chronic social stress, which can form a predisposition to neurodegenerative and/or psychoemotional disorders.

Keywords: chronic social stress; depression; blood plasma proteome; lipid metabolism

1. Introduction

Major depressive disorder (MDD) is a widespread mental illness. In the development of depression, an important role is played by the interactions between genetic, environmental, or stress factors [1]. Numerous studies are being conducted to find peripheral markers of depression in people; such data will help to devise treatment strategies for this disorder, e.g., [2–5]. The analysis of differentially expressed proteins in MDD has revealed significant alterations in lipid transport and metabolic function, including some apolipoproteins and a serine protease inhibitor, in patients in comparison with healthy controls [6]. In that work, the top statistically significant pathways were related to lipid transport, inflammation, and immunity. It is also known that, in patients with MDD, severe psychological distress and anxiety are accompanied by the development of amyloidosis ([7,8], review). Moreover, in humans, depression is considered a common comorbidity of Alzheimer's disease [9,10]. It is important to find peripheral biomarkers of depression in the hope to understand preferential comorbidity of depression with other diseases. This knowledge will help design a strategy for treating the disease and its consequences. Nonetheless, this approach requires

preliminary studies in animal models. To this end, it is necessary to conduct preliminary research on animal models that can maximally reproduce the psychoemotional state and somatic changes characteristic of people having a diagnosis of a depressive disorder.

It has been shown earlier in experimental studies that chronic social defeat stress caused by negative social experiences in daily agonistic interactions leads to a depression-like state accompanied by chronic anxiety in male mice [11–14]. This model has been identified as the most relevant for researching depression and the consequences of chronic social stress [15,16] and is widely used for experimental work [17,18]. This is because this state has been confirmed by all validity criteria proposed for experimental models of depression [19]: the similarity of etiology, symptoms, sensitivity to antidepressants and anxiolytics, and neurochemical changes in the brain similar to those in depressed patients. In various tests after chronic social stress, the defeated mice have demonstrated new types of behavior that are similar to symptoms in depressed patients: a general behavioral deficit in various tests, helplessness, generalized anxiety, immobility, freezing, avoidance of social contacts, decreased sociability, indifference, an anhedonia-like state, weight loss, and long persistence of depression- and anxiety-like states [13,14]. Chronic social stress and anxiety are considered the main psychopathogenic factors.

It has also been shown in experiments that the mechanism of the depression-like state is accompanied by the development of immunosuppression (review, [20]), manifesting itself as a decrease in overall resistance to diseases, impairments of humoral and cellular immunity and of proliferation-related and apoptotic processes in immunocompetent organs, and intensification of oncogenic processes. Primary treatment with anxiolytics and antidepressants to correct psychoemotional status has been found to be more effective in comprehensive reversal of psychoneuroimmune impairments than attempts to restore immune metrics with the aim of producing therapeutic effects on anxious–depressive states and immunity (review, [20]). These data indicate a deep connection between changes in the brain and the peripheral pathophysiology of depression accompanied by immune disorders. This notion has given hope of detection, for example, of the stage and depth of development of psychoemotional disorders using peripheral biomarkers.

The aim of this study was to search for proteins of various peripheral-blood-plasma components in depressed mice using the chronic social conflict model [12–14] and, for comparison, in the aggressive mice after such daily agonistic interactions resulting in the development of psychosis-like behavior that is accompanied by hyperactivity, enhanced aggressiveness, aggressive motivation, development of stereotypies, and a disturbance of social recognition [21,22]. A comparison of the peripheral changes in mice with different psychoemotional-like disorders will reveal which changes are specific to which condition.

2. Materials and Methods

2.1. Animals

Adult C57BL/6 male mice were obtained from the Animal Breeding Facility (Stolbovaya, Moscow region, Russia). The animals were housed under standard conditions (at a constant temperature of 22 ± 2 °C, under a 12:12 h light/dark regimen starting at 8:00 a.m., with food in pellets and water available ad libitum). Mice were weaned at 3 weeks of age and housed in groups of 8–10 in standard plastic cages (36 × 23 × 12 cm). The experiments were performed on 10–12-week-old animals. All procedures were in compliance with the European Communities Council Directive 210/63/EU of 22 September 2010. The protocol of this study was approved by the Scientific Council No. 9 of the Institute of Cytology and Genetics SB RAS of 24 March 2010, decision No. 613 (Novosibirsk, http://spf.bionet.nsc.ru; accessed on 9 October 2023).

2.2. The Chronic Social Conflict Model

Repeated positive and negative social experiences in male mice were induced by daily agonistic interactions through the use of the sensory contact model [21], which was later renamed as the "chronic social conflict model" and is described in detail in some

reviews [14,22]. Pairs of male mice were each placed in a cage (28 × 14 × 10 cm) bisected by a transparent perforated partition, allowing the animals to see, hear, and smell each other but preventing physical contact (Figure 1).

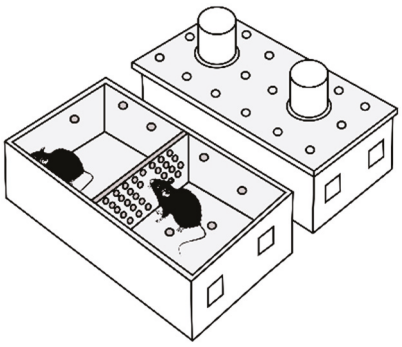


Figure 1. The experimental cage.

The animals were left undisturbed for 2 days to adapt to the new housing conditions and sensory contact before they were subjected to agonistic encounters. After that, every afternoon (3:00–5:00 p.m. local time), the cage cover was replaced by a transparent one, and 5 min later (the period necessary for activation), the partition was removed for 10 min to encourage agonistic interactions. The superiority of one of the mice was established within two or three encounters with the same opponent. The superior mouse would be chasing, biting, and attacking the other, who would be demonstrating only defensive behavior (e.g., upright or sideways postures, withdrawal, and freezing). To prevent damage to the defeated mouse, the aggressive interactions between males were discontinued by lowering the partition if the strong attacking behavior lasted for 3 min (in some cases less). Each defeated mouse (loser) was exposed to the same aggressive mouse for 3 days, whereas afterwards, each loser was placed into an unfamiliar cage containing an unfamiliar winning partner behind the partition. Each aggressive mouse (winner) remained in its own cage. This procedure was performed once a day for 20 days and yielded equal numbers of losers and winners.

In this experiment, three groups of animals were analyzed: (1) controls, i.e., mice without a consecutive experience of agonistic interactions; (2) losers, depressed mice, i.e., a group of repeatedly defeated mice during 20 days of agonistic interactions; (3) winners, i.e., a group of repeatedly aggressive and winning mice during 20 days. The latter group was examined to determine the group specificity of changes in comparison with the losers. The number of mice used for the proteomic analysis was 6 per group. The winners and losers the day after the last agonistic interaction and the control animals were decapitated simultaneously. Blood plasma was collected, frozen, and stored at −80 °C until the proteomic procedures.

2.3. The Partition Test

This test is used for studying the level of communicativeness (sociability) toward a partner. The partition test estimates a mouse behavioral reaction to a conspecific behind the transparent perforated partition dividing the experimental cage into equal parts. It has been demonstrated earlier ([23], review) that a decrease in some parameters measured by the partition test correlates with indices of anxiety-like behavior estimated in the elevated plus-maze test in the losers. The behavior of winners near the partition as a reaction to a partner in the neighboring compartment has correlated with parameters of aggression, such as total attacking time, the number of attacks, and average time of one attack, reflecting the level of aggressive motivation [23].

In the current work, the number of approaches to the partition and the total time spent near it (e.g., moving near the partition, smelling and touching it with the nose or with one or two paws, and sticking the nose into the holes) were scored during 5 min as indices

of reacting to the partner. The duration of a sideways position or "turning away" near the partition was not included in the total time of the test. On the testing day, the cage lid was replaced with a transparent one; five minutes later (period of activation), the behavioral responses of the losers and winners toward the partner in the adjacent compartment were recorded for 5 min.

2.4. Proteomic Data Processing and Analysis

The proteomic analysis was carried out at the Advanced Mass Spectrometry Core Facility of the Institute of Science and Technology (Moscow, Russia). The aim of this study was to compare the protein profile of plasma samples between the groups of mice using label-free quantitative proteomics (LFQ). The plasma samples were trypsin-digested by the manufacturer's S-trap protocol [S-Trap™ micro use 4.7, ProtiFi LLC, https://files.protifi.com/protocols/; accessed on 20 June 2022]. Eighteen samples of trypsinized mouse blood plasma were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) similarly to a previously published study [24]. The proteomic analysis was performed by means of the MaxQuant software (ver. 1.6.3.4) using the SwissProt sequence database for *Mus musculus* (mouse). Proteins were identified with the application of criteria defined by the international proteomic organization HUPO (Human Proteome Organization [25]). A total of 249 mouse proteins were found in the samples (Supplement, Table S1). Proteins were identified in at least two technical replicates by means of two or more peptides, whose sequences were confirmed by a tandem mass spectrum.

2.5. Functional Annotation of the Proteins

This procedure was performed using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) gene annotation tool [https://david.ncifcrf.gov/; accessed on 25 December 2023]. The *Mus musculus* genome served as the background list for over-representation analysis. The Gene Ontology (GO) option in DAVID as well as the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [https://www.genome.jp/kegg/pathway.html; accessed on 16 October 2023] were used to identify significantly ($p < 0.05$) enriched biological processes and metabolic pathways. An atlas of combinatorial transcriptional regulation in mice and humans was employed to identify differentially expressed genes encoding transcription factor genes [26]. The STRING: functional protein association networks [https://string-db.org/; accessed on 25 December 2023] and GeneCards: The Human Gene Database [https://www.genecards.org; accessed on 25 September 2023] were used to construct a functional protein association network.

2.6. Statistical Analysis

Statistical analysis of the data obtained during the protein identification was carried out in the Perseus v.1.6.15.0 software. The data were first filtered to select the most significant points: Possible contaminating and decoy proteins were removed, and proteins that were present in two of three technical replicates of each sample were retained. The plasma samples were grouped according to the behavioral experiences of the mice: "winners, aggressive mice," "losers, depressed mice," and the control group of mice. Statistical analysis of the samples based on the LFQ values of the identified proteins made it possible to find patterns in the distribution of proteins in the samples. Proteins were identified in at least two technical replicates by means of two or more peptides whose sequences were confirmed by a tandem mass spectrum. Principal component analysis (PCA) was performed to test the possibility of clustering of the data about the identified proteins of the mouse groups. To detect significant differences between the proteins of the mouse groups, a pairwise comparison of groups by the LFQ protein content was made using a Volcano plot based on the t test with a false discovery rate (FDR) adjustment of Benjamini and Hochberg (with q = 5%). STATISTICA 12.0 (StatSoft, Tulsa, OK, USA), JACOBI4 (software for multivariate analysis of biological data) [w1. JACOBI4: https://jacobi4.ru; accessed on

21 November 2019], and XlStat (xlstat.com; accessed on 16 October 2023) software were employed for the data analysis and presentation.

3. Results

3.1. Animal Weight

The defeated mice (losers) and aggressive mice (winners) were weighed before and after the 20 days of agonistic interactions (Table 1). Before the experiments, the weight of the mice did not differ between the groups. After the 20 days of the experiment, the weight of the losers was significantly less than that of the winners.

Table 1. Weight of mice before and after 20 days of social conflicts.

	Losers	**Winners**	
Before	27.4 ± 0.42	27.8 ± 0.33	ns
After	28.9 ± 0.32 $p < 0.001$	30.2 ± 0.39 $p < 0.001$	$p < 0.05$

ns: not significant.

3.2. The Partition Test

Behavioral data in the partition test (Figure 2), as in our previous experiments [23], confirmed that the number of approaches to the partition (as a reaction to the partner in an adjacent compartment) and total and mean time near the partition are significantly less in the depressed losers than in the winners. These results indicated that the losers demonstrated a high level of anxiety (by avoiding the socialization) and low motor activity in comparison with the winners, which exhibited high motor activity and strong aggressive motivation toward the partner in the neighboring compartment.

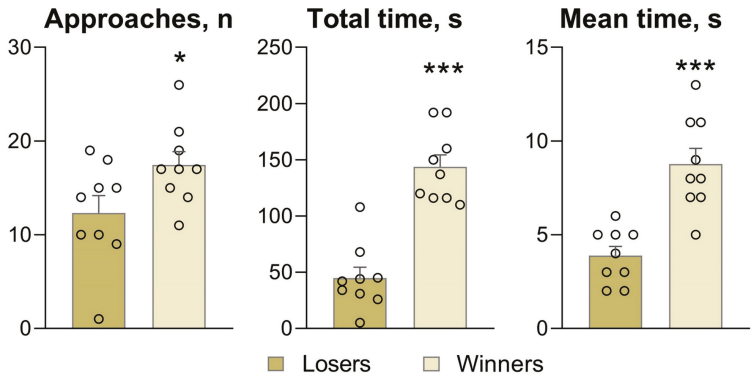


Figure 2. Social reactions of losing and winning mice to a partner in an adjacent compartment of the cage in the partition test. * $p < 0.05$ and *** $p < 0.001$ for a comparison of winners with losers. Statistical significance was set at an FDR-adjusted p value < 5%.

3.3. Analysis of Differentially Expressed Proteins in Blood Plasma of Male Mice

Based on the statistical analysis of the samples in the Perseus v.1.6.15.0 software, 15 proteins were found that were reproducibly present in technical replicates and whose amounts differed significantly between groups of plasma samples (complied according to behavioral experience in the daily agonistic interactions). Furthermore, we paid special attention to the proteins that significantly differed in expression between the groups in the t test corrected for the FDR at $p < 0.05$. Table S2 (Supplement) presents the description of the functions of proteins and genes associated with diseases according to STRING databases for *Mus musculus*.

Judging by our data (Figure 3), the plasma samples from the losers were separated from the control group and winners. The control and winner groups of samples were not distinguishable by PCA.

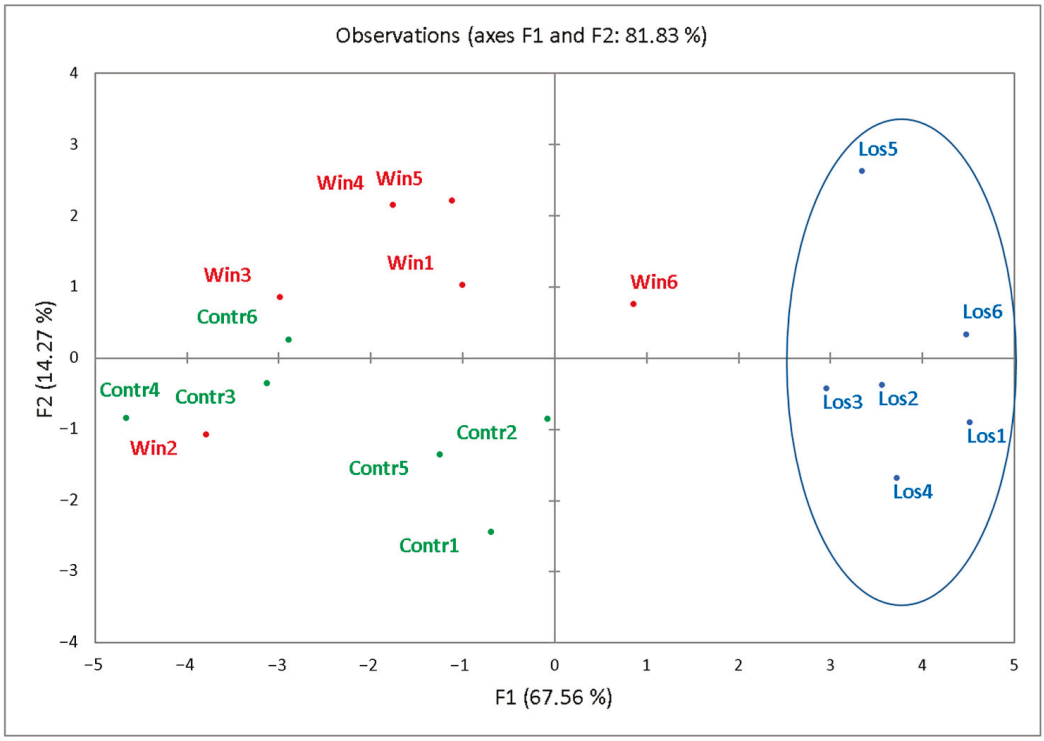


Figure 3. A PCA plot of the distribution of samples according to the average LFQ values of mouse plasma proteins differentially expressed ($q < 0.05$) in at least one of the mouse groups. Green dots denote the control group (Contr), red dots represent the winners (Win), and blue dots represent the losers (Los) in the comparison "Contr–Los," "Contr–Win," or "Los–Win."

No significant differences in the protein content were found between the controls and winners. The depressed mice differed from the control group and the winners in 11 proteins (Figure 4). The data showed that, in blood plasma, the main families of proteins that showed an association between the depression-like state and altered expression were amyloid, apolipoproteins, trypsin inhibitors, serine peptidase inhibitors, complement components, and globular proteins.

For a detailed analysis, we selected the proteins that changed expression only according to FDR-adjusted ($q < 0.05$) values. These were amyloid protein SAA4 as well as SAMP: serum amyloid A-4 protein and serum amyloid P-component, encoded by genes *Saa4* and *Apcs*, respectively (Figure 5). The level of the SAA1 protein, encoded by genes *Saa1,* was significantly higher in the depressed mice than in the winners ($p < 0.000$; $q < 0.0082$). The level of the SAA4 protein was significantly higher in the depressed mice in comparison with the control mice ($p < 0.0015$; $q < 0.0303$) or the winners ($p < 0.0037$; $q < 0.0457$). The SAMP protein was significantly overexpressed in the depressed mice in comparison with the control mice ($p < 0.0011$; $q < 0.0286$) and winners ($p < 0.0105$).

Higher levels in the blood plasma of the depressed mice in comparison with the control mice were registered for the proteins APOC3 ($p < 0.0001$; $q < 0.0089$) and APOD ($p < 0.0002$; $q < 0.0089$), and lower levels were registered for ADIPO proteins ($p < 0.0021$; $q < 0.0333$) (apolipoprotein C-III, apolipoprotein D, and adiponectin, encoded by genes *Apoc3*, *Apod*, and *Adipoq*, respectively). The level of APOA2 (apolipoprotein A-II, encoded by the *Apoa2* gene) was higher in comparison with the winners ($p < 0.0026$; $q < 0.0447$) (Figure 5).

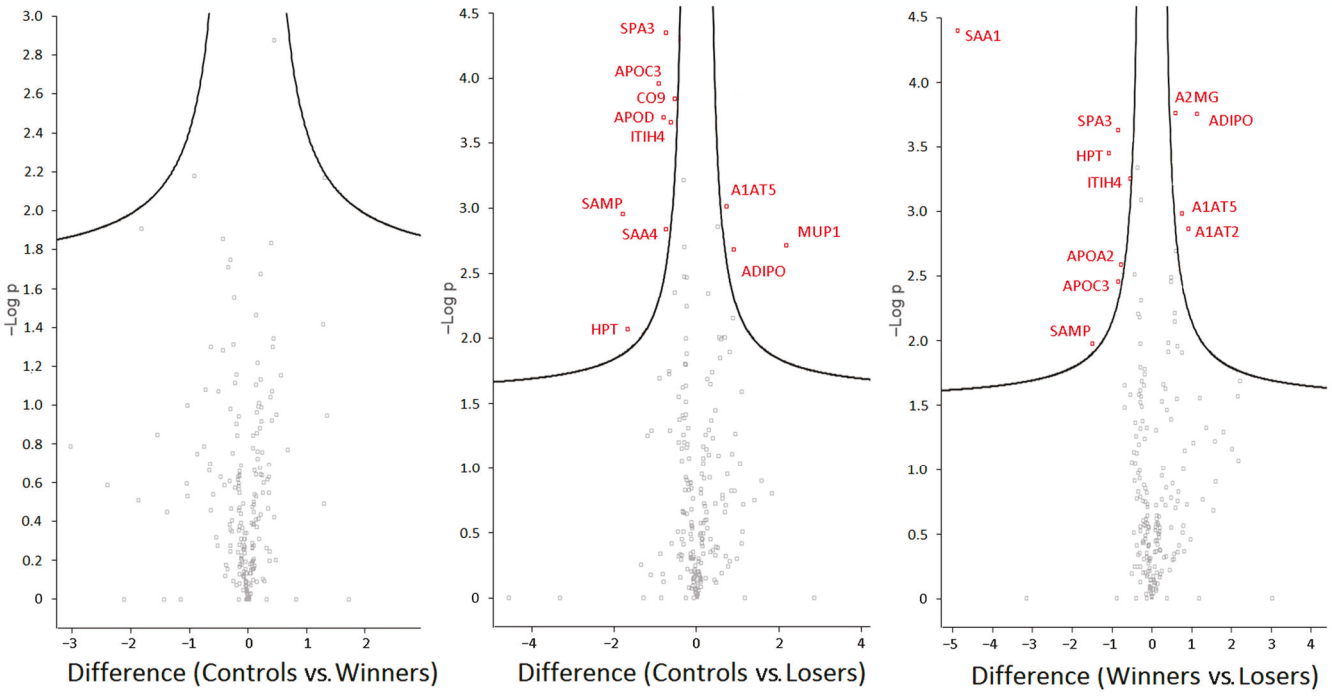


Figure 4. The volcano plot comparing proteins of different groups of mice (Controls vs. Winners, Controls vs. Losers, or Winners vs. Losers). The x-axis is the range of differences; the y-axis is the statistical significance. Red: proteins with statistically significant differences, gray: proteins with statistically insignificant differences.

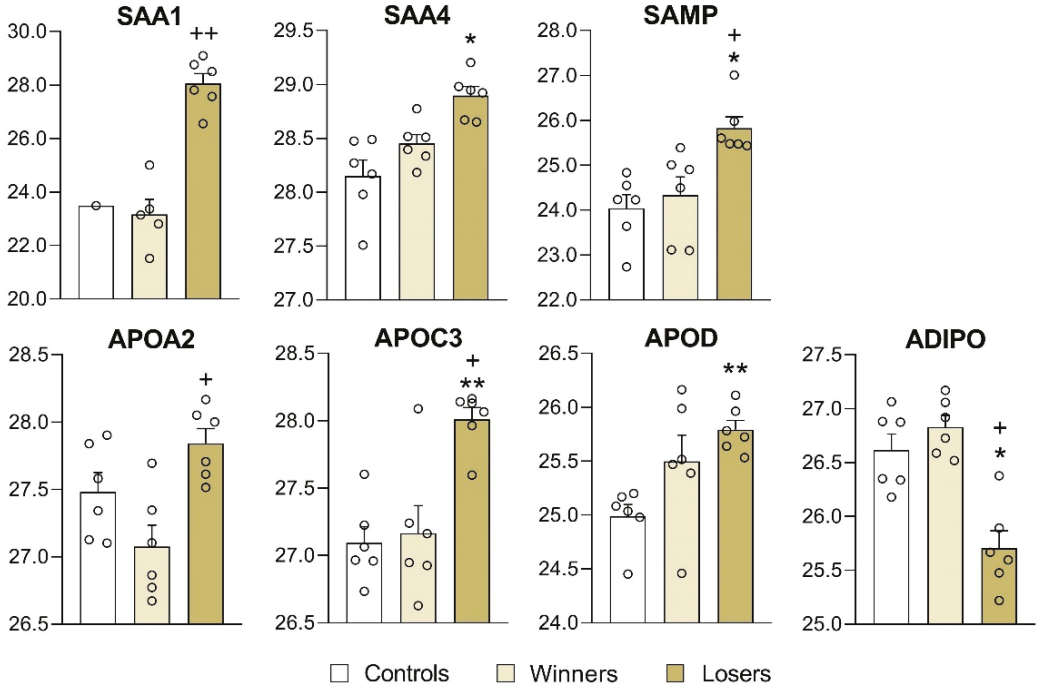


Figure 5. Plasma amyloid proteins and apolipoproteins of interest in the controls, winners, and losers (depressed mice). Controls vs. Losers: * $q < 0.05$; ** $q < 0.01$; and Winners vs. Losers: $^{+}$ $q < 0.05$; $^{++}$ $q < 0.01$. Data are presented as log2 LFQ intensity values. Statistical significance was set at an FDR-adjusted p value < 5%. All data on differentially expressed proteins are presented in Supplement: Table S3 for the Controls vs. Losers comparison, Table S4 for the Controls vs. Winners, and Table S5 for the Winners vs. Losers.

The proteins ITIH4, SPA3 (SPA3N, SPA3A, and SPA3G), A1AT2, and A1AT5, which are encoded by genes *Itih4*, *Serpina1b*, *Serpina1e*, and *Serpina3* (*Serpina3n*, *Serpina3A*, and *Serpina3G*), respectively, are broad-spectrum protease inhibitors (Figure 6). Higher levels of these proteins were found in the depressed mice in comparison with the controls for ITIH4 ($p < 0.0002$; $q < 0.0089$), SPA3 (SPA3N) ($p < 0.0000$; $q < 0.0089$), CO9 ($p < 0.0001$; $q < 0.0089$), and HPT ($p < 0.0087$), and lower levels were found for A1AT5 ($p < 0.0010$; $q < 0.0286$), and A2MG (PZP) ($p < 0.0014$; $q < 0.0303$). The "winners vs. losers" comparison revealed higher concentrations of ITIH4 ($p < 0.0006$; $q < 0.0165$), SPA3 (SPA3N) ($p < 0.0002$; $q < 0.0120$), and HPT ($p < 0.0004$; $q < 0.0146$) and lower concentrations of A1AT2 ($p < 0.0014$; $q < 0.0282$), A1AT5 ($p < 0.0010$; $q < 0.0238$), and A2MG (PZP) ($p < 0.0002$; $q < 0.0120$).

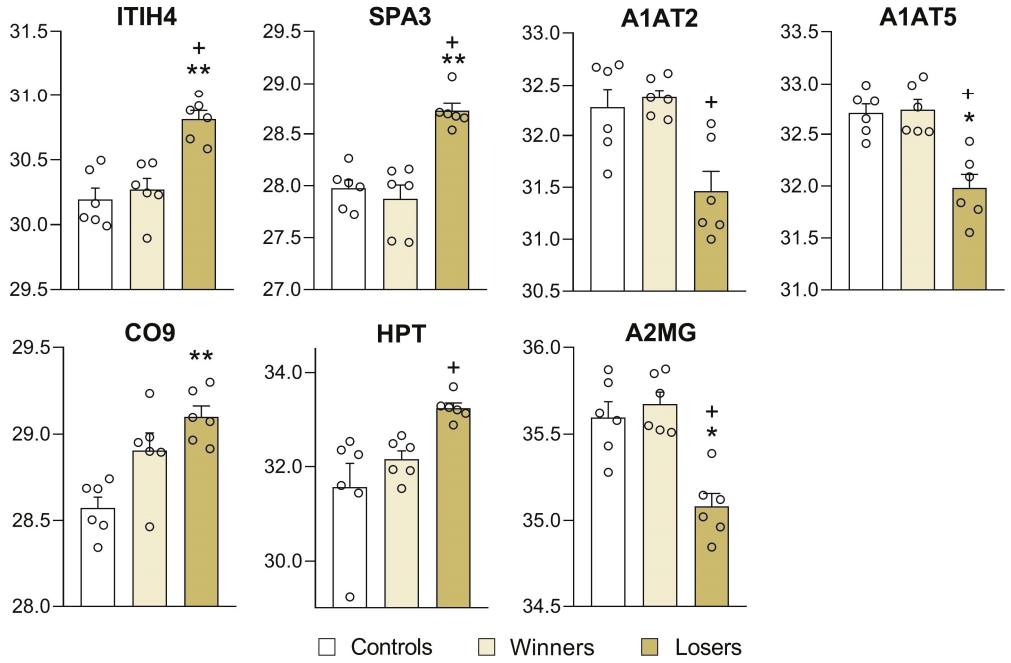


Figure 6. Plasma serine protease inhibitors (proteins) and globulins in the controls, winners, and losers (depressed mice). Controls vs. Losers: * $q < 0.05$; ** $q < 0.01$; Winners vs. Losers: $^{+}$ $q < 0.05$; Data are presented as log2 LFQ intensity values. Statistical significance was set at an FDR-adjusted p value < 5%. All data on differentially expressed proteins are presented in Supplement: Table S3 for the Controls vs. Losers comparison, Table S4 for Controls vs. Winners, and Table S5 for Winners vs. Losers.

For five out of the six samples for the SAA1 protein in the control mice, either expression changes were not detectable, or the protein concentration was so low that the protein simply could not be detected because of individual variation in the protein's levels under different conditions. Because the samples were analyzed in duplicate (two replicates), these data were reliable. We included these proteins in the figure because significant differences were identified between aggressive and depressed animals.

The MUP1 protein (major urinary protein 1) was removed from the analysis owing to high variation in experimental groups and to its major function. According to the STRING database (Table S2, Supplement), MUP1 is primarily responsible for binding pheromones that are released from drying urine of mice. These pheromones affect the sexual behavior of females.

To avoid losing potentially useful information, we also examined differentially expressed proteins with $p < 0.05$. We analyzed proteins with altered expression in the losers compared with the controls and found 47 differentially expressed proteins (Supplement, Table S3): two amyloids, four apolipoproteins, three inter-alpha trypsin inhibitors, eight serine peptidase inhibitors, four complement components, nine globulins, and fourteen others. In the winners compared with the controls, we found 13 proteins

(*p* value) (Supplement, Table S4): one apolipoprotein, two serine peptidase inhibitors, two complement components, and eight others. In the comparison of winners vs. losers (Supplement, Table S5), 57 differentially expressed proteins (*p* and/or *q* values) were found: three amyloids, four apolipoproteins, three inter-alpha trypsin inhibitors, eleven serine peptidase inhibitors, nine complement components, three globular proteins, and twenty other proteins. These findings confirmed that the protein expression changes were strongly specific to the depressed mice in comparison with the chronically aggressive mice.

Thus, the proteomic analysis of the plasma samples showed that the expression of proteins in the blood plasma of the depressed mice specifically changed under the chronic social defeat stress that was accompanied by the development of the depression-like state. These are the amyloid proteins SAA1, SAA4, and SAMP and apolipoproteins APOA2, APOC3, and APOD. Changed levels of serine protease inhibitors and/or proteins associated with lipid metabolism, inflammation, or immune function were also found in this comparison: upregulation of the proteins ITIH4, SPA3, HTP (HP), and CO9 (C9) and downregulation of the proteins A1AT2, A1AT5, and A2MG.

4. Discussion

It has been well confirmed that, after 20 days of agonistic interactions, a psychosis-like state develops in the winners ([12,22], reviews) and depression-like states develop in the losers ([14], review). The aim of this work was to study blood plasma markers in these social groups of mice having different pathological states. Our analysis of the blood plasma samples by mass spectrometry allowed for us to identify 14 proteins whose amounts differed significantly between samples grouped according to the social experience in the daily agonistic interactions in mice: "aggressive mice, winners" and "depressed mice, losers" in comparison with each other and with the control group of mice. These proteins are the most relevant and represent amyloids, apolipoproteins, trypsin and serine peptidase inhibitors, complement components, and globulins.

Amyloidosis is known to be a rare disease that occurs when amyloid proteins accumulate in systems of organs such as the nervous system and digestive tract and can lead to disturbances of their functions. High levels of amyloid proteins are associated with Alzheimer's disease, which is accompanied by progression of cognitive deficits [27]. Extreme fatigue and weakness and weight loss or gain are also symptoms of depression in Alzheimer's patients, implying comorbidity of these disorders. Moreover, serum amyloid A (SAA) participates in many immunomodulatory mechanisms, and its level increases in many inflammatory diseases [28]. In addition, SAA proteins affect the transport and absorption of cholesterol [29,30].

In the depressed mice compared with the control and aggressive mice, the levels of proteins were mostly higher (Figures 5 and 6, Supplement, Tables S3–S5). From the overexpression of the amyloid proteins SAA4 and SAMP (encoded by genes *Saa4*, and *Apcs*) in the losers compared with the controls, we can deduce the formation of a predisposition to amyloidosis under chronic social stress in depressed mice to a greater extent as compared to the winners (which had a positive fighting experience), which did not differ from the control in these proteins' levels.

Of note, the absence ("zero level") of the SAA1 proteins (in five out of six samples) in blood plasma was found only in the control mice. According to GeneCards, diseases associated with the *Saa1* gene include serum amyloid A1 amyloidosis and amyloidosis. This protein also plays an important part in the metabolism of high-density lipoproteins (HDLs) and cholesterol homeostasis. High levels of this protein are linked with chronic inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and Alzheimer's disease, and are also a potential biomarker of certain tumors ([31], review). This is a very important fact in itself and shows that these proteins change their expression as a rule under the influence of experimental conditions to various degrees depending on a pathological state of mice, especially in depressed mice. Their presence in blood plasma can serve as a marker of an altered state and even progression of such a pathology.

Thus, the mostly elevated levels of amyloid proteins and apolipoproteins imply a psychopathogenic role of chronic social stress, which may cause a predisposition to disturbances in lipid metabolism. These data are consistent with those showing that overexpression of serum amyloid A1 induces depressive-like behavior in mice [32].

The most changed protein levels were documented for apolipoproteins ([33], review). The function of apolipoproteins is to bind lipids, including oil-soluble substances such as fats, cholesterol, and fat-soluble vitamins, to form lipoproteins. They transport lipids in the blood, cerebrospinal fluid, and lymph. Lipid components of lipoproteins are insoluble in water. Apolipoproteins and other biomolecules (such as phospholipids) can surround lipids, thus creating a lipoprotein particle that is itself water-soluble and can, therefore, be carried through body fluids (i.e., blood and lymph). Additionally, apolipoproteins interact with lipoprotein receptors and lipid transport proteins, thereby participating in lipoprotein uptake and clearance. They also serve as enzyme cofactors for specific enzymes involved in the metabolism of lipoproteins [33].

In the depressed mice, higher concentrations of proteins in the blood plasma were found for the apolipoproteins APOC3 and APOD, and lower levels were found for ADIPO proteins (apolipoprotein A-II, apolipoprotein C-III, and apolipoprotein D) encoded by genes *Apod*, and *Adipoq*, respectively. According to GeneCards, diseases associated with APOA2 proteins, encoded by genes *Apoa2*, include hypercholesterolemia and some types of amyloidosis. Among pathways related to APOA2, there are statin inhibition of cholesterol production as well as plasma lipoprotein assembly, remodeling, and clearance. Similarly to APOA2-related pathways, diseases associated with APOC3 include apolipoprotein C-III deficiency and coronary heart disease. GO annotations related to these genes include lipid and cholesterol binding [33].

Among the pathways related to APOD (apolipoprotein D), GO annotations related to this gene include transporter activity and cholesterol binding. *ApoD* gene expression is affected in several pathologies, such as HDL (high-density lipoprotein) familial deficiency and many others including central nervous system disorders and cancer.

Diseases associated with ADIPO proteins include adiponectin deficiency and fatty liver disease. Among the pathways related to ADIPO, there are AMP-activated protein kinase signaling and energy metabolism. The *Adipoq* gene is expressed in adipose tissue exclusively. The encoded protein circulates in plasma and is involved in metabolic and hormonal processes.

A comparison of plasma protein expression between patients with depression and healthy controls has revealed that differentially expressed proteins, including apolipoproteins such as the APOE, APOC4, and APOA5, lead to significant alterations of lipid and metabolic functions [6]. According to pathway analysis, the top statistically significant pathways were related to lipid transport, inflammation, and immunity. Network analysis of integration of differential expression of proteins and metabolites indicated that a disturbance of phospholipid metabolism may contribute to inflammation in the central nervous system [6]. It can be hypothesized that the diminished level of the ADIPO protein and the overexpression of the APOC3 protein in comparison with the controls and winners can be considered markers of a depression-like state.

We also found changes in the expression of serpin proteins, which are a widespread family of protease inhibitors that use conformational alterations to inhibit target enzymes [34–36]. Serpins play a central role in the control of many important proteolytic cascades. Serpins are conformationally labile, and many disease-associated serpins result in the misfolding or formation of pathogenic, inactive biopolymers. Approximately two-thirds of human serpins perform extracellular functions by inhibiting proteases in the bloodstream to modulate their activities [36]. Deficiencies of serpins have been implicated in dementia, thrombosis, chronic fatigue, essential hypertension, hypothyroidism, tumor progression, and other health problems [35–41]. Consequently, serpins join a growing number of structurally distinct biomolecules that can cause major degenerative diseases

(such as prion disease, which is a type of proteopathy) or the amyloid proteins that form inclusions in Alzheimer's disease [35,41].

In the depressed mice, changed levels of proteins associated with serpinopathies were registered in the plasma in comparison with the controls: elevated levels of the proteins ITIH4 and SPA3 (SPA3N, SPA3A, and SPA3G) and decreased levels of A1AT5 proteins and A2MG proteins, which are broad-spectrum protease inhibitors. These proteins are serine protease inhibitors and are encoded by genes *Itih4*, *Serpina3* (*Serpina3N*, *Serpina3A*, and *Serpina3G*), *Serpina1b*, and *Serpina1e*, respectively. There are many diseases associated with serpins and serpinopathies [41–46]. For example, the proteins A1AT2, A1AT5, and A2MG participate in alpha-1-antitrypsin deficiency in humans and are associated with liver disease and inflammation. GO annotations related to these genes include binding of an identical protein and protease.

The SPA3 protein, also called α-1-antichymotrypsin (AACT, ACT), is one of the inhibitors of serine proteases. It plays an important part in the anti-inflammatory and antiviral response. Elevated levels of SERPINA3 (encoded by the *SerpinA3* gene) have been observed in heart failure and neurological diseases. Many studies have linked the observed increased expression levels of the *SerpinA3* gene with its crucial role in several pathologies in various types of cancer, such as glioblastoma, breast cancer, colorectal cancer, and melanoma [35,47]. The brain disease most strongly associated with the *SerpinA3* gene is Alzheimer's disease. It has been reported that the SERPINA3 protein may bind to β-amyloid proteins and, thus, makes up a component of amyloid deposits. Another brain disease linked to this protein's upregulation is prion disease [34,35], which includes rapidly developing dementia, muscle stiffness, difficulty walking, and fatigue.

The analysis of plasma proteins in patients indicates that one of the candidate diagnostic biomarkers of depression may be the SERPIND1 protein, which is upregulated in depressed patients [48]. The data from our model of depression match these data, confirming the development of a depression-like state in mice under chronic social defeat stress [11–13].

Higher concentrations of the proteins alpha 1-antitrypsin and haptoglobin (HTP, HP) have been found in the plasma of depressed patients [49–52]. These findings are consistent with the hypothesis that MDD is accompanied by inflammatory shifts with higher levels of the APP protein (amyloid beta precursor protein). It has been reported that elevated amounts of the proteins haptoglobin (HP) and alpha-1-antichymotrypsin (SERPINA3) highly correlate with each other and with the severity of depression and negatively correlate with the thyroid-stimulating hormone response to thyrotropin [49]. These findings provide further evidence for an inflammatory response during depression.

Increased levels of the ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) protein are associated with such disorders as human immunodeficiency and virus infection. It has been shown that, in the blood plasma of patients with MDD in comparison with healthy controls, the ITIH4 protein concentration is significantly higher but not in patients with schizophrenia or bipolar disorder [5]. Moreover, the consistency of these results has been verified by proteomic analysis of postmortem dorsolateral prefrontal cortex samples of brain tissue from 16 MDD patients. These data suggest that plasma ITIH4 protein levels may be a potential diagnostic biomarker of MDD [52] with high specificity. In our depressed mice, the level of the ITIH4 protein was higher. Thus, these data confirm that this protein may be used as a peripheral biomarker of depression in humans and in our experiment—in depressed mice.

Complement component 9 (CO9, C9) is involved in the complement system, which is a part of the innate immune system [53]. It is thought that, in plasma, exosomal proteins CO9 together with other proteins are biomarkers helping to differentiate Alzheimer' patients from healthy individuals [54–56]. Similarly to these findings, abnormal levels of complement component C9 and haptoglobin alpha chain (HPT) were found here in the depressed mice in comparison with the controls. These proteins are also associated with inflammation and immune function.

The level of the A2M protein (in mice: A2MG; alpha-2-macroglobulin) was lower in the depressed mice than in the chronically aggressive mice but not different from the controls. The α-macroglobulin (αM) family of proteins includes a broad-spectrum protease inhibitor that correlates with an elevated risk of Alzheimer's disease [57–59] and is associated with all functions of α2-macroglobulin in the context of inflammation, immunity, and infections [59]. In mice the MUP1 proteins which is primarily responsible for binding pheromones, was identified also as a regulator for glucose and lipid metabolism [60].

Therefore, our study showed that, in mice, chronic social stress induces the development of a depression-like state that is accompanied by elevated levels of amyloid proteins and apolipoproteins in the blood plasma of depressed mice. Higher levels in the blood plasma of the depressed mice were documented for the amyloid proteins SAA1 and SAA4, for SAMP, and for apolipoproteins APOC3 and APOD, and lower levels were documented for ADIPO proteins. The proteins ITIH4, SPA3, A1AT5, A2MG, and CO9 (C9) are serine protease inhibitors and/or are associated with inflammation and innate immune function. As markers of a depression-like state, good candidates are the proteins SAA1, ITIH4, SPA3, and A1AT5, and are broad-spectrum protease inhibitors.

Right now, it is very difficult to interpret the changes in protein levels in the blood plasma of mice. In any case, however, these data indicate a psychopathogenic role of chronic social stress, which may create a predisposition to Alzheimer's disease in humans during depression accompanied very often by immunodeficiency [15]. Our experimental approach may be useful for searches for peripheral biomarkers of depression in the hope to understand the comorbidity of depression with other diseases and to devise an appropriate treatment for the patients.

5. Conclusions

As described in many studies and reviews [61,62], there are many experimental approaches to study the mechanisms of psychoemotional disorders, in particular depression and anxiety, which are either a cause or a consequence of the development of other diseases. Our research confirms the existing ideas about the comorbidity of depression and anxiety and indicates that these pathological conditions are accompanied by many other diseases, creating a predisposition to Alzheimer's disease, immune disorders, etc.

Our experimental data make it possible to understand the relation between chronic social stress, depression-like states, and immune function in male mice. The identification of specific proteins in blood plasma that show altered levels under chronic social defeat stress allows for us to study the molecular pathogenesis of depression and to identify potential biomarkers of comorbidities. If experimental studies reveal a correlation between proteins in blood plasma and changes in the brain, then in the future, this knowledge may help make a more accurate diagnosis, determine the stage of the disease, and predict consequences of this pathological condition. We think that this study opens up new possibilities for the design of novel therapeutic strategies against depression.

Limitation: There is much criticism of the use of animal models (reviews, [63,64]) to study pathological processes in depressed individuals. This is explained by the fact that there are many types of depression, which can be accompanied by various mental changes and somatic consequences developing concurrently with various psychoemotional aberrations and concomitant disorders and depending on heredity and life events. Naturally, researchers must take into account that currently available animal models are of limited use for these purposes because no one animal model fully reproduces this complex disease. However, it is obvious and inevitable that it is necessary to follow the path described in the excellent review "*From Laboratory to Life*" [61]: from experimental studies using adequate models of neurological and psychiatric diseases to the treatment of patients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines12071485/s1, Supplementary Table S1: All proteins that were identified by LC–MS/MS in blood plasma of male mice; Supplementary Table S2: 15 Proteins of interest associated with diseases according to the STRING database for *Mus musculus*;

Supplementary Table S3: The comparison of protein levels in blood plasma of mice (Controls vs. Losers, depressed mice); Supplementary Table S4: The comparison of protein levels in blood plasma of mice (Controls vs. Winners, aggressive mice); Supplementary Table S5: The comparison of protein levels in blood plasma of mice (Winners vs. Losers).

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Data Availability Statement: All relevant data are available in the Supplementary Materials. Raw data are available from the authors upon request.

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From Mild Cognitive Impairment to Dementia: The Impact of Comorbid Conditions on Disease Conversion

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Abstract: The conversion from mild cognitive impairment (MCI) to dementia is influenced by several factors, including comorbid conditions such as metabolic and vascular diseases. Understanding the impact of these comorbidities can help in the disease management of patients with a higher risk of progressing to dementia, improving outcomes. In the current study, we aimed to analyze data from a large cohort of MCI (n = 188) by principal component analysis (PCA) and cluster analysis (CA) to classify patients into distinct groups based on their comorbidity profile and to predict the risk of conversion to dementia. From our analysis, four clusters emerged. CA showed a significantly higher rate of disease progression for Cluster 1, which was predominantly characterized by extremely high obesity and diabetes compared to other clusters. In contrast, Cluster 3, which was defined by a lower prevalence of all comorbidities, had a lower conversion rate. Cluster 2, mainly including subjects with traumatic brain injuries, showed the lowest rate of conversion. Lastly, Cluster 4, including a high load of hearing loss and depression, showed an intermediate risk of conversion. This study underscores the significant impact of specific comorbidity profiles on the progression from MCI to dementia, highlighting the need for targeted interventions and management strategies for individuals with these comorbidity profiles to potentially delay or prevent the onset of dementia.

Keywords: mild cognitive impairment; dementia; Alzheimer's disease; conversion; comorbidities; depression; biomarkers; metabolic diseases; disease management; neurodegenerative diseases; vascular dementia

1. Introduction

Around 50 million people live with dementia worldwide, and this number is projected to almost triplicate by 2050. The number of people with dementia is rising, and it is imperative to identify potentially modifiable risk factors and tackle comorbid conditions since it has been estimated that treating twelve risk factors might prevent up to 40% of dementia [1]. Patients with dementia generally manifest more comorbidities than those without dementia, having an independent impact on outcomes and a consistent socio-economic burden [2]. Several studies reported that comorbidities, including cerebral vascular disease, depression, and chronic obstructive pulmonary disease, may affect the progression of dementia, reducing the quality of life and showing a potential relationship between the underlying disease [3–5]. In addition, comorbidities at the time of dementia diagnosis have been associated with decreased survival [6]. Lastly, it has been suggested that treating

the comorbid conditions may ameliorate dementia symptoms [7]. Thus, recognizing the importance of comorbidities for dementia care is critical in disease management.

Mild cognitive impairment (MCI) is a heterogeneous condition characterized by a cognitive impairment that does not affect independence in activities of daily living, thus not fulfilling the criteria for dementia. MCI is usually considered a transitional phase between typical aging and dementia. About 10–20% of individuals with MCI annually convert to dementia; thus, a considerable proportion of these individuals will ultimately convert to dementia over 2–5 years [8–11]. The risk of developing dementia may be affected by concomitant pathologies [1], but the global impact of comorbidities on the conversion from MCI to dementia still needs to be understood. Distinct clinical phenotypes exist, with different risks of progression to dementia, including the amnestic MCI (aMCI) and nonamnestic MCI (naMCI); both include a single domain and a multiple domain subtype [12]. The aMCI is characterized by a prominent or isolated decline in memory. It is usually considered the prodromal phase of Alzheimer's dementia (AD), despite a not negligible percentage of aMCI subjects remaining stable over time [13–15].

The heterogeneity of the MCI condition complicates the precise stratification of subjects according to the expected progression. Identifying MCI subjects at risk for developing dementia is of utmost importance since clinical trials testing disease-modifying therapies should include only participants who will progress to a more severe clinical stage. Research into innovative strategies for managing AD is flourishing, with several therapies currently under investigation [16]. In the last few years, monoclonal antibodies targeting pathological amyloid beta aggregates have been tested in randomized clinical trials, showing an evident effect on clearing beta-amyloid accumulation but without clinical efficacy [17]. There are several reasons behind the unsatisfying results of these trials, including issues related to the participants, since subjects who received the treatments, despite being selected as MCI or mild dementia, could be already in a too advanced stage of the disease, and related to the target, considering the complexity of AD pathology and associated neurodegenerative mechanisms [18]. There is a need in clinical practice and research settings for tools and strategies to recognize patients at risk of developing dementia, even in the preclinical and prodromal stages, to evaluate the efficacy of new possible treatments.

Since its prevalence increases with age, MCI is a condition of the elderly [19]. Coherently, MCI subjects are often affected by multiple comorbidities. In a progressively aging population, an increase in MCI and other comorbidities must be expected. Most of these conditions have been associated with more complicated management, faster development of dementia, or more severe cognitive decline [20–25]. Among these conditions, vascular risk factors (VRFs) play a key role in the development of cognitive impairment [26]. In MCI populations, VRF not only contributes to cognitive decline but also promotes conversion to dementia [27]. In addition, the presence of VRF seems associated with the development of multiple-domain impairment, influencing the clinical MCI phenotype [28]. Also, in healthy subjects, VRF participates in neurodegeneration, with an addictive effect on amyloid deposition [29].

Depression is currently considered one of the main risk factors associated with the development of dementia. In particular, late-onset depression, usually involving individuals over 65 years of age, appears to be an accelerating factor for cognitive deterioration [30]. Depression is observed in about 50% of people with AD, and it is directly correlated with the accelerated progression of dementia [31]. In MCI cohorts, depression was shown to affect outcomes and reduce quality of life [32,33]. Depression in MCI contributes to neurodegenerative changes and corresponding clinical impact, being associated with a higher degree of cortical atrophy [34] and with the development of impairment of specific abilities, such as financial capacity [35].

Traumatic brain injury has been recently included, together with alcohol consumption and air pollution, in the group of potentially modifiable risk factors for dementia [1]. Traumatic encephalopathy, which is generally related to chronic sports head injuries, describes a broad range of neuropathology [36]. The history of traumatic brain injury

was associated with increased odds of a diagnosis of MCI even after adjusting for age, education, genotype, and VRF. However, the association was attenuated when correcting for the presence of depression, suggesting the need for further confirmation [37].

Self-reported hearing problems are associated with an increased risk of disability and dementia, which is mitigated by the use of hearing aids [38]. Hearing loss is linked with a higher risk of MCI and accelerated cognitive decline [39]. The importance of considering hearing loss as a crucial treatable risk factor for dementia also lies in its vast prevalence since it involves one-third of individuals aged 65 years and older [40].

Preventing, recognizing, and managing comorbidities throughout all stages of life can significantly reduce the risk of developing dementia later in life. This should be one of the most ambitious and promising goals for the global healthcare system. Strategies include not only medications, such as those to reduce blood pressure, but also specific lifestyle modification interventions. The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) is a multicenter, randomized, controlled study aiming at identifying intervention strategies to prevent the onset of cognitive decline and dementia [41]. The study involved more than one thousand individuals at risk of cognitive decline. Participants underwent a multidomain intervention consisting of diet, exercise, cognitive training, social activity, and control of vascular risk factors. The project demonstrated that administering multidomain interventions is feasible and effective, reporting significant benefits on change in global cognitive performances in older adults at risk for developing dementia [42].

Starting from these premises, several studies explored the prognostic significance of comorbidities in MCI populations, using various statistical approaches and reporting inconsistent results [43–45]. A recent study based on the Alzheimer's Disease Neuroimaging Initiative (ADNI) dataset classified MCI subjects into clinically relevant subtypes according to multiple features, including comorbidities and genetic, imaging, and neuropsychological data, demonstrating the crucial importance of considering comorbidities in the prognostic stratification [46].

This study aimed to identify clinical groups of aMCI subjects with different risks of progression to dementia. We performed a principal component analysis (PCA) and a subsequent cluster analysis (CA) exploring comorbidities in a population referring to the Centres for Dementia and Cognitive Disorders (CDCD) of the University of Piemonte Orientale, Piedmont, Italy, having a diagnosis of aMCI and a suitable follow-up. Subjects were clustered according to comorbid conditions and compared regarding the severity of the progression of cognitive decline and the conversion to dementia, together with other clinical variables, to underline the peculiarity of each cluster.

2. Materials and Methods

2.1. Sample Selection

We selected participants whom expert neurologists consecutively evaluated at the CDCD at the Sant'Andrea Hospital, Vercelli, or at the Maggiore della Carità University Hospital, Novara, Italy (University of Piemonte Orientale). We included only subjects aged $\geq$ 55 years old who were diagnosed with a clinical diagnosis of aMCI using the Petersen criteria [47] from January 2018 to January 2022. All enrolled aMCI subjects had a clinical dementia rating (CDR) scale of 0.5 (questionable dementia), normal cognitive function, and preserved activities of daily living assessed by the evaluation of the Mini-Mental State Examination (MMSE), activity of daily living (ADL), and instrumental activity of daily living (IADL) scores. Cognitive and functional scores (MMSE, ADL, and IADL) were available both at the baseline and the follow-up visits. Since a part of MCI subjects convert to dementia every year, with a variable annual conversion rate of about 10–20%, to avoid bias related to the observational time, other inclusion criteria were considered: (a) symptoms' onset within three years before the first evaluation (baseline); (b) at least one available follow-up visit between two and five years after the baseline evaluation [48]. We excluded subjects with a diagnosis other than aMCI (naMCI, dementia, psychiatric

disturbance, subjective cognitive decline) or with a comorbid neurological condition such as normal pressure hydrocephalus and extrapyramidal syndromes.

2.2. Demographic and Clinical Variables

Demographics included age at baseline, sex, educational level, and years from symptoms' onset. Additional features were familiarity and voluptuary habits, including smoking and alcohol consumption. Among comorbidities, we considered eight recognized AD-related comorbidities [46]: (1) hypertension; (2) diabetes; (3) high cholesterol; (4) depression; (5) obesity; (6) cardiovascular disease; (7) hearing loss; (8) traumatic brain injury (TBI).

We established the presence of depression based on either an anamnestic criterion (reported by the patient or caregiver as a previous diagnosis of depression and with ad hoc questions and in line with the Diagnostic and Statistical Manual of Mental Disorders V criteria) or upon the prescription of antidepressant treatments [49]. For TBI, we based the inclusion on the International Classification of Disease, which defines severe TBI as a skull fracture, edema, brain injury, or bleeding.

2.3. Clinical, Cognitive, and Functional Assessment

Subjects received cognitive and functional assessments using the following scales: MMSE to assess global cognition [50], Katz's Index of ADL [51], and IADL index [52] to assess the impact of cognitive impairment on everyday living and the correlation between functional and cognitive impairment [53]. For each subject, at the last available follow-up, we collected the established clinical diagnosis, classifying subjects as aMCI (individuals with stable clinical and cognitive status and preserved activities of daily living) and dementia patients (aMCI converted to any type of dementia). Conversion to dementia was considered a binary outcome variable of interest. To assess the degree of cognitive decline over time in each subject, we calculated the index of progression using the formula: follow-up MMSE—baseline MMSE/years of follow-up. The index of progression was used as a continuous variable of interest to compare the degree of cognitive decline over time in different groups of cognitively impaired individuals [54,55].

2.4. Statistical Analysis

IBM SPSS software (version 25.0, IBM Corporation, Armonk, NY, USA) was used for the statistical analysis, and GraphPad PRISM (Version 9.0, GraphPad Software, Inc., San Diego, CA, USA) was used for the figures. Statistical significance was set at a p-value < 0.05.

All continuous data are presented as the mean $\pm$ standard deviation (SD). All categorical variables are presented as numbers (percentages). For continuous variables, we used the Kolmogorov–Smirnov test to explore the normality of the distribution of data. Based on the variable distribution of most variables, the ANOVA and Kruskal–Wallis were used for continuous data comparison. A chi-square test was performed to compare categorical variables. PCA was performed to highlight eigenvalues and loading factors. An eigenvalue reflects the amount of variance captured by a given principal component (PC). The eigenvalue-one criterion (eigenvalue ≥ 1) was used to decide how many PCs were to be retained [56]. A factor loading of one independent variable is considered large if its absolute value exceeds 0.45. Then, k-means clustering was run to identify discrete clusters within the PCA data, and the obtained clusters were compared to each other.

We considered as main outcome variables the index of progression, indicating the degree of cognitive decline over time and conversion to dementia at follow-up, identifying two conditions: (1) aMCI and (2) dementia. To investigate comorbid-related clusters associated with conversion to dementia, we conducted a Cox proportional hazards regression model. For each aMCI subject, we considered time 0 as the baseline assessment, the initial event, the diagnosis of aMCI, and the endpoint event, the diagnosis of dementia; the clusters obtained with PCA data were considered covariates.

This study was approved by the local Ethic Committees ("Comitato Etico Interaziendale Alessandria" and "Comitato Etico Territoriale Interaziendale AOU Maggiore della CArità, Novara") and performed in compliance with the Declaration of Helsinki.

3. Results

3.1. Patient Characteristics

The final sample included n = 188 participants with the aMCI diagnosis who met the inclusion criteria. Table 1 summarizes the main demographics and clinical and cognitive characteristics of all participants. The median age of participants was 76.00 (IQR: 72.00–79.00), with 91 males (49%) and 97 females (51%). The baseline MMSE score was 25.33 $\pm$ 2.60 points; at follow-up, participants scored on average 21.97 $\pm$ 4.94 MMSE points, with a mean of -1.13 ± 1.45 points per year lost.

Table 1. Subject demographic, clinical, and cognitive characteristics.

Sample Characteristics	MCI Cohort (n = 188)
Age at the first evaluation (years)	76.00 (IQR: 72.00–79.00)
Male/female	91 (49%)/97 (51%)
Years of education	5.00 (IQR: 5.00–8.00)
Symptoms onset (months)	12.00 (IQR: 6.00–24.00)
Follow-up duration (months)	36.00 (IQR: 30.00–45.00)
Baseline MMSE corrected score	25.33 (SD: 2.60)
Follow-up MMSE corrected score	21.97 (SD: 4.94)
Index of progression (loss of MMSE score/year)	−1.13 (SD: 1.45)

MCI: mild cognitive impairment; IQR: interquartile range; SD: standard deviation; MMSE: mini-mental state examination.

3.2. Comorbidities

The mean of comorbidities for each patient in our cohort was 2.00 (SD: 1.31). A total of 118 MCI patients (which corresponds to 63% of the whole cohort) had more than one comorbidity. Table 2 shows the prevalence of the eight considered comorbidities.

Table 2. Prevalence of comorbidities in MCI cohort.

Comorbidity	Number of Patients (%)
Hypertension	120 (64%)
Diabetes	51 (27%)
Hypercholesterolemia	83 (44%)
Hearing loss	15 (8%)
Depression	43 (23%)
Obesity	6 (3%)
Cardiovascular diseases	55 (29%)
Traumatic brain injury	9 (5%)

3.3. Comorbidities PCA

In this study, we used all comorbidity data to calculate the eigenvalues and the eigenvectors required to obtain the PCs, aiming to determine the main factors for conversion. Since we considered eight factors, eight PCs were generated. Only the eigenvalues of the first four PCs were >1 (1.619, 1.258, 1.112, and 1.007, respectively) and considered for subsequent analysis. These first four PCs explained about 62% of the total variation. Subsequently, with a parallel analysis, only PC1 was selected. PC1 appears to be primarily influenced by factors related to metabolic and cardiovascular health issues, including high cholesterol (loading: −0.663), diabetes (loading: −0.641), cardiovascular diseases (loading: −0.652), and hypertension (loading: −0.551). Other included variables, such as depression (loading: 0.194), hearing loss (loading: −0.029), obesity (loading: −0.026), and

TBI (loading: −0.012), have less significant influence on PC1. The eigenvalues obtained and the cumulated variance for each PC are shown in Figure 1.

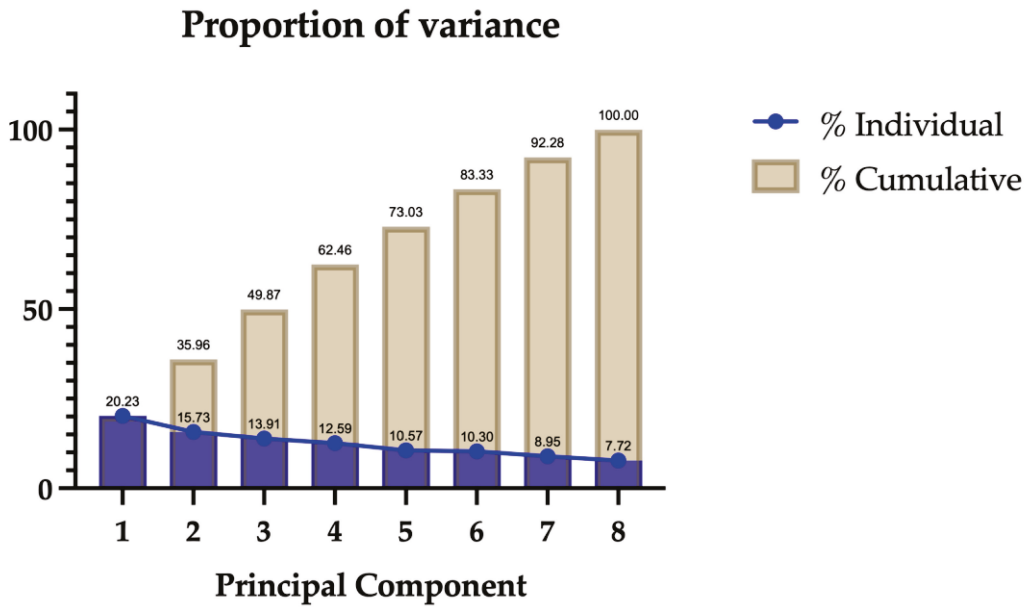


Figure 1. PCA results. Blue: Eigenvalues. Brown: cumulated variance. The *x*-axis represents the principal components, which are the new variables created from the original data. These are ordered by the amount of variance they explain, with PC1 explaining the most variance, followed by PC2, and so on. The *y*-axis represents the proportion of the total variance explained by each principal component. This is shown both individually (for each component) and cumulatively (accumulating the variance explained by all components up to that point). The blue line represents the eigenvalues or the proportion of variance explained by each individual principal component, while the brown bars show the cumulative proportion of variance explained by the principal components.

3.4. Cluster Analysis

From PCA data, we conducted the k-means analysis, obtaining four distinct clusters, which are Cluster 1, Cluster 2, Cluster 3, and Cluster 4. Table 3 presents the final cluster centers derived from *k*-means clustering on standardized PCA scores for various health conditions across four clusters. Each value represents the mean score of the corresponding variable within that cluster.

Table 3. Final clusters center for each comorbidity. Each row represents a comorbidity, and each column under "Cluster" represents the center value of that comorbidity within a particular cluster.

Comorbidity	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Hypertension	0.06	0.29	−0.06	0.45
Diabetes	0.51	−0.11	0.01	−0.29
Hypercholesterolemia	−0.55	0.06	0.04	−0.17
Hearing loss	0.32	−0.29	−0.29	3.38
Depression	−0.15	−0.28	−0.04	0.64
Obesity	5.49	−0.18	−0.18	−0.18
Cardiovascular diseases	0.09	−0.15	−0.01	0.14
Traumatic brain injury	−0.22	4.44	−0.22	−0.22

The *k*-means clustering results highlighted distinct profiles within the patient population (Figure 2):

- Cluster 1: predominantly characterized by extremely high obesity and diabetes;
- Cluster 2: marked by a very high TBI;

- Cluster 3: a mixed cluster with slight increases in several conditions but generally lower for others;
- Cluster 4: notable for very high levels of hearing loss and depression.

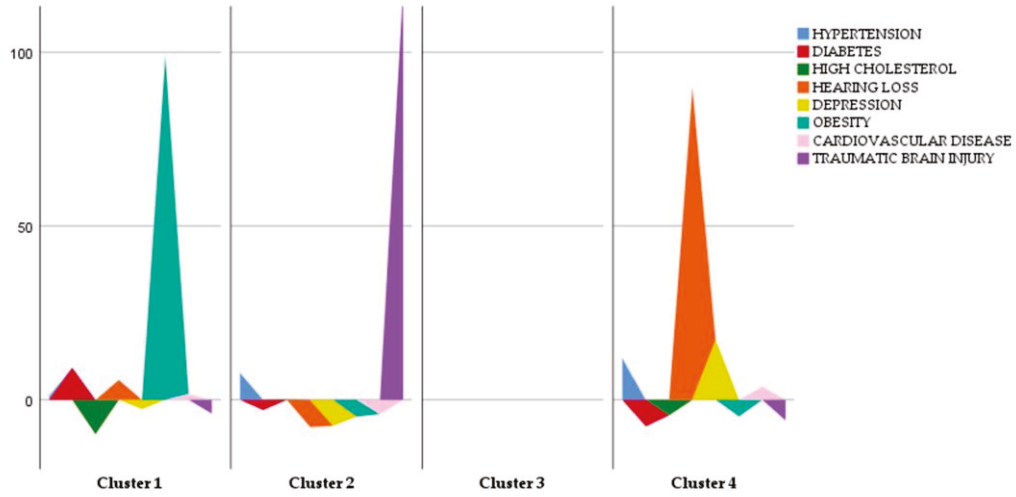


Figure 2. Distinct comorbidity profiles along clusters.

Table 4 shows a summary of the sociodemographic and clinical features in each of the four clusters. All the clusters had similar average values for age, gender, MMSE at baseline, and follow-up duration.

Table 4. Cluster sociodemographic characteristics. This table presents the sociodemographic characteristics and clinical data of patients in each cluster identified through the k-means clustering analysis.

Cluster	N. of Cases	Age (Years)	Gender (M/F)	MMSE at Baseline	Follow-Up (Years)	ADL/ IADL	Index of Progression	% of Converters
1	6	79.33	1/5	25.58	2.54	2.83/0.83	−3.48	90
2	9	72.11	4/5	24.62	3.28	5.33/3.75	−0.73	33
3	159	75.83	81/78	25.25	3.16	4.38/3.47	−1.01	51
4	14	76.36	5/9	25.32	2.92	4.78/3.67	−1.64	57

M: male; F: female; MMSE: mini-mental state examination; ADL and IADL are intended for follow-up; *p*-value for index of progression: <0.001; other *p*-values > 0.05.

When comparing cognitive and functional outcomes, significant differences among clusters emerged. In the whole group, after a mean follow-up of 3.13 years, 98 aMCI subjects (52%) converted to dementia. In Cluster 1, 90% of subjects converted to dementia, while the rate of conversion was 33%, 51%, and 57%, respectively, in Clusters 2, 3, and 4. The Cox proportional hazards regression model showed that cluster 1 had a significant effect on the progression from aMCI to dementia (p-value = 0.004). The result is also supported by the rate of the index of progression, where post hoc tests showed that Cluster 1 exhibited a significantly higher index of progression compared to Cluster 2, Cluster 3, and Cluster 4, indicating faster cognitive decline (F = 7.003; p-value < 0.001).

Moreover, regarding functional abilities, at the follow-up, we observed that patients belonging to Cluster 1 had higher ADL/IADL impairments, showing a statistically significant difference in the IADL score when compared with other clusters (F = 7.93, p-value < 0.001) and a statistical trend in the ADL score comparison (F = 2.33, p-value = 0.057) (see Figure 3).

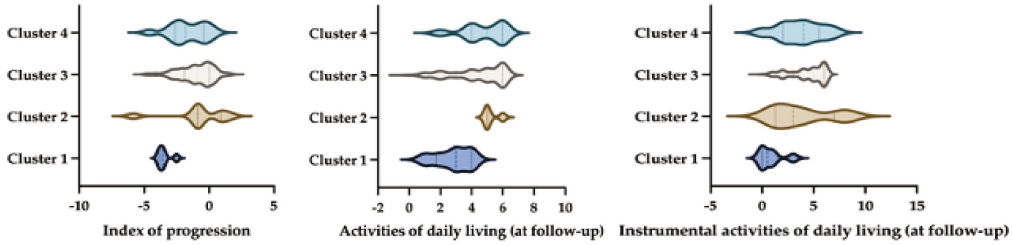


Figure 3. Representation of cluster differences for an index of progression (**left**), activities of daily living (**middle**), and instrumental activities of daily living (**right**). The image illustrates the differences between clusters in three key areas. On the left, it shows an index of progression, which likely measures how dementia progresses over time. In the middle, it presents activities of daily living (ADLs), which include basic self-care tasks. On the right, it displays instrumental activities of daily living (IADLs), which are more complex activities necessary for independent living. By comparing these clusters, the image highlights how different groups vary in terms of disease progression and their ability to perform daily and instrumental activities.

4. Discussion

Our study investigated the impact of comorbidities on the progression of cognitive decline in a large, amnestic MCI population. We explored eight comorbid conditions recognized to play a role in dementia workup [46]. The PCA stratified MCI subjects according to the weight of each comorbidity, and the subsequent CA allowed us to identify different risk profiles for cognitive impairment progression. Our main results showed that some comorbid conditions associated with high cerebrovascular risk, including obesity and diabetes, have the most critical impact on dementia progression, confirming the importance of considering comorbidities when stratifying subjects with cognitive decline for prognostic considerations.

Substantial evidence testified to the cumulative effect of the association between chronic conditions and dementia, reporting that comorbidities including cardiovascular disease, diabetes, depression, bowel, renal, and respiratory diseases may influence cognitive decline [20,56–58]. Consistent with clinical observations, pathological studies confirmed the occurrence of co-pathologies, contributing to a detrimental effect and faster disease progression, steeper cognitive decline, or atypical presentations [22]. In addition, the presence of comorbid conditions may have an impact on the management of patients with cognitive decline and dementia, adding complexity to the therapeutic workup.

Cluster analysis successfully separated individuals showing different conversion risks to dementia and presenting significantly different clinical courses. Cluster 1 was predominantly characterized by a higher weight of two comorbidities, namely obesity and diabetes. Individuals in Cluster 1 had the highest conversion rate and the fastest cognitive decline over time, losing 3.48 MMSE points per year over the considered follow-up. In addition, aMCI in Cluster 1 has suffered from the most important functional decline over time, as revealed by the lowest scores in the ADL and IADL scales. Cluster 2 was dominated by TBI and showed the lowest conversion rate and index of progression despite having the longest follow-up time (3.28 years). Besides TBI, only hypertension, which was equally distributed among clusters, appears to contribute relatively to this cluster. We can thus consider Cluster 2 a particular sample of our aMCI population, characterized by a low weight of comorbid conditions, which is also supported by the youngest age of the group. In addition, although the Lancet Commission [1] considers severe TBI as a significant risk factor for dementia development in the early period after TBI, the pathogenic link remains elusive, and a study involving more than 2500 MCI subjects showed that TBI was not clearly associated with progression to AD over a very long follow-up (8 years) [59]. In line with our results from the Global Burden of Disease Study 2019, the relative risk for dementia in TBI patients was the lowest in the youngest group and decreased across the age range. In addition, considering the strong age gradient, this study reported that less than 2% of the global prevalence of dementia was related to TBI [60].

A slight occurrence of several comorbidities characterized Cluster 3, but without a prominent condition. Lastly, Cluster 4 was characterized by a high prevalence of hearing loss and depression. Individuals in Cluster 4 showed the second most rapid cognitive decline over time among clusters (1.64 points lost per year) and the second highest conversion rate, with 57% of subjects converting after the considered follow-up.

Our study entails important practical repercussions. In the overall management of individuals with MCI, special efforts should be made to eliminate potentially modifiable risk factors associated with a higher risk of progression of cognitive decline. In our analysis, obesity and diabetes were shown to be associated with a significant risk of conversion.

Obesity has been associated with the development of cognitive deficits and dementia [61]. Specifically, obesity in midlife is a significant risk factor for developing dementia in old age [62]. In addition, a relationship between obesity and impairment of ADL and IADL has been reported in older adults, suggesting that obese subjects have a higher degree of disability as compared to individuals with typical body weight [63]. However, contrasting results have also been reported [63,64]. A recent study delineated no difference in the rate of decline in global cognition between obese participants and participants with typical weights in a pooled data analysis of 28,867 participants, even with lower baseline cognitive scores in obese individuals [65]. The molecular link between obesity and dementia has been explored in several studies. A pathogenic mechanism linked to hormonal pathways and neuroinflammatory responses has been hypothesized. However, further investigation is needed to reveal the underlying processes [66]. The cross-talk between neuroinflammation, diet, and nutrition has received growing attention due to factors representing easily accessible and modifiable lifestyle components impacting AD and other dementia development [67].

Diabetes is another cerebrovascular risk factor associated with the development of both AD and vascular dementia [68], and diabetic patients have a higher risk of developing cognitive decline than non-diabetic individuals [69,70]. Diabetes is generally associated with accelerated cognitive decline [71]; however, there are also studies reporting no association between diabetes and AD [72,73]. Pathogenic mechanisms related to vascular abnormalities, neuroinflammation, and amyloidosis have been advocated to explain the link between diabetes and cognitive impairment [74], and antidiabetic therapies have been proposed as potential AD therapies [75,76]. Along with the increased risk of cognitive dysfunction, patients with diabetes have an increased risk of functional disability as measured by ADL and IADL impairment compared to subjects without diabetes [77]. Since increased disability reflects diabetic complications and is associated with higher social costs, preventive and therapeutic interventions for metabolic dysfunctions are required in order to achieve healthy brain function.

Cluster 4, showing an intermediate risk of conversion to dementia and mild progression of cognitive decline and functional impairment over time, was characterized by a high load of hearing loss and depression.

Hearing loss has been suggested as a risk factor for dementia [78,79], and it has been estimated to account for 9% of cases of dementia [1]. The link between hearing loss and dementia has been explained by the impoverished environment associated with hearing impairment, which leads to altered cortical architecture and decreased cognitive reserve [80]. In this vein, hearing screening and treatment may represent a successful strategy for mitigating this risk factor and preventing cognitive decline.

A history of depression has been related to the development of dementia later in life [22]. The association between depression and AD significantly impacts quality of life and autonomy in activities of daily living [81]. Several studies reported an association between depression and AD; depression may precede the onset of dementia, manifesting already in the preclinical phase and representing the earliest sign of dementia [82,83]. Evidence suggests that antidepressant therapy may influence the development and progression of cognitive decline by stimulating neurogenesis in the hippocampus, modulating neuroin-

flammatory responses, and inhibiting amyloid deposition [84]. Thus, the recognition of depression is a crucial step in the management of patients with cognitive impairment.

Since MCI and dementia are conditions associated with advanced age, and aging is often associated with other medical conditions and comorbidities, the impact of comorbid conditions on cognitive impairment is not trivial. Comorbidities are often treatable and sometimes reversible; thus, preventive programs are warranted to counter cognitive decline associated with or exacerbated by concomitant conditions.

We recognize some limitations in our study. The most critical point regards the lack of biomarker inclusion, which would have allowed a more precise subject stratification and a more accurate prognostic prediction. We aimed to identify clinical variables that are easily recognizable and to test their impact on the progression of cognitive decline from the clinical condition of amnestic MCI to dementia. To reduce variability in the outcome, we included only subjects with a follow-up between two and five years. This allowed us to compare cognitive and functional scores between subjects in different clusters. As the results confirmed, the clusters were matched for clinical variables other than comorbidities, including age, educational level, sex, cognitive and functional scores at baseline, and follow-up duration. Thus, we can infer that differences in cognitive decline progression were related to the comorbidity-related classification. However, we used comorbidities as binary variables (presence/absence) without considering the severity of each condition, which would have influenced results, especially functional effects. Further longitudinal analysis with a larger sample and more detailed clinical and biomarker characterization is needed to confirm our data. Lastly, the small sample of some clusters might influence some results; as an example, most subjects belonged to Cluster 3, which was characterized by the presence of several comorbidities but without a prominent condition. It would be interesting to both better stratify participants in Cluster 3 and implement the cohorts included in the other clusters. Despite these limitations, our study provides an original stratification of amnestic MCI subjects related to comorbidities, showing that different clusters have different risk profiles. Our study points out the essential role of comorbidities in contributing to cognitive decline and progression to dementia.

5. Conclusions

In the current study, we performed a multi-modal analysis of comorbid conditions in aMCI, delineating clusters of individuals with significantly different clinical progression. The highest risk of cognitive and functional decline over time was observed in the cluster associated with obesity and diabetes, followed by the cluster associated with hearing loss and depression. Identifying aMCI groups with different risk profiles and rates of progression is of utmost importance in prognostic stratification and management of patients with cognitive decline, as well as affecting the design of clinical trials since the inclusion of subjects with mixed pathology might impact the outcomes. This study underlines the importance of preventing comorbid conditions, especially those related to cerebrovascular risk, to counter cognitive decline.

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were in accordance with the Declaration of Helsinki. There were no clinical or therapeutic changes for the patients included in the current study.

Informed Consent Statement: Informed consent was retrospectively obtained from all subjects involved in the study when possible.

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Periodontal Indices as Predictors of Cognitive Decline: Insights from the PerioMind Colombia Cohort

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Abstract: Background: Poor oral health and periodontitis have been epidemiologically linked to cognitive decline and mild cognitive impairment (MCI) in older adults. However, specific metrics directly linking these clinical signs are exceedingly limited. **Methods**: To address this gap and develop novel tools to help clinicians identify individuals at risk of cognitive decline, we established the PerioMind Colombia Cohort, comprising elderly Colombian subjects who underwent comprehensive neurocognitive and periodontal evaluations. **Results**: The results revealed that subjects diagnosed with MCI exhibited significantly higher scores in specific periodontal indices, including gingival erythema and pocket depth parameters. The predictive model identified positive associations with MCI, with gingival erythema showing the strongest correlation, followed by the presence of periodontitis and variations in pocket depth measurements. Additionally, lower educational attainment was associated with a higher likelihood of being classified in the periodontitis-MCI group. **Conclusions**: Here, we show that specific altered periodontal metrics are associated with MCI diagnosis, and the generated results provide defined metric ranges for identifying individuals at risk. Upon validation in larger cohorts, the findings reported here could offer dental practitioners and clinicians innovative tools to identify individuals at risk of MCI and age-related dementias through routine oral health assessments, thereby enabling more accessible and highly sought-after early intervention strategies in both developing and developed countries.

Keywords: dentistry; periodontitis; cognitive decline; mild cognitive impairment; oral health; prodromal Alzheimer's disease; risk factors; predictive models; cohort studies; periodontal indices

1. Introduction

The global prevalence of dementia was estimated at approximately 55 million individuals in 2021, with projections indicating an increase to 78 million by 2030 and 139 million by 2050 [1]. This substantial rise is primarily driven by population aging, particularly in

low- and middle-income countries, which currently account for approximately 60% of individuals living with dementia [2]. In Latin America, the prevalence of aging-associated dementias is estimated to range between 5% and 10% among individuals over 60 years of age, with projections indicating a significant upward trend consistent with global patterns [3–5]. Recent studies emphasize the significance of conducting research within this specific elderly population, as misdiagnosis and underdiagnosis remain prevalent [6]. These challenges are primarily attributed to limited access to culturally appropriate diagnostic tools and persistent healthcare disparities across countries in the region [7], as well as the influence of the mestizo population's genetic diversity, which complicates dementia characterization [7]. Similarly, most research is based on European ancestry, potentially leading to biases in understanding and treating dementia in mestizo populations [7].

Colombia presents an optimal setting for investigating mestizo populations within Latin America owing to its heterogeneous genetic and cultural heritage, which exemplifies the broader demographic patterns of the region [7]. Furthermore, Colombia's increasing emphasis on public health research, in conjunction with its healthcare challenges, renders it a significant context for examining global health disparities and population dynamics [6]. Dementia prevalence in Colombia ranges from 5% to 7% in individuals over 60 years of age, aligning with the aforementioned broader Latin American trends [6]. However, specific geographical areas within the country have exhibited an exceptionally high prevalence of 23.6% in individuals over 60 years old, attributed to socioeconomic factors and poorly defined chronic comorbidities [8].

Mild cognitive impairment (MCI) is an early stage of cognitive decline [9–12], characterized by noticeable but mild impairments in memory, language, or spatial perception, while daily tasks remain largely unaffected [12]. Individuals with MCI exhibit an elevated risk of progressing to dementia, with lifestyle factors, genetic predispositions, and comorbidities playing pivotal roles in this progression [13,14]. Therefore, the identification of MCI in elderly populations is crucial for decelerating the progression of this debilitating and fatal syndrome, thereby facilitating timely therapeutic interventions to enhance patient outcomes and address healthcare challenges associated with aging populations [15].

Conversely, periodontitis is a progressive inflammatory disease characterized by the destruction of the soft and hard tissues of the periodontal complex, driven by interactions between dysbiotic microbial communities and aberrant immune responses [16]. A critical determinant of periodontitis development and progression is the elevated concentration of pathogenic bacteria within the dental biofilm, which elicits a detrimental immune response [17,18]. Notably, the deleterious effects of periodontitis extend beyond the oral cavity and have been associated with systemic health issues, as we and other colleagues have previously elucidated [19–21]. Furthermore, this disease appears to be a chronic comorbidity commonly observed in individuals affected by aging-related dementias [22]. Emerging epidemiological data from various countries suggest that periodontitis may play a significant role in influencing the onset and progression of age-related dementias [23]. This association is likely attributable to the systemic inflammation and immune responses elicited by chronic periodontal infections, which could exacerbate neuroinflammatory processes linked to cognitive decline [23]. Additionally, the prevalence of periodontitis in aging populations underscores its potential as a modifiable risk factor for dementia [22]. Elucidating this connection could facilitate the development of targeted preventive strategies to mitigate dementia's burden in susceptible populations.

Considering these factors, this study evaluates the potential interactions between MCI and periodontitis-associated pathogenic factors in elderly individuals from a mestizo population. To accomplish this objective, we established the PerioMind Colombia Cohort, conducting comprehensive assessments of both neurocognitive and periodontal

parameters associated with MCI and periodontitis. Our findings reveal significant and specific relationships between periodontal disease and MCI, providing insights into novel diagnostic tools. By identifying key periodontal metrics strongly associated with MCI, we propose that routine oral examinations, which are more readily available than specialized neurocognitive assessments in Colombia and internationally, could serve as an effective method for identifying individuals at risk of cognitive decline. Therefore, this study aims to address a significant gap in understanding the relationship between oral and cognitive health by providing novel data and diagnostic tools that advance early intervention strategies for aging populations globally.

2. Materials and Methods

2.1. Study Design and Participants

The establishment of novel cohorts to investigate diverse and specific aspects of MCI in elderly populations is imperative for advancing dementia prevention strategies [24]. Therefore, as previously referred [25], cohort-based studies provide valuable insights into region-specific challenges, facilitating the development of culturally tailored healthcare interventions.

A cross-sectional observational study was designed and conducted at the Institute of Genetics, Universidad Nacional de Colombia. This research design was selected due to its suitability for exploratory investigations aimed at identifying specific clinical associations among variables, as previously delineated [26]. This approach allows for the efficient collection of representative data within resource constraints, providing preliminary evidence to guide future longitudinal studies for causal confirmation [26]. Participant recruitment occurred from January to December 2023. The study enrolled a total of 70 participants. The cohort comprised Colombian men and women, predominantly residents of Bogotá, aged 65 years and older. All participants provided informed consent prior to study inclusion. Subsequently, subjects underwent comprehensive neurocognitive evaluations and oral periodontal examinations.

To ensure the integrity of the results, stringent exclusion criteria were implemented. Subjects were excluded if they were undergoing periodontal or orthodontic treatment or had received such treatment within the preceding three months were at risk for endocarditis due to periodontal probing, or had received antibiotic treatment within the previous three months. Participants with a clinical diagnosis of dementia, including Parkinson's or Alzheimer's disease, were also excluded. Based on these criteria, a total of 30 participants were included in the final study cohort. This timeframe of three months was chosen to minimize confounding effects from acute medical conditions, recent systemic infections, or dental treatments that could influence both periodontal health and cognitive assessments.

The study protocol received approval from the Ethics Committee of the Universidad Nacional de Colombia (Act No. 11-23 of 2023), ensuring adherence to the ethical principles delineated in the Declaration of Helsinki by the World Medical Association. Informed consent documents were developed to ensure transparency and comprehensive disclosure to potential participants. These documents provided detailed information regarding the study's objectives, methodology, potential risks, and anticipated benefits, enabling participants to make informed decisions concerning their involvement. All participants provided written consent, affirming their voluntary participation and comprehension of the study's procedures and ethical safeguards. Of note, data privacy measures were rigorously implemented in this study. All collected data were anonymized and stored securely in digital databases. Data sharing adhered to institutional and international ethical guidelines, ensuring that only de-identified datasets were shared for collaborative research purposes and solely with explicit approvals.

2.2. Neurocognitive Assessment

The study participants underwent comprehensive evaluations conducted by clinical professionals from the Neurosciences Department at the Universidad Nacional de Colombia. Detailed clinical histories were obtained. Cognitive function was assessed utilizing the Montreal Cognitive Assessment (MoCA) test, a validated instrument developed by Nasreddine et al. [27]. MoCA is widely recognized as an appropriate tool for identifying MCI [28]. It was specifically designed to detect MCI and has been shown to have greater predictive accuracy compared to other cognitive assessments like the Mini-Mental State Examination (MMSE) [29]. MoCA has been recently reported to exhibit a sensitivity of 73.5% to 83.8% and a specificity of 70.8% to 91.3% for detecting MCI, contingent upon the utilized cutoff scores [30]. This assessment instrument evaluates multiple cognitive domains, encompassing attention, concentration, executive function (including abstraction), memory, language, visuoconstructive abilities, calculation, and orientation [30]. Based on the score ranges in the MoCa test recently validated for MCI [31,32], this study categorizes cognitive function as follows: scores of 26 or higher indicate normal cognition, scores between 18 and 25 indicate MCI, scores between 10 and 17 indicate moderate cognitive impairment, and scores below 10 points indicate severe cognitive impairment.

2.3. Determination of the Educational Level

Participants' educational levels were systematically recorded and categorized into three groups to investigate potential associations with MCI, given that education is a critical factor in assessing predisposition to developing MCI, as indicated by previous research [33]. Basic education referred to individuals who had completed primary school but had not progressed beyond secondary education, intermediate education encompassed participants who had successfully completed secondary education without pursuing tertiary education, and advanced education comprised those who had obtained a university degree, either at the undergraduate or postgraduate level, as well as individuals with technical certifications.

2.4. Periodontal Examination

A comprehensive dental examination was conducted by the faculty and residents of the Periodontics Postgraduate Program at the Faculty of Dentistry, Universidad Nacional de Colombia. All examiners underwent calibration training using the Polson criteria to ensure consistency and accuracy in their assessments [34]. The periodontal parameters evaluated comprised the following: (1) number of teeth present; (2) gingival erythema (inter-examiner Kappa Index [IE], 0.654); (3) pocket depth (PD) (IE, 0.609); (4) gingival margin position (IE, 0.432); (5) bleeding on probing (BoP) (IE, 0.583); and (6) plaque index (PI) (as described by O'Leary IE, 0.543). These parameters were systematically measured on six surfaces of each tooth, including distal vestibular [DV], vestibular [V], mesial vestibular [MV], distal lingual [DL], lingual [L], and mesial lingual [ML], excluding teeth restored with implants and third molars. A University of North Carolina probe was employed for all measurements to ensure precision and reproducibility. The rigorous calibration process, based on the Polson criteria, included training that encompassed theoretical sessions on periodontal parameters and practical exercises utilizing the University of North Carolina probe to measure PD, gingival margin position, and PI. Inter-examiner reliability was evaluated using Kappa Index values, demonstrating moderate to substantial agreement (e.g., PD = 0.609, PI = 0.543). Periodic recalibration and feedback addressed variability, ensuring standardized application of CDC/AAP guidelines for periodontitis diagnosis.

The diagnosis of periodontitis was established based on two internationally recognized classification systems. According to the Centers for Disease Control and Prevention/American Academy of Periodontology (CDC/AAP) guidelines [35], participants

were categorized based on clinical attachment loss (CAL) and PD. Mild periodontitis was characterized by the presence of at least two interproximal surfaces with a CAL of three millimeters or more, at least two interproximal surfaces with a PD of four millimeters or more (on different teeth), or one surface with a PD of five millimeters or more. Moderate periodontitis was defined by at least two interproximal surfaces with a CAL of four millimeters or more (on different teeth) or two interproximal surfaces with a PD of five millimeters or more. Severe periodontitis was diagnosed when at least two interproximal surfaces exhibited a CAL of six millimeters or more (on different teeth), and at least one interproximal surface presented a PD of five millimeters or more [36].

According to the classification established by the American Academy of Periodontology in association with the European Federation of Periodontology during the 2017 World Workshop (AAP/EFP) [37,38], periodontitis was diagnosed when interproximal CAL was observed in at least two non-adjacent teeth or when vestibular or lingual CAL of three millimeters or more was accompanied by a PD exceeding three millimeters in at least two teeth [37]. This system further categorized periodontal health as exhibiting less than 10% BoP, with PD of three millimeters or less, and no interdental CAL or gingivitis. Gingivitis was defined as BoP of 10% or more, with PD of three millimeters or less, and no interdental CAL. Periodontitis stages ranged from stage I, characterized by interdental CAL of one to two millimeters, to stage IV, which involved CAL of five millimeters or more, with substantial tooth loss attributed to periodontitis [37].

2.5. Statistical Analyses

The Shapiro–Wilk test was employed to assess the normality of data distributions across the study groups. Clinical and sociodemographic variables related to cognitive function were analyzed using the Chi-squared (Chi^2) test and Fisher's exact test, as appropriate. For the analysis of periodontal parameters based on cognitive function, two statistical approaches were implemented depending on the distribution of the data. For non-normally distributed data, the Mann–Whitney U test was utilized to compare median values of periodontal parameters. Conversely, for normally distributed data, the Student's T-test was applied.

Multiple linear regression analysis was performed using R software (version 4.4.0) to identify the most significant periodontal parameters for inclusion in the predictive model. The selection process involved evaluating various combinations of periodontal parameters and their contributions to predicting cognitive outcomes. The selected parameters were subsequently incorporated into a cluster prediction model analysis using MassHunter Mass Profiler Professional Software (Agilent Technologies, Santa Clara, CA, USA). The Partial Least Squares Discriminant (PLSD) model was applied for its suitability in analyzing complex, high-dimensional data and its effectiveness in distinguishing between categorical outcomes, such as control and MCI groups [39].

Furthermore, considering that Naïve Bayes models have been extensively utilized for clinical variables modeling due to their simplicity and efficacy and that these models have demonstrated promising results in predicting patient outcomes, particularly in the context of Alzheimer's disease [40], the Naïve Bayes model was employed as a complementary approach, given its parsimony and utility in classification tasks. All statistical analyses were conducted with a 95% confidence level using Stata version 14. A p-value of ≤ 0.05 was considered indicative of statistical significance for all tests performed [41,42].

3. Results

3.1. Gathering and Evaluation of the PerioMind Colombia Cohort

The recruitment and experimental design of the study are summarized in Figure 1. A total of 70 participants were initially assessed, but 40 were excluded based on predetermined criteria, leaving a final cohort of 30 participants. These individuals were classified into two groups according to their cognitive function: the Control group (n = 20) and the MCI group (n = 10), as detailed in Table 1. The mean age of the participants was 70 ± 5 years (Table 1). In the Control group, the median age (IQR) was 67 (66–72), while in the MCI group it was 69 (68–72) (Table 2). A notable demographic feature was the predominance of women over men, with a 2:1 ratio in both groups. Comprehensive demographic and cohort assessment data are provided in Tables 1 and 2.

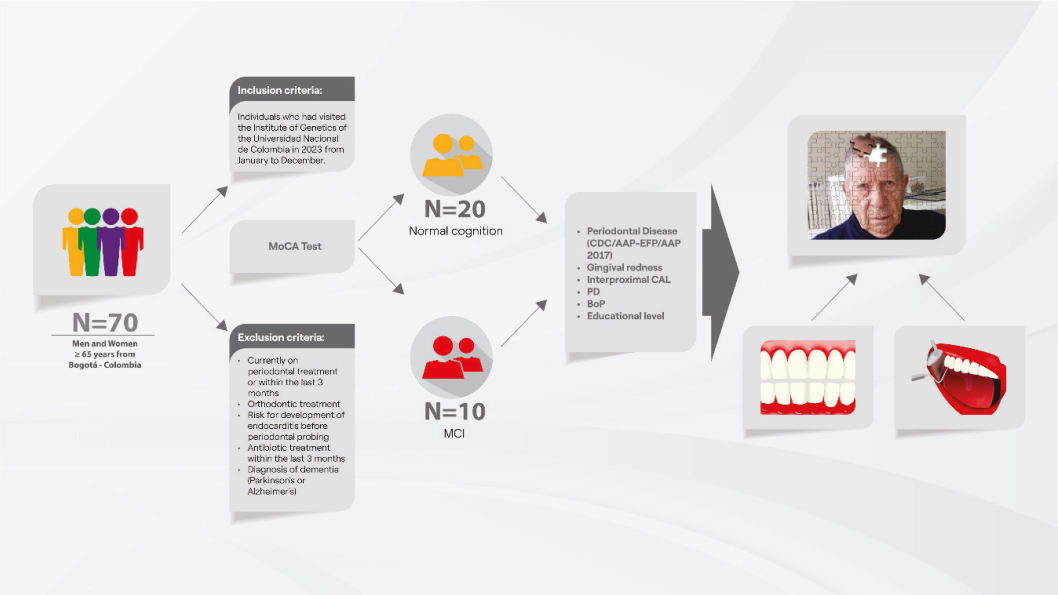


Figure 1. Workflow and main findings of the study. The diagram illustrates the comprehensive workflow of the study, including cohort selection, experimental design, data acquisition through periodontal and neuropsychological assessments, data analysis, and the clinical relevance of periodontal screening in identifying associations with unhealthy cognitive aging.

Table 1. Descriptive analysis of socio-demographic and clinical variables of the PerioMind Colombia Cohort. The mean ± standard deviation (SD) is provided for age, while absolute frequencies (n) and percentages (%) are used for categorical variables such as sex, educational level, and cognitive function. The cohort includes 30 elderly individuals stratified by cognitive function into control and mild cognitive impairment (MCI) groups.

Variable		Mean/Frequency	Percentage (%)
Age		70 ± 5	-
Sex	Female	20	66.7
	Male	10	33.3
Educational level	Basic	11	36.7
	Intermediate	12	40.0
	Advanced	7	23.3
Cognitive function	Control	20	66.7
	Mild Cognitive Impairment (MCI)	10	33.3

Table 2. Clinical and sociodemographic variables of participants of the PerioMind Colombian Cohort grouped into Control and MCI categories. The data include median values and interquartile ranges (IQR) for age and number of teeth (overall, upper jaw, and lower jaw), along with the distribution of sex and educational levels across groups.

		Control		MCI	
		n = 20	**Median (IQR)**	**n = 10**	**Median (IQR)**
Age		-	67 (66–72)	-	69 (68–72)
Sex	Female	13	-	7	-
	Male	7	-	3	-
Educational level	Basic	7	-	4	-
	Intermediate	7	-	5	-
	Advanced	6	-	1	-
Number of teeth		-	22 (16–25)	-	20 (16–27)
Number of teeth (Upper Jaw)		-	10 (7–12.5)	-	9 (5–12)
Number of teeth (Lower Jaw)		-	11 (9.5–13.5)	-	11 (9.5–13.5)

Neuropsychological and dental assessments suggested that participants in the MCI group were less likely to have an advanced educational level compared to the Control group (Table 2). Additionally, a lower median number of teeth was observed in the MCI group compared to the Control group, with similar patterns noted for the upper jaw. For the lower jaw, tooth counts appeared comparable between groups. While these differences reflect trends in the data, they did not reach statistical significance (Table 2).

3.2. Periodontal and Cognitive-Linked Parameters in the PerioMind Colombia Cohort

The analysis of periodontal parameters demonstrated significant differences between the Control and MCI groups. Individuals in the MCI group exhibited higher median values for gingival redness, PD at 4 mm, and PD$\geq$ 4 mm (Table 3). Although not statistically significant, a trend was observed for total PD (Table 3). Interestingly, a higher proportion of participants in the Control group exhibited PI levels within the 0–20% range compared to the MCI group. This trend approached statistical significance, suggesting potential differences in oral hygiene habits or outcomes between the groups (Table 3).

Table 3. Comparison of periodontal clinical variables based on cognitive function between Control and mild cognitive impairment (MCI) groups. Values for median (IQR) and mean $\pm$ SD are provided, along with corresponding *p*-values for statistical analyses. Significant differences ($p < 0.05$) are marked with †. Medians were compared using the Mann–Whitney U test, Chi^2 test, and Fisher's exact test, whereas means were compared using the *t*-test. Abbreviations: MoCA, Montreal Cognitive Assessment; PI, plaque index; BoP, bleeding on probing; PD, pocket depth; CAL, clinical attachment loss.

		Cognitive Function		***p* Value**
		Control	**MCI**	
MoCA Test	Median (IQR)	27 (26–28)	21 (21–23)	† 0.000
PI	Mean $\pm$ SD	0.4 $\pm$ 0.31	0.4 $\pm$ 0.1	0.996
PI	Median (IQR) 0–20%	n = 6 (100%)	n = 0 (0%)	0.053
	Median (IQR) > 20%	n = 14 (58.33%)	n = 10 (41.67%)	
Gingival redness	Median (IQR)	0.0 (0.0–0.16)	0.39 (0.31–0.59)	† 0.003
BoP	Mean $\pm$ SD	0.34 $\pm$ 0.22	0.31 $\pm$ 0.15	0.672
Total PD	Median (IQR)	2.19 (1.88–2.5)	2.51 (2.22–2.91)	0.074
PD $\geq$ 3 mm	Mean $\pm$ SD	0.38 $\pm$ 0.24	0.51 $\pm$ 0.16	0.143
PD = 4 mm	Median (IQR)	0.03 (0.02–0.07)	0.1 (0.05–0.18)	† 0.015
PD $\geq$4 mm	Median (IQR)	0.05 (0.02–0.1)	0.18 (0.05–0.2)	† 0.044

Table 3. *Cont.*

		Cognitive Function		*p* Value
		Control	MCI	
PD = 5 mm	Median (IQR)	0.01 (0.0–0.02)	0.04 (0.0–0.06)	0.169
PD ≥ 5 mm	Median (IQR)	0.01 (0.0–0.04)	0.05 (0.0–0.12)	0.350
PD ≥ 6 mm	Median (IQR)	0.0 (0.0–0.02)	0.01 (0.0–0.04)	0.713
Interproximal CAL 1/2 mm	Mean ± SD	0.4 ± 0.2	0.43 ± 0.2	0.687
Interproximal CAL 3/4 mm	Mean ± SD	0.32 ± 0.18	0.27 ± 0.09	0.352
Interproximal CAL ≥ 5 mm	Median (IQR)	0.1 (0.04–0.15)	0.12 (0.1–0.2)	0.350
Interproximal CAL ≥ 6 mm	Median (IQR)	0.03 (0–0.07)	0.05 (0.03–0.09)	0.169

3.3. Generation of Class-Predictive Models for Identifying Mild Cognitive Impairment Risk

To develop a screening tool for identifying MCI risk based on periodontal parameters, predictive models were constructed using data from the PerioMind Colombia Cohort. The Partial Least Squares Discriminant (PLSD) and Naïve Bayes models were applied to analyze the selected periodontal parameters and generate class-predictive models (Table 4). These models aim to assist dental practitioners and oral health professionals in identifying individuals at risk of MCI. The models demonstrated high accuracy, correctly classifying participants into either the control or MCI group, with 100% accuracy in the Naïve Bayes model and 96.7% accuracy in the PLSD model (Supplementary Tables S1–S3). These results highlight the robustness of these methods in correctly classifying outcomes, supporting their applicability in predictive analyses. Among the periodontal parameters, gingival redness and PD ≥ 4 mm were the most effective at distinguishing individuals with MCI. Specifically, individuals with pronounced gingival redness and PD ≥ 4 mm greater than 0.3 exhibited 3.15 and 2.59 times greater odds of belonging to the MCI group than age-matched controls, respectively (Table 4). The model parameters exhibited positive interactions, with gingival redness showing the strongest predictive interaction (18.73%), followed by periodontitis according to the CDC/AAP classification (14.07%), moderate or severe periodontitis (6.13%), and PD ≥ 4 mm (4.54%) (Table 4). Notably, all participants classified as having periodontitis based on the CDC/AAP criteria, as well as those in advanced periodontal severity stages (stage III or IV in the EFP/AAP classification and moderate or severe periodontitis in the CDC/AAP classification), were consistently categorized in the MCI group. Lower educational attainment was also strongly associated with a higher probability of being classified in the MCI group. Comprehensive predictive performance metrics for both the Naïve Bayes and PLSD models are detailed in Supplementary Tables S1–S3.

Table 4. Results of predictive models for identifying MCI risk based on periodontal and educational parameters applied to the PerioMind Colombia Cohort. Parameter ranges for Control and mild cognitive impairment (MCI) groups, the ratio of MCI to Control, interaction percentages, and interaction directions are detailed. Predictive values were generated using Naïve Bayes and Partial Least Squares Discriminant (PLSD) models. * Strong positive interactions are denoted by "p". Abbreviations: CDC/AAP, Centers for Disease Control and Prevention in association with the American Academy of Periodontology; EFP/AAP, European Federation of Periodontology in association with the American Academy of Periodontology.

	Range Control	Range MCI	Ratio MCI/Control	Interaction (%)	Interaction Direction *
Education level	2.37–2.77	<2.37	0.87	-	-
Stages Periodontal disease (EFP/AAP)	2.65–3.08	>3.08	1.06	-	-
Stages Periodontal disease (CDC/AAP)	1.34–1.97	>1.97	1.11	6.13	p
Periodontal Disease (CDC/AAP)	≤1	1	1.11	14.07	p
Gingival redness	0–0.281	>0.281	3.15	18.73	p
PD ≥ 4 mm	0–0.30	>0.3	2.59	4.54	p

4. Discussion

Epidemiological studies suggest that conditions such as periodontitis, tooth loss, and other oral diseases may contribute to MCI [43], potentially through mechanisms involving systemic inflammation and neuroinflammatory processes [44]. Recent findings indicate a bidirectional relationship between oral health and cognitive dysfunction, emphasizing the potential for poor oral health to exacerbate cognitive impairment and vice versa [43]. Of note, a recent systematic review and meta-analysis found a significant association between periodontitis and an increased risk of MCI, with a pooled odds ratio of 1.70, indicating that individuals with periodontitis are more likely to develop MCI compared to those without the condition [45].

Our research contributes to those above, expanding the corpus of evidence from a novel perspective by focusing on specific periodontal parameters and their associations with MCI, an area that has not been previously explored in depth to the best of our knowledge. Through the evaluation of periodontal indices in the context of MCI risk within a diverse elderly population, we provide new insights into the role of oral health in cognitive decline. This research adds a layer of specificity to the understanding of how periodontal health may influence cognitive function, offering potential new avenues for risk assessment and early diagnosis of MCI.

The data collected here indicates that subjects diagnosed with MCI had a lower average number of teeth compared to those with normal cognitive function, particularly in the upper jaw. This aligns with previous studies showing that participants with fewer than 20 teeth exhibited reduced cognitive capacity [46] and that partial or total tooth loss is associated with lower cognitive scores [47]. Research has also highlighted that chewing with natural teeth supports brain activity, cerebral blood flow, and hippocampal and cortical function, while tooth loss reduces sensory input and alters neurotransmission [48,49]. Additionally, individuals with fewer teeth, particularly edentulous individuals, have an increased risk of cognitive impairment [50], as observed in older adults with fewer than 10 teeth performing worse on cognitive assessments [51]. These findings underscore the importance of preserving natural teeth and addressing tooth loss as part of strategies to mitigate cognitive decline. Further research is needed to explore factors such as dental prosthesis use and its potential impact on cognitive health [52].

Similarly, educational level is a well-recognized determinant of cognitive reserve and an essential factor influencing the onset and progression of MCI [53]. Higher educational attainment is often associated with enhanced cognitive resilience [53], enabling individuals to better compensate for neuropathological changes. Conversely, lower educational levels have been linked to increased vulnerability to MCI [54], often compounded by socioeconomic disparities, limited access to healthcare, and lifestyle factors. Despite its significance, educational level frequently acts as a confounding variable in studies investigating MCI, complicating efforts to isolate the influence of other risk factors [55], such as health comorbidities, including periodontal disease.

Our study, conducted within the PerioMind Colombia Cohort, highlights the critical interplay between educational level and periodontal disease in predicting MCI. Participants with stage IV periodontitis were more likely to have basic education, consistent with national surveys reporting a higher prevalence of severe periodontitis among individuals with lower educational levels [35]. Limited education may contribute to poorer oral health literacy and access to preventive care, compounding the risk of both periodontal disease and cognitive decline [56]. By incorporating educational attainment into predictive models, we identified specific periodontal metrics, such as gingival redness and severe periodontitis, strongly associated with MCI in individuals with lower education levels. These findings underscore that cognitive reserve, influenced by educational attainment, may modulate

the impact of periodontal disease on cognitive outcomes. Notably, our results indicate that routine periodontal examinations, in conjunction with an understanding of educational disparities, could function as cost-effective mechanisms for identifying individuals at risk of MCI, thereby facilitating targeted early interventions and comprehensive public health strategies.

In a related context, one of the most significant associations identified in this study was between increased gingival erythema and MCI, rendering it the most prominent factor associated with cognitive decline in our cohort. Previous research corroborates the role of gingival inflammation, rather than hemorrhage, as a significant predictor of cognitive impairment [57]. This relationship may be influenced by advanced age and underlying cardiovascular conditions, which are known to contribute to systemic inflammation, neurodegeneration, and potentially gingival health [58,59].

Here, we also provide significant thresholds for periodontal parameters, such as gingival erythema and PD, derived from class-predictive models that demonstrate a substantial correlation with the risk of MCI in individuals over 70 years of age. These parameters function as practical indicators during routine periodontal examinations, offering a methodology to stratify patients at risk of dementia in clinical practice. The predictive models developed in this research exhibited potential for preliminary screening purposes. However, they necessitate further validation and testing in larger and more diverse populations to confirm their reliability and applicability. Expanding these efforts will enhance their utility and contribute to effective early detection and intervention strategies, ultimately improving outcomes for older adults globally [9,41,60–62].

It is also crucial to emphasize that in both developed and developing nations, dental clinics frequently serve as accessible healthcare points for older adults, providing routine and preventive treatments. This accessibility offers an opportunity to integrate periodontal assessments into broader healthcare strategies to identify individuals at risk for cognitive decline. Research has demonstrated the feasibility of linking oral health services with systemic healthcare in regions such as Nigeria, Brazil, and North America [63–65]. Nevertheless, neuropsychological evaluations remain less accessible due to their complexity, associated costs, and limited availability of trained professionals and diagnostic instruments [66]. Enabling dental professionals to identify patients who may benefit from specialized care could address this disparity. Early detection of individuals with MCI or early dementia stages facilitates timely intervention, potentially decelerating cognitive decline, enhancing quality of life, and delaying the progression to severe dementia [59,67].

5. Conclusions

This study introduces a novel approach to clinically leverage the link between specific periodontal parameters and MCI. By examining clinically measurable indicators like gingival erythema and PD, we identify significant MCI-associated thresholds that can be applied in routine periodontal examinations. Consequently, based on the findings elucidated herein, individuals within the predicted MCI risk thresholds should undergo neuropsychological evaluations to rule out potential MCI.

The data obtained also underscore the potential of oral health screenings to address cognitive healthcare disparities, especially in underserved populations. Predictive models emphasize the significance of interdisciplinary approaches, integrating dental and cognitive assessments for a comprehensive strategy to address cognitive decline. Consideration of educational attainment is strongly recommended, as elucidated in the findings. Further investigations to validate and expand these results could substantially enhance early intervention practices and improve outcomes for elderly individuals at risk of age-related dementia globally.

6. Limitations of the Study

The authors acknowledge that the relatively small sample size, despite extensive recruitment efforts and comprehensive neuropsychological and periodontal assessments, could be considered a limitation of this study. While the results provide valuable proof of concept, further analysis in larger cohorts is needed to validate the findings. Nevertheless, it is important to highlight that the number of participants included was sufficient to allow for meaningful comparisons of various periodontal parameters with an acceptable level of statistical confidence. Furthermore, we acknowledge the limitations of the MoCA in populations with low literacy levels. To address this constraint, we incorporated specific measurements of literacy levels and educational attainment in our cohort. These factors were subsequently integrated into our analysis to account for their potential influence on cognitive outcomes, thereby ensuring a more robust and context-sensitive evaluation.

It is also imperative to acknowledge that lifestyle factors and healthcare accessibility may exert significant influence on both periodontitis and cognitive outcomes. Subsequent investigations should take into account the variability of these factors in the relationship between periodontitis and MCI, as elucidated by the findings presented in this study.

Additionally, because most participants were examined in an institution lacking access to comprehensive dental assessment equipment, intraoral radiographs could not be obtained. However, the interdental CAL considered in this investigation is the gold standard for determining the severity of periodontitis. It allows for the placement of the patient into Stage I to IV, as discussed in the online Journal of Clinical Periodontology within the framework of the AAP/EFP16. X-rays are also unnecessary for appropriate classification according to the CDC/AAP case definition [35]. This approach ensured that the evaluation remained robust and clinically relevant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines13010205/s1, Table S1: Naïve Bayes model predictive results for Control and mild cognitive impairment (MCI) groups in the PerioMind Colombia cohort; Table S2: Partial Least Squares Discrimination (PLSD) model predictive results for Control and mild cognitive impairment (MCI) groups in the PerioMind Colombia cohort; Table S3: Predictive model performance of the Naïve Bayes and Partial Least Squares Discriminant (PLSD) models in predicting cognitive status (Control or mild cognitive impairment (MCI)) among participants in the PerioMind Colombia cohort.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All raw clinical data generated in this study from the PerioMind Colombia Cohort has been made publicly available through the clinical data repository https://data.mendeley.com/datasets/c3jk6nwr56/1 (accessed on 19 December 2024) under the identifier: Arévalo Caro, Catalina María; López, Diego; Sánchez Milán, Jose Antonio; Lorca, Cristina; Mulet, María; Arboleda, Humberto; Losada, Sergio; Gallart-Palau, Xavier; Serra, Aida (2024), "Anonymised PerioMind Colombia cohort database", Mendeley Data, V1, doi: 10.17632/c3jk6nwr56.1. This ensures the open and prospective use of the data by the scientific community in accordance with the recommendations outlined in the research group's publication [60].

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Physical Inactivity Amplifies the Link Between Anxiety, Depression, and Neck Pain in Breast Cancer Survivors

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Abstract: Background and Objectives: Breast cancer can cause significant changes in both physical and mental health, leading to conditions such as low back pain, neck pain (musculoskeletal pain), anxiety, and depression. Women with this diagnosis tend to subsequently decrease their levels of physical activity, which can contribute to the emergence of musculoskeletal dysfunction. With this in mind, the aim of the present study was to analyze the relationship between physical activity levels (active vs. inactive), neck and low back pain in women breast cancer survivors in association with anxiety and depression. **Methods:** This cross-sectional study was conducted with 128 women breast cancer survivors. The prevalence of neck pain stratified by anxiety and depression status was higher in women physically inactive than in active women; however, there was no statistically significant difference. **Results:** Symptoms of anxiety and depression were associated with neck pain only in physically inactive women. No associations were observed between anxiety and depression and low back pain regardless of physical activity levels. **Conclusions:** This study demonstrates significant results for the association between physical activity level, anxiety and depression, and neck pain in women breast cancer survivors. However, the same association was not found for low back pain.

Keywords: breast cancer; low back pain; neck pain; physical activity; anxiety; depression

1. Introduction

Breast cancer (BC) is the most common malignant neoplasm among women worldwide, with over 2 million new cases, and more than 600,000 deaths from this cancer, in 2024 [1,2]. It is predicted that the worldwide incidence of female BC will reach approximately 3.2 million new cases per year by 2050 [3]. The global breast cancer mortality rate has increased by 0.23% per year, with statistically significant rises observed in individuals under 50 and over 70 years of age [4]. The incidence and mortality rates of BC have increased over the past three decades due to shifts in risk factor profiles, improvements in cancer registration systems, and advancements in cancer detection [5].

It is also observed that although the prevalence of this neoplasm is higher in developed countries, the highest mortality rates are observed in less developed regions [6]. Among the numerous risk factors for this disease, variables such as sex, aging, the female hormone (estrogen), family history, genetic mutations, and an unhealthy lifestyle can increase the possibility of developing BC [7–9].

The diagnosis of BC and the possible treatments used can have a large impact on quality of life, which may lead to the development of systemic side effects, perceived shoulder pain, and anxiety and/or depression, due to the stigma related to the pathology and the impacts of the therapy on body appearance and female identity [10–14].

Anxiety is an emotional state defined by tension, persistent worry, and physiological responses, such as elevated blood pressure. While anxiety and fear are distinct concepts, they are frequently used interchangeably. Anxiety is regarded as a future-focused and prolonged reaction to a vague or nonspecific threat [15–17]. Depression is described as a mood disorder that causes a persistent feeling of sadness and loss of interest, and which controls patients' attitudes by altering their perception of themselves, so that they start to view situations in their life as major problems. This disorder presents characteristics that may indicate a serious pathology or simply another manifestation of the condition when faced with a real-life situation [18–21].

The condition of BC and the development of associated psychological symptoms tend to lead to decreases in the levels of physical activity in affected individuals, which can contribute to the emergence of musculoskeletal dysfunctions, including neck pain and low back pain [22].

Women more frequently report neck pain than men [23]. The causes of neck pain vary widely, with the main causes being poor ergonomics at work, when sitting, and maintaining neck posture in a non-physiological position for long periods of time [24]. There is, however, greater evidence for some risk factors, such as a lack of physical activity, duration of daily computer use, perceived stress, and being female [25]. As there is a tendency for neck pain to become a chronic problem, it is important to identify risk factors to enable its prevention and early diagnosis [26].

The biopsychosocial model postulates low back pain as a dynamic interaction between social, psychological, and biological factors that can predispose to and result from injury [27]. Low back pain is the leading cause of disability and loss of productivity worldwide, with a lifetime prevalence of up to 84% for the adult population [28]. This condition can be classified as mechanical, radicular (neuropathic), or due to increased sensitivity of the central nervous system, and the distinctions affect the treatment decisions [29].

Low back and neck pain are multifactorial diseases and a major problem in modern society, as well as being the leading and fourth leading causes of years lived with disability (YLDs) worldwide, respectively. Moreover, the prevalence of neck pain (NP) is exceeded only by depressive disorders and other musculoskeletal conditions [30].

It is widely recognized that physical activity contributes to improving symptoms of anxiety and depression and reducing musculoskeletal pain [31]. Ribeiro et al. [13], in a study involving BC women survivors, observed that the practice of physical activity during leisure time and commuting physical activity were inversely related to depressive symptoms. Mirandola et al. [32] observed that physical activity through exercise favored greater upper limb mobility in BC survivors, which could reduce the chances of musculoskeletal pain. The gap in our knowledge about the associations of low back and neck pain with anxiety and depression in women who have recovered from BC demonstrates a clear need for a study on the association of both conditions, since studies in this context are still scarce. The current work differs from previous studies, as it aims to understand if there is an association between the aforementioned symptoms and the decrease in the practice of

physical activities. In this way, the results can help to identify the best therapeutic approach to promote biopsychosocial well-being. Therefore, the aim of the current study is to evaluate the association between anxiety and depression with low back and neck pain related to women BC survivors according to physical activity levels.

2. Materials and Methods

2.1. Study Design and Sample Selection Process

This is a cross-sectional study, approved by the Research Ethics Committee of FCT/UNESP (CAAE: 54169416.6.0000.5402). All volunteers who agreed to participate in the study signed an informed consent form. To calculate the sample, a correlation of r = 0.30 was considered between anxiety symptoms and low back pain based on the study by Hung et al., with an alpha error of 5% and a sample power of 80%. Anticipating possible losses, an additional 20% was added to the sample size, requiring a minimum of 101 participants [33].

2.2. Selection Criteria

The study was conducted with 128 female breast cancer survivors. Participant recruitment was conducted through institutions that support breast cancer and referrals from mastologists in the city. Data collection was carried out through face-to-face interviews conducted by previously trained researchers.

The selected inclusion criteria were women who had survived breast cancer and being over 18 years of age. The exclusion factors were having a pacemaker or metal plate, due to bioelectrical impedance, or any type of mental disorder that would prevent the woman from answering the questionnaires.

2.3. Data Collection

Personal information, such as name, age, marital status, and education, and clinical data, such as date of diagnosis and types of surgical treatment and alternative intervention were collected.

2.4. Physical Activity

The degree of physical activity was assessed using the Baecke questionnaire [34] which presents good reliability against gold standard methods like double-labeled water [30] and was ratified for the study population [35]. This evaluation method contains 16 questions divided into three areas of habitual physical activity, collected in relation to the year prior to the study [36]. In Section 1 of the questionnaire, the occupational score of physical activity is evaluated through eight questions that cover aspects of exercises performed during domestic services or work, such as amounts of weight carried, among others, checking for the presence of fatigue [36]. The second section is related to sports practices during leisure-time or physical exercises. It consists of four questions that classify the intensity (light, moderate or intense), frequency, and time attributed to the activities performed [34]. The composition of the points also considers comparisons in relation to leisure or work carried out by people of the same age and level of activity, while the final question considers the performance of exercises outside the leisure period. In Section 3, the degree of physical activity in leisure time and during transportation is measured by means of four questions about the frequency in which transportation is employed during these practices. This part considers the time spent cycling, watching TV, walking, and also the time it takes to shop or go to work. This tool provides an unfounded score through a specific equation for each sphere evaluated and the sum of the three points of the sphere establishes the total practice of physical activity, which can vary from 0 to 15 points [36]. As the instrument

developed by Baecke et al. [34] does not provide specific cut-off points to classify physical activity levels, quartiles were used in the analysis. Women in the highest quartile (quartile 4) were classified as active ($\geq$8.92 points according to Baecke score), while those in the lower quartiles were classified as inactive (<8.91 points according to Baecke score).

2.5. Socioeconomic Status

The Brazilian Economic Classification Criteria (ABEP) [37] was used to classify economic status. This tool performs an evaluation according to educational level, the number and type of rooms in the home, and the consumer goods in the household, in working order, regardless of how the goods were acquired. The instrument generates a score, which indicates the socioeconomic classification, stratified from the highest to the lowest classes (A, B1, B2, C1, C2, D, E). Based on these criteria, the participants in the current study were separated into classes: high (A and B1), medium (B2, C1 and C2), and low (D and E) [36].

2.6. Depressive Symptoms

The Hospital Anxiety and Depression Scale (HADS) was used to assess the presence of symptoms of anxiety and depression [38]. The main advantage of the HADS is its validity for use in clinical populations, as it was specifically designed to exclude items that could be generated due to physical conditions rather than psychological states [39]. The HADS questionnaire contains 14 multiple-choice questions, which separately assess symptoms of anxiety (seven questions) and depression (seven questions). Each item receives a score from 0 to 3, with a higher score representing a higher occurrence of anxiety and depression symptoms. The range for each outcome (i.e., anxiety and depression) is from 0 to 21 [40,41]. A score greater than or equal to nine indicates a potential case of anxiety and/or depression [42]. In the present study, the individual item scores were analyzed rather than solely considering the cut-off point for each symptom.

2.7. Musculoskeletal Pain

NP and LBP were assessed using the Nordic Musculoskeletal Questionnaire, designed to report musculoskeletal symptoms [43]. The questionnaire is divided into two parts: the first part is a general survey where the participant indicates the body part(s) in which they experience pain. The second part is a specific questionnaire where the participant answers questions about the duration of symptoms throughout their life, in the previous 12 months, and in the previous 7 days. In addition to symptom duration, the questionnaire also assesses the impact of pain on work and leisure activities, the presence of medical leave, and symptom duration. In this instrument, there are two possible answers: yes or no. In the present study, responses reporting cervical and/or lumbar pain in the last seven days were considered.

2.8. Statistical Analysis

The sample characterization variables are presented as mean and standard deviation for continuous variables and frequency for categorical analyses. The independent t-test was used to compare continuous variables, and the chi-square test was used to compare categorical variables. The association between anxiety and depression with neck and low back pain according to the physical activity levels of BC survivors was performed by Binary Logistic Regression in the unadjusted model and in the adjusted model (age, marital status, type of surgery, and socioeconomic classification). The statistical significance was set at 5% and the confidence interval was 95%.

3. Results

Table 1 presents the variables used to characterize the sample according to the levels of physical activity. The mean age of the inactive women was 59.06 (±10.03) and of the active women was 55.88 (±8.58). Most patients were married and had undergone a quadrantectomy. The prevalence of neck pain was 34.6%. Low back pain was reported by 56.2% of the study participants. No statistically significant differences were observed in the sample characterization variables when compared to physically active and inactive BC survivors.

Table 1. Characteristics of the sample according to levels of physical activity (*n* = 128).

Variables	Inactive (*n* = 95)	Active (*n* = 33)	*p*
Age (years), Mean (SD)	59.06 (±10.03)	55.88 (±8.58)	0.110
Weight (kg), Mean (SD)	55.10 (±5.13)	54.60 (±6.05)	0.718
Height (cm), Mean (SD)	156.63 (±6.01)	156.72 (±6.39)	0.767
Frequency (%)			
Marital status			
Married, %	70.5	56.3	0.072
Single, %	8.4	9.4	
Divorced, %	13.7	15.6	
Widow, %	7.4	18.8	
Surgery type			
Quadrantectomy, %	52.1	56.3	0.843
Mastectomy, %	47.9	43.8	
Anxiety, %	25.3	21.9	0.882
Depression, %	13.7	18.8	0.683
Neck pain, %	34.7	37.5	0.945
Low back pain, %	57.9	53.1	0.791

In Figure 1, it can be seen that the prevalence of neck pain was higher in physically inactive patients than in physically active patients; however, there was no statistically significant difference. In relation to low back pain, the prevalence rates were similar.

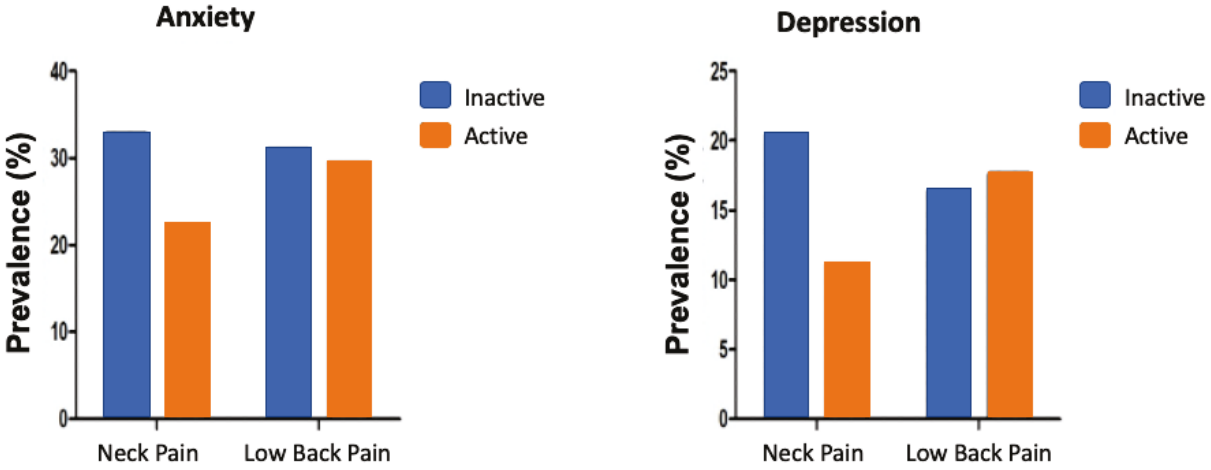


Figure 1. Prevalence of neck and low back pain stratified by anxiety and depression status in physically inactive and active breast cancer survivors.

Similar results were observed when considering depression, in which the prevalence of neck pain was higher in the physically inactive women when compared to the physically active women. However, the prevalence of low back pain was similar in both groups of patients.

Table 2 presents the associations between anxiety and depression with neck and low back pain according to physical activity levels. In BC survivors classified as inactive, anxiety was significantly associated with neck pain (p = 0.030). In women classified as physically active, no association was observed between anxiety and neck pain. Considering the relationship between depression and neck pain, associations were observed in BC survivors classified as inactive, but not in those classified as active. Regarding low back pain, anxiety and depression were not associated, regardless of the level of physical activity of the women BC survivors.

Table 2. Association between anxiety and depression with neck pain and low back pain according to levels of physical activity in BC survivors (n = 128).

	OR	95%CI	p
		Neck Pain	
Anxiety			
Inactive			
Crude model	3.01	1.16–7.38	0.024
Adjusted model	3.10	1.11–8.67	0.030
Active			
Crude model	1.33	0.24–7.33	0.741
Adjusted model	1.15	0.10–13.07	0.909
Depression			
Inactive			
Crude model	3.01	1.16–7.83	0.024
Adjusted model	5.10	1.35–19.27	0.016
Active			
Crude model	1.33	0.24–7.33	0.741
Adjusted model	0.86	0.09–8.24	0.900
		Low Back pain	
Anxiety			
Inactive			
Crude model	2.10	0.76–5.71	0.142
Adjusted model	2.37	0.80–7.01	0.117
Active			
Crude model	2.70	0.40–16.88	0.842
Adjusted model	1.87	0.12–29.52	0.655
Depression			
Inactive			
Crude model	1.76	0.50–6.18	0.377
Adjusted model	1.62	0.43–6.04	0.467
Active			
Crude model	0.86	0.14–5.06	0.865
Adjusted model	1.58	0.13–18.72	0.713

Adjusted for age, socioeconomic status, marital status, and type of surgery.

4. Discussion

The current study aimed to examine the relationship between anxiety, depression, and neck and back pain in breast cancer survivors, considering the potential influence of physical activity levels. Our findings suggest that higher levels of anxiety and depression are associated with increased neck pain in this population, and that women BC survivors report a high prevalence of neck and low back pain. Additionally, physical activity levels appeared to moderate these relationships, although the effect sizes observed indicate the need for further investigation. Moreover, while the association between anxiety, depression, and neck pain was evident, no statistically significant difference was found for low back

pain across physical activity levels, suggesting that different mechanisms may be involved in pain perception at distinct anatomical sites.

The relationship between BC, anxiety, and depression is complex. In this sense, symptoms of anxiety and depression may be associated with episodes of musculoskeletal pain. In a systematic review, Liu et al. [44] observed that symptoms of anxiety and depression were more common in patients with severe neck pain. Similarly, our findings align with this evidence, reinforcing the notion that psychological distress may exacerbate musculoskeletal symptoms in breast cancer survivors. Another study identified significant associations between fatigue, passive shoulder flexion, pressure pain sensitivity, depression, and the intensity of neck and shoulder pain in breast cancer survivors. Depression, neck pain, and passive shoulder flexion were significant predictors of fatigue in this population. These findings suggest that self-reported psychological factors, along with neck pain and shoulder mobility, play a relevant role in cancer-related fatigue [43]. While our study did not directly assess fatigue, the observed associations between anxiety, depression, and neck pain suggest that similar mechanisms could be involved. One factor that may be responsible for this association is that symptoms of anxiety and depression are generally associated with a higher inflammatory profile [45,46], and studies have shown that inflammation is one of the factors linked to a greater chance of experiencing pain [47]. In breast cancer survivors, chronic inflammation resulting from cancer treatments, along with the psychological burden of the disease, could contribute to heightened pain sensitivity [48].

Kim et al. [49] conducted a systematic review of possible factors for the development of neck pain and observed a greatly increased risk of developing neck pain in individuals with high levels of stress and catastrophizing. It is widely known that being diagnosed with BC leads to a high level of stress and negative thoughts like catastrophizing [50,51], which could also explain this association between neck pain and anxiety and depression.

Physical activity has been used as a tool to reduce symptoms of anxiety and depression, as well as episodes of pain. In the current study, the association of anxiety and depression symptoms with cervical pain was observed in the group of women BC survivors classified as physically inactive. Nazari et al. [52] observed that exercises for the cervical flexor muscle reduced pain and episodes of anxiety and depression in patients with chronic neck pain. Christofaro et al. [53], in a study conducted with more than 1800 Brazilian adults, observed that symptoms of depression were associated with musculoskeletal pain, mainly in physically inactive participants. One of the possible mechanisms to explain these findings is the analgesic role that physical activity could play. The different hormones released during physical activity include β-endorphins [54] and serotonin [55], which could contribute to reducing symptoms of anxiety, depression, and, subsequently, episodes of pain [56].

Another relevant aspect is the lack of a significant association between low back pain and physical activity level in our study. This finding contrasts with previous research that showed an inverse relationship between physical activity and musculoskeletal pain [22,57]. Unlike neck pain, which is often associated with stress-related muscle tension and postural imbalances, low back pain may result from structural abnormalities, such as intervertebral disc degeneration, spinal instability, or paraspinal muscle dysfunction [58]. Additionally, some studies suggest that chronic low back pain is more strongly influenced by central sensitization mechanisms rather than peripheral inflammation alone [59]. This could explain why the current study did not find a clear association between physical activity levels and low back pain in breast cancer survivors. Future studies should investigate whether specific types of exercise interventions, such as core stabilization exercises or neuromuscular training, may have a greater impact on low back pain management in this population.

When considering the population of female BC survivors, some important aspects must be considered. A study conducted by Lavallée et al. [60] showed that the debilitating side effects of treatment and the fear of causing irreversible harm serve as barriers to physical activity. Engaging in physical activity within a supportive environment may provide a distraction, allowing individuals to feel "normal" and begin to redefine themselves, shifting their focus toward health and well-being rather than cancer. Providing timely, accurate, and personalized information about the benefits of incorporating physical activity into the treatment regimen, along with guidance from experienced physical activity instructors, may facilitate engagement.

The findings of the current study demonstrated an association that should be considered when promoting interventions for the treatment of these conditions, since the level of physical activity was found to be directly related to symptoms of anxiety, depression, and neck pain [61]. It is also worth highlighting the importance of a multi-disciplinary team, including therapists and specialist nurses working with breast cancer patients, in order to optimize fatigue management. Implementing multidimensional interventions incorporating pain management strategies, mobility exercises, and cognitive behavioral therapy may enhance the patient's ability to cope with a cancer-related condition [43]. Additionally, individualized exercise prescriptions that consider patient preferences, limitations, and motivational factors could improve adherence and long-term benefits.

Among the main limitations of this study, the observational design prevents cause and effect analyses from being performed. Another point is that the subjective measurement of physical activity is less accurate for identifying the different intensities of physical activity when compared to direct measurement methods, such as accelerometry. Future studies employing objective measures, such as wearable activity trackers or laboratory-based assessments, could provide a more precise evaluation of physical activity levels in breast cancer survivors. Despite this, the current study is pioneering in investigating the relationship between the level of physical activity, anxiety and depression, and musculoskeletal dysfunctions (neck and low back pain), because previous studies have only analyzed these variables in isolation [22,57].

As practical applications of the present study, we emphasize the importance of encouraging the practice of physical activity in female cancer survivors, mainly with the aim of mitigating the relationship between musculoskeletal pain and symptoms of anxiety and depression. Future studies should explore structured physical activity programs tailored for breast cancer survivors, evaluating their effectiveness in controlling symptoms of anxiety and depression and musculoskeletal conditions.

5. Conclusions

Our findings revealed a significant association between anxiety, depression, and neck pain specifically in physically inactive women. Conversely, this association was not observed in physically active women, nor was a significant link found between anxiety, depression, and low back pain, irrespective of physical activity level. These results suggest that physical activity may modulate the relationship between psychological distress and neck pain in this population. High prevalence of neck and low back pain was noted, reinforcing their clinical relevance, with the differential findings for neck versus low back pain suggesting distinct underlying mechanisms. The primary contribution of this work is identifying this potential moderating role of physical activity. Practically, these findings underscore the importance of encouraging physical activity among breast cancer survivors to mitigate the link between psychological symptoms and neck pain, highlighting the value of integrated, multidisciplinary care. Study limitations, such as the observational design preventing causal inference and subjective physical activity measurement, should be

acknowledged. Future research should employ objective methods and longitudinal designs to clarify causality, exploring targeted interventions, including specific exercise programs.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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Review

Neuroinflammation and Natural Antidepressants: Balancing Fire with Flora

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Abstract: Background/Objectives: Major depressive disorder (MDD) is a major global health concern that is intimately linked to neuroinflammation, oxidative stress, mitochondrial dysfunction, and complicated metabolic abnormalities. Traditional antidepressants frequently fall short, highlighting the urgent need for new, safer, and more acceptable therapeutic techniques. Phytochemicals, i.e., natural antidepressants derived from plants, are emerging as powerful plant-based therapies capable of targeting many pathogenic pathways at the same time. **Summary**: This narrative review synthesizes evidence from preclinical and clinical studies on the efficacy of phytochemicals such as curcumin, polyphenols, flavonoids, and alkaloids in lowering depressed symptoms. Consistent data show that these substances have neuroprotective, anti-inflammatory, and antioxidant properties, altering neuroimmune interactions, reducing oxidative damage, and improving mitochondrial resilience. Particularly, polyphenols and flavonoids have great therapeutic potential because of their capacity to penetrate the blood–brain barrier, inhibit cytokine activity, and encourage neuroplasticity mediated by brain-derived neurotrophic factor (BDNF). Despite promising results, the heterogeneity in study designs, phytochemical formulations, and patient demographics highlights the importance of thorough, standardized clinical studies. **Conclusions**: This review identifies phytochemicals as compelling adjuvant or independent therapies in depression treatment, providing multimodal mechanisms and enhanced tolerability. Additional research into improved dosage, pharmacokinetics, long-term safety, and integrative therapy approaches is essential. Using phytotherapeutics could considerably improve holistic and customized depression care, encouraging new research routes in integrative neuroscience and clinical psychiatry.

Keywords: major depressive disorder (MDD); plant-based therapeutics; neuroinflammation; oxidative stress; natural antidepressants; tryptophan; kynurenine; brain-derived neurotrophic factor (BDNF); mitochondrial dysfunction; curcumin

1. Introduction

Major depressive disorder (MDD) is a common and debilitating mental condition characterized by persistent low mood, anhedonia, cognitive dysfunction, and severe impairments in occupational, social, and interpersonal functioning [1]. Aside from its impact on quality of life, MDD remains the leading psychiatric contributor to global suicide mortality, with core symptoms including diminished self-worth, excessive guilt, psychomotor changes, and sleep disturbances—factors that collectively contribute to increased all-cause mortality rates [2]. Patients with MDD often show persistent negative cognitive biases, social withdrawal, and impaired emotional regulation [3]. Epidemiological data reveal an increasing global burden, presently impacting over 350 million individuals, with World Health Organization forecasts indicating that MDD will emerge as the foremost cause of disability worldwide by 2030 [4,5]. Vulnerable populations, such as pregnant women, the elderly, and children, have disproportionately high incidence rates, emphasizing the role of psychological stressors, genetic predisposition, and environmental adversity in its etiology [6–9]. Chronic stress, in particular, has been linked to the pathophysiology of MDD because of its ability to disrupt neuroplastic processes, resulting in neuronal atrophy, synaptic loss, and volumetric changes in key brain regions such as the hippocampus and prefrontal cortex, all of which contribute to the disorder's affective, cognitive, and behavioral symptoms [10–12]. Figure 1 summarizes the primary neurobiological mechanisms involved in MDD pathogenesis. The purpose of this state-of-the-art critical narrative review is to integrate disparate mechanistic, pre-clinical, and clinical data into a coherent conceptual model of phytochemical action in depression rather than to catalogue every published study.

Emerging evidence underscores the critical role of neuroinflammatory processes in the pathogenesis of MDD, positioning central nervous system (CNS) inflammation as a key mechanistic driver in a subset of patients [13]. A growing body of research implicates dysregulation within the kynurenine pathway of tryptophan metabolism—a principal biosynthetic route for serotonin—in contributing to both decreased serotonergic tone and increased production of neurotoxic metabolites [14]. These metabolic changes not only disrupt neurotransmitter homeostasis, but they also increase susceptibility to mood dysregulation and cognitive impairment, which are hallmarks of depressive pathology [15–18]. Furthermore, metabolomic profiling investigations in MDD patients regularly reveal abnormalities in a variety of circulating metabolites, including tryptophan, tyrosine, methionine, valine, phenylalanine, pyruvate, kynurenic acid, and deoxycholic acid [19]. These metabolic disruptions are closely associated with impaired neuroprotective mechanisms, increased oxidative burden, dysregulated apoptotic signaling, and chronic low-grade inflammation [20–22]. Collectively, these pathophysiological alterations foster a neurobiological environment conducive to the initiation and persistence of depressive symptoms, particularly through the destabilization of neuronal resilience and synaptic integrity [23].

While traditional pharmacological treatments, particularly tricyclic antidepressants (TCAs) such as imipramine, amitriptyline, clomipramine, desipramine, and doxepin, remain essential in the treatment of MDD, their therapeutic efficacy is primarily mediated by modulation of monoaminergic neurotransmission, specifically by increasing synaptic concentrations of serotonin and norepinephrine [4]. Despite their clinical value, emerging evidence suggests that while antidepressants are considered first-line therapies, they are not the only effective modality for managing MDD [2]. Notably, in pediatric and adolescent populations, non-pharmacological strategies such as psychological support, scheduled physical activity, and dietary optimization have shown clinically relevant preventative and therapeutic outcomes [24]. Nonetheless, a large proportion of people show partial or complete resistance to these therapies, emphasizing the importance of complementary or

alternative therapy approaches [25]. Phytotherapeutics have recently rekindled scientific attention due to their historical use, biological plausibility, cost effectiveness, and acceptable side-effect profiles. Medicinal plants have historically provided bioactive chemicals with neuroprotective, anti-inflammatory, and neuromodulatory effects [26]. These natural compounds persist in guiding the discovery and development of innovative pharmacological medicines, presenting a potentially beneficial adjunctive strategy for persons with treatment-resistant or recurrent depression [27,28].

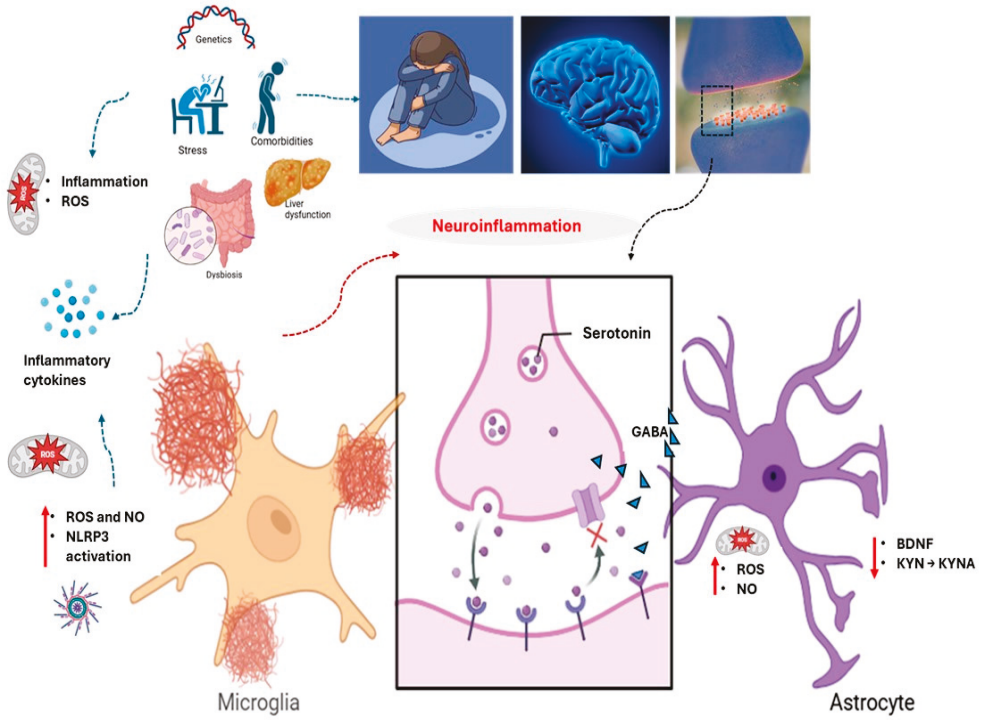


Figure 1. Neurobiological contributors to the onset and progression of major depressive disorder (MDD). Multiple interacting systems—including genetic predisposition, chronic psychosocial stress, systemic comorbidities, and immune dysregulation—converge to disrupt neuroimmune homeostasis. Central to this pathophysiology are oxidative stress, liver dysfunction, intestinal dysbiosis, and neuroinflammation, driven by activated microglia and astrocytes. These cells increase reactive nitrogen and oxygen species (NO and ROS), impair mitochondrial function, and alter kynurenine (KYN) pathway dynamics, reducing kynurenic acid (KYNA) and promoting neurotoxicity. Dysregulated serotonin and gamma-aminobutyric acid (GABA) signaling, alongside diminished brain-derived neurotrophic factor (BDNF), further impair synaptic plasticity and emotional regulation. ↑: increase; ↓: decrease.

Phytochemicals are naturally occurring bioactive molecules present in a variety of plants and food sources that influence their pigmentation, fragrance, and flavor profiles [29]. These chemicals, which include alkaloids, flavonoids, steroids, coumarins, and terpenoids like eucalyptol, are increasingly recognized for their therapeutic effects, particularly in situations characterized by oxidative stress and chronic inflammation [30]. Aside from their well-established antioxidant and anti-inflammatory properties, phytochemicals also have vasoprotective and cardiometabolic effects, making them prospective agents in the prevention and treatment of complex, multifactorial illnesses [31]. Phytocompounds have received a lot of attention in neuropsychiatry because of their possible role in altering important neurobiological processes involved in neurodegeneration and mood disorders. Compounds derived from plants such as *Aizoaceae*, *Acorus*, *Korthalsella*, *Astragalus membranaceus*, *Sophora flavescens*, and *Ononis spinosa* have long been employed in traditional medicine systems for their psychotropic and neuromodulatory effects [32–34]. Preclinical research and ongoing

clinical trials indicate that several of these botanicals have antidepressant-like properties via processes involving monoaminergic regulation, neurotrophic signaling, and neuroinflammatory cascade reduction [35]. As a result, the incorporation of phytocompounds into therapeutic frameworks for MDD is increasingly being investigated both as standalone treatments and as adjuncts to traditional pharmacotherapy [36]. Their ability to address many pathogenic domains at once makes them especially useful in the context of MDD, which has complex and heterogeneous neuronal bases [37].

It is well established that neurodevelopment intricately shapes emotional regulation and cognitive function, both of which are highly susceptible to disruption by psychiatric and neurological disorders [38]. In recent decades, the global burden of neuropsychiatric conditions—particularly those with complex, multifactorial etiologies such as MDD—has escalated significantly, placing substantial pressure on healthcare systems and highlighting the urgent need for innovative, accessible, and well-tolerated therapeutic strategies [39–41]. Although conventional antidepressants remain central to current treatment paradigms, their limitations—namely delayed onset of action, treatment resistance, and undesirable side-effect profiles—have driven the search for alternative and adjunctive interventions that can engage broader neurobiological targets with improved safety margins [42,43].

In this regard, phytocompounds have emerged as intriguing candidates due to their pleiotropic modes of action, which include anti-inflammatory, antioxidant, and neuromodulatory capabilities [44]. Recent research has highlighted the intricate connection between phytochemicals, neuroinflammation, and mitochondrial health [22,45]. Yet, no previous analysis has definitively identified the clinical trial landscape assessing the effectiveness of general phytocompounds in MDD despite growing preclinical evidence and sporadic clinical research suggesting their potential usefulness. Notably, recent reviews contend that not all trials have yielded favorable outcomes, reinforcing the need for further research to clarify underlying mechanisms, optimize dosage regimens, assess long-term safety, and determine the comparative effectiveness of medicinal plants in the treatment of depression [46]. Addressing this gap, this comprehensive narrative review seeks to illuminate the diverse roles that phytotherapeutics may play in depression management. We hope these insights will not only guide future investigations on optimal usage and mechanisms of action but also encourage the meaningful integration of plant-based interventions into established, evidence-based psychiatric care.

We compiled an **expert-guided evidence map**: senior authors snowballed references from seminal phytochemical-depression papers and maintained weekly PubMed, Scopus, and Web of Science keyword alerts ("phytochemical* AND depression OR MDD") through 28 February 2025. Records were double-screened and retained only when they advanced mechanistic understanding or held translational relevance, deliberately privileging depth over exhaustive coverage. This theory-driven selection underpins the narrative synthesis while avoiding the rigidities of systematic-review protocols. Our theory-driven, expert-guided evidence map inevitably introduces selection bias; some pertinent studies may therefore be absent. This trade-off was accepted to prioritize analytic depth over exhaustive coverage. To improve clarity and avoid redundancy, overlapping descriptions of key mechanisms such as oxidative stress, mitochondrial dysfunction, and neuroinflammation were streamlined. These interconnected processes are herein discussed in focused sections with minimal repetition, ensuring conceptual continuity without diluting analytical depth.

2. Neuroinflammation: A Core Driver of Severe Depression

Emerging data suggest that neuroinflammation plays a critical role in the pathophysiology of MDD, particularly in its more severe and treatment-resistant forms [47]. With immune-mediated processes significantly influencing brain function, inflammation in the

CNS is now understood to be a primary cause of depressive illness rather than a supporting actor [48]. This section investigates how neuroinflammatory processes, which are characterized by immunological activation, cytokine dysregulation, and metabolic imbalance, interact with brain areas involved in mood regulation. Novel therapeutic approaches that target these inflammatory pathways, such as phytochemical therapies, are becoming more popular as promising adjuncts in the treatment of depression as knowledge grows.

Neuroinflammation, driven by immune activity in the CNS, is intimately associated with depression through elevated levels of pro-inflammatory cytokines and enhanced microglial activation in the prefrontal cortex, anterior cingulate cortex, and insula [49–54]. Among the key cytokines, interleukin (IL)-1β can interfere with neurotransmitter systems and trigger more inflammatory substances, while IL-6 often increases in people with depression, affecting the normal function of the hypothalamic–pituitary–adrenal axis [55,56]. Tumor necrosis factor-α (TNF-α), known for its potent pro-inflammatory role, influences synaptic transmission and neural plasticity [57]. Interferon-gamma (IFN-γ) boosts inflammation in the brain, while IL-2, IL-8, and IL-18 create a harmful inflammatory situation [58,59]. In the prefrontal cortex, microglia adopt a pro-inflammatory phenotype, producing high levels of cytokines that may impair neuronal health [60]. When these cellular sentinels remain on high alert, they release toxic mediators that compromise neuronal functioning and hamper communication between circuits [60]. Similar inflammatory activity in the anterior cingulate cortex affects emotional processing, and overactive microglia in the insula intensify negative emotional states [61]. The hippocampus and amygdala also display maladaptive immune reactivity, undermining neuroplasticity and fueling the progression of depressive symptoms [62].

Approximately 30% of persons with MDD have substantial neuroinflammation in the CNS, which is associated with increased symptom intensity and resistance to standard treatments [47,63]. Neuroinflammation is a key factor in the pathogenesis of MDD, with activation of the NLRP3 inflammasome leading to elevated pro-inflammatory cytokines like IL-1β and IL-18, which are linked to depressive symptoms [64–66]. Activation of pattern recognition receptors, such as Toll-like receptor 4 (TLR4) on microglia and astrocytes, can induce NF-κB-driven cytokine production when damage or pathogens are detected [67]. Peripheral immune cells invade the CNS via a weakened blood–brain barrier, exacerbating local inflammation and neurotoxicity [68]. Chronic inflammation exacerbates oxidative and nitrosative stress, damages mitochondria, and enhances glutamate excitotoxicity through reduced astrocytic reuptake, resulting in neuronal injury [69]. Dysfunctional mitochondria produce pro-inflammatory chemicals, which promote inflammation [70]. Chronic stress-induced cortisol elevations activate microglia, whereas gut–brain axis abnormalities allow bacterial endotoxins to worsen systemic inflammation [71]. Last but not least, inflammatory kynurenine pathway activation lowers serotonin synthesis, which feeds a vicious cycle of depression [72].

Inflammatory cascades, which are characterized by sustained activation of cytokines and immune responses, are not limited to depression; they are also found in various chronic infections, neurodegenerative disorders such as multiple sclerosis, and perinatal depression, demonstrating their widespread influence across pathologies [73–79]. Chronic infections continue to excite immune cells, resulting in elevated amounts of pro-inflammatory cytokines, which contribute to mood and cognitive impairment [80]. Analogously, autoimmune-mediated inflammation is a feature of neurodegenerative diseases like multiple sclerosis, in which protracted immune responses harm brain tissue, aggravating symptoms of depression [75]. In prenatal depression, immunological activation triggered by hormonal and physiological changes greatly increases inflammation, affecting both maternal mood and child outcomes [81]. Furthermore, immunological dysregula-

tion frequently interacts with metabolic changes such as neuroendocrine dysfunction, gut microbiota dysbiosis, and mitochondrial abnormalities, all of which exacerbate depressive states [82–85]. Neuroendocrine disruptions, such as cortisol imbalance due to hypothalamic–pituitary–adrenal axis dysfunction, exacerbate inflammation and depressive symptoms [86]. Gut dysbiosis promotes systemic inflammation by increasing intestinal permeability and endotoxin exposure, whereas mitochondrial dysfunction impairs cellular energy metabolism and increases oxidative stress [87]. These interconnected inflammatory and metabolic abnormalities highlight the complicated, diverse pathophysiology of depression.

Given the importance of neuroinflammation in the pathogenesis of severe and treatment-resistant depression, identifying effective therapeutic strategies that target inflammation is critical [47,88]. Notably, accumulating evidence suggests that phytochemical therapies, such as polyphenols, may provide neuroprotective advantages by inhibiting inflammatory pathways and lowering neuronal death, making them a promising adjunct method for treating MD [73,74]. Natural substances can protect neurons by modulating inflammation, inhibiting NF-κB activation, and reducing oxidative stress. Furthermore, polyphenols may repair dysregulated neurotransmission and increase mitochondrial function, addressing the basic metabolic abnormalities seen in depressed states [89]. Because neuroinflammation is so tightly linked to metabolic dysfunction, immunological dysregulation, and neuronal degeneration, therapies that target numerous inflammatory and apoptotic pathways at the same time may produce better therapeutic results [90]. Future research should emphasize investigating the entire therapeutic potential of phytochemicals and comparable molecules as supplemental treatments, which could improve outcomes for people suffering from severe, inflammation-related depression.

3. Oxidative Stress: A Silent Driver of Depression

Amid the complex web of MDD pathology, oxidative stress has emerged as a subtle yet powerful contributor deserving closer scrutiny [90]. Characterized by an imbalance between reactive oxygen species (ROS) and antioxidant defenses, oxidative stress quietly undermines neuronal integrity and brain function [91]. Intriguingly, mounting evidence suggests that this biochemical imbalance not only parallels depressive symptoms but may actively drive their persistence and severity [92]. From antioxidant depletion to mitochondrial dysfunction, oxidative stress orchestrates a cascade of cellular disruptions [93]. In this section, we explore the biochemical undercurrents of oxidative stress and its far-reaching implications in the development and progression of depression.

Oxidative stress occurs when ROS and other free radicals exceed the body's capacity to neutralize them through antioxidant defenses, leading to cellular harm via the degradation of essential biomolecules such as proteins, lipids, and nucleic acids [94,95]. In individuals with depression, this imbalance is particularly pronounced due to significantly reduced levels of key antioxidants, including zinc, vitamin E, and coenzyme Q10, amplifying cellular vulnerability and intensifying neurodegenerative processes [91,96]. These antioxidant deficits facilitate oxidative damage to neuronal membranes, impair mitochondrial function, and disrupt neurotransmission, further exacerbating depressive symptoms [95]. Zinc deficiency, for example, diminishes neuroplasticity and impairs glutamatergic signaling, whereas insufficient vitamin E reduces neuronal protection against lipid peroxidation [97]. Likewise, low levels of coenzyme Q10 impair mitochondrial respiration, increasing ROS production and energy deficits in neurons [98]. Collectively, this heightened oxidative state promotes neuronal apoptosis and inflammation, reinforcing a destructive cycle that sustains and deepens depressive pathology [89]. Thus, addressing oxidative imbalances

through antioxidant supplementation or dietary interventions could potentially mitigate cellular damage and improve therapeutic outcomes in MDD.

Alterations in tryptophan metabolism significantly contribute to oxidative stress in MDD, primarily by shifting this amino acid toward the kynurenine pathway rather than serotonin synthesis. When inflammation activates enzymes such as indoleamine 2,3-dioxygenase (IDO), tryptophan is increasingly converted into kynurenine metabolites, which not only reduce serotonin availability but also generate neurotoxic byproducts like quinolinic acid [19,99,100]. These toxic intermediates promote neuronal damage through enhanced generation of ROS, exacerbating oxidative stress and accelerating cellular injury within vulnerable brain regions [91]. Furthermore, reduced tryptophan availability itself can intensify the formation of free radicals, damaging essential biomolecules including deoxyribonucleic acid (DNA), lipids, and proteins [93]. This cascade creates a destructive cycle of oxidative damage, inflammation, and impaired neurotransmission, significantly correlating with increased severity of depressive symptoms [91]. The resulting neuronal dysfunction, loss of synaptic plasticity, and impaired mitochondrial performance contribute to the pathophysiology of depression [101]. Therapeutic interventions targeting the kynurenine pathway or enhancing tryptophan availability thus hold considerable potential for reducing oxidative damage and alleviating depressive symptoms.

Mitochondrial dysfunction—including structural alterations and disrupted energy metabolism—significantly escalates ROS production, directly promoting neuroinflammation and cognitive decline in depressive disorders [102–105]. Accumulation of dysfunctional mitochondria further exacerbates neuronal damage, perpetuating chronic inflammatory cycles within the brain [106,107]. Specifically, impaired mitophagy hampers the removal of damaged mitochondria, allowing toxic debris to accumulate and activate inflammatory complexes, notably the NLRP3 inflammasome, leading to increased pro-inflammatory cytokine production [108]. Disrupted mitochondrial calcium buffering elevates intracellular calcium, triggering oxidative damage, microglial activation, and amplified inflammatory signaling [109]. Moreover, mitochondrial energy deficits dysregulate AMP-activated protein kinase (AMPK) signaling, causing aberrant mammalian target of rapamycin (mTOR) activation that sustains inflammatory responses [110]. Increased ROS production triggers transcription factors such as NF-κB, leading to ongoing release of inflammatory cytokines (IL-6 and TNF-α) [111]. Reduced mitochondrial health also lowers NAD^+ levels, which weakens protective sirtuin signaling, while imbalanced iron levels encourage ferroptosis, worsening oxidative damage [90,112]. Consequently, impaired neurogenesis and reduced synaptic plasticity intensify cognitive deficits, reinforcing a destructive feedback loop of mitochondrial-driven inflammation in depression [107].

Obesity-induced inflammation significantly heightens oxidative stress by increasing adipokine secretion—such as IL-6, IL-1, and tumor necrosis factor-alpha—while simultaneously impairing insulin regulation, thus elevating risks for type 2 diabetes and depression [113–115]. Chronic obesity also fosters leptin resistance, amplifying microglial activation and further promoting inflammatory cascades within the brain [116]. Moreover, lifestyle factors, particularly high-fat diets and sedentary behaviors, escalate ROS production through enhanced mitochondrial β-oxidation and activation of nuclear factor kappa B (NF-κB) signaling, reinforcing inflammation and neuronal stress pathways [117–121]. Dietary saturated fats directly stimulate inflammasome complexes like NLRP3, causing excessive IL-1β release and perpetuating neuroinflammation [122]. Additionally, obesity-related hyperglycemia accelerates formation of advanced glycation end-products (AGEs), activating inflammatory responses via AGE receptors on microglia [123]. High-fat diets exacerbate gut microbiota disruption, increasing endotoxin leakage into circulation and systemic inflammation while simultaneously lowering BDNF levels, impairing neuronal

resilience [124]. These interconnected metabolic, inflammatory, and behavioral mechanisms underscore the critical role of dietary antioxidants and regular physical exercise in mitigating inflammation-induced depressive pathology.

Oxidative stress stands as a potent yet often overlooked force driving the progression and persistence of MDD [95]. Rather than being a mere consequence of other pathological changes, it plays an active role in linking mitochondrial dysfunction, immune activation, metabolic disturbances, and adverse lifestyle factors [125]. Through sustained production of ROS and insufficient antioxidant defenses, oxidative stress damages cellular structures, fuels neuroinflammation, and disrupts key neural circuits involved in mood regulation [95]. Its impact extends from molecular disruption to large-scale cognitive decline and emotional dysregulation [126]. Addressing this underlying imbalance—via antioxidant therapies, metabolic support, and targeted lifestyle changes—offers a promising direction for enhancing treatment response and improving long-term outcomes in individuals with depression.

4. Depression's Mitochondrial Roots and Repair Strategies

Far beyond their role as mere energy suppliers, mitochondria are now recognized as pivotal regulators of brain health and emotional resilience [127]. In the context of MDD, mitochondrial dysfunction has garnered growing attention as a hidden yet potent driver of neurobiological disruption [112]. From impaired energy metabolism to excessive ROS production, mitochondrial abnormalities appear to fuel the progression of depressive symptoms [89]. This section delves into the emerging landscape of mitochondrial pathology in depression—alongside innovative repair strategies—highlighting a compelling shift in how we understand and potentially treat this multifaceted disorder.

Mitochondria also play a pivotal role in regulating neuroinflammation, positioning them as key contributors to both the onset and persistence of MDD [85]. In individuals with MDD, mitochondrial abnormalities—such as structural degradation, disrupted mitochondrial DNA (mtDNA), and impaired electron transport chain (ETC) function—lead to excessive production of ROS, destabilizing neurotransmitter systems and exacerbating depressive symptoms [103,128–130]. These disturbances are intensified by additional mitochondrial impairments [131]. Oxidative damage to mtDNA impairs the synthesis of ETC components, while dysregulated biogenesis reduces the formation of healthy mitochondria [132]. Imbalances in fusion and fission dynamics further fragment the mitochondrial network, diminishing metabolic capacity and increasing susceptibility to cell death [133]. Chronic stress-related calcium overload, inhibition of ETC complexes, and glucocorticoid-induced suppression of mitochondrial maintenance genes all contribute to dysfunction [134]. Moreover, a disrupted NAD^+/NADH ratio and persistent exposure to pro-inflammatory cytokines impair mitochondrial respiration, DNA repair, and anti-inflammatory signaling [135]. Together, these interconnected mechanisms establish a vicious cycle of mitochondrial breakdown, oxidative stress, and neuroinflammation that drives the neurobiological deterioration seen in MDD.

While psychosocial stressors undoubtedly contribute to the onset of MDD, a growing body of evidence highlights the role of biochemical markers—particularly those tied to mitochondrial health—in its pathophysiology [102]. One such marker, methylmalonic acid (MMA), is elevated in cases of vitamin B12 deficiency and has been shown to impair succinate dehydrogenase activity, thereby increasing ROS production and promoting neuronal injury [136–140]. Additional biomarkers further implicate mitochondrial dysfunction in MDD. Elevated homocysteine, another consequence of B-vitamin deficiency, induces oxidative stress and compromises mitochondrial DNA repair [141]. Increased brain lactate and pyruvate levels suggest a shift from oxidative phosphorylation to inefficient anaerobic metabolism [142]. Accumulation of acylcarnitines reflects disrupted fatty acid oxidation,

while elevated ammonia impairs mitochondrial respiration and contributes to neurotoxicity [143]. Depletion of glutathione (GSH), the mitochondria's frontline antioxidant, leaves cells vulnerable to ROS, and heightened levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) indicate mtDNA damage [144]. Deficiencies in coenzyme Q10 and imbalances in the NAD^+/NADH ratio further hinder ATP production and stress resilience [145]. Together, these markers underscore the biochemical complexity and mitochondrial vulnerability underlying depressive disorders. These observations resonate with broader neurodegenerative research frameworks, which emphasize the integration of imaging biomarkers, inflammatory profiles, and metabolic signatures to characterize disease subtypes and guide personalized intervention strategies [146].

Recent studies underscore several promising therapeutic strategies aimed at correcting mitochondrial dysfunction in MDD [147]. Among them, mitochondrial transplantation—either by replacing damaged mitochondria or introducing healthy mitochondrial DNA and related proteins—has emerged as a novel approach to restoring cellular energy balance and reversing neuronal damage [148]. Similarly, inhibiting the renin–angiotensin system with angiotensin receptor blockers has shown potential in reducing neuroinflammation and oxidative stress, both key drivers of depressive pathology [149,150]. Beyond these, mitochondria-targeted antioxidants like MitoQ and SkQ1 directly neutralize ROS within mitochondria, improving membrane potential and behavioral outcomes [151]. Supplementation with NAD^+ precursors (e.g., nicotinamide riboside) enhances mitochondrial biogenesis and DNA repair via sirtuin activation [152]. Coenzyme Q10 boosts ATP production, while PGC-1α activators such as resveratrol and AMPK activators promote mitochondrial renewal [153,154]. Other emerging strategies include inhibition of the mitochondrial permeability transition pore (mPTP), ketogenic diets to optimize mitochondrial fuel use, sirtuin activators to dampen inflammation, and L-carnitine derivatives that support fatty acid oxidation [155–158]. Gene therapies targeting mtDNA mutations offer future avenues for correcting mitochondrial defects at their source [159].

Ultimately, restoring mitochondrial health offers a compelling path forward in the treatment of MDD. By integrating phytochemicals and other multimodal strategies—ranging from targeted antioxidants to metabolic and genetic interventions—it may be possible to reduce oxidative damage, enhance energy metabolism, and reinforce neuronal resilience [102]. These approaches hold promise not only for symptom relief but also for addressing the cellular dysfunctions at the core of depression's pathology. Importantly, mitochondrial fragility does not occur in isolation. Caloric excess, insulin resistance, and adipose-derived cytokines impose a chronic oxidative–inflammatory load that further erodes mitochondrial ATP output and calcium buffering in limbic neurons. In turn, energy-starved mitochondria dysregulate appetite and glucose homeostasis via hypothalamic circuits, creating a vicious cycle in which metabolic inflammation and mitochondrial dysfunction co-amplify depressive pathology. This bidirectional loop sets the stage for examining obesity-linked inflammation in greater detail.

5. Nutrition, Inflammation, and Depressive Spirals

While nutrition and lifestyle factors clearly shape metabolic risk and modulate inflammation relevant to MDD, a detailed discussion lies beyond the core focus of this review. We briefly highlight key findings to underscore how diet quality, physical activity, and sleep patterns influence neuroimmune health. These modifiable behaviors may complement pharmacological and phytochemical strategies, but a full exploration of their therapeutic applications warrants separate, dedicated analysis. Nutrition lies at the crossroads of physical and mental health, yet its role in MDD is often underestimated [160]. Increasingly, research points to a vicious cycle in which poor dietary habits not only reflect depressive states but

actively intensify them through metabolic disruption and chronic inflammation [161]. As nutrient deficiencies and unhealthy food choices fuel neurobiological stress, depressive symptoms may deepen, further eroding motivation for self-care [162]. This section explores how diet, lifestyle, and inflammation intertwine in self-perpetuating spirals—offering both a cautionary tale and a hopeful lens on how strategic nutritional interventions may help break the cycle.

Recent lines of evidence highlight that dietary behaviors, modulated by psychological factors, significantly shape obesity risk and depressive symptoms by influencing nutrient availability and metabolic function [163–165]. In particular, inadequate consumption of vitamin B12, zinc, magnesium, folic acid, and vitamin B6 has been associated with elevated susceptibility to MDD [15]. Moreover, diets dominated by high-fat convenience foods often coincide with lower intake of fruits, vegetables, lean proteins, and whole grains, fueling systemic inflammation and oxidative stress linked to depression pathogenesis [166–168]. These findings underscore the multifaceted interplay among dietary patterns, nutritional deficiencies, and mood disorders, suggesting that suboptimal eating habits may potentiate neurobiological pathways underlying depression [169]. By integrating these insights into clinical practice, researchers and clinicians can more effectively target modifiable lifestyle factors in conjunction with pharmacotherapy or psychotherapy [170]. Ultimately, developing evidence-based nutritional interventions, particularly those emphasizing nutrient-rich whole foods, could mitigate depressive symptom severity while reducing broader metabolic risk [171]. This comprehensive approach holds promise for reducing disease burden and improving patient outcomes in MDD.

Obesity is increasingly recognized as a critical comorbidity in depression, given that excess adiposity promotes a pro-inflammatory milieu and metabolic dysregulation, potentially exacerbating psychiatric symptomatology [83,84]. In parallel, emerging data indicate that individuals with severe depressive disorders often exhibit behaviors that intensify these physiological burdens, such as smoking, physical inactivity, and insufficient sleep [172,173]. These lifestyle factors further elevate systemic inflammation and oxidative stress, reinforcing a feedback loop that intensifies both mood disturbances and metabolic risk [47]. Notably, recent studies underscore that even modest reductions in body mass index or targeted interventions to improve sleep quality can substantially attenuate neuroinflammatory processes linked to depression [174]. By highlighting these interdependent mechanisms, the present review affirms the need for comprehensive management approaches that address not only psychological distress but also modifiable health behaviors [170]. Tailored interventions that incorporate nutritional counseling, physical activity regimens, and sleep hygiene may thus hold therapeutic promise, mitigating the dual burden of metabolic and mental health complications [175,176]. Advancing this integrative perspective could ultimately refine treatment algorithms and inform future research directions in translational psychiatric care.

Emerging data consistently underscore the potential for lifestyle modifications to alleviate mood-related symptomatology and address comorbid metabolic disorders in individuals with depression [170]. Notably, adopting healthier diets characterized by nutrient-dense foods, minimized refined sugars, and balanced macronutrient intake appears to diminish systemic inflammation and improve metabolic regulation [177]. When combined with regular physical activity, such dietary patterns may enhance neuroplasticity through increased neurotrophic factor release, supporting neuronal resilience [178]. Stress management techniques, including mindfulness-based practices and cognitive–behavioral strategies, further mitigate hypothalamic–pituitary–adrenal axis dysregulation, thereby moderating pro-inflammatory responses and bolstering mental health [165,179,180]. Although these findings remain preliminary, they collectively suggest that targeted lifestyle

programs can serve as essential components of comprehensive treatment plans [181]. Moreover, recent randomized controlled trials affirm that multifaceted approaches integrating nutritional counseling, behavioral interventions, and exercise regimens yield meaningful improvements in depressive symptoms and metabolic risk profiles [182]. This perspective highlights the need to broaden therapeutic frameworks beyond pharmacological paradigms to address the complex interplay among diet, behavior, and psychological well-being in depression [183].

Collectively, these findings illuminate how poor dietary habits can instigate a vicious cycle in which chronic inflammation exacerbates depressive pathology, further eroding motivation for healthy behavior [161]. By perpetuating neurobiological stress, this spiral intensifies symptom severity and increases metabolic risk, reinforcing suboptimal eating patterns [161]. Adopting balanced, nutrient-rich diets, regular physical activity, and evidence-based stress management may disrupt this harmful feedback loop, thereby enhancing neurotransmitter balance, mitigating systemic inflammation, and restoring metabolic homeostasis [170]. Although rigorous trials remain necessary to pinpoint optimal protocols, strategic nutritional interventions hold considerable promise for curtailing the depressive spiral and advancing patient-centered treatment paradigms in MDD [184].

6. Rethinking Depression Care: Drugs and Natural Options

As our understanding of MDD deepens, so too does the landscape of its treatment—now evolving beyond traditional pharmacotherapy toward more integrative, personalized care [185]. While established medications like SSRIs and SNRIs remain central, their side-effect profiles and limitations prompt a reexamination of what comprehensive care can look like [186]. Intriguingly, nature itself may offer untapped solutions [187]. From time-honored botanicals to novel phytochemicals, natural compounds are gaining recognition as adjunctive or alternative treatments with promising efficacy and improved tolerability [188]. This section explores the growing synergy between conventional drugs and natural options in the quest for more holistic depression care.

Pharmacological and non-pharmacological therapies for MDD are increasingly being delivered through multidisciplinary care teams, reflecting a shift toward more holistic, patient-centered treatment models [189,190]. These teams typically include psychiatrists—who oversee diagnosis, medication management, and complex case coordination—and clinical psychologists or psychotherapists, who deliver evidence-based therapies such as CBT, IPT, and mindfulness-based interventions [191,192]. Nutritionists or dietitians also play a key role by addressing nutritional deficiencies and promoting anti-inflammatory dietary patterns that support mitochondrial and mental health [191,193]. Exercise physiologists or physical therapists prescribe tailored physical activity regimens to reduce depressive symptoms and improve neurobiological function [191,192]. Primary care physicians manage medical comorbidities that often worsen mood disorders, while pharmacists ensure medication safety and adherence [194]. Complementary and integrative health practitioners may introduce acupuncture, yoga, or nutraceutical support to reduce oxidative stress [195]. Social workers and case managers address psychosocial barriers and improve continuity of care, and peer support specialists offer lived-experience insights that build trust and engagement [196]. This interdisciplinary approach is especially effective in treatment-resistant or complex cases, offering a comprehensive strategy that integrates biological, psychological, and social dimensions of depression [197–199].

Among pharmacological options for MDD, selective serotonin reuptake inhibitors (SSRIs) remain the preferred first-line treatment due to their efficacy and relative tolerability. However, alternative classes such as serotonin–norepinephrine reuptake inhibitors (SNRIs), TCAs, monoamine oxidase inhibitors (MAOIs), and N-methyl-D-aspartate

(NMDA) antagonists provide additional therapeutic pathways, especially in treatment-resistant cases [10,200–202]. Despite their benefits, these agents are associated with a range of adverse effects [203]. SSRIs can cause gastrointestinal disturbances, insomnia, sexual dysfunction, and in some cases, QTc prolongation—particularly with citalopram [204]. SNRIs like venlafaxine and duloxetine may induce hypertension, hepatotoxicity, and withdrawal symptoms [205]. TCAs carry anticholinergic burdens (e.g., dry mouth, constipation) and significant cardiovascular risks, including arrhythmias [206]. MAOIs, though effective, are limited by dietary restrictions and the risk of hypertensive crises or serotonin syndrome [207]. Atypical antidepressants such as bupropion and mirtazapine offer varied mechanisms but introduce risks like insomnia, weight gain, or seizure potential [208]. Novel agents, including NMDA antagonists like esketamine, present dissociative and cardiovascular side effects [209]. Given these diverse profiles, careful, individualized treatment planning is essential [208,210–213]. The limitations of conventional antidepressants, such as the risk of hyponatremia in older adults and individuals taking diuretics, underscore the need for safer and more tolerable treatment options [214,215].

These safety concerns have prompted increasing interest in alternative and complementary strategies that can enhance efficacy while reducing adverse effects [216]. Herbal medicines are gaining recognition as potential adjuncts in the treatment of MDD [217]. These natural compounds may exert neuroprotective, anti-inflammatory, and mood-regulating effects while offering better tolerability and fewer side effects compared to standard medications [218–220]. By improving safety profiles and supporting long-term adherence, herbal therapies may help address some of the limitations associated with conventional pharmacological approaches [221]. Their integration into clinical practice could also support a more holistic and individualized model of care [222]. As research continues to explore these compounds' mechanisms and clinical potential, combining pharmacological and natural interventions may represent a promising path toward more comprehensive and patient-centered depression management.

7. Plant-Based Therapies: Natural Allies Against Depression

As science turns its gaze toward nature's pharmacopoeia, plant-based therapies are emerging as exciting frontiers in the treatment of MDD [223]. Rich in bioactive compounds, plants offer a diverse array of phytochemicals capable of modulating the same neurobiological pathways targeted by conventional antidepressants—often with fewer side effects [224]. From polyphenols and flavonoids to carotenoids and alkaloids, these natural agents show potential to combat oxidative stress, inflammation, and neurodegeneration [225]. This section delves into the growing body of evidence supporting phytotherapies as integrative, multifaceted allies in the fight against depression—opening the door to greener, gentler innovations in mental health care.

Phytochemicals, as bioactive compounds naturally derived from plants, have attracted increasing interest for their ability to modulate key neurobiological pathways involved in MDD [37,226]. Among them, polyphenols are particularly noteworthy for their ability to cross the blood–brain barrier and exert neuroprotective, antioxidant, and anti-inflammatory effects, thereby attenuating cytokine activity and reducing neuronal apoptosis [74,227]. Within this class, flavonoids such as luteolin effectively scavenge free radicals and suppress neuroinflammation [227–229], while carotenoids offer similar antidepressant benefits through antioxidant and anti-inflammatory mechanisms [230–233]. These effects align with the growing evidence that enhancing BDNF signaling improves synaptic plasticity and emotional regulation. Compounds like curcumin and resveratrol have demonstrated the ability to upregulate BDNF in animal models, contributing to mood stabilization and resilience [234–236]. Additional polyphenols—including quercetin, EGCG, fisetin, apigenin,

and baicalein—further reinforce these effects by modulating inflammatory signaling, reducing oxidative stress, and promoting mitochondrial function [237–240]. Collectively, these natural agents represent promising adjunctive therapies for improving clinical outcomes in MDD through multimodal neuroprotective actions.

Additional phytochemicals found in medicinal plants such as *Panax ginseng*, *Mitragyna speciosa*, *Astragalus membranaceus*, and species within the *Acorus* genus have shown antidepressant-like effects primarily through their anti-inflammatory and antioxidant properties [32,34,241–243]. These effects are echoed in other plant-derived compounds that act on convergent molecular pathways [130]. Withanolides from *Withania somnifera* modulate the HPA axis, reduce pro-inflammatory cytokines like IL-6 and TNF-α, and elevate BDNF levels, enhancing stress resilience [244]. Hericenones and erinacines from *Hericium erinaceus* stimulate nerve growth factor synthesis, reduce oxidative stress, and improve monoaminergic balance [245]. Ginsenosides from *Panax ginseng* and bacopasides from *Bacopa monnieri* both boost mitochondrial efficiency, suppress neuroinflammation, and promote neuroplasticity [130,246]. Other promising agents include honokiol from *Magnolia officinalis*, paeoniflorin from *Paeonia lactiflora*, and berberine from *Berberis* species, all of which influence oxidative, inflammatory, and neurotransmitter systems [247–249]. Rhynchophylline and salidroside further contribute to this phytochemical network by protecting neurons and regulating mood circuits [250]. Collectively, these compounds offer diverse mechanisms for mitigating the multifaceted pathways underlying mood disorders by reducing inflammation, alleviating oxidative stress, fine-tuning neurotransmitter balance, and fostering neuroplasticity, thereby highlighting their integrative therapeutic potential.

Although plant-based interventions often exhibit fewer side effects than conventional antidepressants, their clinical application still requires deeper investigation to refine optimal dosing, clarify underlying mechanisms, and ensure long-term safety [251,252]. Nonetheless, mounting evidence supports the potential of phytotherapeutics as effective, integrative strategies for addressing the complex and multifactorial nature of MDD [1]. By targeting neuroinflammation, oxidative stress, mitochondrial dysfunction, and neurotransmitter imbalances, these compounds offer a multi-pronged approach to mood regulation [253]. Their ability to enhance neurotrophic factors such as BDNF further strengthens their therapeutic value [254]. As the field of psychoneuropharmacology evolves, incorporating plant-derived compounds into treatment frameworks may improve outcomes for patients who are unresponsive or intolerant to traditional therapies [255]. Collectively, these findings highlight the critical need for continued clinical research on phytomedicines in mood disorder management [256]. Figure 2 illustrates select phytochemicals and their modulatory effects on brain metabolism and neural function, underscoring their relevance as natural allies in depression care.

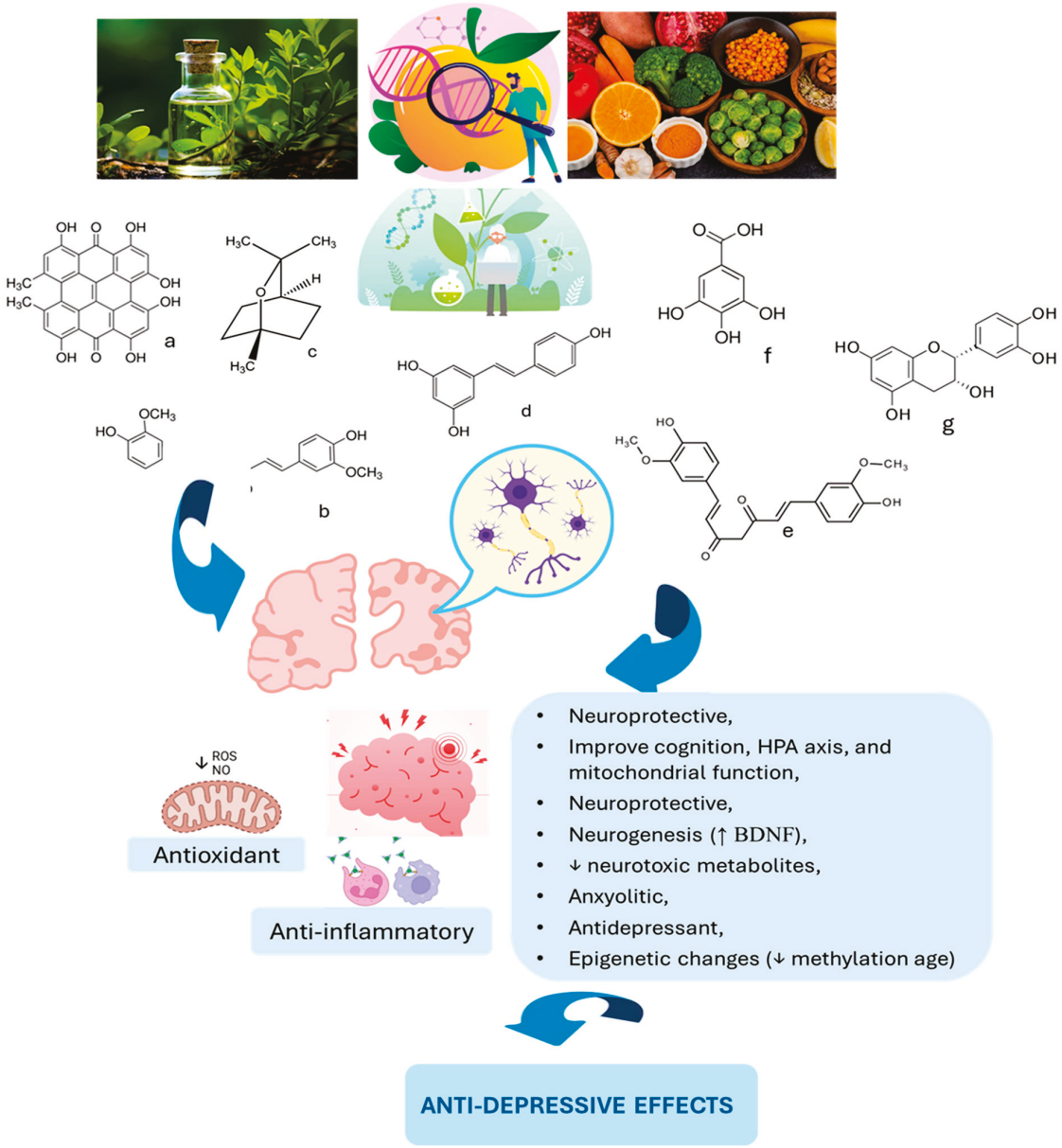


Figure 2. Phytochemicals and the role in brain health and reduction in depression. HPA: hypothalamic–pituitary–adrenal; BDNF: brain-derived neurotrophic factor; a: hypericin; b: coumarin; c: eucalyptol; d: resveratrol; e: curcumin; f: gallic acid; g: epicatechin. ↑: increase; ↓: decrease.

8. From Curcumin to Cocoa: Plant-Based Mood Solutions

The following interventional studies reveal a promising though heterogeneous body of evidence regarding the therapeutic potential of phytocompounds in MDD. These trials examined a wide range of phytochemicals, including curcumin, quercetin, polyphenols, anthocyanins, isoflavones, proanthocyanidins, and flavonoid-rich extracts, often administered in monotherapy or adjunctive formats. Although sample sizes, duration, and design quality varied, most studies used validated depression scales and biochemical endpoints, supporting a neurobiological rationale for clinical translation.

In a well-structured randomized controlled trial, Soltani et al. [25] evaluated the impact of nanocurcumin supplementation in individuals with coronary slow flow phenomenon (CSFP). The intervention led to improvements in depressive symptoms, physical and psychological health-related quality of life, and cardiometabolic parameters, with biochemical assays confirming anti-inflammatory and antioxidant effects [257]. In another

study examining quercetin effects in patients who have experienced myocardial infarction, the authors did failed to not find significant antidepressant effects [258].

Methodological strengths such as stratified randomization and biomarker analysis were evident in the study by Hajiluian et al. [34], which demonstrated reduced depressive symptoms in individuals with multiple sclerosis following polyphenol-rich interventions. However, its narrow population limits generalizability to broader MDD cohorts [259]. In a similarly well-structured clinical trial, Choi et al. [27] examined the effects of flavonoid-enriched orange juice in healthy adults. Improvements in depressive symptoms were paralleled by increased serum BDNF and reduced zonulin levels, indicating neuromodulatory and gut–brain axis effects. Despite robust biochemical correlates, the sample size of 40 reduced statistical power [27].

Maeda-Yamamoto et al. [35] assessed the impact of anthocyanin-rich *Solanum tuberosum* L. on stress and mesenchymal stem cell proliferation. Although qualitative outcomes were positive, the trial was limited by its very small sample (n = 15) and short duration (8 weeks), which precluded long-term conclusions. Similarly, Barfoot et al. [36] conducted a randomized study during the COVID-19 pandemic, which introduced significant confounding related to global psychological stress. While randomization via software strengthened validity, high dropout rates impaired data reliability.

Parilli-Moser et al. [37] explored the effects of botanical cognitive enhancers on depressive symptoms. Although standardized cognitive assessments were used, low group sizes and a lack of blinding limited interpretive strength and COVID-19-related disruptions likely impacted outcome validity. In contrast, the RISTOMED study by Bourdel-Marchasson et al. [38] assessed a dietary protocol rich in antioxidants and polyphenols. After 2 months, reductions in depressive symptoms were observed, but these were found to be independent of inflammatory biomarker changes, suggesting mechanisms beyond systemic inflammation.

Kontogianni et al. [260] investigated high versus low polyphenol diets in the PPhIT trial, noting improved psychological well-being in the high-polyphenol group using standardized lifestyle and mood assessments. Park, Choi, and Lee [260] similarly conducted a placebo-controlled flavonoid study using orange juice and observed improvements in depression alongside increased serum serotonin, BDNF, and reduced CRP—though the small sample again limits external validity.

Smetanka et al. [260] focused on the potential for pycnogenol to mitigate SSRI-induced sexual dysfunction in patients taking escitalopram. However, the open-label design and simultaneous pharmacologic interventions created confounding that weakens causal inference. In the curcumin trial by Kanchanatawan et al. [40], methodological strengths included matched placebo capsules and double-blinding, although prior treatments continued during the trial, potentially masking true curcumin effects.

The study by Esmaily et al. [41] observed only marginal reductions in anxiety and depressive symptoms in response to saffron extract, with effects limited to a single outcome measure—suggesting potential underpowering or scale insensitivity. In a postmenopausal population, Terauchi et al. [33] demonstrated that grape seed proanthocyanidins reduced depressive symptoms in a dose-dependent manner, using validated questionnaires. However, the short duration (8 weeks) and modest sample size limit generalizability.

Equol and resveratrol were evaluated in a longitudinal 12-week study on menopausal women aged 50–55, where supplementation improved mood as assessed by the Hamilton depression rating scale (HAM-D) [261]. Hirose et al. [262] explored low-dose isoflavone aglycone for postmenopausal symptoms, including depression and anxiety, using HADS and AIS. Despite positive outcomes, lack of adverse event documentation and small sample size limit clinical confidence.

Cognitive-affective benefits of blueberry-derived flavonoids were tested in a crossover design by Khalid et al. [262], involving both children and young adults. Stratified analysis revealed reductions in depressogenic cognitive patterns two hours after consumption, supporting the acute neuromodulatory potential of these compounds.

Lastly, Sathyapalan et al. [262] evaluated chocolate rich in cocoa liquor and polyphenols versus low-polyphenol control chocolate in patients with chronic fatigue syndrome. Participants in the high-cocoa group reported improvements in depressive and fatigue symptoms, supporting flavonoid efficacy in neuropsychological syndromes. Pase et al. [11] reported mood-enhancing effects of cocoa polyphenols but no cognitive improvements after 30 days of supplementation.

Overall, these reviewed studies underscore the emerging promise of phytocompounds as adjunctive or standalone interventions for MDD (Table 1). While most trials reported clinically meaningful reductions in depressive symptoms, variability in methodological rigor, sample size, and treatment duration limited the generalizability of these findings. Notably, studies incorporating double-blind, placebo-controlled designs and objective biomarkers (e.g., inflammatory mediators neurotrophic factors) provided stronger evidence for neuromodulatory and neuroprotective mechanisms. Nevertheless, several investigations reported inconclusive or modest effects, indicating the necessity of improved trial designs and larger, more diverse samples. Given the heterogeneous nature of depression, a multitargeted approach—such as that offered by phytochemicals—holds particular relevance. Future research efforts should focus on optimizing dosage regimens, exploring synergistic effects with standard antidepressants, and elucidating long-term safety profiles to solidify phytocompounds' place in evidence-based psychiatric care.

Table 1. Summary of Interventional Studies Evaluating the Therapeutic Effects of Phytocompounds in Major Depressive Disorder (MDD). This table presents a structured synthesis of the 19 clinical trials included in the systematic review, selected according to PRISMA guidelines. Each study is characterized by its design, geographic location, sample demographics, intervention type, control conditions, outcome measures, and reported adverse events. The interventions include a range of phytocompounds—such as curcumin, flavonoids, anthocyanins, and isoflavones—administered in monotherapy or adjunctive formats. Outcomes were assessed via validated psychometric scales and, where applicable, supported by biochemical and neurotrophic markers. This compilation provides a comparative overview of study quality, efficacy, and safety in the clinical application of phytochemicals for MDD.

Study [Ref.]	Country	Population	Intervention	Outcomes	Side Effects
Soltani et al. [257]	Iran	42 patients (CSFP) aged 35–70	Nano-curcumin 80 mg/day, 12 weeks	Improved depression and quality of life	Nausea, headache, and diarrhea
Dehghani et al. [258]	Iran	76 individuals aged 35–65	Quercetin 500 mg/day, 8 weeks	Marginal depression improvement	Headache, joint pain, and abdominal discomfort
Hajiluian et al. [259]	Iran	50 MS patients with depression, aged 18–55	Ellagic acid 180 mg/day, 12 weeks	Reduced inflammation and depression	None significant
Choi et al. [263]	Republic of Korea	40 young adults aged 18–29 with MDD	Flavonoid-rich orange juice, 8 weeks	Significant improvement in depressive scores	None significant
Maeda-Yamamoto et al. [264]	Japan	15 older adults aged 50–70	Anthocyanin-rich potatoes, 8 weeks	Improved psychological stress response	None significant

Table 1. *Cont.*

Study [Ref.]	Country	Population	Intervention	Outcomes	Side Effects
Parilli-Moser et al. [265]	U.K.	38 new mothers	High-flavonoid diet, 2 weeks	Reduced anxiety and improved social relationships	None significant
Barfoot et al. [266]	Spain	63 young overweight adults	Roasted peanuts and peanut butter, 6–7 months	Significant depression reduction	Digestive symptoms and softer stools
Bourdel-Marchasson et al. [267]	Europe	125 elderly adults aged ~70	Antioxidant-rich diet, 2 months	Reduced depressive symptoms	None serious
Kontogianni et al. [260]	U.K.	99 mildly hypertensive adults aged 40–65	High vs. low polyphenol diet, 8 weeks	Improved depressive symptoms	Not reported
Park et al. [268]	Republic of Korea	40 healthy adults (20–30 years old)	Flavonoid-rich (FR) or flavonoid-low (FL) drinks, 190 mL twice daily for 8 week	↓ Depressive symptoms (CES-D < 20)	None significant
Smetanka et al. [269]	Slovakia	67 adults with MDD, aged 18–65	Pycnogenol with escitalopram, 12 weeks	No additional benefit compared to escitalopram alone	None significant
Kanchanatawan et al. [270]	Multinational	61 adults aged 18–63 with MDD history	Curcumin 500–1500 mg/day, 12–16 weeks	Improved depression scores (MADRS)	Dizziness, nausea, insomnia, and diarrhea
Terauchi et al. [261]	Italy	60 menopausal women aged 50–55	Equol and resveratrol, 12 weeks	Reduced depressive symptoms (HAM-D)	Mild diarrhea
Khalid et al. [271]	U.K.	Young adults and children	Wild blueberry drink (flavonoids), acute administration	Improved positive affect shortly after consumption	None significant
Hirose et al. [262]	Japan	87 menopausal women aged 40–60	Isoflavone aglycones, 8 weeks	Improved anxiety and modest depression changes	None significant
Sathyapalan et al. [272]	U.K., Iran	30 obese adults	Curcumin 1 g/day, 30 days	Reduced anxiety and no significant depression improvement	None significant
Terauch et al. [273]	Japan	91 menopausal women (40–60 years old)	GSPE (100 or 200 mg/day) vs. placebo, 8 weeks	↓ Anxiety (dose-dependent); no effect on depression	None significant
Pase et al. [11]	Australia	Adults aged 40–65	Cocoa polyphenols 250–500 mg/day, 30 days	Improved mood and calmness	None
Clinical pilot [274]	U.K.	10 adults	High-polyphenol chocolate, crossover	Improved anxiety and depression scores	None

AE: adverse events; AIS: Athens Insomnia Scale; BAI: Beck Anxiety Inventory; BDI: Beck Depression Inventory; BDI-II: Beck Depression Inventory-II; BDNF: brain-derived neurotrophic factor; CB: control butter; CES-D: Center for Epidemiological Studies Depression Scale; EDSS: expanded disability status scales; ESC: escitalopram; FL: low-flavonoid orange cordial group; FR: group of orange juice rich in flavonoids; GSPE: grape seed proanthocyanidin extract; HADS: Hospital Anxiety and Depression Scale; HAMD/HAMD -17: Hamilton Depression Rating Scale; IFN-γ: gamma interferon; IQR: range between quartiles; MADRS: Montgomery–Asberg Depression Rating Scale; MDD: major depressive disorder; MSC: mesenchymal stem cells; MSS: Menopause Symptom Scale; MS: multiple sclerosis; NO: nitric oxide; PA: positive affect; PB: peanut butter; PR: time interval between the beginning of atrial depolarization and the beginning of ventricular depolarization; PYC: pycnogenol; QRS: depolarization of the ventricles, consisting of Q, R, and S waves; SQ: Shadow Queen; SRP: roasted peanuts in shell; WBB: wild blueberry drink.

9. Discussion

MDD remains a complex, multifaceted psychiatric condition that resists simple explanations or treatments [275]. The primary goal of this review was to explore how phytochemicals—naturally occurring plant-derived compounds—may offer a biologically plausible and clinically relevant approach to modulating the intricate neurobiological disruptions observed in MDD [1]. Central to the disorder's pathophysiology are sustained neuroinflammation, oxidative stress, mitochondrial dysfunction, and metabolic disturbances, which interact to compromise neuronal integrity, synaptic plasticity, and emotional regulation [276]. While monoaminergic deficits have long dominated depression theories, current evidence indicates these downstream effects may be fueled by deeper cellular and immune dysregulation [277]. Phytocompounds such as polyphenols, flavonoids, and alkaloids appear to influence these upstream processes by attenuating pro-inflammatory cytokines, scavenging ROS, restoring mitochondrial dynamics, and modulating neurotrophic signaling [136]. In doing so, they challenge conventional paradigms and suggest a more integrative neuropsychiatric model—one that views depression through the lens of systemic inflammation and neuroenergetic compromise rather than solely neurotransmitter depletion [278].

A central finding of this review is the growing body of evidence supporting the antidepressant potential of phytochemicals, particularly polyphenols, flavonoids, and alkaloid-rich plant extracts. Compounds such as curcumin, resveratrol, quercetin, and luteolin consistently demonstrate antidepressant-like effects across preclinical models and emerging clinical studies. These benefits are mediated through a combination of neuroprotective, anti-inflammatory, and antioxidant mechanisms. Notably, curcumin has been shown to downregulate pro-inflammatory cytokines (e.g., IL-1β and TNF-α), reduce lipid peroxidation, and enhance BDNF expression—key pathways implicated in MDD. Similarly, flavonoids such as quercetin and luteolin attenuate microglial activation, restore mitochondrial function, and improve synaptic plasticity. These multi-target effects distinguish phytochemicals from traditional antidepressants, which primarily modulate monoamine reuptake and often require several weeks to achieve symptom relief. Moreover, phytochemicals tend to have more favorable safety profiles, presenting a lower risk of side effects such as sexual dysfunction, weight gain, or hyponatremia. For individuals with treatment-resistant depression or intolerance to conventional therapies, these natural agents may offer a promising adjunct or alternative approach. Their ability to modulate upstream drivers of MDD rather than solely downstream neurotransmitter imbalances reflects a potentially paradigm-shifting advance in how we conceptualize and manage depression.

The therapeutic potential of phytochemicals in MDD is rooted in their ability to engage a broad array of molecular targets implicated in the neurobiology of depression [279]. One of the most prominent mechanisms is the upregulation of BDNF, a key molecule in synaptic plasticity, neurogenesis, and neuronal survival [226]. Phytocompounds such as curcumin, resveratrol, and apigenin have been shown to elevate BDNF expression in preclinical models, countering the synaptic deficits commonly observed in depression [280]. Another critical mechanism involves the inhibition of the NLRP3 inflammasome, a key driver of neuroinflammation [276]. Polyphenols like quercetin and baicalein suppress this pathway, leading to decreased release of pro-inflammatory cytokines such as IL-1β and IL-18 [281]. These compounds also reduce oxidative stress by enhancing endogenous antioxidant defenses (e.g., upregulation of Nrf2 and glutathione pathways), protecting neurons from ROS-induced apoptosis [282]. Importantly, many of these phytochemicals—especially flavonoids and terpenoids—demonstrate the capacity to cross the blood–brain barrier due to their lipophilic structures and low molecular weight [283]. Once in the CNS, they can stabilize mitochondrial function, modulate calcium homeostasis, and maintain mem-

brane potential, all of which are disrupted in MDD [284]. These mechanistic actions align closely with contemporary neurobiological models of depression, which emphasize the roles of neuroinflammation, mitochondrial dysfunction, and impaired neuroplasticity over simplistic monoamine depletion theories [89]. By targeting upstream cellular stressors and restoring homeostatic balance across neuroimmune and neurometabolic pathways, phytochemicals represent a compelling therapeutic modality that resonates with the current shift toward systems-based approaches in psychiatric research. Complementing this systems-level approach, recent insights into quinoline-based drug design suggest that subtle structural modifications, such as halogenation or esterification, can meaningfully modulate excitotoxicity and immunoactivity signaling relevant to depression pathophysiology [285].

The incorporation of phytochemicals into clinical treatment strategies for MDD presents several promising implications for practice [1]. Unlike conventional antidepressants, which often carry a high burden of side effects—such as sexual dysfunction, weight gain, insomnia, or gastrointestinal disturbances—phytochemicals typically exhibit favorable safety and tolerability profiles [256]. This reduced side-effect burden may significantly enhance patient adherence, particularly among individuals who are medication-sensitive, elderly, or managing comorbid conditions [224]. Furthermore, many phytocompounds, such as curcumin, resveratrol, and quercetin, are available in standardized formulations and have shown efficacy when used alongside standard antidepressant regimens, supporting their role as adjunctive therapies [286]. Their natural origin also makes them appealing to patients seeking holistic or integrative approaches to mental health [287]. To maximize the benefits of phytochemical interventions, multidisciplinary care teams—comprising psychiatrists, psychologists, nutritionists, pharmacists, and exercise specialists—are essential [288]. These teams can tailor phytochemical strategies to the individual's biological and psychosocial profile, monitor for herb–drug interactions, and integrate them into broader lifestyle and behavioral interventions [289]. This integrative model not only broadens therapeutic options but aligns with growing interest in personalized medicine, where treatment is adapted to the individual's unique physiological and lifestyle context [290].

Despite encouraging findings, several challenges and limitations temper the immediate clinical translation of phytochemicals for depression [252]. A primary concern lies in the methodological variability across studies [256]. Many preclinical and clinical trials differ widely in terms of dosage, treatment duration, and formulation, making it difficult to establish standardized protocols [1]. Sample sizes are often small, with limited representation across age groups, genders, and ethnic backgrounds, reducing the generalizability of results [291]. Moreover, key pharmacokinetic parameters—such as bioavailability, half-life, and tissue distribution—remain poorly characterized for many phytochemicals [292]. This complicates efforts to optimize dosage and timing for maximum therapeutic effect [293]. Additionally, the long-term safety of chronic phytochemical use is not well understood, particularly when used in combination with conventional antidepressants, raising concerns about potential herb–drug interactions [294]. These knowledge gaps highlight the need for rigorously designed, large-scale randomized, controlled trials that incorporate standardized extract preparations, dose-ranging studies, and biomarker-based outcome measures [295]. Future research should also emphasize population diversity and assess pharmacogenomic factors that may influence individual responses [296]. Addressing these limitations is essential for moving phytochemicals from promising adjuncts to reliable, evidence-based components of depression treatment protocols.

Future research on phytochemicals in the treatment of MDD should prioritize rigorously designed clinical trials and translational studies that bridge the gap between laboratory findings and real-world clinical application [279]. Large-scale, placebo-controlled

trials are essential to validate the antidepressant efficacy of individual phytochemicals, establish optimal dosing strategies, and assess long-term safety [297]. In addition to studying single compounds, future investigations should explore the therapeutic potential of phytochemical combinations [252]. Many plant-derived compounds act on overlapping molecular targets, and their synergistic effects on neuroinflammation, oxidative stress, and mitochondrial dysfunction may offer enhanced efficacy compared to isolated agents [298]. Combinatorial approaches could mirror the polypharmacological nature of depression, targeting its multifactorial pathophysiology more effectively [225]. Moreover, research should move toward precision medicine models that account for individual differences in genetic makeup, metabolic profiles, gut microbiota composition, and inflammatory status [299]. Stratifying participants based on these biomarkers could identify responders and non-responders to specific phytochemicals, allowing for more personalized treatment [300]. Integrating omics technologies—such as metabolomics, transcriptomics, and pharmacogenomics—into trial designs will enhance mechanistic understanding and treatment customization [301]. Finally, future studies should include robust secondary endpoints that assess cognitive function, quality of life, and functional recovery, not just symptom reduction [199]. By embracing these strategies, the field can move toward the development of targeted, effective, and biologically grounded phytochemical interventions that expand the therapeutic landscape for depression.

Effective management of MDD increasingly requires a multidisciplinary approach that integrates biological, psychological, and lifestyle-based strategies [302]. Collaborative care teams—comprising psychiatrists, psychologists, nutritionists, pharmacists, and exercise specialists—are well positioned to deliver comprehensive, personalized treatment plans [303]. Phytochemical supplementation can be particularly valuable when embedded within this broader therapeutic framework [304]. Nutritionists can guide dietary modifications that reinforce the anti-inflammatory and antioxidant actions of phytocompounds, while exercise specialists can prescribe physical activity regimens that synergize with phytochemicals to enhance mitochondrial health and neuroplasticity [305]. Psychologists can provide cognitive–behavioral or mindfulness-based therapies that further modulate stress-related pathways implicated in MDD [306]. The literature from integrated care models demonstrates that combining pharmacological and non-pharmacological interventions—such as in collaborative care or lifestyle psychiatry frameworks—improves clinical outcomes, enhances patient satisfaction, and reduces relapse rates [307]. For example, interventions that pair dietary polyphenols with structured aerobic exercise and therapy have shown additive effects on depressive symptom reduction and cognitive function [308]. This integrated model not only reflects the multifactorial nature of depression but also offers a pragmatic pathway to improve treatment adherence, reduce polypharmacy, and promote long-term mental wellness [309]. Phytochemicals, when coordinated with other interventions, may serve as powerful components of such a multidimensional care strategy.

This review illuminates the multifaceted nature of MDD and the potential utility of phytocompounds in its management. Recent evidence reveals that MDD involves not only monoaminergic dysregulation but also neuroinflammatory processes, oxidative stress, and mitochondrial dysfunction, implicating diverse pathophysiological pathways [223]. By evaluating interventional studies centered on phytochemicals—including polyphenols, flavonoids, and alkaloids—this review highlights their potential neuroprotective, anti-inflammatory, and antioxidant properties [22]. These effects align with emerging translational research suggesting that multi-targeted strategies may offer enhanced therapeutic outcomes in complex mood disorders [79,310]. Nevertheless, the heterogeneity of study designs, phytocompound formulations, and patient populations underscores the

need for more robust trials with standardized protocols [311–313]. This discussion aims to synthesize the mechanistic underpinnings and clinical implications of phytocompound use in MDD, situating these findings within broader neuropsychiatric research and outlining key directions for future investigation. Taken together, these insights reaffirm the value of phytochemicals as complementary or alternative therapies in MDD, particularly given their favorable safety profiles, multimodal mechanisms of action, and promise for treatment-resistant populations. Continued clinical exploration of these natural interventions may significantly enhance holistic, personalized approaches to depression care and expand the future of integrative psychiatric treatment.

10. Conclusions

Emerging data suggest that specific plant-derived compounds can influence the neuro–immune interface and mitochondrial health in major depressive disorder, yet these signals remain preliminary. Wide variation in extract standardization, dosing protocols, study designs, and participant profiles hampers direct comparison, and most trials enroll modest, geographically limited samples. To translate early promise into clinical practice, large phase-III studies with rigorous pharmacokinetic assessment, active comparators, and extended safety follow-up are essential. Nonetheless, the literature reviewed here highlights the distinctive neuroprotective, anti-inflammatory, and antioxidant capacities of phytochemicals—attributes that align well with the complex biology of depression, extending therapeutic thinking beyond monoamine modulation alone. Although several investigations report meaningful symptom relief with minimal adverse effects, methodological shortcomings—small cohorts, heterogeneous interventions, and inconsistent endpoints—preclude definitive guidance. Future research must emphasize harmonized formulations, adequately powered populations, and mechanistic biomarkers to clarify durability, optimal dosing, and synergy with standard antidepressants. Collectively, the current evidence positions phytocompounds as promising adjuncts, warranting continued, methodologically robust exploration to fully harness their translational potential.

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Abbreviations

The following abbreviations are used in this manuscript:

BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DNA	Deoxyribonucleic acid
HAM-D	Hamilton depression rating scale
IL	Interleukin
MMA	Methylmalonic acid
MDD	Major depressive disorder

mtDNA	Mitochondrial deoxyribonucleic acid
NF-κB	Nuclear factor kappa B
ROS	Reactive oxygen species
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant

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Unidirectional Crosstalk Between NTRK1 and IGF2 Drives ER Stress in Chronic Pain

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Abstract: Background: Chronic postsurgical pain (CPSP) poses a major clinical challenge due to unresolved links between neurotrophic pathways and endoplasmic reticulum (ER) stress. While Neurotrophic Tyrosine Kinase Receptor Type 1 (NTRK1) modulates ER stress in neuropathic pain, its interaction with Insulin-Like Growth Factor II (IGF2) in CPSP remains uncharacterized, impeding targeted therapy. This study defined the spinal NTRK1-IGF2-ER stress axis in CPSP. **Methods**: Using a skin/muscle incision–retraction (SMIR) rat model, we integrated molecular analyses and intrathecal targeting of NTRK1 (GW441756) or IGF2 (siRNA). **Results:** SMIR surgery upregulated spinal NTRK1, IGF2, and ER stress mediators. NTRK1 inhibition reduced both NTRK1/IGF2 expression and ER stress, reversing mechanical allodynia. IGF2 silencing attenuated ER stress and pain but did not affect NTRK1, revealing a unidirectional signaling cascade where NTRK1 drives IGF2-dependent ER stress amplification. These findings expand understanding of stress-response networks in chronic pain. **Conclusions**: We show that spinal NTRK1 drives IGF2-mediated ER stress to sustain CPSP. The NTRK1-IGF2-ER stress axis represents a novel therapeutic target; NTRK1 inhibitors and IGF2 biologics offer non-opioid strategies for precision analgesia. This work advances CPSP management and demonstrates how decoding unidirectional signaling hierarchies can transform neurological disorder interventions.

Keywords: Neurotrophic Tyrosine Kinase Receptor Type 1; Insulin-Like Growth Factor II; Endoplasmic reticulum stress; Chronic Post-surgical Pain

1. Introduction

Chronic Post-surgical Pain (CPSP), defined as persistent pain lasting ≥3 months post-surgery, affects 10–50% of patients across procedures like thoracotomy (30–60% incidence) and amputation [1–4]. This condition imposes profound socioeconomic burdens, with 30% of patients developing long-term disability refractory to conventional analgesics, including opioids and Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) [4,5]. While neuroinflammation and central sensitization are established contributors, accumulating evidence positions endoplasmic reticulum (ER) stress as a pivotal yet under-characterized mechanism in CPSP pathogenesis [6,7].

ER stress orchestrates maladaptive pain signaling through the unfolded protein response (UPR) [8–10]. Activation of its three sensor pathways—PKR-like ER Kinase

(PERK)/eukaryotic Initiation Factor 2α (eIF2α), Inositol-Requiring Enzyme 1α (IRE1α)/X-box Binding Protein 1 X (XBP1), and Activating Transcription Factor 6 (ATF6)—initially promotes cellular adaptation but ultimately drives pathological outcomes when sustained [8,9]. Specifically, PERK/eIF2α induces C/EBP Homologous Protein C/EBP (CHOP)-mediated apoptosis of dorsal horn inhibitory neurons [11], IRE1α/XBP1 triggers microglial TNF-α/IL-1β release, amplifying neuroinflammation [12,13], ATF6 upregulates chaperones (e.g., Glucose-Regulated Protein 78 (GRP78)) to restore proteostasis [14], though this fails in chronic pain states [15–17]. Critically, neurotrophic factors dynamically modulate ER stress resilience [10]. In Alzheimer's models, Brain-Derived Neurotrophic Factor (BDNF) enhances ATF6-mediated adaptive UPR [18,19], while, in diabetic neuropathy, Glial Cell Line-Derived Neurotrophic Factor (GDNF) suppresses PERK hyperactivation [20]. Notably, although UPR markers GRP78 and CHOP are elevated in CPSP models [21–23], the regulatory role of neurotrophic signals in this process remains uncharacterized [24].

Neurotrophic Tyrosine Kinase Receptor Type 1 (NTRK1), a high-affinity receptor for Nerve Growth Factor (NGF), exhibits dual functionality in pain modulation [25–27]. In acute nociception, NTRK1 promotes neuronal hyperexcitability by upregulating voltage-gated sodium channel Nav1.7 and sensitizing transient receptor potential vanilloid 1 (TRPV1) [27–29], while concurrently activating pro-inflammatory nuclear factor kappa-B (NF-κB) signaling [30–34]. Conversely, in chronic pain states, NTRK1 displays paradoxical functions: it exerts neuroprotective effects through PI3K (Phosphatidylinositol 3-Kinase)/Akt (Protein Kinase B)-mediated suppression of apoptosis [6,7], yet sustains pathological ER stress via persistent PERK/eIF2α-CHOP cascade activation [16,35,36]. This functional divergence is increasingly attributed to NTRK1's capacity for transcriptional reprogramming, wherein it orchestrates cell-type-specific stress responses [34]. Emerging evidence reveals that NTRK1 signaling induces distinct transcriptional programs across pathologies [34]—in glioblastoma, NTRK1 complexes drive Insulin-Like Growth Factor II (IGF2) expression to promote tumor survival [15], while, in neuropathic pain, NTRK1- cAMP responsive element binding protein (CREB) signaling upregulates matrix metalloproteinase-9 (MMP9), disrupting blood–nerve barriers [37,38]. Single-cell epigenomic studies further identify NTRK1-binding motifs within stress-response genes (ATF4, XBP1) [24], underscoring its broad influence on proteostatic adaptation [15,24]. Nevertheless, whether surgical injury engages NTRK1 to orchestrate IGF2-dependent ER stress resolution in CPSP—a mechanism conserved in oncology—remains unexplored, presenting a critical translational gap in targeting postoperative maladaptations.

Insulin-Like Growth Factor II (IGF2), a pleiotropic neurotrophic factor [39–41] traditionally studied in development and metabolism, now emerges as a critical ER stress regulator across neurological contexts [42–45]. IGF2 orchestrates ER proteostasis through dual mechanisms: it suppresses pro-apoptotic effectors (CHOP, GRP78) while enhancing adaptive unfolded protein response (UPR) signaling via ATF4 activation and XBP1 splicing, thereby preserving neuronal viability in Alzheimer's models [8,15,18,46–48]. Beyond its anti-apoptotic role, IGF2 exhibits cell-type-specific functionalities—driving oxidative phosphorylation in macrophages to resolve inflammation while stabilizing HIF-1α in tumor microenvironments to exacerbate hypoxic stress [49]. Recent advances further identify TMED10 as an essential cargo receptor for IGF2 ER-to-Golgi trafficking, a process disrupted in chronic diseases [40,42,47]. Critically, spinal IGF2 overexpression reverses neuropathic pain by attenuating ER stress, implicating its therapeutic potential [50,51]. Intriguingly, NTRK1 activation upregulates IGF2 transcription in neuronal stress models [15], suggesting a feedforward loop wherein IGF2 mediates NTRK1's ER-modulatory effects [15]. Despite these mechanistic insights in neurodegeneration and neuropathy, IGF2's spatiotemporal regulation in CPSP—particularly its interplay with NTRK1 in the context of surgical

proteostatic disruption—remains uncharacterized, posing a translational barrier for precision analgesia.

Building upon the conserved role of IGF2 in stress adaptation across neurological and oncological contexts, we hypothesize that spinal IGF2 mediates NTRK1-dependent resolution of ER stress to attenuate CPSP progression. To test this, we aim to (1) map spatiotemporal activation of the NTRK1-IGF2-ER stress axis in a clinically relevant skin/muscle incision–retraction (SMIR) model; (2) determine IGF2's necessity and sufficiency for NTRK1-mediated ER proteostasis using loss- and gain-of-function approaches; and (3) evaluate the therapeutic potential of IGF2 pathway modulation via siRNA and recombinant protein strategies. By defining this axis, our work bridges fundamental neurotrophic signaling with surgical stress biology, advancing precision analgesia strategies that target maladaptive proteostatic responses—a critical unmet need in perioperative medicine.

2. Materials and Methods

2.1. Study Design Overview

This mechanistic study employed a randomized, blinded design to investigate the NTRK1-IGF2-ER stress axis in a rat CPSP model. Experimental timelines, interventions, and endpoint assessments are summarized in Figure 1. Key methodological choices were based on established protocols for neuropathic pain research [52,53].

2.2. Animals and Ethical Compliance

Male Sprague–Dawley (SD) rats (7–8 weeks, 220–250 g; Tongji Medical College Animal Center, Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Certificate No. SYXK(E)2023-0139) were housed under SPF conditions (22–25 °C, 45–65% humidity, 12 h light–dark cycle) with enrichment (PVC tunnels, nesting material).

Rats were randomly assigned to groups (n = 18 or 6/group) using computer-generated codes (GraphPad QuickCalcs). Investigators performing behavioral tests and molecular analyses were blinded to group allocation. Allocation concealment was maintained via coded cage labels. Power analysis was performed using G*Power 3.1 (α = 0.05, β = 0.2, effect size = 1.2), based on established effect magnitudes in prior SMIR studies [52,53] This yielded a minimum required sample size of n = 6/group; To account for potential attrition, 20% additional animals were included (final n = 8/group).

All procedures complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and are reported in compliance with the ARRIVE 2.0 guidelines (Animal Research: Reporting of In Vivo Experiments; https://arriveguidelines.org, 20 September 2022). All experimental protocols involving animals were approved by Tongji Medical College, Huazhong University of Science and Technology (Approval ID: TJH-202211016).

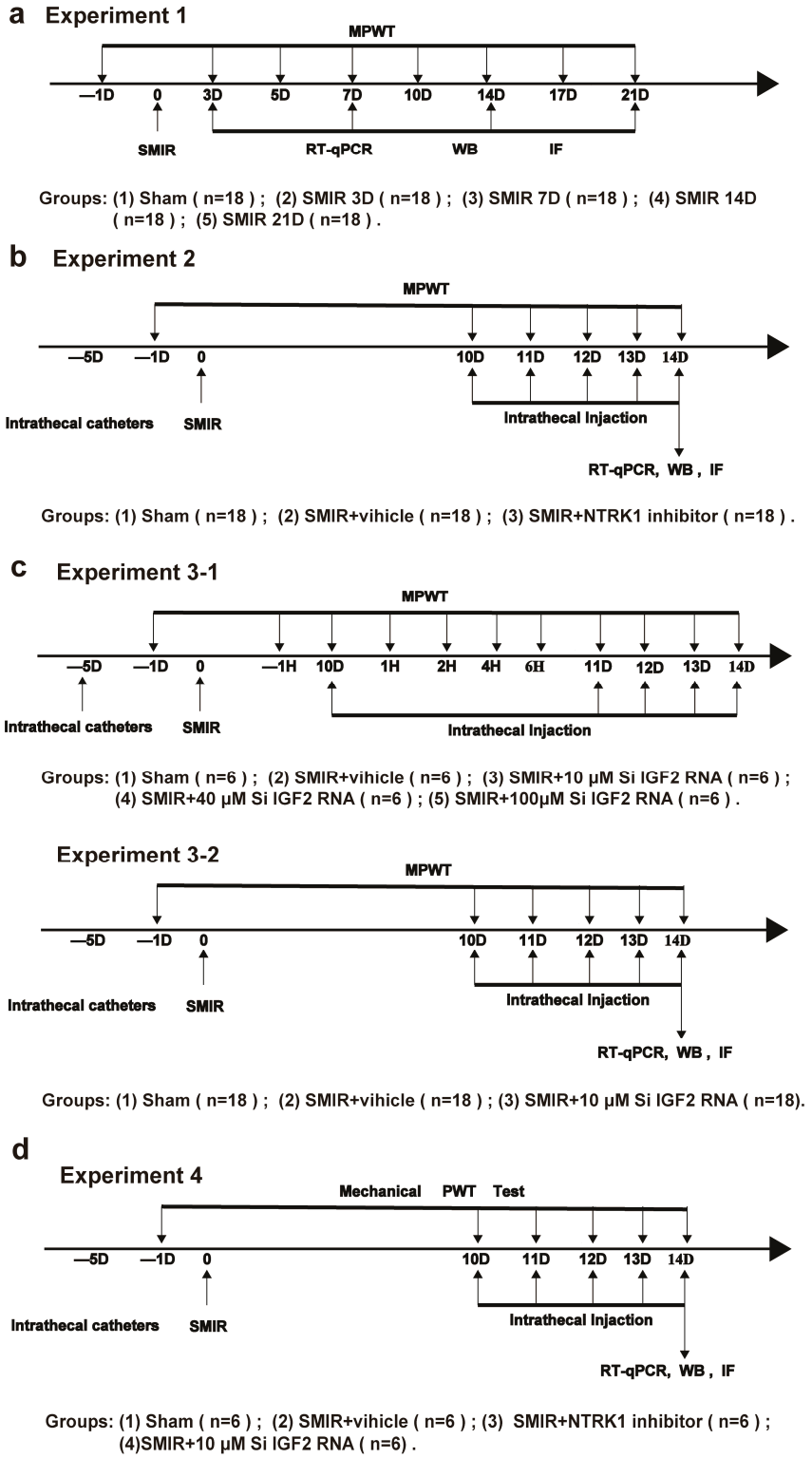


Figure 1. Experimental designs and animal groups. (**a**) Experiment 1: Assessment of NTRK1, ER stress, and IGF2 alterations in the spinal cord of rats with SMIR. (**b**) Experiment 2: Evaluation of NTRK1 inhibitor effects on mechanical allodynia and ER stress expression in rats with SMIR. (**c**) Experiment 3-1: Determination of the effective dose of IGF2 siRNA on mechanical allodynia in rats with SMIR; Experiment 3-2: Effect of IGF2 siRNA on mechanical allodynia and ER stress expression in SMIR rats. (**d**) Experiment 4: Comparison of the effects of NTRK1 inhibitor and IGF2 siRNA on the expression of IGF2 and NTRK1, and their relationship in rats with SMIR. SMIR: skin–muscle incision and retraction; MPWT: mechanical paw withdrawal threshold measurement; RT-qPCR: real-time quantitative polymerase chain reaction; WB: Western blot; IF: Immunofluorescence staining.

2.3. Skin/Muscle Incision and Retraction (SMIR) Model

The SMIR model was established as described by Flatters et al. [52], with modifications to optimize analgesia and aseptic conditions. Under sodium pentobarbital anesthesia (50 mg/kg i.p., pedal reflex loss confirmed), the right medial thigh was shaved and sterilized with povidone-iodine. A 1.8 cm skin incision exposed the gracilis muscle, followed by an 8 mm superficial muscle incision. A microdissection retractor (70% ethanol-sterilized) was inserted to maintain 2 cm tissue retraction for 60 min, with saline-moistened sterile gauze preventing dehydration. Muscle and skin were closed with 5-0 absorbable sutures in anatomical layers. Sham controls underwent identical procedures, excluding retraction. Postoperatively, rats received subcutaneous lactated Ringer's solution (5 mL/100 g) and were monitored for 72 h for infection or distress, with buprenorphine analgesia (0.05 mg/kg s.c. q12h) administered throughout.

2.4. Intrathecal Catheterization

Intrathecal catheterization was performed 5 days prior to SMIR surgery under sodium pentobarbital anesthesia (50 mg/kg i.p.) using established techniques [53]. Polyethylene catheters (PE-10; inner diameter 0.28 mm, outer diameter 0.61 mm) were aseptically inserted through the L5-L6 intervertebral space and advanced 2.0 cm rostrally into the intrathecal space. Correct placement was verified by dual methods: immediate tail-flick response upon insertion and transient bilateral hindlimb paralysis following intrathecal lidocaine administration (2%, 10 μL). Rats exhibiting persistent motor dysfunction (>2 h post-procedure) or cerebrospinal fluid leakage were excluded per predefined criteria (no exclusions required in this cohort).

2.5. Drug Administration

Pharmacological interventions were administered intrathecally with rigorous validation and blinding protocols. The NTRK1 inhibitor GW441756 (Selleck Chemicals #S2789) was dissolved in saline containing $\leq$1% DMSO, with the 100 μM selected based on pilot dose-response experiments establishing ED_{80} efficacy (10–200 μM range) in Table 1 [28]. For IGF2 knockdown, commercially synthesized SiRNA targeting rat IGF2 (sense: 5′-GCAAGUUCUUCAAAUUCGA-3′; antisense: 5′-UCGAAUUUGAAGAACUUGC-3′, Tsingke Biotechnology) was complexed with polyethyleneimine (1:5 *w*/*w* in 5% glucose) for 10 min before administration. This siRNA sequence demonstrated >70% mRNA reduction (RT-qPCR) and >65% protein knockdown (Western blot) versus scramble controls, with specificity confirmed by BLAST 2.14.0 analysis. Both single-dose (day 10) and repeated-dose (days 10–14) regimens were evaluated at concentrations of 10–100 μM [51]. The vehicle controls included (1) saline + $\leq$1% DMSO for the GW441756 groups, and (2) PEI/glucose solution for the siRNA groups. All treatments were assigned via computer-randomized sealed envelopes opened by an independent technician to maintain blinding integrity.

2.6. Mechanical Allodynia Assessment

Mechanical paw withdrawal thresholds (PWTs) were quantified by a blinded investigator using calibrated von Frey filaments (0.4–15.0 g; Stoelting #58011) following the up–down method [54] with strict environmental controls (22–25 °C, 45–65% humidity, background noise < 50 dB). Rats underwent 30 min habituation in opaque plexiglass chambers (20 × 25 × 15 cm) on an elevated mesh platform. Filaments were applied perpendicularly to the mid-plantar hindpaw for 6 s, with positive responses (rapid withdrawal, licking, or shaking) prompting testing of the next finer filament, and negative responses leading to the next coarser filament after a 5 min interval. The 50% threshold was calculated using

Dixon's formula [54,55]. Testing occurred between 9:00 and 12:00 to minimize circadian influences, with exclusion criteria for aberrant responses (<0.4 g or >15.0 g baselines) [55].

Table 1. Pharmacological interventions.

Agent	Formulation	Administration	Dose Rationale
GW441756 (NTRK1i; Selleck #S2789)	100 μM in saline + 1% DMSO	i.t. daily, days 10–14	Dose-response pilot [28]
IGF2 siRNA (Tsingke Biotechnology)	10–100 μM in 5% glucose + PEI (1:5 *w*/*w*)	i.t. single dose (day 10)	Prior efficacy in neuropathic pain [51]
IGF2 siRNA (Tsingke Biotechnology)	10 μM in 5% glucose + PEI (1:5 *w*/*w*)	i.t. daily, days 10–14	Prior efficacy in neuropathic pain [51]
Vehicle controls	Saline + 1% DMSO or PEI/glucose	Matched volumes/timing	N/A

2.7. Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed following MIQE guidelines to ensure rigor [56]. Total RNA was extracted from L4-L6 spinal cord segments using TRIzol reagent (Takara, Cat# 9109), with RNA integrity verified (RIN > 8.0, Agilent Bioanalyzer 2100, Santa Clara, CA, USA) and purity confirmed spectrophotometrically (NanoDrop 2000, Wilmington, DE, USA; A260/A280: 1.8–2.0). After DNase I treatment (Vazyme Biotech Co., Ltd, Nanjing, China, Ca # EN401), 1 μg RNA was reverse-transcribed using HiScript III RT SuperMix (Vazyme, Cat# R323-01) with genomic DNA wiper (Vazyme, Nanjing, China, Cat# R323-01). qPCR reactions were run in triplicate on a QuantStudio 5 system (Applied Biosystems (Thermo Fisher Scientific), Waltham, MA, USA) using ChamQ Universal SYBR mix (Vazyme #Q711-02) with 200 nM primers. Gene-specific primers (Table 2) were designed via NCBI Primer-BLAST and validated by (1) single-peak melt curves (65–95 °C ramp), (2) 2% agarose gel confirmation of amplicon size, and (3) BLAST verification against rat transcriptome. Amplification efficiency (95–105%) was established through five-point serial cDNA dilutions ($R^2 > 0.99$). The thermocycling parameters were 95 °C for 30 s (initial denaturation); 40 cycles of 95 °C for 10 s and 60 °C for 30 s; followed by melt curve analysis (95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s). Data were normalized to Gapdh (geNorm stability value M < 0.5) using the $2^{(-\Delta\Delta Ct)}$ method [57], with no-template and no-RT controls included per plate.

Table 2. The primer sequences used for quantitative PCR assay.

Gene	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)
GAPDH	GAAGGTCGGTGTGAACGGAT	CCCATTTGATGTTAGCGGGAT
NTRK1	AGGAGGATTTGTGTGGTGTGTAT	GAGTCATTGGGCATCTGGATCTT
IGF2	GGGAAGTCGATGTTGGTGCT	AAGCAGCACTCTTCCACGAT
GRP78	GGTTGGCGGATCTACTCGAATTC	AAGAGGACACACATCAAGCAGAA
XBP1	CCCAGAACATCTTCCCATGGATT	CAGAGAAAGGGAGGCTGGTAAG
CHOP	TTCATACACCACCACACCTGAAA	TAGGGATGCAGGGTCAAGAGTAG
ATF6	AGCAAGATTCCAGGAGAGTGAAA	TGACATGGAGGTGGAGGGATATA
PERK	TTGGAAGGTCATGGCGTTTAGTA	TGGCCTCTGTACATCCCTAAGTA
IRE1α	TCAAGGCGATGATCTCAGACTTT	GTTGCCCTCAGAGATGACATAGT

2.8. Western Blot Analysis

Western blot analysis was performed under blinded conditions with rigorous validation controls. L4-L6 spinal cord tissues were homogenized in ice-cold RIPA lysis buffer

(Boster Biological Technology, Pleasanton, CA, USA, Cat #AR0102) containing 1× protease/phosphatase inhibitor cocktail (Boster, Cat #AR1182) and 1 mM PMSF. Lysates were centrifuged at 12,000× *g* for 30 min at 4 °C, and supernatants were quantified via BCA assay (Boster, Cat #AR0146) against BSA standards ($R^2 > 0.99$). Proteins (30 μg/lane) were separated on 10% SDS-PAGE gels (Bio-Rad, Cat #4561096) and transferred to 0.45 μm PVDF membranes (Millipore, Burlington, MA, USA, Cat #IPVH00010) using semi-dry transfer (Bio-Rad; 75 V, 30 min). Membranes were blocked in 5% BSA (phospho-antibodies) or 5% non-fat milk (non-phospho) in TBST for 1 h, followed by overnight incubation at 4 °C with validated primary antibodies (Table 3). Antibody specificity was confirmed through (1) siRNA knockdown (>50% target reduction), (2) peptide blocking controls, and (3) λ-phosphatase treatment for phospho-epitopes. After TBST washes, membranes were incubated with HRP-conjugated secondary antibodies (1:5000; CST #7074) for 2 h at RT. Signals were detected using SuperLumia ECL Plus (Abbkine Scientific Co., Ltd, Wuhan, China; #K22030) and imaged on a ChemiDoc XRS+ (Bio-Rad) within the linear dynamic range. Band intensities were quantified in Image Lab 6.1 (Bio-Rad), normalized to β-actin (Cell Signaling Technology, Danvers, MA, USA; #4970; 1:1000), with three technical replicates per sample.

Table 3. The detailed information on antibodies.

Antibodies	Source	Identifier	Dilution	Applications
Anti-TrkA antibody	ABclonal	A15618	1:1000,1:50	WB,IF
Anti-IGF2 antibody	CST	Ab9574	1:2000	WB
Anti-IGF2 antibody	ABclonal	A2086	1:50	IF
Anti-GRP78 antibody	Affinity	Cat# AF5366	1:1000,1:50	WB,IF
Anti-β-actin antibody	Proteintech	Cat# 81115-1-RR	1:5000	WB
Anti-p-PERK antibody	Affinity	Cat# DF7576	1:100	WB
Anti-PERK antibody	ABclonal	Cat# A21255	1:1000	WB
Anti-p-IRE1α antibody	Affinity	Cat# AF7150	1:100	WB
Anti-IRE1α antibody	Proteintech	Cat# 27528-1-AP	1:1000	WB
Anti-p-eIF antibody	Affinity	Cat# AF3087	1:100	WB
Anti-eIF antibody	Proteintech	Cat# 11170-1-AP	1:1000	WB
Anti-XBP1 antibody	Proteintech	Cat# 24868-1-AP	1:1000	WB
Anti-CHOP antibody	ABclonal	A20987	1:1000	WB
Anti-ATF6 antibody	Affinity	Cat# DF6009	1:1000	WB
Anti-NeuN antibody	Abcam	Ab104224	1:200	IF
Anti-GFAP antibody	CST	Cat# 3670	1:200	IF
Anti-IBA1 antibody	Abcam	Ab5076	1:200	IF
oraLite594 anti-rabbit IgG	Proteintech	Cat# SA00013-8	1:200	IF
oraLite488 anti-mouse IgG	Proteintech	Cat# SA00013-5	1:200	IF
FITC affinipure anti-goat IgG	Proteintech	Cat# SA00003-3	1:200	IF
Anti-rabbit IgG HRP	Abbkine	Cat# A21020	1:5000	WB
Anti-goat IgG HRP	ABclonal	Cat# AS031	1:5000	WB

2.9. Immunofluorescence Staining

Immunofluorescence staining was performed under blinded conditions with comprehensive antibody validation. Following transcardial perfusion (0.1 M PBS → 4% PFA), L4-L6 spinal cord segments were post-fixed in 4% PFA for 24 h at 4 °C, cryoprotected in 30% sucrose (72 h), and embedded in OCT. Coronal sections (20 μm) were cut on a cryostat (Leica Microsystems, Wetzlar, Germany; CM1900) and mounted on Superfrost Plus slides. After antigen retrieval (10 mM citrate buffer, 95 °C, 15 min for nuclear targets), sections were permeabilized (0.3% Triton X-100, 15 min) and blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA, Cat #S-1000) for 1 hr. Primary antibodies

(Table 3) were incubated overnight at 4 °C after validation with (1) knockout tissue controls, (2) isotype-matched IgG, and (3) peptide absorption tests. After PBS washes, sections were incubated with Alexa Fluor-conjugated secondaries (1:500; Invitrogen ™ (Thermo Fisher Scientific), Carlsbad, CA, USA, Cat #A-11008) for 2 h at RT, followed by DAPI nuclear counterstain (1 μg/mL, 10 min). Images were captured on an Olympus BX51 microscope with 20×/0.75 NA objective using standardized settings (exposure: 200 ms, gain: 2×, z-stack: 1 μm intervals). Quantification was performed using ImageJ (Fiji v2.3) with rolling-ball background subtraction (50-pixel radius) [58] and threshold-based object detection (3 sections/rat, 3 fields/section, dorsal horn laminae I–IV).

2.10. Experimental Designs and Animal Groups

This study employed a randomized, blinded design across four sequential experiments investigating the NTRK1-IGF2-ER stress axis in CPSP pathogenesis (summarized in Figure 1). All procedures complied with ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care Committee (TJH-202211016).

Experiment 1: Assessment of NTRK1, ER stress, and IGF2 alterations in the spinal cord of rats with SMIR.

Temporal Profiling (n = 90 rats) assessed dynamic changes in the NTRK1-IGF2-ER stress axis post-SMIR. Rats were randomized to Sham (n = 18), SMIR-3D (n = 18), SMIR-7D (n = 18), SMIR-14D (n = 18), and SMIR-21D (n = 18). Mechanical allodynia was tested at baseline (−1 d) and days 1, 3, 5, 7, 10, 14, 17, 21 post-surgery, with spinal cord collection at terminal timepoints for molecular analyses.

Experiment 2: Evaluation of NTRK1 inhibitor effects on mechanical allodynia and ER stress expression in rats with SMIR.

GW441756-mediated NTRK1 inhibition was evaluated in 54 rats. The groups included Sham+Veh (n = 18), SMIR+Veh (n = 18), and SMIR+NTRK1i (100 μM, i.t. days 10–14; n = 18). Behavioral tests occurred 2 h post-injection on days 10–14, with tissue harvest on day 14. The 100 μM dose was selected based on pilot ED_{80} determination (10–200 μM range) [28].

Experiment 3: Determination of the effective dose of IGF2 siRNA on mechanical allodynia in rats with SMIR

Experiment 3-1 Dose-Finding

Dose-Finding (n = 30 rats): Sham+Veh (n = 6), SMIR+Veh (n = 6), SMIR+siIGF2-L/M/H (10/40/100 μM single i.t. dose day 10; n = 6/group). PWT was measured at −1 h, +1 h, +2 h, +4 h, and +6 h post-injection.

Experiment 3-2 Chronic Efficacy

Chronic Efficacy (n = 54 rats): Sham+Veh (n = 18), SMIR+Veh (n = 18), SMIR+siIGF2 (10 μM i.t. days 10–14; n = 18). Tests occurred 2 h post-injection (days 10–14), with day 14 tissue collection.

Experiment 4: Comparison of the effects of NTRK1 inhibitor and IGF2 siRNA on the expression of IGF2 and NTRK1, and their relationship in rats with SMIR

A total of 24 rats were randomly divided into the following groups to examine NTRK1→IGF2 directionality: Sham+Veh (n = 6), SMIR+Veh (n = 6), SMIR+NTRK1i (100 μM; n = 6), and SMIR+siIGF2 (10 μM; n = 6). Drugs were administered on days 10–14 (i.t.), with behavioral tests 2 h post-injection and tissue analysis on day 14.

Blinded investigators performed all outcome assessments, with randomization via computer-generated codes and allocation concealment through coded cages. Sample sizes

were predetermined by power analysis ($\alpha = 0.05$, $\beta = 0.2$, effect size = 1.2) with a 20% attrition buffer.

2.11. Statistical Analyses

Data were analyzed using GraphPad Prism 9.0 and SPSS 26.0 after verifying normality (Shapiro–Wilk) and variance homogeneity (Brown–Forsythe). Parametric data (presented as mean ± SEM) were assessed by two-way RM-ANOVA with Geisser–Greenhouse correction for longitudinal behavior, and one-way ANOVA with Tukey/Games–Howell post hoc tests for molecular data. The significance threshold was * $p < 0.05$.

3. Results

3.1. Temporal Upregulation of Neuronal NTRK1 Drives CPSP Pathogenesis

The SMIR model consistently induced chronic postsurgical pain, manifested as progressive mechanical hypersensitivity developing postoperatively (Figure 2d). Immunofluorescence analysis confirmed predominant neuronal localization of TrkA within $NeuN^+$ cells of the spinal dorsal horn, with minimal expression in $GFAP^+$ astrocytes or $IBA1^+$ microglia (Figure 2a–c). Quantitative assessment revealed enhanced TrkA/NeuN co-localization intensity across postoperative timepoints, indicating neuronal-specific NTRK1 overexpression that correlated with pain severity (Figure 2e). Molecular profiling demonstrated time-dependent upregulation of NTRK1 signaling, with transcriptional activation peaking at day 14 post-SMIR and corresponding protein elevation mirroring this trajectory (Figure 2g,i). Crucially, strong inverse correlations existed between NTRK1 expression levels and mechanical pain thresholds throughout the observation period. These findings establish temporal induction of neuronal NTRK1 as a central pathogenic driver in CPSP, with molecular-behavioral correlations confirming its critical role in pain maintenance.

3.2. NTRK1 Inhibition Reverses Mechanical Hypersensitivity

Intrathecal administration of the NTRK1 inhibitor GW441756 (100 μM) significantly attenuated mechanical allodynia in SMIR rats within 2 h post-injection, as evidenced by normalized paw withdrawal thresholds (Figure 3a) [28,59]. This analgesic effect coincided with concomitant suppression of NTRK1 signaling at multiple levels: reduced *NTRK1* transcription, diminished TrkA protein expression, and decreased neuronal TrkA/NeuN co-localization intensity (Figure 3b,d,e), confirming comprehensive target engagement. Dose-dependent inverse correlations were observed between the magnitude of NTRK1 pathway suppression and mechanical hypersensitivity resolution across all timepoints. These findings collectively establish NTRK1 signaling as a critical pathogenic driver in CPSP, demonstrating that its pharmacological inhibition simultaneously reverses both behavioral manifestations and associated molecular pathology.

3.3. Neuronal ER Stress Activation Underlies CPSP Pathophysiology

SMIR surgery triggered progressive ER stress activation in spinal dorsal horn neurons, characterized by temporally coordinated upregulation of the master UPR regulator GRP78. Immunofluorescence analysis confirmed predominant neuronal localization of GRP78 within $NeuN^+$ cells, with minimal detection in $GFAP^+$ astrocytes or $IBA1^+$ microglia (Figure 4a–c). Quantitative assessment revealed sustained GRP78 induction at both transcriptional and protein levels, peaking at postoperative day 14 and remaining elevated through day 21 (Figure 4d,e). Enhanced GRP78/NeuN co-localization intensity paralleled mechanical hypersensitivity development, with maximal neuronal expression at day 14 demonstrating a strong inverse correlation with pain thresholds (Figure 4f,g).

Comprehensive pathway analysis confirmed simultaneous activation of all three UPR branches: PERK/eIF2α axis: Phosphorylation cascade involving PERK and eIF2α with transcriptional amplification (Figure 5b,g,h); IRE1α/XBP1 pathway: Increased IRE1α phosphorylation and XBP1s generation at translational and transcriptional levels (Figure 5c,k); ATF6/CHOP cascade: Nuclear translocation of ATF6 and CHOP induction with concordant mRNA elevation (Figure 5d,f,j,l).

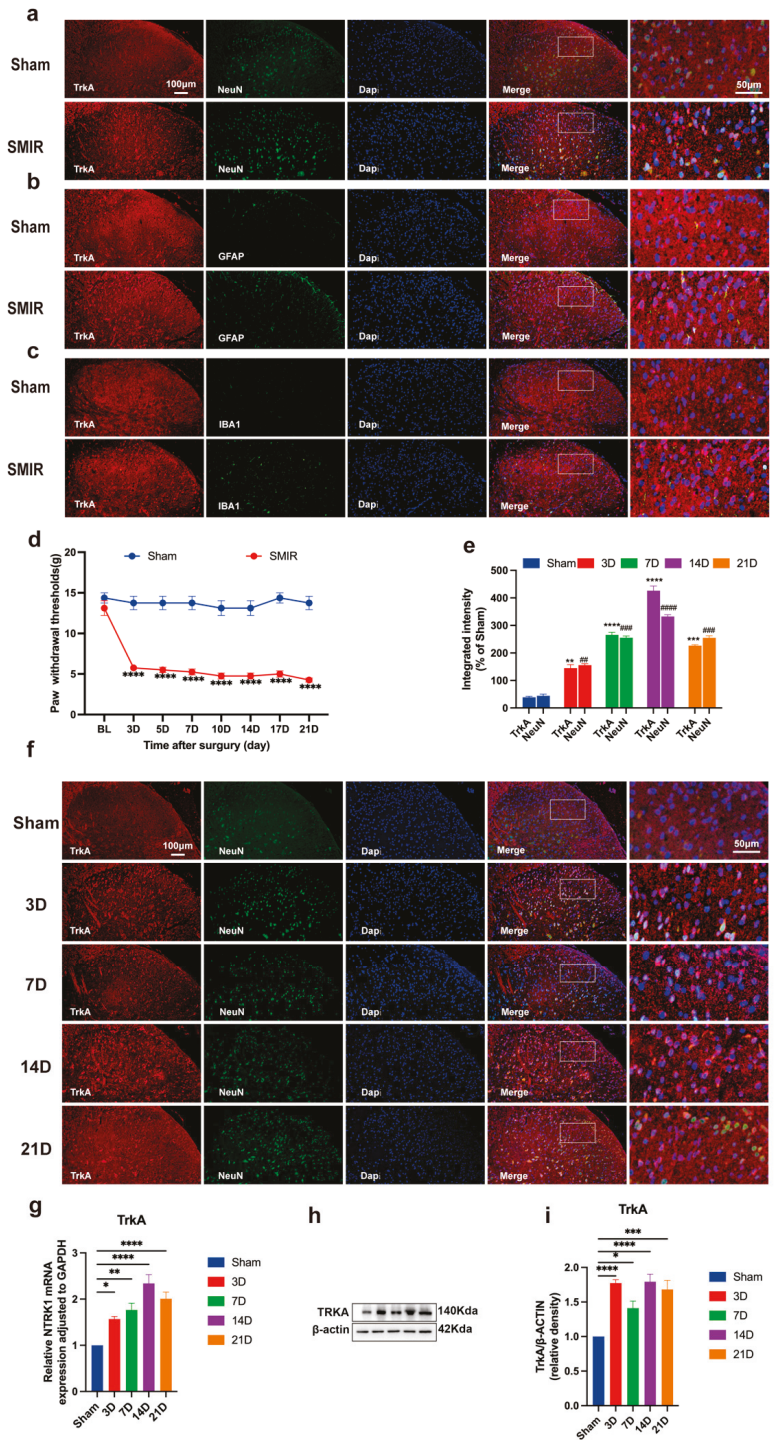


Figure 2. Temporal upregulation of NTRK1 in spinal dorsal horn neurons following SMIR surgery. (**a**–**c**) Representative immunofluorescence images showing NTRK1 (TrkA) co-localization with (**a**) neuronal (NeuN), (**b**) astrocytic (GFAP), and (**c**) microglial (IBA1) markers in spinal cord sections.

Scale bars: 100 μm (overview), 50 μm (insets). (**d**) Mechanical hypersensitivity progression measured by paw withdrawal threshold (PWT) **** $p < 0.0001$ compared with the Sham group, n = 6/group). (**e**) Quantification of TrkA/NeuN co-localization intensity across postoperative timepoints (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with the Sham group; ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ compared with the Sham group; $n = 3$/group) (**f**) Immunofluorescence visualization of NTRK1 expression dynamics in dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). (**g**) qPCR analysis of *NTRK1* transcriptional regulation (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared with the Sham group, $n = 6$/group).(**h**,**i**) Western blot assessment of NTRK1 protein expression (representative blots and quantification, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ compared with the Sham group, n = 6/group). SMIR, Skin/Muscle Incision and Retraction.

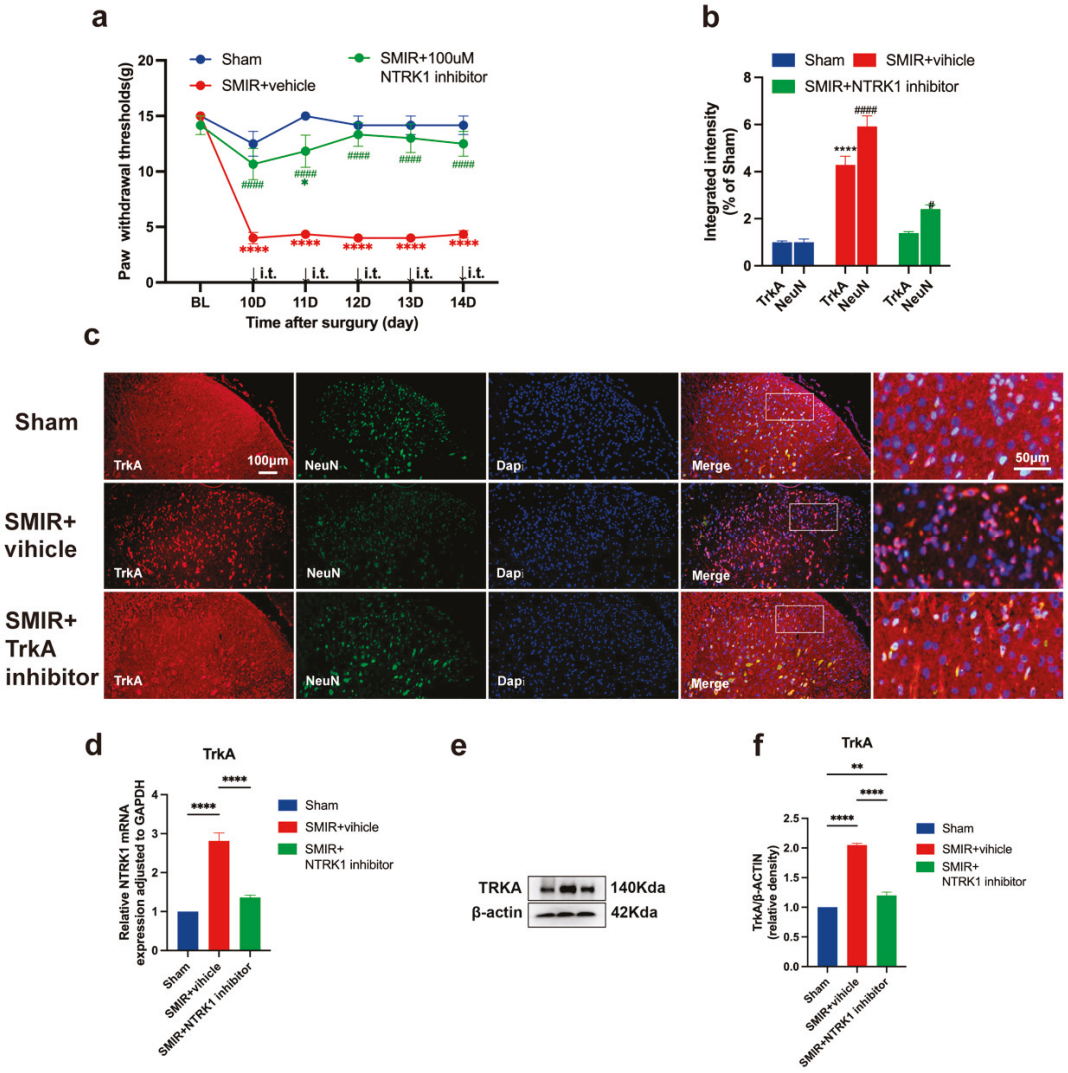


Figure 3. Therapeutic impact of intrathecal NTRK1 inhibition on mechanical allodynia and NTRK1 expression in SMIR rats. (**a**) Mechanical hypersensitivity measured by paw withdrawal threshold (PWT) following intrathecal administration of NTRK1 inhibitor (green) versus vehicle (red) (* $p < 0.05$, **** $p < 0.0001$ compared with the Sham group; #### $p < 0.001$ compared with the SMIR + Vehicle group; n = 6/group). (**b**) Quantification of TrkA/NeuN co-localization intensity across treatment groups (**** $p < 0.0001$ compared with the Sham group; # $p < 0.05$, #### $p < 0.0001$ compared with the Sham group; $n = 3$/group). (**c**) Representative immunofluorescence images showing NTRK1 expression in the spinal dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). (**d**) qPCR analysis of *NTRK1* transcriptional regulation (**** $p < 0.0001$; $n = 6$/group). (**e**,**f**) Western blot assessment of NTRK1 protein expression (representative blots and quantification, ** $p < 0.01$, **** $p < 0.0001$; $n = 6$/group). SMIR, Skin/Muscle Incision and Retraction.

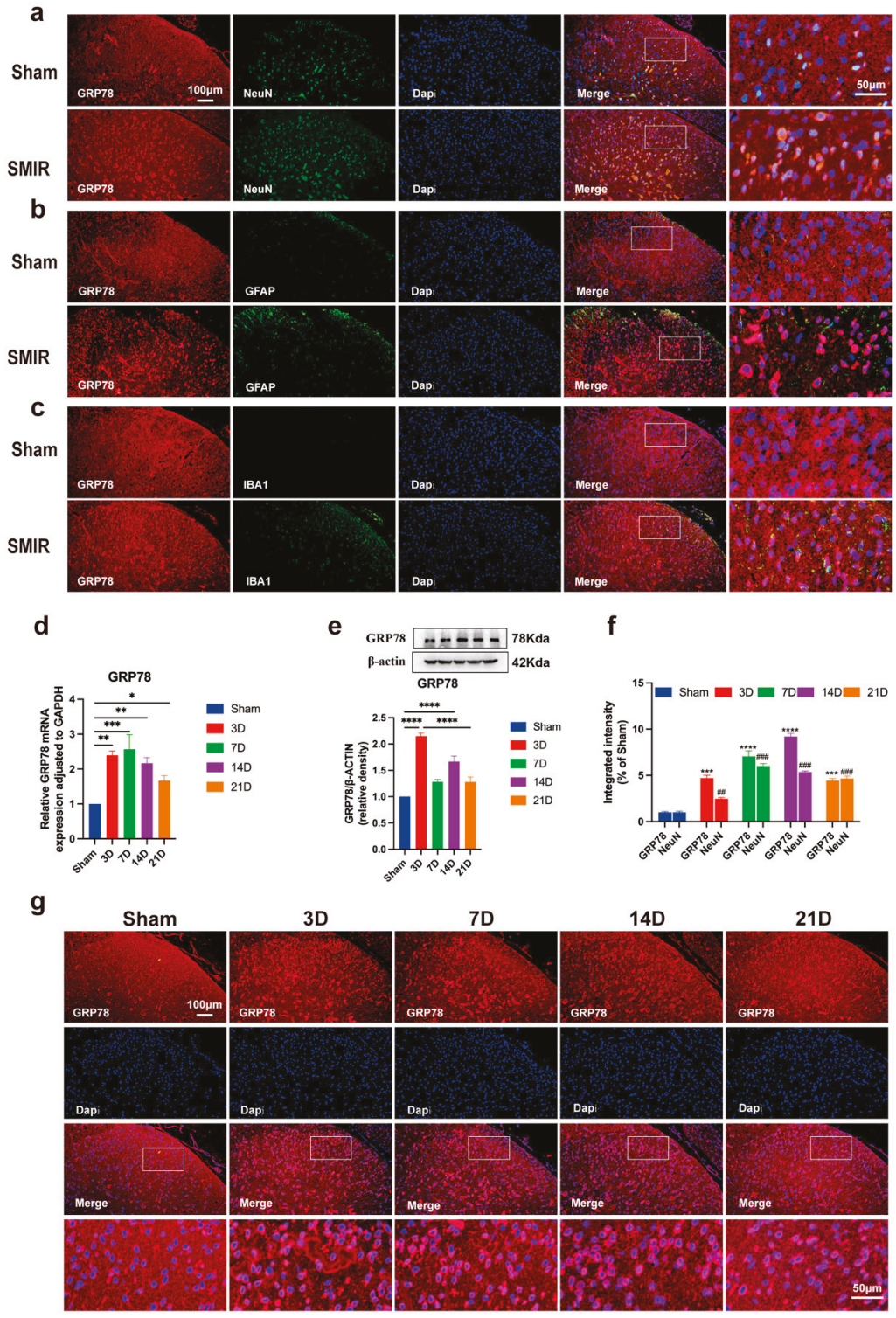


Figure 4. Temporal upregulation of GRP78 in spinal dorsal horn following SMIR surgery. (**a**–**c**) Representative immunofluorescence images showing GRP78 co-localization with (**a**) neuronal (NeuN), (**b**) astrocytic (GFAP), and (**c**) microglial (IBA1) markers in spinal cord sections. Scale bars: 100 μm (overview), 50 μm (insets). (**d**) qPCR analysis of *GRP78* transcriptional regulation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 6/group). (**e**) Western blot assessment of GRP78 protein expression (representative blots and quantification, **** $p < 0.0001$; n = 6/group). (**f**) Quantification of GRP78/NeuN co-localization intensity across postoperative timepoints (*** $p < 0.001$, **** $p < 0.0001$ compared with the Sham group; ## $p < 0.01$, ### $p < 0.001$, compared with the Sham group; n = 3/group). (**g**) Immunofluorescence visualization of GRP78 expression dynamics in the dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). SMIR, Skin/Muscle Incision and Retraction.

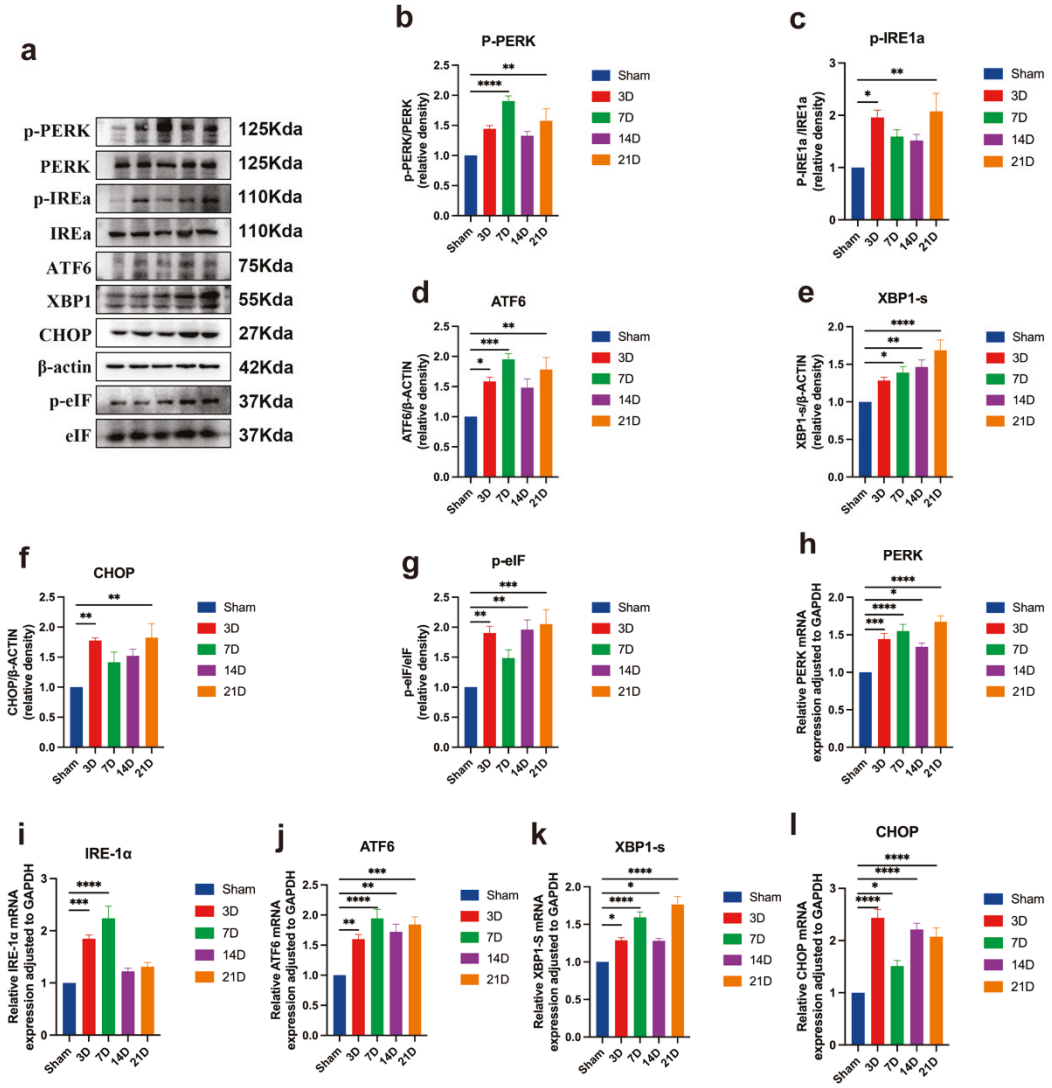


Figure 5. Temporal activation of endoplasmic reticulum stress pathways in spinal cord of SMIR rats. (**a**) Representative western blots of UPR signaling proteins. (**b**–**g**) Quantification of key ER stress markers: p-PERK, p-IRE1α, p-eIF2α, XBP1s, ATF6, and CHOP. Temporal profiles show progressive upregulation from days 3 to 21 post-SMIR (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**h**–**l**) qPCR analysis of corresponding transcriptional regulation: *PERK*, *IRE1α*, *XBP1s*, *ATF6*, and *CHOP* (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). SMIR, Skin/Muscle Incision and Retraction.

These coordinated molecular responses establish robust ER stress activation as a fundamental pathophysiological mechanism in CPSP. The temporal concordance between UPR marker elevation and behavioral hypersensitivity, substantiated by strong molecular-behavioral correlations, demonstrates that neuronal proteotoxic stress drives CPSP pathogenesis.

3.4. NTRK1 Inhibition Attenuates ER Stress in Spinal Dorsal Horn

To elucidate the NTRK1-ER stress interplay in CPSP pathogenesis, we assessed the impact of intrathecal NTRK1 inhibition (GW441756) on UPR activation. SMIR+vehicle rats exhibited substantial GRP78 upregulation at transcriptional and protein levels compared to sham controls, which was markedly attenuated by GW441756 treatment (Figure 6a,b). Immunofluorescence analysis confirmed reduced GRP78 neuronal expression and diminished co-localization with $NeuN^+$ neurons following NTRK1 inhibition (Figure 6c,d).

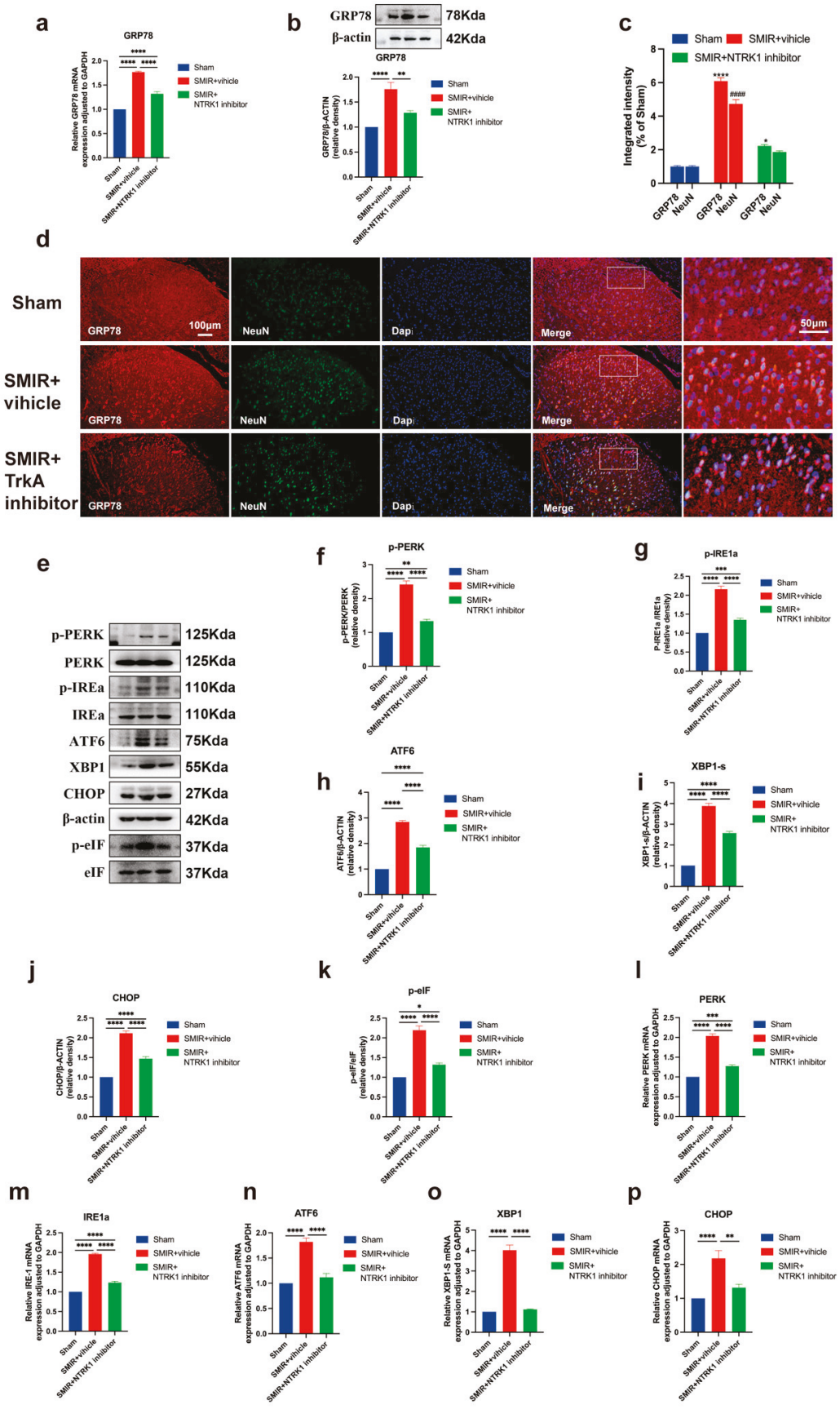


Figure 6. NTRK1 inhibition suppresses ER stress activation in SMIR rats. (**a**) qPCR analysis of *GRP78* transcriptional regulation following intrathecal NTRK1 inhibitor administration (**** $p < 0.0001$;

n = 6/group). (**b**) Western blot assessment of GRP78 protein expression (representative blots and quantification, ** $p < 0.01$, **** $p < 0.0001$; n = 6/group). (**c**) Quantification of GRP78/NeuN co-localization intensity across treatment groups (* $p < 0.05$, **** $p < 0.0001$ compared with the Sham group; #### $p < 0.0001$ compared with the Sham group; n = 3/group). (**d**) Immunofluorescence visualization of GRP78 expression in the spinal dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). (**e**) Representative western blots of UPR signaling proteins. (**f**–**k**) Quantification of key ER stress markers: p-PERK, p-IRE1α, p-eIF2α, XBP1s, ATF6, and CHOP. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**l**–**p**) qPCR analysis of corresponding transcriptional changes: *PERK*, *IRE1α*, *XBP1s*, *ATF6*, and *CHOP* (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group).

Comprehensive pathway evaluation revealed that SMIR-induced phosphorylation of PERK/eIF2α, IRE1α activation, XBP1s generation, and ATF6/CHOP induction were significantly reversed by NTRK1 inhibition at both translational and transcriptional levels (Figure 6e–p). Crucially, the magnitude of ER stress marker suppression strongly correlated with mechanical pain threshold recovery, with key markers including GRP78, p-PERK, and CHOP.

These findings demonstrate that NTRK1 drives ER stress through coordinated PERK, IRE1α, and ATF6 pathway activation in CPSP, and that pharmacological inhibition concurrently alleviates proteotoxic stress and pain behavior through quantifiable molecular-behavioral linkages.

3.5. SMIR Surgery Upregulates Neuronal IGF2 in Spinal Dorsal Horn

Spatiotemporal profiling revealed significant IGF2 induction in spinal dorsal horn neurons following SMIR surgery. Immunofluorescence analysis confirmed predominant neuronal localization of IGF2, with robust co-localization in $NeuN^+$ cells and minimal detection in $GFAP^+$ astrocytes or $IBA1^+$ microglia (Figure 7a–c). Quantitative assessment demonstrated progressive IGF2 upregulation from postoperative day 3 to peak expression at day 14, persisting through day 21 at both transcriptional and protein levels (Figure 7d,e). Enhanced IGF2/NeuN co-localization intensity directly correlated with mechanical allodynia severity (Figure 7f,g), establishing IGF2 as a molecular correlate of CPSP progression.

These findings delineate a neuron-specific IGF2 induction pattern that temporally parallels pain hypersensitivity development, positioning IGF2 as both a biomarker and potential mediator of CPSP pathogenesis.

3.6. IGF2 Silencing Reverses Mechanical Hypersensitivity

To establish IGF2's functional role in CPSP, we administered intrathecal IGF2 siRNA and quantified behavioral responses. A single injection (10–100 μM) produced dose-dependent attenuation of mechanical allodynia within 2 h, with maximal efficacy at 40–100 μM, restoring paw withdrawal thresholds (PWTs) to sham levels (Figure 8a). The 100 μM dose provided no additional benefit over 40 μM, while 10 μM achieved partial efficacy, establishing 40 μM as the optimal concentration for subsequent experiments.

Continuous daily administration of 40 μM IGF2 siRNA (postoperative days 10–14) sustained analgesic effects, maintaining PWT near sham levels throughout the treatment period (Figure 8b). Molecular analysis confirmed target engagement, with siRNA significantly reducing spinal IGF2 expression at both transcriptional and translational levels (Figure 8c–e). The magnitude of IGF2 suppression strongly correlated with mechanical threshold recovery (r = 0.91, 95% CI 0.85–0.95), with Cohen's d effect sizes >1.8 for all molecular endpoints.

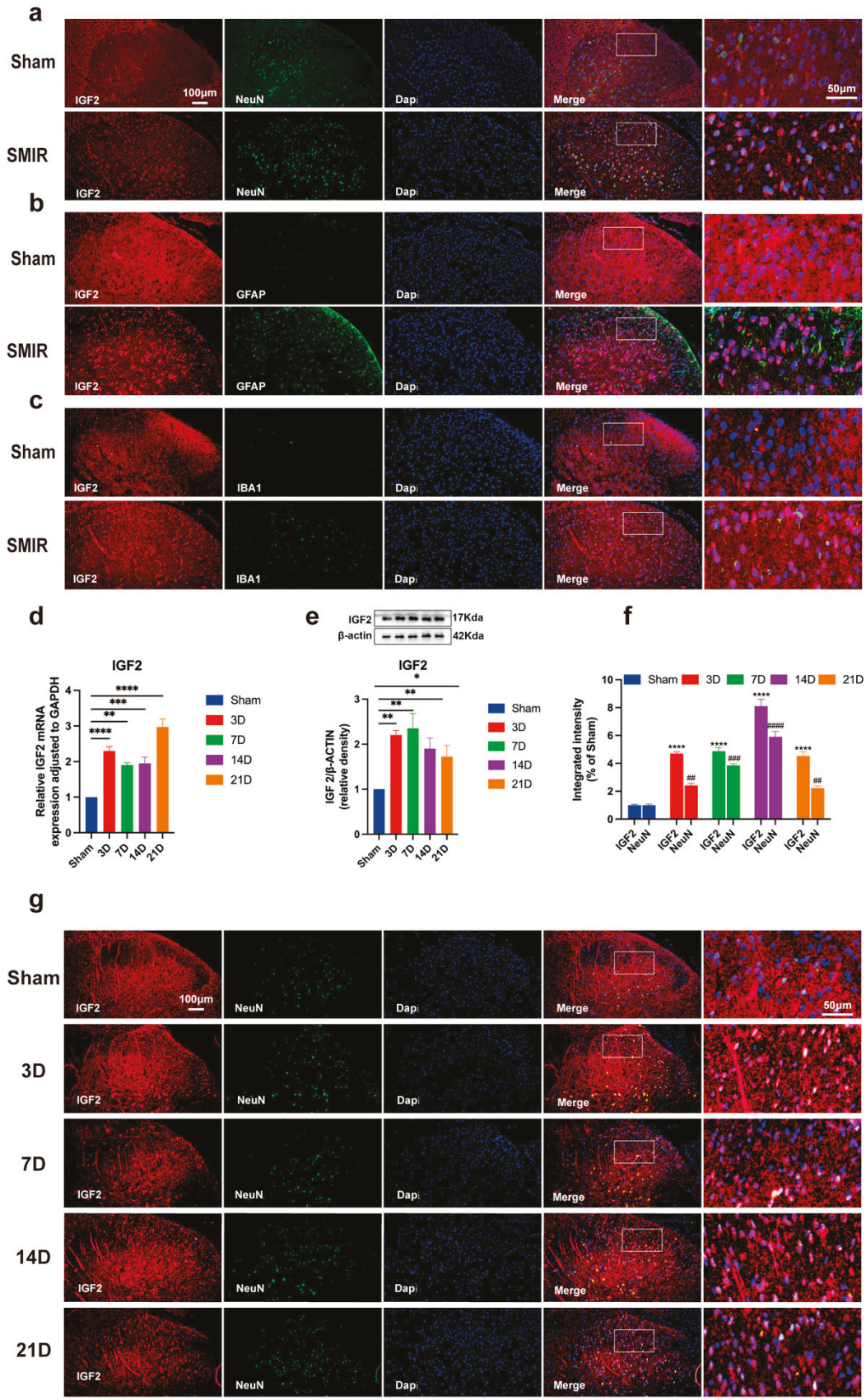


Figure 7. Temporal upregulation of IGF2 in spinal dorsal horn following SMIR surgery. (**a**–**c**) Representative immunofluorescence images showing IGF2 co-localization with (**a**) neuronal (NeuN),

(**b**) astrocytic (GFAP), and (**c**) microglial (IBA1) markers in spinal cord sections. Scale bars: 100 μm (overview), 50 μm (insets). (n = 3/group). (**d**) qPCR analysis of *IGF2* transcriptional regulation (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**e**) Western blot assessment of IGF2 protein expression (representative blots and quantification, * $p < 0.05$ ** $p < 0.01$; n = 6/group). (**f**) Quantification of IGF2/NeuN co-localization intensity across postoperative timepoints (**** $p < 0.0001$ compared with the Sham group; ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ compared with the Sham group; n = 3/group). (**g**) Immunofluorescence visualization of IGF2 expression dynamics in the dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). (n = 3/group).

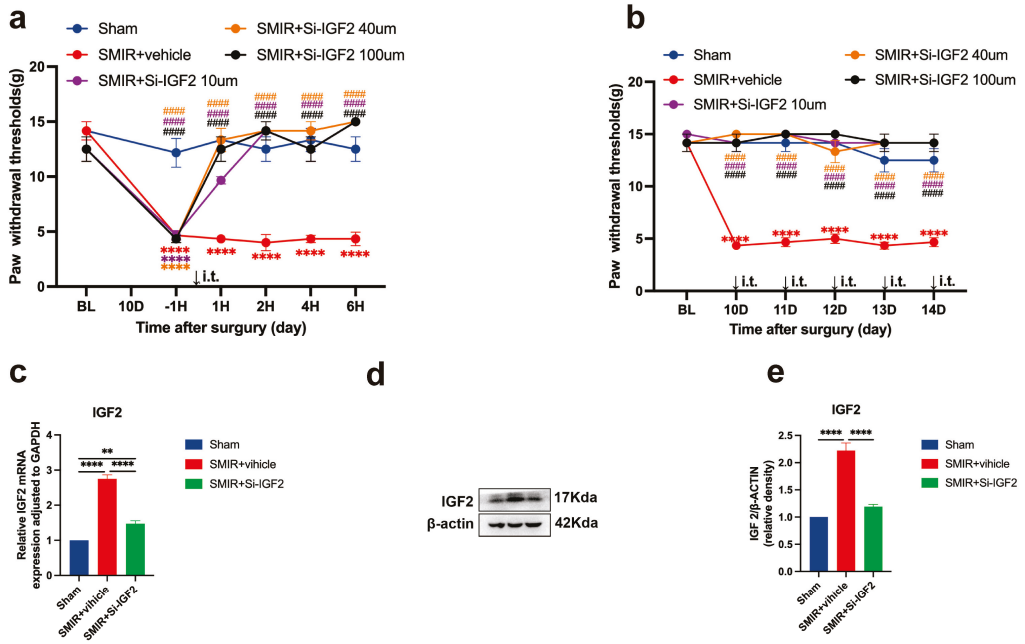


Figure 8. Therapeutic impact of IGF2 silencing on mechanical allodynia and IGF2 expression in SMIR rats. (**a**) Dose-dependent effects of single intrathecal IGF2 siRNA administration (10 μM, 40 μM, 100 μM) on mechanical hypersensitivity. (**** $p < 0.0001$ compared with the Sham group; #### $p < 0.0001$ compared with the SMIR + Vehicle group; n = 6/group). (**b**) Dose-response profile of continuous intrathecal IGF2 siRNA treatment on pain behavior. (**** $p < 0.0001$ compared with the Sham group; #### $p < 0.0001$ compared with the SMIR + Vehicle group; n = 6/group). (**c**) qPCR analysis of IGF2 transcriptional suppression. (** $p < 0.01$, **** $p < 0.0001$ compared with the Sham group; n = 6/group). (**d**,**e**) Western blot assessment of IGF2 protein expression (representative blots and quantification). (**** $p < 0.0001$ compared with the Sham group; n = 6/group).

These findings demonstrate that IGF2 silencing produces potent, dose-dependent antinociception in CPSP, with continuous administration achieving sustained analgesia through effective target suppression.

3.7. IGF2 Knockdown Attenuates ER Stress via GRP78 and UPR Pathways

To establish the mechanistic link between IGF2 and ER stress in CPSP, we evaluated the effects of IGF2 silencing on UPR activation. SMIR+vehicle rats exhibited substantial GRP78 upregulation at both transcriptional and protein levels compared to sham controls, which was markedly reversed by intrathecal IGF2 siRNA administration (Figure 9a–c). Immunofluorescence analysis confirmed reduced GRP78 neuronal expression in the spinal dorsal horn following IGF2 knockdown (Figure 9d).

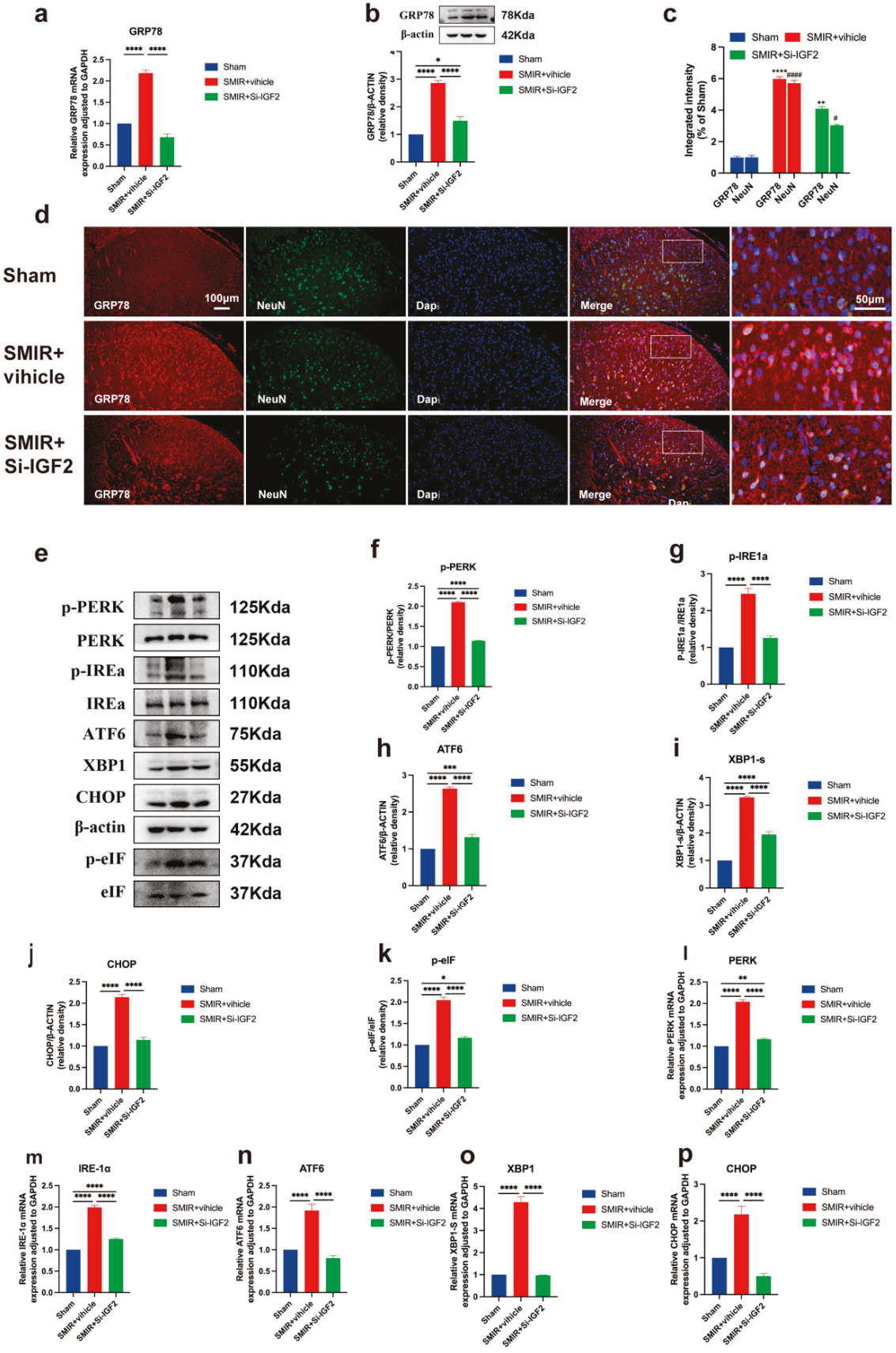


Figure 9. IGF2 silencing attenuates ER stress activation in SMIR rats. (**a**) qPCR analysis of *GRP78* transcriptional suppression following IGF2 siRNA administration (**** $p < 0.0001$ compared with the sham group; n = 6/group). (**b**) Western blot assessment of GRP78 protein expression (representative

blots and quantification, * $p < 0.05$, **** $p < 0.0001$ compared with the sham group; n = 6/group). (**c**) Quantification of GRP78/NeuN co-localization intensity across treatment groups (** $p < 0.01$, **** $p < 0.0001$ compared with the Sham group; $^{\#}$ $p < 0.05$, $^{\#\#\#\#}$ $p < 0.0001$ compared with the Sham group; n = 3/group). (**d**) Immunofluorescence visualization of GRP78 expression in the spinal dorsal horn. Scale bars: 100 μm (overview), 50 μm (insets). (**e**) Representative western blots of UPR signaling proteins. (**f**–**k**) Quantification of key ER stress markers: p-PERK, p-IRE1α, p-eIF2α, XBP1s, ATF6, and CHOP (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**l**–**p**) qPCR analysis of corresponding transcriptional changes: *PERK*, *IRE1α*, *XBP1s*, *ATF6*, and *CHOP* (** $p < 0.01$, **** $p < 0.0001$; n = 6/group).

Comprehensive pathway evaluation demonstrated that IGF2 silencing significantly attenuated SMIR-induced activation of all three UPR branches: PERK/eIF2α axis: Reduced phosphorylation of PERK and eIF2α (Figure 9f,j), IRE1α/XBP1 pathway: Diminished IRE1α phosphorylation and XBP1s generation (Figure 9g,k); ATF6/CHOP cascade: Suppressed ATF6 cleavage and CHOP induction (Figure 9h,i). Transcriptional analysis corroborated these protein-level changes, showing concordant downregulation of UPR effector genes (Figure 9l–p). Crucially, the magnitude of IGF2 suppression strongly correlated with a reduction in key ER stress markers.

These findings establish IGF2 as an upstream regulator of ER stress in CPSP, demonstrating that SMIR surgery upregulates neuronal IGF2 expression, IGF2 amplifies proteotoxic stress through GRP78 induction, IGF2 coordinates activation of all three UPR branches, and targeted IGF2 knockdown reverses this signaling cascade at translational and transcriptional levels. The robust correlation between IGF2 reduction and UPR attenuation confirms IGF2's central role in driving maladaptive proteostasis in CPSP pathogenesis.

3.8. Unidirectional NTRK1→IGF2 Regulation in CPSP Pathogenesis

Pharmacological inhibition of NTRK1 (GW441756, 100 μM) significantly attenuated SMIR-induced IGF2 upregulation in spinal dorsal horn neurons. Quantitative analysis demonstrated dose-dependent reduction in IGF2 at transcriptional (Figure 10a) and translational levels (Figure 10b), with diminished IGF2/NeuN co-localization intensity confirming neuronal-specific suppression (Figure 10c,d). The magnitude of NTRK1 inhibition strongly correlated with IGF2 reduction, establishing NTRK1 as an upstream regulator of IGF2-mediated ER stress.

In reciprocal experiments, IGF2 silencing failed to alter NTRK1 signaling despite complete target engagement. SMIR-induced elevations in NTRK1 transcript (Figure 10e) and TrkA protein expression (Figure 10f) remained unchanged following IGF2 siRNA administration, with comparable TrkA/NeuN co-localization intensity between treatment groups (Figure 10g,h).

These results demonstrate that the NTRK1-IGF2 axis operates unidirectionally in CPSP, with no feedback regulation of NTRK1 by IGF2.

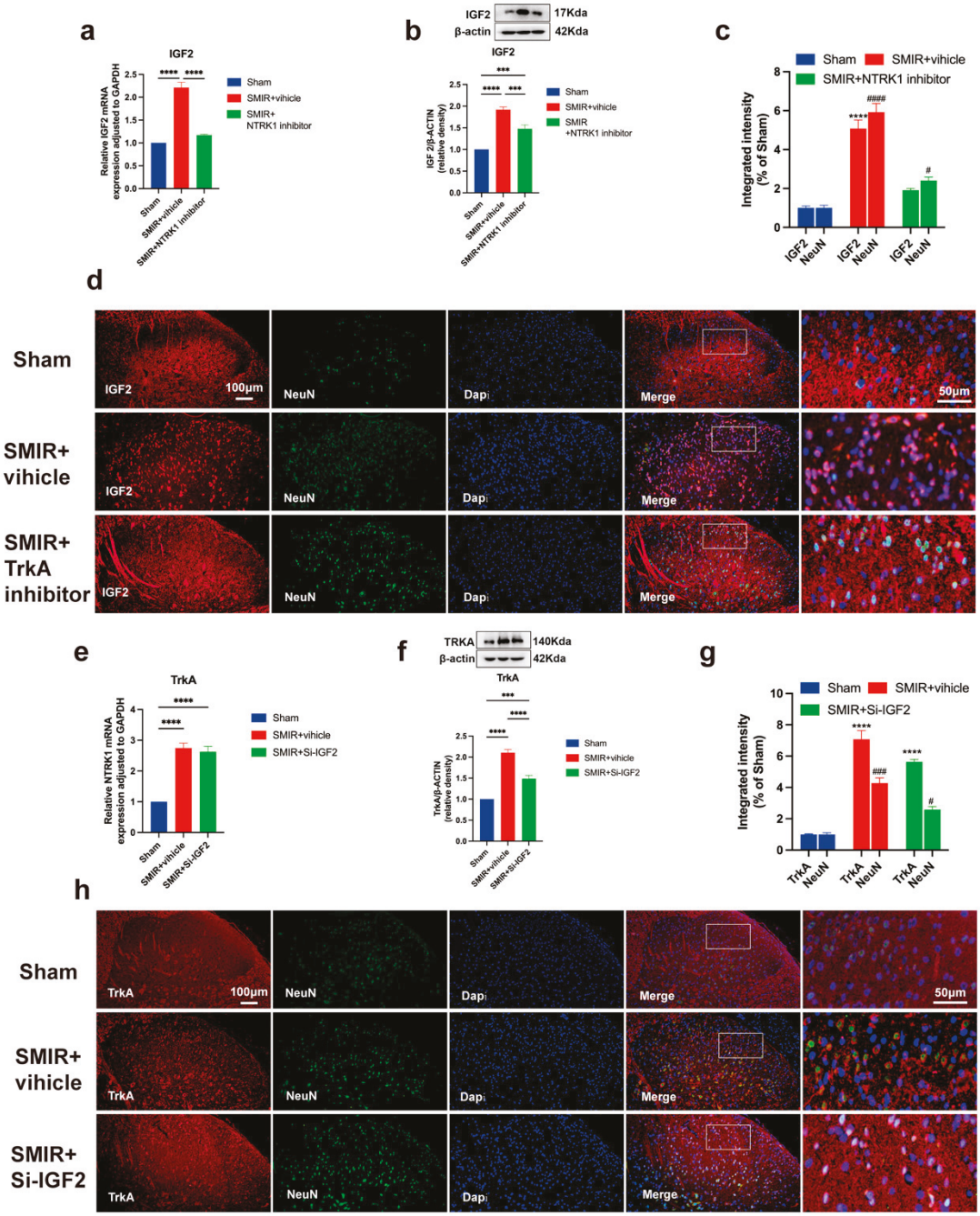


Figure 10. Unidirectional regulation within the NTRK1-IGF2 axis in CPSP. (**a**) qPCR analysis of *IGF2* transcriptional changes following NTRK1 inhibition (**** $p < 0.0001$; n = 6/group). (**b**) Western blot assessment of IGF2 protein expression after NTRK1 inhibitor treatment (representative blots and quantification, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**c**) Quantification of IGF2/NeuN co-localization intensity across treatment groups (**** $p < 0.0001$ compared with the Sham group; $^{\#}$ $p < 0.05$, $^{\#\#\#\#}$ $p < 0.0001$ compared with the Sham group; n = 3/group). (**d**) Immunofluorescence visualization of neuronal IGF2 expression. Scale bars: 100 μm (overview), 50 μm (insets). (**e**) qPCR analysis of *NTRK1* transcriptional changes following IGF2 silencing (**** $p < 0.0001$; n = 6/group). (**f**) Western blot assessment of TrkA protein expression after IGF2 siRNA administration (representative blots and quantification, *** $p < 0.001$, **** $p < 0.0001$; n = 6/group). (**g**) Quantification of TrkA/NeuN co-localization intensity (**** $p < 0.0001$ compared with the Sham group; $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ compared with the Sham group; n = 3/group). (**h**) Immunofluorescence visualization of neuronal TrkA expression. Scale bars: 100 μm (overview), 50 μm (insets).

4. Discussion

Our study delineated a maladaptive signaling axis wherein spinal NTRK1 orchestrates IGF2-dependent endoplasmic reticulum (ER) stress to drive chronic postsurgical pain (CPSP) pathogenesis. Employing the validated skin/muscle incision–retraction (SMIR) model [52], we demonstrated that surgical injury compels NTRK1 upregulation in dorsal horn neurons, consequently amplifying key ER stress mediators—including GRP78, PERK/eIF2α, IRE1α/XBP1s, and ATF6—with temporal progression paralleling mechanical allodynia [28,31,52]. Critically, intrathecal inhibition of NTRK1 (GW441756) or targeted IGF2 silencing not only alleviated established pain hypersensitivity but also suppressed ER stress, reconciling NTRK1's seemingly contradictory functions in neuroprotection and stress exacerbation. Crucially, IGF2 knockdown attenuates ER stress and behavioral sensitization without altering NTRK1 expression, revealing a strictly unidirectional cascade: NTRK1 transcriptionally governs IGF2 to amplify proteotoxic stress. This mechanism extends NTRK1's recognized role in neuropathic pain states to surgical pain paradigms while unveiling its capacity to reprogram stress responses through IGF2-dependent transcriptional networks [60–63].

Endoplasmic reticulum (ER) stress has evolved from a recognized cellular response to a pivotal maladaptive signaling hub in chronic pain pathogenesis [64], serving as a critical driver of central sensitization in CPSP that transcends etiological boundaries, including spinal nerve ligation, diabetic neuropathy, and formalin-induced pain models [9,19,65,66]. Our data demonstrated that SMIR surgery induces profound ER stress in spinal dorsal horn neurons, characterized by coordinated upregulation of the PERK/eIF2α/CHOP and IRE1α/XBP1 pathways alongside elevated GRP78 expression (Figures 4 and 5). This mirrors pathognomonic signatures in human neuropathic pain biopsies, where GRP78 overexpression correlates with therapeutic resistance and pain chronicity [67,68], suggesting conserved pathomechanisms across species. Mechanistically, these pathways drive nociception through distinct yet complementary mechanisms [8,9]. Specifically, the PERK/eIF2α axis exacerbates nociception via ATF4/CHOP [8,9]-mediated synaptic inhibition and dysregulated GABAergic transmission [46,69]—a mechanism corroborated by studies showing that GABA neuron hypofunction in spinal and supraspinal circuits directly facilitates pain persistence and comorbid anxiety [70,71]. Conversely, IRE1α/XBP1 activation orchestrates lipid metabolism reprogramming, autophagic flux impairment, and immune evasion through downstream effectors, like TRAF2 and JNK [10,14,72]. Notably, IRE1α's recent identification as a dynamic scaffold that coalesces with stress granules amplifies its RNAse efficiency under ER stress [10], accelerating XBP1 splicing and potentiating nociceptive signaling [10]—a process conserved across neuropathic pain and cancer [64,68]. These findings contextualize prior observations of persistent ER lumen dilation and UPR marker elevation (GRP78, CHOP, XBP1s) following nerve injury [10,64], while advancing mechanistic insights: ER stress integrates metabolic–immune crosstalk in CPSP and mediates spinal–supraspinal disinhibition [68,73]. The evolutionary conservation of the ER stress response system—from its physiological role in proteostasis to its pathological hijacking in chronic pain [68,73]—underscores its significance as a therapeutic target.

The nerve growth factor (NGF)-NTRK1 axis is indispensable for neuronal development and nociceptive signaling [74,75], with its dysregulation implicated in pathologies spanning congenital insensitivity to pain (CIPA) due to loss-of-function mutations to chronic pain hypersensitivity from gain-of-function signaling [75,76]. During embryogenesis, NGF-NTRK1 interactions orchestrate axonal growth and functional pain circuitry through TrkA-dependent survival signaling in sensory and sympathetic neurons [75,76], while, in adulthood, this pathway transitions to modulating nociceptive plasticity, where sustained NGF-NTRK1 activation drives central sensitization via enhanced TRPV1 traf-

ficking and transcriptional upregulation of nociceptive mediators [77,78]. Crucially, our demonstration that SMIR-induced spinal NTRK1 upregulation (Figure 2e–g) recapitulates patterns observed in diabetic neuropathy models reveals evolutionary conservation of this pathway—a finding substantiated by human genetic studies linking NTRK1 polymorphisms to neuropathic pain susceptibility [60,77,78]. Beyond establishing NTRK1's central role in CPSP pathogenesis [77], our mechanistic studies reveal that the pharmacological inhibition of NTRK1 significantly attenuates ER stress pathways in vivo (Figure 6). This aligns with prior evidence demonstrating that classical ER stress inhibitors (e.g., 4-PBA) reverse mechanical allodynia by restoring proteostasis in dorsal horn neurons [21,66]. Crucially, we identified that NTRK1 signaling amplifies GRP78 expression—a master regulator of UPR sensors—leading to hyperactivation of IRE1α/XBP-1, CHOP, and ATF6 cascades [79].

Notably, pathogenic *NTRK1* mutations disrupt chaperone-assisted protein folding, triggering self-sustaining ER stress loops that impair autophagic flux and mitochondrial homeostasis [7,29]—processes implicated in chemotherapy-induced neuropathy and neurodegeneration [7,29]. Our earlier RNA-seq profiling of NTRK1-overexpressing SH-SY5Y cells substantiated this axis, showing profound enrichment of ER protein processing pathways alongside UPR effector genes (*HSPA5*, *DDIT3*, *ERN1*) [24]. Furthermore, we verified that NTRK1 triggers the IRE1α-XBP1 and PERK pathways, facilitating ER function restoration and inhibiting apoptosis [15]. Paradoxically, while acute NTRK1 activation transiently enhances IRE1α-XBP1 and PERK signaling to restore ER function, chronic stimulation—as occurs in CPSP—exhausts adaptive UPR capacity [77,78]. This establishes a mechanistic bridge between neurotrophic signaling and proteostasis failure, specifically chronic NTRK1 hyperactivation. Thus, our data position ER stress modulators as compelling candidates for CPSP intervention.

Therapeutically, our findings positioned IGF2 as a high-value target for chronic pain intervention. IGF2, a pivotal neurotrophic factor, is extensively distributed throughout various tissues and the nervous system [43,44]. Research has unveiled IGF2's significant involvement in neurodegenerative diseases, brain injury, schizophrenia, and other pathological conditions through its modulation of ER stress [8,18,42]. Mechanistically, this process entails activation of signaling pathways, including GRP78, IRE-1α, and JNK, alongside regulation of factors such as PERK, ATF4, and CHOP, which collectively inhibit neuronal apoptosis and impede disease progression [15,48]. Previous studies have highlighted the mutual involvement of NTRK1 and IGF2 in development and cancer [15,33,34,80,81]. Notably, IGF2 functionality relies heavily on NTRK1 activation within signaling pathways like TrkA/Akt and downstream cascades [45]. Our study uncovers a unidirectional regulatory axis wherein NTRK1 governs IGF2 expression in CPSP. This paradigm, observed in glioblastoma but previously unreported in pain neuroscience [15], manifests maladaptively in CPSP as NTRK1-induced IGF2 overexpression exacerbates ER stress, creating a pathogenic feedforward loop. Several lines of evidence support this hierarchical relationship: SMIR surgery triggered IGF2 overexpression specifically in spinal dorsal horn neurons (Figure 7), coinciding with UPR marker elevation. Intrathecal NTRK1 inhibitors robustly suppressed spinal IGF2 (Figure 10a–d), whereas IGF2 siRNA failed to alter NTRK1 expression (Figure 10e–h), confirming NTRK1's upstream position. IGF2 elevation correlated with PERK/eIF2α and IRE1α/XBP1 pathway hyperactivation, contrasting with its protective role in acute neuronal stress. This aligns with RNA-seq data from NTRK1-overexpressing SH-SY5Y cells showing ER pathway enrichment, suggesting chronic NTRK1-IGF2 signaling exhausts adaptive UPR capacity [24]. Intrathecal IGF2 siRNA not only reduced ER stress markers (GRP78, p-PERK, ATF6; Figure 9) but also alleviated mechanical allodynia (Figure 8), replicating efficacy in neuropathic pain models where IGF2 blockade restored

GABAergic inhibition [50,51]. In CPSP, this axis appears maladaptive: NTRK1-driven IGF2 overexpression exacerbates ER stress [15], creating a feedforward loop that sustains pain.

Notwithstanding the strengths of our multi-modal validation approach—encompassing behavioral phenotyping, molecular pathway analyses, and histological quantification—and the significant discovery of an oncogenic NTRK1-IGF2 mechanism repurposed in surgical pain, three substantive limitations necessitate contextualization. First, the exclusive use of male rodent models precludes examination of sex-specific mechanisms, a critical gap given clinical evidence demonstrating 30–40% higher CPSP incidence in women following hysterectomy or mastectomy [4]. Second, while we establish IGF2 as an ER stress amplifier, its precise mechanistic interface with UPR sensors remains incompletely resolved. Third, therapeutic interventions assessed at 10–14 days post-SMIR may not adequately model established CPSP (>3 months duration) [4,16]. To address these limitations and advance clinical translation, we propose the following: First, a comprehensive investigation of sexual dimorphism integrating ovariectomized models with clinical cohorts of surgical menopause patients. Second, deep functional phenotyping of NTRK1/IGF2 haplotypes in existing pain GWAS resources. Third, longitudinal CSF biomarker profiling measuring dynamic IGF2 trajectories in high-risk surgical cohorts.

5. Conclusions

This study establishes the spinal NTRK1-IGF2-ER stress signaling axis as a fundamental pathogenic driver of CPSP. We demonstrate a unidirectional regulatory hierarchy wherein NTRK1 transcriptionally governs IGF2 to amplify proteotoxic stress, a mechanism previously documented in glioblastoma but newly implicated in pain neuroscience. This maladaptive feedforward loop positions NTRK1 inhibitors and IGF2-targeted therapeutics as compelling intervention strategies. Therapeutically, our findings foreground IGF2 as a high-value yet context-dependent target: while its suppression alleviates CPSP, its dualistic roles in neuroprotection demand precision approaches such as neuron-restricted delivery or epigenetic modulation to mitigate off-target risks. As ER stress emerges as a cross-disease mechanism, strategies targeting this pathway may yield broad benefits beyond pain management. This study establishes a strong foundation for comprehending the pathogenesis of CPSP and devising effective therapeutic strategies, offering valuable insights for future investigations.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further data are available from the corresponding author upon reasonable request at ourpain@163.com.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ATF6	Activating Transcription Factor 6
CHOP	C/EBP Homologous Protein C/EBP
CPSP	Chronic Post-surgical Pain
e-IF2α	Eukaryotic Initiation Factor 2α
ER stress	Endoplasmic Reticulum stress
GRP78	Glucose-Regulated Protein 78
IF	Immunofluorescence
IGF2	Insulin-Like Growth Factor II
IRE1α	Inositol-Requiring Enzyme 1α
PERK	PKR-like ER Kinase
RT-qPCR	Real Time Quantitative polymerase chain reaction
MPWT	Mechanical paw withdraw threshold
NTRK1	Neurotrophic Tyrosine Kinase Receptor Type 1
SMIR	Skin/Muscle Incision and Retraction
TrkA	Tyrosine kinase receptor A
UPR	Unfolded Protein Response
WB	Western Blot
XBP1	X-box Binding Protein 1 X

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