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MESTRADO INTEGRADO EM MEDICINA

The Influence of Biological Mediators and Pharmacological Agents on Sleep in Chronic Pain Patients: A Systematic Review

Bento Emanuel Oliveira Alves

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FACULDADE DE **MEDICINA**

Bento Emanuel Oliveira Alves

The Influence of Biological Mediators and Pharmacological Agents on Sleep in Chronic Pain Patients: A Systematic Review

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Professor Doutor Daniel Humberto Pozza

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UC Dissertação - DECLARAÇÃO DE INTEGRIDADE

Eu, Bento Emanuel Oliveira Alves, abaixo assinado, nº mecanográfico 201606594, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter actuado com absoluta integridade na elaboração do meu trabalho de Dissertação ou Monografia.

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Medicina Básica

TÍTULO DISSERTAÇÃO/~~MONOGRAFIA~~ (riscar o que não interessa)

The Influence of Biological Mediators and Pharmacological Agents on Sleep in Chronic Pain Patients: A Systematic Review

ORIENTADOR

Professor Doutor Daniel Humberto Pozza

COORIENTADOR (se aplicável)

Professora Doutora Isaura Ferreira Tavares

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTE TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.	☐
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UC Dissertação - DECLARAÇÃO DE TRANSPARÊNCIA RELATIVAMENTE À UTILIZAÇÃO DE FERRAMENTAS DE CHATBOT GENERATIVO BASEADAS EM LARGE LANGUAGE MODELS

Eu, Bento Emanuel Oliveira Ales, abaixo assinado, nº mecanográfico 201606594, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro que:

☒ Não procedi à utilização de ferramentas de *chatbox* generativo baseadas em *large language models* para nenhuma das tarefas no contexto do meu trabalho de Dissertação ou Monografia

☐ Procedi à utilização de ferramentas de *chatbox* generativo baseadas em *large language models* no contexto do meu trabalho de Dissertação ou Monografia, encontrando-se todas as interacções (*prompts* e respostas) transcritas em anexo bem como a indicação das aplicações utilizadas.

Neste sentido, confirmo que a eventual utilização de ferramentas de *chatbox* generativo baseadas em *large language models* no contexto do meu trabalho de Dissertação ou Monografia foi exclusivamente descrita na sequência de *prompts* e respostas transcritos em anexo e nas aplicações indicadas.

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Um muito obrigado

à minha mãe, por me ter suportado e apoiado durante todos estes anos, por nunca me pressionar e por sempre acreditar em mim

ao meu pai, por me ensinar o valor do trabalho

às minhas irmãs e ao meu irmão, por me mostrarem o que podemos conseguir se nunca desistirmos, demostrando perseverança frente a qualquer obstáculo

aos meus amigos, por me acompanharem neste percurso e por todos os momentos inesquecíveis que tornaram esta jornada ainda mais especial

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Review

The Influence of Biological Mediators and Pharmacological Agents on Sleep in Chronic Pain Patients: A Systematic Review

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Abstract: Chronic pain and sleep disorders significantly impact the overall quality of life, triggering a cascade of physical, emotional, and social challenges. This systematic review synthesizes current evidence on the role of biological mediators and pharmacological agents influence on sleep and chronic pain. The main results demonstrated that various inflammatory cytokines and biological markers were elevated in patients with chronic pain conditions, showing associations with sleep disorders. Different drugs, including opioids, antidepressants, and antiepileptics, were commonly used and had varied effects on sleep stages, sleep quality, and pain management. While some drugs improved sleep efficiency and reduced pain intensity, others had mixed or negative effects on sleep quality and pain severity. The complexity pathophysiology of chronic pain and sleep disorders negatively affects the neurophysiology of the patient, being influenced by drugs and dependent on the inflammatory status of the patient.

Keywords: Molecular biomarkers; Neurophysiological systems; Locus Coeruleus; Opioid system; Monoaminergic system; Immune system; Melatonin; Insomnia; Obstructive sleep apnea; Fibromyalgia.

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1. Introduction

Pain is defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage. After it persists beyond the expected healing period, or 3 months according to the International Classification of Diseases and IASP, it becomes pathologic and its considered chronic pain [1, 2]. Opposite to acute pain, chronic pain is associated with detrimental changes like peripheral and central sensitization, development of abnormal neural connections and dysfunction of established neuronal circuits leading to pathology-specific brain alterations [2, 3]. Chronic pain conditions have been linked to elevated levels of inflammatory cytokines, having an influential role in the central sensitization and heightened pain sensitivity often present [4, 5]. Some examples are tumor necrosis factor alfa (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) having the capacity to directly induce central sensitization [6, 7]. In a similar way, sleep disturbance is also linked with higher levels of biological markers like C-reactive protein (CRP) [8]. Cortisol, tau, β-amyloid 42, and fasting glucose are linked to both sleep disorders and chronic pain conditions, such as restless leg syndrome, fibromyalgia, and chronic low back pain [9-11].

Sleep quality is a complex construct composed of both subjective and objective aspects [12]. It possesses five attributes that can be measured: sleep efficiency, sleep

disturbance, sleep latency, sleep duration and wake after sleep onset (WASO) [13-16]. Major sleep disorders are the main example of sleep disturbances and can be categorized into insomnia, circadian rhythm sleep-wake disorders, sleep-related breathing disorder, central disorders of hypersomnolence, parasomnias and sleep-related movement disorders [17]. Sleep disorders, which are common and debilitating, particularly affect individuals with chronic pain conditions, often manifesting as insomnia, restless legs syndrome and obstructive sleep apnea (OSA) [18-22]. OSA can hypersensitize nociceptors and affect antinociceptive treatment. The complex pathophysiology of OSA, including sleep fragmentation, sleep loss, and nocturnal hypoxia, enhances pain sensitivity through inflammatory mediators and hypoxia markers. Opioids may cause sleep-disordered breathing by impairing upper airway function, with μ-opioid receptor stimulation potentially increasing the risk of airway collapse. The impact of OSA on pain involves multiple factors, including inflammatory signaling pathways and hypoxemic changes [23].

The bidirectional relationship between chronic pain and sleep involves multiple neurophysiological systems, including the opioid, monoaminergic, orexinergic, and immune systems, as well as the HPA axis and signaling molecules like melatonin, adenosine, and nitric oxide [24-27]. Notably, norepinephrine from the Locus Coeruleus (LC-NE) system modulates pain perception by inhibiting nociceptive transmission [28]. Pain can interfere with sleep by making it difficult to fall or stay asleep, reducing sleep quality, and disrupting sleep architecture [29, 30]. Conversely, short or disturbed sleep lowers pain thresholds and heightens pain intensity, creating a vicious cycle that further impairs sleep [31-33]. Together, these factors negatively affect both physical and psychological functions, affecting overall quality of life [21].

Medications for managing pain and sleep disorders profoundly influence sleep pattern and overall sleep quality, though their effects vary widely [34, 35]. For chronic pain—including neuropathic pain, fibromyalgia, and low back pain—non-opioid agents such as tricyclic antidepressants, serotonin–norepinephrine reuptake inhibitors, and antiepileptics are commonly used [36-40]. In fibromyalgia, for example, pro-inflammatory Th1 and Th17 helper T cells increase pain sensitivity via cytokines like IL-17, a mechanism that may be modulated by some antidepressants and by pregabalin, which also reduces proinflammatory cytokines [39, 41, 42]. Regarding sleep, opioids tend to reduce slow-wave and REM sleep while increasing stage N2 and may raise the risk of sleep apnea, whereas amitriptyline suppresses REM sleep. In contrast, trazodone and pregabalin enhance slow-wave sleep and reduce sleep onset latency and wake after sleep onset, promoting deeper, more restorative sleep [26, 43-55].

Given the undesirable impact of chronic pain and sleep on overall health, it is crucial to understand how these variables interact and respond to pharmacological agents. This systematic review aims to synthesize current evidence on the effects of various biological mediators and pharmacological agents on sleep in individuals with chronic pain.

2. Materials and Methods

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [56, 57]. The PICO question was: “How biological mediators and pharmacological agents affect the relation between chronic pain and sleep in human patients?”

The study protocol was registered in PROSPERO under the code CRD42024555479. A search in three electronic bibliographic databases, Web of Science, PubMed, and Scopus, was carried out on 5 June 2024. The search parameters were: “chronic pain AND sleep wake disorders” for Web of Science, "Chronic Pain"[Mesh]) AND "Sleep Wake Disorders"[Mesh for PubMed (] and “chronic AND pain AND sleep AND wake AND disorders” for Scopus).

Titles and abstracts were screened by two authors using the Rayyan tool in blind mode to determine eligible studies. The inclusion criteria comprised articles written in

English involving chronic pain patients, indicating drug involvement or measuring biological mediators, and analyzing perceived sleep quality and/or other sleep parameters as outcomes. The exclusion criteria included chronic pain of oncologic origin, other substances (E.g., alcohol and cannabis) and review articles. In the second stage, the full-text articles of the eligible studies were examined for a more comprehensive understanding.

Data extraction was manually performed, and the information sought included the type of study, population characteristics such as age and sex, type of chronic pain, sleep disturbance studied, drug involved or biological mediator measured, type and duration of intervention, main results, and complications observed.

Due to differences between results based on drug involvement and those based on biological mediator measurements, the data was sorted into two separate groups. All the information studied here was summarized and inserted into one of the two tables created in the results section.

Search, selection, assessment, and data extraction procedures were performed by two authors. The Kappa test was conducted to verify the level of agreement. Data synthesis was performed by one of the authors and verified by the other two. All authors collaborated in the analysis and classification of the results. Any disagreements that arose were resolved through consensus among the three authors.

The assessment of potential bias across individual studies was done by two authors utilizing appropriate tools in accordance with the study design. For randomized trials the revised Cochrane Risk of Bias tool (RoB 2) [58] and for non-randomized studies the Risk Of Bias In Non-randomized Studies – of Exposures (ROBINS-E) [59] or the Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) [60] if the study evaluated the effect of exposures or interventions. The final assessment for all studies was summarized using the Risk-of-bias VISualization tool (robvis) [61].

3. Results

The initial search in the databases (5 June 2024) resulted in 1,279 publications: 512 in PubMed, 656 in Scopus, and 111 in Web of Science. After duplicate removal, 1,007 studies were screened through reading the titles and abstracts and subsequently 43 were selected. Six studies raised different opinions of the two authors during selection. After conflict resolution (3 included and 3 excluded), 40 were retrieved for full text evaluation (8 July 2024). Kappa test was 0.93, meaning a high level of agreement. Subsequently, 1 manuscript was not retrieved, and 6 studies were excluded: one article in cannabinoids, one in alcohol, one article because it was not possible to find the full text; one was a review of literature; one did not include sleep parameters in results; one was a study protocol, and one was a preliminary study. Thus, 33 manuscripts were included in this systematic review. This process is summarized in Figure 1.

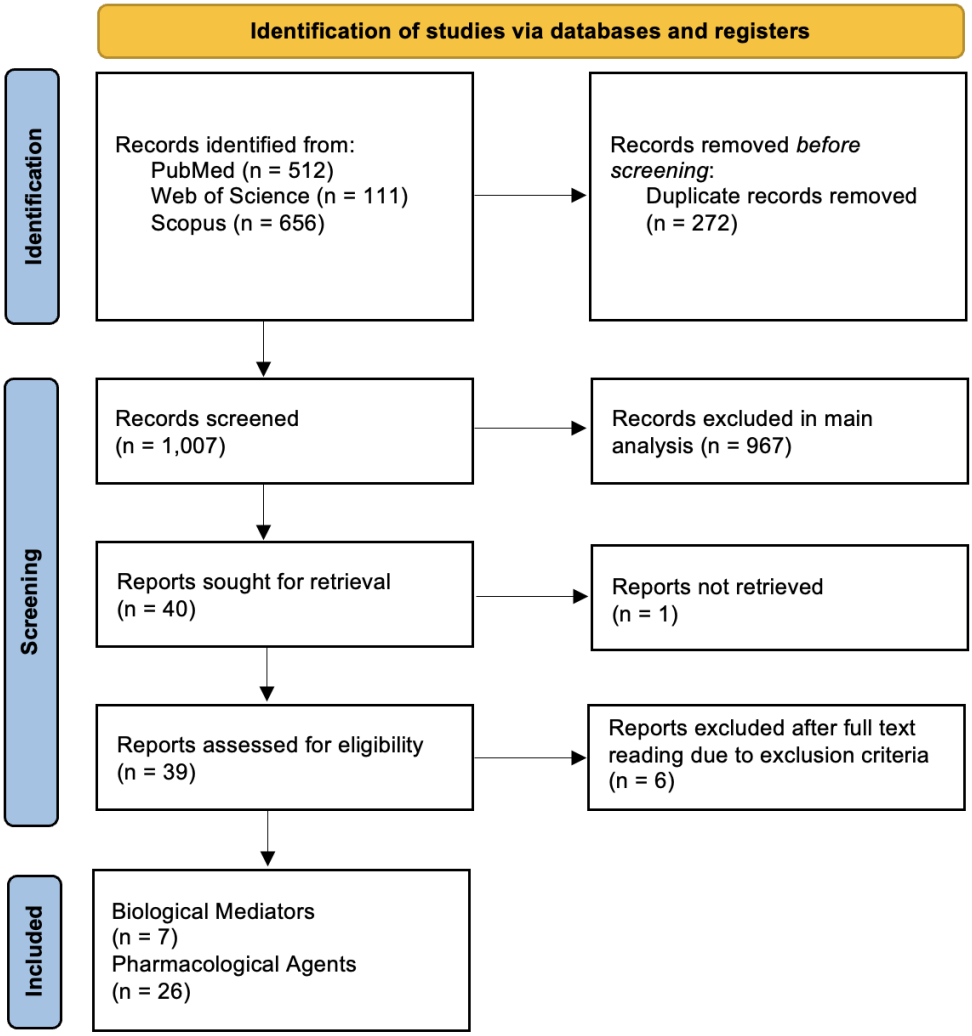

Figure 1 – PRISMA flowchart of manuscript selection.

The analysis of the results was divided into two categories: The first category encompassed studies examining biological mediator in similar populations (table 1), consisting of 7 studies: 2 observational studies [62, 63], 3 case-control studies [10, 11, 64], 1 cohort study [65] and 1 cross-sectional study [9]. The second category encompassed studies on drug effects in a population of chronic pain patients (table 2) comprising 11 experimental studies [66-76], 15 cross-sectional studies [46, 77-88], 1 cohort study [89] and 1 case-control study [90].

Table 1. Comparative Overview of Clinical Studies Involving Molecules on Sleep Disturbance and Chronic Pain

Study	Patients (Age)	Chronic Pain	Sleep Disturbance	Molecule	Treatments	Main Results
(Ho et al., 2023) [65] Cohort study	12348 (various subsamples)	Chronic Low Back Pain and Lower Limb Pain (≥ 3 months)	Insomnia	High-sensitive C-reactive protein (hsCRP)	Observational (11 years follow-up)	RR = 1.37 of insomnia in chronic low back and lower limb pain patients RR = 1.06 of chronic low back and lower limb pain in insomnia patients RR = 1.20 of chronic low back pain in insomnia patients no amplifying effects of hsCRP
(Park & Chung, 2016) [64] Case-control study	High disability: 20F (33.0 ± 12.2 years) Low disability: 20F (32.9 ± 14.5 years) Control: 20F (32.4 ± 12.0 years)	Temporomandibular Disorder	Perceived sleep quality	C-reactive protein, interleukins, tumor necrosis factor	Observational	↑ESS in high disability ↑PSQI ↑IL-1β, IL-6, IL-10, TNF-α ↑cytokine related ↑PSQI ↑IL-1β and IL-10 related ↑ESS ↑disability level related ↑IL-10 and TNF-α
(Lerman et al., 2022) [62] Observational study	insomnia + short sleep (ISSD) 31F (40.4 ±11.1 years) insomnia 97F (34.9 ± 11.0 years)	Temporomandibular Disorder (≥ 3 months, pain ≥ 3/10 last week and last ≥ 10/30 days)	Insomnia (≥ 3x week)	Interleukin-6	Observational (2 weeks follow-up)	↑pain severity and functional limitation of the jaw in ISSD ↑generalized pain sensitivity, central sensitization characteristics and ↓cold pressor tolerance in ISSD ↑IL-6 in ISSD
(Hunt et al., 2022) [63] Observational study	110F (35.6 ± 11.1 years)	Temporomandibular Joint Disorder (pain ≥ 3/10 last week)	Insomnia	Interleukin-6	Observational (14 days follow-up)	↑IL-6 related ↑pain sensitivity ↑SOL predicted ↑IL-6 no effect of PA on resting or pain-invoked IL-6 ↑PA predicted ↓IL-6 at higher levels of TST and SE and at lower levels of SOL and WASO ↑PA predicted ↑IL-6 on poor sleep

(Stehlik et al., 2018) [10] Case-control study	31F (57 ± 8 years) Control: 23F (57 ± 10 years)	Chronic Widespread Pain	Restless Leg Syndrome (RLS) and perceived sleep quality	Cortisol, Glucose, Ferritin	Observational	↑RLS and severe RLS ↑cortisol and fasting glucose no difference in ferritin RLS severity not associated to cortisol or glucose levels ↑PSQI and ESS ↑airflow limitation and ↑pulse wave attenuation RLS associated ↑ESS Chronic Widespread Pain associated anxiety symptoms RLS associated depression
(Thi Nguy et al., 2022) [11] Case-control study	21F, 1M (49.5 ± 8.2 years) Control: 22F (47.6 ± 7.4 years)	Fibromyalgia	Perceived sleep quality	Tau, β-amyloid 42	Observational	↑serum tau and β-amyloid 42 ↑serum tau related ↑PSQI ↑serum tau x β-amyloid 42 related ↑PSQI serum β-amyloid 42 not related to PSQI
(Aroke et al., 2023) [9] Cross-sectional study	No pain: 24F, 25M (39.94 ± 14.52 years) Low-impact pain: 14F, 18M (44.81 ± 13.49 years) High-impact pain: 21F, 16M (47.30 ± 13.39 years)	Chronic Low Back Pain	Insomnia	DNA methylation	Observational	↑biological aging in high-impact pain ↑DNA met. associated ↑insomnia severity and ↓functional performance association of ↑insomnia severity and ↑pain and of ↑insomnia severity and ↓functional performance mediated by biological aging

Legend: ↑: increase; ↓: decrease; F: female; M: male; CRP: C-reactive protein; ESS: Epworth sleepiness scale; IL: interleukin; INSD: insomnia with objective normal sleep duration; ISSD: insomnia with objective short sleep duration (< 6 hour at night); PA: trait-like positive affect; PSQI: Pittsburgh sleep quality index; RR: risk ratio; SE: sleep efficiency; SOL: sleep onset latency; TNF: tumor necrosis factor; TST: total sleep time; WASO: wake after sleep onset

Table 2. Comparative Overview of Clinical Studies Involving Drugs on Sleep Disturbance and Chronic Pain

Study	Patients (Age)	Chronic Pain	Sleep Disturbance	Drug	Treatments	Main Results	Complications
(Rosenthal et al., 2007) [66] Single-blind study	27F, 7M (53.7 years)	Osteoarthritis at hip/knee joint average pain ≥ 4 on BPI scale	Chronic pain secondary to osteoarthritis	Opioids: Morphine sulphate	14 days 30mg or 7 days 30mg → 14 days 60mg	30mg x14: ↑S2, S3/4 ↑TST, SE ↓LPS, WASO, awakenings 60mg x14: less consistent ↑hours of sleep and perceived sleep quality ↓BPI average pain ↑cognition	Adverse effects: Nausea (n= 10) Sedation (n= 5) Constipation (n= 5) Vomiting (n= 4) Pruritus (n= 4) Severe sedation and unresponsiveness (n= 1)
(Peles et al., 2009) [84] Cross-sectional study	Chronic Pain: 1F, 17M (46.6 ± 9.0 years) Non-chronic Pain: 8F, 17M (45.5 ± 10.3 years)	Moderate to severe chronic pain ≥ 6 months	Perceived sleep quality and daytime sleepiness	Opioids: high (> 150mg) or low (< 80mg) daily Methadone	Maintenance treatment (≥ 3 months)	= methadone serum level ↑PSQI = ESS ↓SE, TST ↑stage wake and ↓S2, REM ↑>3min awakenings	None reported
(Jungquist et al., 2012) [46] Cross-sectional study	No Pain: 171, 37%F (48.0 ± 12 years) Pain + No Opioid: 187, 55%F (50.7 ± 12 years) Pain + Opioid: 61, 64%F (50.4 ± 11 years)	Chronic pain ≥ 6 months	Obstructive Sleep Apnea and Central Sleep Apnea	Opioids: Morphine equivalent doses of 5-60mg, 61-200mg, or 201-960mg	Observational	↑apnea in pain + opioids ↑morphine-equivalent dose related ↑apnea and ↑S3/4 pain intensity a predictor of apnea opioid class not a predictor of apnea	None reported

(Rose et al., 2014) [90] Case-control study	Opioid: 12F, 12M (52.4 ± 9.4 years) Control healthy: 5F, 15M (50.6 ± 10.1 years) Control sleep clinic: 9F, 11M (52.9 ± 9.8 years)	Opioid-treated chronic pain	Sleep Disordered Breathing (SBD)	Opioids: Morphine 40-500 mg/day Oxycodone 30-350 mg/day Methadone 20-100 mg/day	Observational	↑apnea and ↓arousal index ↑severe SDB and ↑%sleep time SpO2 < 90% in opioid and control sleep clinic slower reaction time and ↑lapses in PVT mean PaCO2 in the upper limit ↑opioid dose related to ↑apnea ↑wake PaCO2 related to ↑%sleep time SpO2 < 90% ↑%sleep time SpO2 < 90% related slower reaction times	None reported
(Morasco et al., 2014) [83] Cross-sectional study	Pain + Opioid: 6F, 66M (54.9 ± 6.3 years) Pain + No Opioid: 6F, 98M (54.6 ± 8.6 years) No Pain + No Opioid: 4F, 87M (52.8 ± 9.1 years)	Arthritis, Fibromyalgia, Low Back Pain, Migraine headache, Neck or Join Pain, Neuropathy (12.8 ± 11.5 years)	Perceived sleep quality	Opioids: average 34.6 ± 54.9 mg/day of Morphine equivalent	Observational	↑sleep apnea diagnosis ↑pain severity and pain interference ↑PSQI (global score, sleep quality and sleep latency) ↑opioid dose related ↑PSQI (global score and sleep latency)	None reported
(Robertson et al., 2016) [86] Cross-sectional study	High-dose Opioid: 2F, 2M (42 ± 28 years) Low-dose Opioid: 7F, 4M (44 ± 22 years) Non-Opioid 3F, 3M (44 ± 28 years) Control: 4F, 6M (44 ± 27 years)	Chronic Back Pain (7.5 ± 8.6 years)	Perceived sleep quality	Opioids: high (~100 mg/day) or low (< 100 mg/day) Morphine equivalent	Observational (14 days follow-up)	↑PSQI, insomnia, fatigue, pain rating, TIB, TST and SOL in chronic pain opioid dose not related with pain rating, PSQI and actigraphic measurements abnormal brain activity during sleep in high-dose opioid	None reported

(Yarlas et al., 2016) [67] Randomized Controlled Trial	Trial I: 298F, 243M (49.4 ± 13.0 years) Trial II: 314F, 346M (50.0 ± 12.6 years)	Chronic Low Back Pain (moderate-to-severe pain for ≥ 12 weeks)	Perceived sleep quality	Opioids: Buprenorphine	Trial I (12 weeks): Buprenorphine 10/20mcg/hour vs placebo in opioid-naïve Trial II (12 weeks): Buprenorphine 20mcg/hour vs 5mcg/hour in opioid-experienced	↑SPI and disturbance domain score of MOS-SS in both trials ↓pain severity related ↑MOS-SS (all domain scores)	None reported
(Miller, Chan, Curtis, et al., 2018) [81] Cross-sectional study	137F, 7M (51.6 ± 11.4 years)	Pain ≥ 10/100 last 14 days	Insomnia (SOL/WASO > 30 min last 14 days)	Opioids	Observational (14 days follow-up)	↑pain related ↑WASO and ↓sleep quality opioid use related ↑TIB opioid use related ↑SOL in mild but not moderate to severe pain	None reported
(Curtis, Miller, Rathinakumar, et al., 2019) [79] Cross-sectional study	Opioid: 60F, 5M (52.75 ± 11.27 years) No Opioid: 122F, 6M (51.13 ± 12.05 years)	Fibromyalgia (≥ 3 months)	Insomnia (SOL/WASO > 30min for ≥ 3 days/week for ≥ 6 months)	Opioids	Observational (14 days follow-up)	opioid use predicted ↑S2 and ↓SWS opioid use predicted ↑SOL in middle-age/older adults high-dose opioid use predicted ↑SOL and ↓SE in older adults opioid use did not predict WASO, TST, S1 or REM opioid dose predicted ↓SWS and ↑arousal index in low pain and ↑slow-wave sleep in higher pain	None reported
(Curtis, Miller, Boissoneault, et al., 2019) [78] Cross-sectional study	188F, 11M (51.46 ± 11.57 years)	Fibromyalgia (≥ 10/100 evening pain)	Insomnia (≥ 30min total wake time)	Opioids	Observational (14 days follow-up)	↑opioid dose associated with better subjective (↓SOL, ↑SE) than objective reports in younger adults and subjective ↑SOL than objective in older adults opioid dose did not predict WASO discrepancies	None reported

(Koffel et al., 2020) [68] Secondary analysis of a randomized controlled trial	Severe Sleep Disturbance: 16F, 64M (53.5 ± 13.9 years) No Severe Sleep Disturbance 15F, 143M (60.6 ± 13.0 years)	Chronic Back Pain and Osteoarthritis of hip or knee (moderate to severe pain almost daily for ≥ 6 months)	Perceived sleep quality	Opioids	12 months opioid therapy vs nonopioid therapy	baseline sleep disturbance did not modify opioid vs nonopioid therapy pain outcomes ↑sleep disorders at baseline predicted less improvement in BPI (interference and severity)	None reported
(Ponce Martinez et al., 2020) [85] Cross-sectional study	33F, 56M (45.18 ± 10.05 years)	Chronic non-specific pain (≥ 3 months)	Perceived sleep quality	Opioids: Methadone (mean daily dose of 81mg)	Observational	↑sleep disturbance associated ↑pain catastrophizing which was associated with ↑pain intensity ↑pain intensity associated ↑pain catastrophizing	None reported
(Miller et al., 2021) [82] Observational study	60F, 5M (52.91 ± 11.16 years)	Fibromyalgia (≥ 3 months)	Insomnia (> 30min to fall asleep or ≥ 30min awake at night 3x/week for 6 months)	Opioids (average daily use of 1.75 ± 0.73 dosage units)	Observational (14 days follow-up)	no effect of opioid use on total wake time or sleep quality ↓sleep quality and ↑opioid use in evenings with greater pain	None reported
(Cody et al., 2021) [77] Cross-sectional study	HIV + Pain 27F, 56M (44.3-54 years) HIV + No Pain 5F, 27M (30-50 years)	≥ 3 months	Insomnia	Opioids	Observational	↑severity of insomnia in HIV + pain ↑↑severity of insomnia in HIV + pain with opioid use ↑pain associated ↑insomnia severity in HIV + pain with opioid use	None reported
(Ellis et al., 2022) [80] Cross-sectional study	61F, 93M (35.13 ± 8.42 years)	Chronic non-specific pain	Insomnia, Sleep Apnea, Sleep Paralysis, Restless Leg Syndrome	Opioids (use disorder treatment)	Observational	↑decline in sleep quality (childhood → adolescence → adulthood) in chronic pain + insomnia, smaller decline in only opioid use disorder persistent sleep disturbance related to chronic pain, insomnia, greater number of treatment episodes and female sex	None reported

(Wilson et al., 2023) [88] Cross-sectional study	213, 58%F (42.8 ± 13.3 years)	Chronic Low Back Pain (≥ 4/10 pain for ≥ 3 months	Perceived sleep quality	Opioids	Observational	↑sleep disturbance, ↑pain severity and ↑pain interference in opioid use ↑sleep disturbance related ↑pain severity, ↑pain interference and ↓physical function opioids moderate greater association of ↑sleep disturbance with ↓physical function and ↑pain interference	None reported
(Lintzeris et al., 2016) [89] Cross-sectional study	1243, 43%M (mean of 59 years)	Chronic Back and Neck Pain, Arthritis or Rheumatism (most common)	Perceived sleep quality	Morphine equivalent 72.7 (36-145) mg/d Benzodiazepines 28.2 (25.6-30.8) Antipsychotics 7.2 (5.8-8.8) Antidepressants 54.4 (51.6-57.4) Antiepileptic 40.9 (38.1-43.8)	Observational (2 years follow-up)	SPI = 47.3 ± 20.9 (25.8 in general population) ↑levels of sleep problems in chronic pain patients with long-term opioids associated with mental health problems and increased medication use higher number of pain conditions associated ↑sleep disturbance, ↓sleep adequacy, ↑daytime somnolence, ↑snoring, ↑awakening short of breath or with headache, ↑total sleep problems, ↓hours slept and ↑BPI sleep interference benzodiazepine use related ↑SPI antidepressant and antipsychotic use related ↓SPI ↑pain interference and antiepileptic and/or antipsychotic use related ↑respiratory impairment (MOS-SS)	None reported
(Saletu et al., 2005) [69] Nonrandomized crossover study	6F, 5M (49.1 ± 11.9 years) Control: 6F, 5M (49.5 ± 11.7 years)	Somatoform Pain Disorder (SPD)	Nonorganic Insomnia related to SPD (3x week for ≥ 1 month)	Antidepressant: Trazodone hydrochloride	3 nights (adaptation night → baseline/placebo night → trazodone night) control: 2 nights (adaptation night → baseline night)	↓awakenings, ↓TSP, ↑latency to S1 and ↑SOL ↑S3 and SWS and ↓S2 and stage shifts ↑minimal O2 saturation and ↓arousal index	None reported

(Calderon et al., 2011) [70] Randomized controlled trial	AMP: 11 (32.4 ± 9.5 years) AMP + CBT: 13 (36.7 ± 13.6 years) Placebo + CBT: 12 (38.2 ± 11.1 years) Placebo: 11 (34.5 ± 11.0 years)	Temporomandibular Disorder (≥ moderate pain for ≥ 6 months, almost daily last month)	Perceived sleep quality	Antidepressant: Amitriptyline (AMP)	7 weeks: AMP 25mg, AMP 25mg + CBT, placebo + CBT or placebo	↓pain for all groups, more in AMP ↓depression and OHIP in placebo + CBT no differences in PSQI	Adverse effects: Visual symptoms (n=1)
(Roth et al., 2012) [71] Randomized crossover study	103F, 16M (48.4 years)	Fibromyalgia	Sleep disorder ≥ 3x week for ≥ 1 month	Anticonvulsant: Pregabalin 300-450mg	Pregabalin (4 weeks) → taper/washout (2 weeks) → placebo (4 weeks) or inverse	↓ WASO, LPS, NAASO ↑ TST, SE, SWS ↓subjective WASO and SOL, tiredness, daily pain ↑subjective TST, sleep quality	Adverse effects: dizziness (n= 32) somnolence (n= 23) headache (n=8) nausea (n=7)
(Silverman et al., 2012) [72] Randomized controlled trial	11F (28-63 years) Control: 7F (26-77 years)	Abdominal Adhesion Pain (pain ≥ 4/10 for ≥ 3 months)	Daily sleep interference	Anticonvulsant: Pregabalin 150-300mg	Pregabalin 150/300mg or placebo (8 weeks) → pregabalin 300mg (4 weeks)	↓pain ↓sleep interference (not significant)	Adverse effects: dizziness (n= 2) night sweats, worsening headaches, hyperactivity, drowsiness, blurred vision, numbness in hands (n= 1)
(Bamgbade et al., 2022) [73] Crossover study	99F, 51M (91 33-64 years and 59 65-88 years)	Spinal or Paraspinal and Non-spinal Limb Pain (frequent and/or significant pain > 3 months)	Insomnia (3x week for > 3 months)	Clonidine, Zopiclone	Zopiclone (3.75/7.5mg) and Clonidine (0.1/0.2mg) on alternate nights for 3 weeks	↓pain, ↓time to fall asleep, ↑feeling rested on waking up, ↑sleep quality, ↑Total Likert sleep score and ↑TST in clonidine ↑adverse effects in zopiclone	Zopiclone: collapse, fall, confusion, amnesia, mood disorder, hallucination, nightmare, nocturnal restlessness, locomotor dysfunction, nausea and headache Clonidine: dry mouth
(Vidor et al., 2013) [74] Randomized Controlled Trial	16F (32.37 ± 4.65 years) Control: 16F (29.47 ± 5.01 years)	Temporomandibular Disorder (pain ≥ 3/10 for 7 days)	Perceived sleep quality	Melatonin	4 weeks Melatonin 5mg or placebo	↓pain and daily analgesic use ↑pressure pain threshold ↑sleep quality	No serious or moderate adverse effects

(Onyeakazi et al., 2024) [75] Randomized crossover trial	Group A: 20F, 10M (62 years) Group B: 16F, 12M (55 years)	Chronic pain ≥ 7/10 for ≥ 3 months	Perceived sleep quality	Melatonin	Melatonin 2mg (6 weeks) → washout (2 weeks) → placebo (6 weeks) or inverse	no differences at 6 weeks but ↓sleep disorder, ↓sleep latency, ↓WASO, ↑sleep quality and ↓effect of pain on sleep at 3 weeks ↓pain intensity no difference in adverse effects	7% reported side-effects
(Roehrs et al., 2020) [76] Crossover study	10F (50.0 ± 9.1 years)	Fibromyalgia	Insomnia	Suvorexant	Suvorexant 20mg (9 nights) → washout (7 days) → placebo (9 nights)	↑TST and ↓WASO ↓duration of awakenings but not number LPS, S1, S3/4 and REM not altered ↓pain sensitivity	Common adverse effects in suvorexant: residual sedation (n= 4) nausea (n= 3)
(Ueno et al., 2024) [87] Retrospective observational study	14F, 7M (63 years)	Chronic non-specific pain	Insomnia	Lemborexant	Lemborexant 5mg (2 weeks) → Lemborexant 2,5/5/10mg (2 weeks)	↓insomnia no change in pain score	5 dropped due to lightheadedness, daytime sleepiness or mood disorder

Legend: ↑: increase; ↓: decrease; =: similar; F: female; M: male; BPI: brief pain inventory; CBT: cognitive- behavioral therapy; ESS: Epworth sleepiness scale; LPS: latency to persistent sleep; MOS-SS: medical outcomes study - sleep scale; NAASO: number of awakenings after sleep onset (wake period of at least 2 epochs' duration); OHIP: oral health impact profile; PSQI: Pittsburgh sleep quality index; PVT: psychomotor vigilance test; REM: rapid eye movement sleep; S2: Stage 2; SE: sleep efficiency; SOL: sleep onset latency; SPI: sleep problems index; SWS: slow-wave-sleep; TIB: time in bed; TSP: total sleep period; TST: total sleep time; WASO: wake after sleep onset

The sample sizes of the selected studies ranged from 10 to 12,348 participants, with most studies including both men and women. Seven studies were limited to women [10, 62-64, 72, 74, 76] and one did not provide information on sex [70]. The majority of studies focused on unspecified chronic pain or included multiples etiologies, while some targeted specific diagnoses, with the most common being fibromyalgia and chronic back pain. Regarding sleep disorders, the most common was insomnia followed by sleep apnea. A comparable number of studies did not focus on a specific sleep problem but instead analyzed the perceived sleep quality of the patients.

Inflammatory cytokines (table 1), including IL-1β, IL-6, IL-10, and TNF-α, were elevated in chronic pain patients. Some of these cytokines also showed a positive association with sleep disorders [62-64]. Cortisol and fasting glucose were found to be increased in chronic widespread pain but had no impact on restless leg syndrome severity [10]. Serum tau and β-amyloid 42 were increased in fibromyalgia patients, with both serum tau and serum tau x β-amyloid 42 ratio showing a positive association with PSQI scores [11]. Biological aging, measured by DNA methylation, was found to be accelerated in patients with high-impact chronic pain with increased insomnia severity and decrease functional performance [9].

The most used drugs were opioids (Table 2), with 16 references: 1 related to morphine [66], 2 to methadone [84, 85], 1 to buprenorphine [67] and 1 to a combination of morphine, oxycodone, and methadone [90]. This was followed by 2 studying antidepressants (1 for trazodone and 1 for amitriptyline [69, 70]), 2 melatonin [74, 75], 2 antiepileptics (both for pregabalin [71, 72]), and 2 orexin antagonists (1 for suvorexant and 1 for lemborexant [76, 87]). Two studies combined several drug groups: one included morphine, benzodiazepines, antipsychotics, antidepressants, and antiepileptics [89], while the other involved hypnotic nonbenzodiazepine drug (zopiclone) and a α2-adrenoreceptor agonist drug (clonidine) [73].

An association between opioid use and sleep onset latency (SOL) was observed in two studies, where opioids predicted a higher SOL in middle age and older adults [79], as well as in cases of mild but not moderate to severe pain [81]. This contrasted with latency to persistent sleep (LPS), where small doses of morphine led to a reduction [66]. In other studies, SOL increased with trazodone use [69], while LPS decreased with pregabalin [71] and was maintained with suvorexant [76].

Sleep efficiency (SE) improved in three studies: one involving opioid use [66], one on opioids, but only in younger adults [78], and one with pregabalin [71]. This opioid study also showed a reduction in the awakenings during sleep. In another opioid study [79], there was a decrease in SE limited to older adults, accompanied by a higher arousal index in low pain patients. A different opioid study [90] demonstrated a reduced arousal index.

Pregabalin had additional benefits, such as reducing wake after sleep onset (WASO) and the number of awakenings [71]. Similar results were seen with other drugs: melatonin reduced WASO [75], while trazodone decreased both awakenings and arousal index [69]. Suvorexant reduced WASO and the duration of awakenings, though not the number of awakenings [76].

Number of hours of sleep or total sleep period (TSP) increased in two studies [66, 81] on opioids but decreased in one study on trazodone [69]. Total sleep time (TST) increased in four studies, each involving a different drug: morphine [66], pregabalin [71], clonidine [73] and suvorexant [76].

Sleep quality showed improvements in 7 studies [66, 67, 71, 73, 75, 83, 87]. Of these, 3 were about opioids [66, 67, 83] reporting increased perceived sleep quality and decreased insomnia-like symptoms, 3 with pregabalin, clonidine and melatonin [71, 73, 75], and 1 with lemborexant [87]. In contrast, 2 other studies on opioids had increased insomnia severity [77] and increased sleep disorders [88]. A multiple-drugs study [89] had different results depending on the type of drug: benzodiazepines improved sleep problems, whereas antidepressants and antipsychotics worsened sleep problems and respiratory impairment.

Higher levels of sleep problems in a population of chronic pain patients with long-term opioids was associated with more mental health problems and increased medication use [89].

A positive association between opioid use and sleep apnea diagnosis was found in three observational studies [46, 83, 90], with two of them also showing an association between the opioid dose and sleep apnea [46, 90]. Similarly, a study on high-sensitivity C-reactive protein (hsCRP) concluded that chronic pain patients had a higher risk of insomnia, and those with insomnia had an increased risk of chronic low back pain, though there was no amplifying effect of hsCRP [65].

Regarding pain control, 7 studies reported reduced pain intensity with the use of opioids [66], amitriptyline [70], pregabalin [71, 72], clonidine [73] and melatonin [74, 75], while use of lemborexant had no effect on pain [87]. Other 2 studies reported an increase in pain severity and pain interference with opioid use [83, 88]. Additionally, both melatonin and suvorexant were found to reduce pain sensitivity [74, 76]. A summary of the results is depicted in Figure 2.

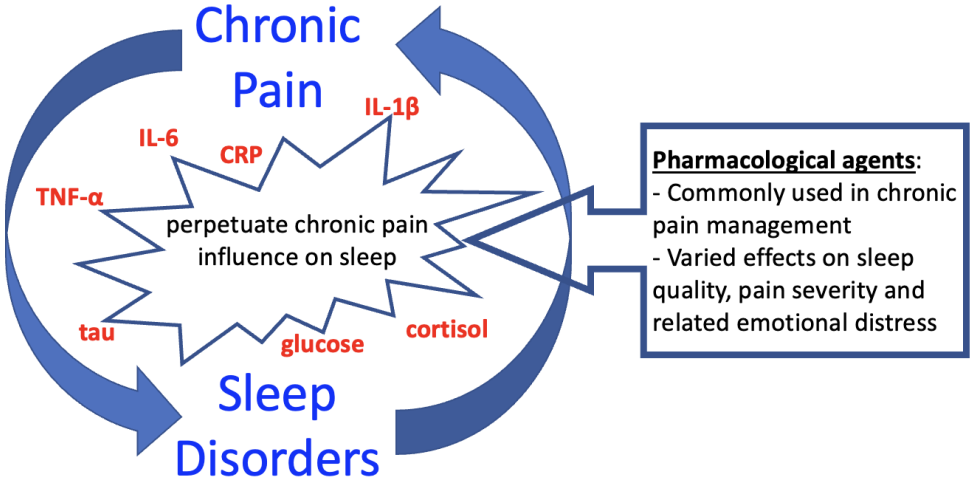

Figure 2 – Graphical abstract of the main results. Legend: IL – Interleukin, CRP – C-reactive protein, TNF – Tumor necrosis factor.

A risk of bias analysis was conducted for each study in accordance with its design. A total of six randomized trials were assessed using RoB 2 (Figure 3) concluding in an overall risk of bias of low in four and some concerns in two. Silverman et al, 2012 [72] raised concerns about missing outcome data and the lack of an appropriate analysis to estimate the intervention's effect, with the latter issue also present in Calderon et al., 2011 [70].

Non-randomized studies were evaluated using ROBINS-E (Figure 4) or ROBINS-I (Figure 5), depending on whether they investigated an intervention (5 studies) or an exposure (22 studies). In this evaluation, important confounding factors that were taken in consideration were age and gender [91], body mass index (BMI) [92], physical activity [93], mental health (anxiety or depressive symptoms) [94], alcohol consumption [95], smoking [96], caffeine use [97], concomitant use of other medications and pain severity and duration [98]. The majority of studies raised concerns about potential bias due to confounding, as they did not account for the important factors mentioned above. Aroke et al., 2023 [9], Peles et al., 2009 [84], Jungquist et al., 2012 [46], Rose et al., 2014 [90] and Ueno et al., 2024 [87] had a high risk of bias due to including only a subset of the relevant confounding factors. Ten studies relied on questionnaires as the primary tool for measuring outcomes, raising concerns about potential bias due to participants' awareness of their exposure.

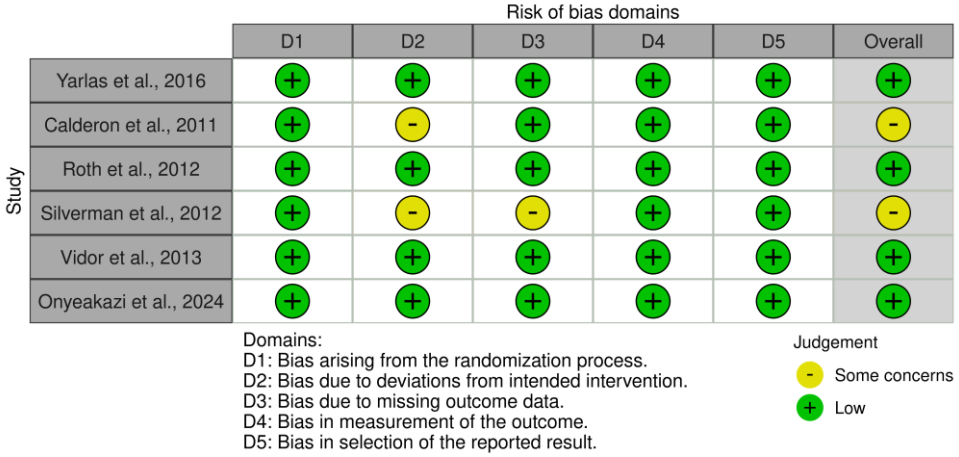

Figure 3 – Risk of bias domains assessment of randomized trials according to Cochrane risk of bias tool.

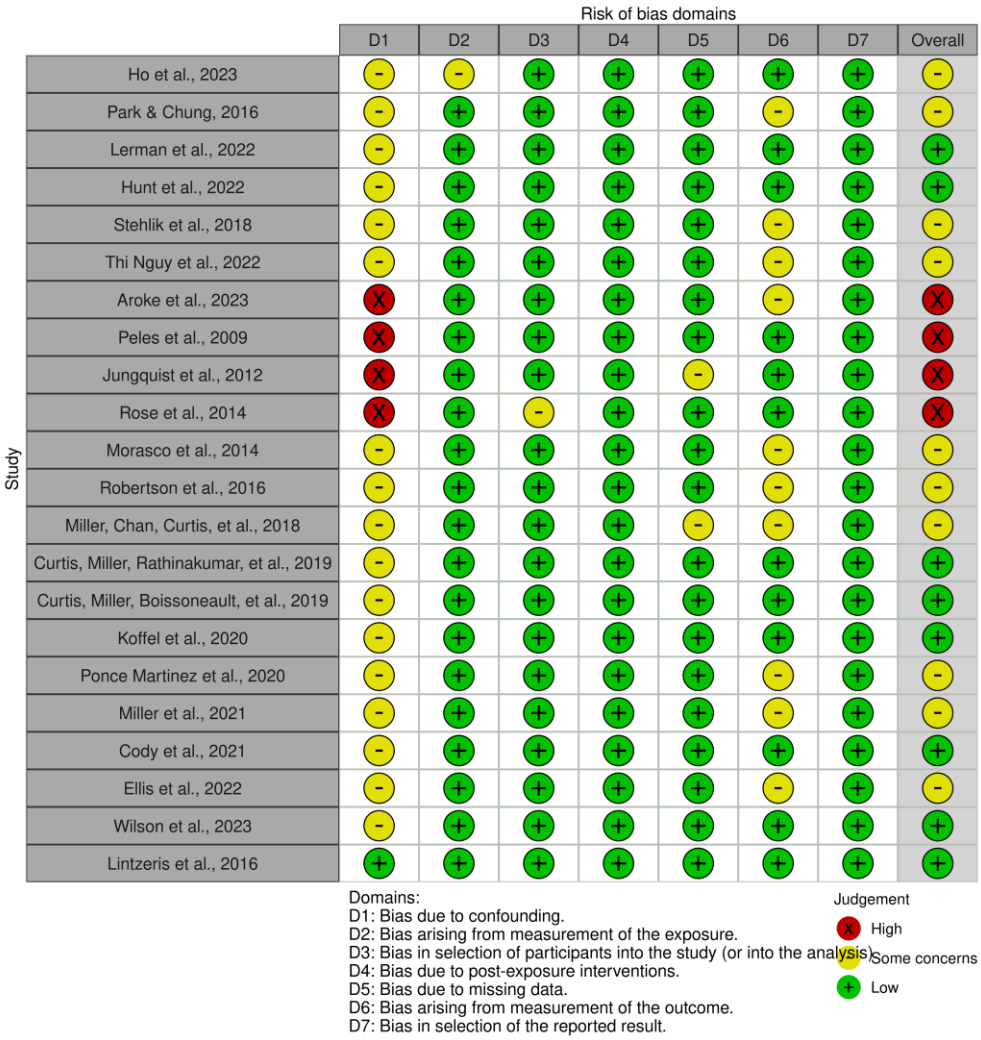

Figure 4 – Assessment of Risk of Bias Domains in Non-Randomized Studies According to the Risk of Bias in Non-Randomized Studies of Exposures (ROBINS-E).

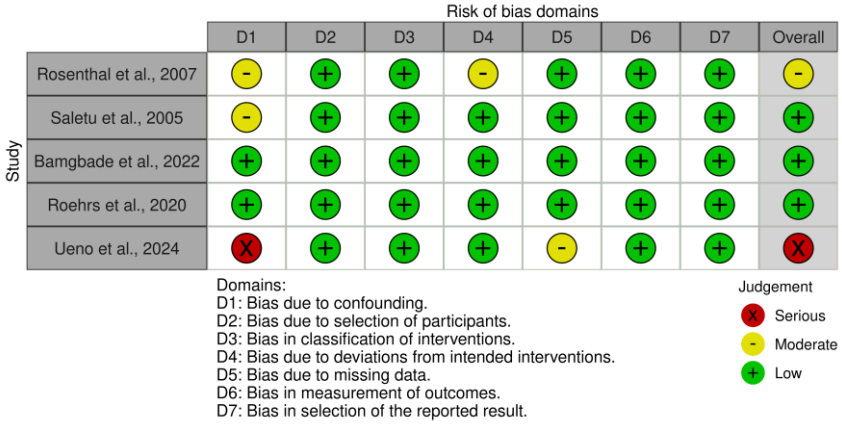

Figure 5 – Assessment of Risk of Bias Domains in Non-Randomized Studies According to the Risk of Bias in Non-Randomized Studies of Interventions (ROBINS-I).

4. Discussion

The main results of the studies analyzed in the systematic review indicate that elevated levels of inflammatory cytokines, particularly IL-6, play a crucial role in the pathophysiology of chronic pain and poor sleep quality [6, 62, 63]. For instance, IL-6 levels are elevated in patients with insomnia, and these higher levels predict increased pain sensitivity [62, 63]. Additionally, patients with high disability and pain from temporomandibular disorder (TMD) exhibit worse perceived sleep quality and elevated levels of IL-1β, IL-6, IL-10, and TNF-α. These cytokines are linked to increased sleep disorders and excessive daytime sleepiness, highlighting a strong connection between inflammation, disability level, and sleep quality in chronic pain patients [64].

In a less sensitive test, C-reactive protein (CRP) presented elevated plasma levels in TMD patients, correlating with sleep quality and suggesting its contribution to sleep disorders in patients with chronic pain conditions [64]. This finding corroborates a previous systematic review in which CRP was linked to sleep disorders but did not explore its interaction with chronic pain [8]. On the other hand, high-sensitive C-reactive protein (hsCRP) did not amplify the effects of insomnia and chronic pain, suggesting that this inflammatory marker may not play a significant role in this bidirectional relation [65]. These differences might be influenced by the specific type and duration of chronic pain, as well as variations in the nature of the sleep disorders and the characteristics of the studied populations.

Therefore, while cytokines such as IL-6, IL-1β, and TNF-α can be present in peripheral sensitization and consequently central pain sensitivity [6, 7], hsCRP has contradictory influence in the specific context of chronic pain and sleep disorders. This discrepancy underscores the complexity of the inflammatory processes underlying chronic pain and sleep disorders. Different biological mediators may have distinct roles depending on the specific pain condition and the type of inflammatory sensitization occurring in the peripheral nervous system having a negative impact in the central nervous system. Additionally, the methods employed in the studies may also have an influence in the results.

There is a complex relationship between metabolic, inflammatory, and neurodegenerative processes in chronic pain patients with sleep disorders. Elevated morning cortisol and fasting glucose levels have been observed in women with chronic widespread pain, caused by increased adrenergic sympathetic activity during sleep, indicating a potential link between chronic pain and metabolic dysregulation [10]. Furthermore, elevated serum levels of tau and β-amyloid have been found in fibromyalgia

patients, with a positive correlation between tau levels and sleep disorders, suggesting that sleep disorders may contribute to the neurodegeneration in fibromyalgia [11].

The shift from acute to chronic pain involves rapid DNA methylation reprogramming, highlighting its potential role in pain chronicity [99]. Chronic pain conditions also contribute to neurodegeneration by accelerating biological aging [100], as indicated by DNA methylation changes. Additionally, higher DNA methylation scores correlates with increased insomnia severity, lower quality of life (functional and activity limitations) and has been linked to differential methylation in core circadian clock genes in individuals with high-impact chronic pain when compared to those without pain [9, 101].

Different drugs, including melatonin, opioids, antidepressants, and antiepileptics, were commonly studied and had varied effects on sleep and pain management. While some drugs improved sleep efficiency and reduced pain intensity, others had mixed or negative effects on sleep quality and pain severity. Melatonin, an endogenous molecule produced by the pineal gland and widely recognized for its role in regulating circadian rhythms and improving sleep quality, can also be used as a pharmacological agent. Neurophysiologically, melatonin administration enhances sleep efficiency (SE) by modulating the suprachiasmatic nucleus of the hypothalamus, which controls the sleep-wake cycle. This modulation reduces wake after sleep onset (WASO) and increases total sleep time (TST), thereby improving sleep continuity. Melatonin also decreases sleep onset latency (SOL) by promoting the onset of sleep through its action on melatonin receptors MT1 and MT2, which are involved in the regulation of circadian rhythms [74, 75].

Chronic pain patients often experience disrupted sleep patterns, which can lead to reduced melatonin levels. Additionally, melatonin has shown transient benefits in reducing pain in patients with severe chronic pain conditions. This dual role is attributed to its anti-inflammatory and analgesic properties, which involve the inhibition of pro-inflammatory cytokines and modulation of pain pathways [102-105]. For instance, melatonin has been effective in improving sleep measures and alleviating pain in patients with conditions such as fibromyalgia and orofacial pain, highlighting its potential in managing both sleep disorders and pain through neurophysiological mechanisms [74, 75].

However, the benefits of melatonin are often transient, necessitating ongoing evaluation of its long-term efficacy and optimal dosing strategies. While melatonin is generally well-tolerated, its effectiveness can vary based on individual characteristics and the underlying causes of sleep disorders. Further research is needed to establish standardized guidelines for melatonin use in chronic pain and sleep disorders, including potential interactions with other medications and long-term safety profiles. Despite these challenges, melatonin remains a valuable option for improving sleep quality and managing pain, particularly when used as part of a more comprehensive treatment plan that includes behavioral and lifestyle interventions [102, 106, 107].

Opioids are an important and well-studied medication in the management of chronic pain. However, they present a complex neurophysiological profile regarding their effects on sleep. Opioids can enhance sleep Stage 2 (S2) by modulating the activity of the central nervous system. However, their effects on slow-wave sleep (SWS) are mixed, with some studies reporting increases and others decreases [43, 45]. Opioids generally increase SOL by affecting the brain's arousal systems but can reduce latency to persistent sleep (LPS) at lower doses, probably due to their sedative properties [66].

Opioids can improve SE by reducing awakenings and the arousal index, particularly in older adults. This is achieved through their action on opioid receptors, which modulate pain and stress responses, thereby promoting more stable sleep patterns [66, 69]. However, chronic opioid use has been identified as a risk factor for central sleep apnea and ataxic breathing, with higher doses potentially exacerbating these conditions due to their depressive effects on respiratory centers in the brain [46-48].

The impact of opioids on sleep is further complicated by their dose-dependent effects. While low doses may improve certain sleep parameters, long-term, higher doses and opioids use disorder can lead to increased insomnia severity, sleep disorders, fatigue, mental health problems, and respiratory complications [66, 77, 80, 83-86, 88-90]. Furthermore, opioids have been found to improve perceived sleep quality more than objective sleep outcomes in younger adults, with the opposite effect observed in older adults, mainly at higher doses [78, 79]. These variabilities underscore the importance of individualized treatment plans and careful monitoring to balance the analgesic benefits with potential adverse effects on sleep and respiratory health [48, 108-110].

It was demonstrated that baseline sleep disorders negatively impact the effectiveness of pain treatments, and even opioids can be ineffective on chronic pain [68]. Pain intensity has also been found to moderate the association between opioid use and insomnia symptoms, with higher pain intensity linked to worse sleep quality and longer wake after sleep onset. Furthermore, evening pain adversely impacted both sleep quality and opioid use [81, 82]. These important nuances highlight the need for considering both subjective and objective measures when evaluating the effectiveness of opioid therapy on the bilateral relationship of pain and sleep.

The bidirectional relationship between pain and sleep disorders generally includes emotional distress. Some antidepressants, such as mirtazapine and trazodone, are sometimes used in sleep disorders for their sedative properties. Mirtazapine improves sleep quality by increasing SWS and reducing WASO, making it beneficial for patients with insomnia and depression. It achieves these effects by antagonizing histamine H1 receptors and serotonin 5-HT2 receptors, which promotes deeper sleep and reduces nighttime awakenings [111-113]. Trazodone, commonly used off-label for insomnia, enhances sleep continuity and reduces SOL by blocking serotonin 5-HT2A receptors and inhibiting serotonin reuptake [114]. This results in increased SWS and SE, contributing to improved sleep quality. However, trazodone can cause next-day drowsiness and other side effects, which need to be managed carefully [55, 69].

Antidepressants can also be employed in the management of chronic pain symptoms. These agents enhance descending inhibitory pain pathways by increasing serotonin and norepinephrine levels, which reduces the perception of pain. Antidepressants can also mitigate the psychological distress often associated with chronic pain, further contributing to an overall improvement in quality of life for these patients [70, 115-117]. In this context, mirtazapine and trazodone, due to their sedative effects, can improve sleep quality in chronic pain patients, addressing the bidirectional relationship between poor sleep and heightened pain sensitivity.

Additionally, certain antidepressants, such as selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors, may decrease S2 sleep and promote respiratory problems [54, 89, 118]. These medications are particularly useful for patients who require both antidepressant and hypnotic effects. However, their use must be tailored to individual patient needs to minimize side effects and maximize therapeutic outcomes [111, 119].

Among anticonvulsants, pregabalin binds to the α2-δ subunit of presynaptic, voltage-dependent calcium channels in the central and peripheral nervous system, reducing the release of neurotransmitters such as glutamate, norepinephrine, and substance P. This action decreases neuronal excitability and transmission of pain signals, making pregabalin effective in managing neuropathic pain [120]. Pregabalin has been shown to increase SWS and SE, reduce SOL and WASO, and enhance overall sleep quality [71]. These effects are particularly beneficial for patients experiencing both pain and sleep disorders, providing a comprehensive therapeutic approach [66, 69]. The dual benefits of anticonvulsants in pain and sleep management, with reduced side effects, highlight their potential as a preferred treatment option in neuropathic pain patients.

Benzodiazepines are commonly used for their anxiolytic and hypnotic properties. Benzodiazepines are known to enhance the inhibitory effects of the neurotransmitter

GABA at the GABA-A receptor, leading to increased neuronal inhibition [121]. This action effectively reduces SOL and increases TST, thereby improving sleep initiation and maintenance [73]. However, long-term use of benzodiazepines can lead to dependence and tolerance due to downregulation of GABA-A receptors and alterations in brain chemistry, necessitating careful management and consideration of alternative therapies for chronic use [122].

Antipsychotics are occasionally used off-label for sleep disorders, though their effects can be variable. They work by antagonizing dopamine D2 receptors and various serotonin receptors, which can promote sedation and improve sleep in some patients [123]. However, antipsychotics are also associated with adverse effects on sleep quality and respiratory function, such as increased risk of sleep apnea [89]. This variability highlights the need for cautious use and thorough evaluation of risks and benefits when considering antipsychotics for sleep management in chronic pain patients.

Orexin receptor antagonists such as suvorexant and lemborexant have shown efficacy in managing insomnia and improving sleep in chronic pain patients. Suvorexant increased TST and reduced WASO in fibromyalgia patients, also decreasing pain sensitivity, though some experienced residual sedation and nausea [76]. Lemborexant improved insomnia symptoms in patients with chronic non-specific pain but did not affect pain scores, with some discontinuing due to lightheadedness and daytime sleepiness [87]. These findings highlight the potential of these medications to enhance sleep quality, with suvorexant also offering pain relief benefits.

Despite the comprehensive analysis, some limitations must be acknowledged. The division of results into biological mediators and drug effects highlights the heterogeneity of study designs and populations. The first category included 7 studies on biological mediators, while the second encompassed 26 studies on drug effects, with varying designs such as observational, case-control, cohort, and cross-sectional studies. This variability may affect the generalizability of the findings. Additionally, seven studies were limited to women, potentially introducing sex bias, and most studies focused on unspecified chronic pain or included multiple etiologies, limiting applicability to broader chronic pain populations. This manuscript does not aim to provide pharmacological recommendations for managing the complexity of chronic pain and sleep disorders. Instead, it seeks to identify the molecular factors influencing and being influenced by this intricate relationship, including emotional variables that further complicate it.

The subjective nature of chronic pain and sleep quality assessments and discrepancies between subjective and objective measures, particularly in opioid studies, highlight the need for standardized evaluation methods. The mixed effects of pharmacological agents on sleep stages, sleep onset latency, and sleep efficiency underscore the complexity of managing sleep disorders in chronic pain patients. The potential for adverse effects, such as increased risk of sleep apnea with opioid use, necessitates careful consideration of treatment risks and benefits. Further research with standardized methodologies and diverse populations is needed to enhance understanding and management of sleep disorders in chronic pain patients.

5. Conclusions

This systematic review highlights the complex interplay between chronic pain and sleep disorders focused on molecular influences. Elevated levels of inflammatory cytokines, particularly IL-6, and accelerated biological aging, are associated with increased pain sensitivity, poor sleep quality, and functional impairments in chronic pain patients. While pharmacological agents such as melatonin, pregabalin, and certain opioids demonstrate benefits in improving sleep efficiency, reducing wake after sleep onset, and alleviating pain, their effects are variable and dose dependent. Adverse outcomes, including increased insomnia severity, respiratory complications, and reduced sleep quality, were observed with certain medications, mainly at higher doses and being age dependent. The choice of medication should be tailored to the individual patient's needs,

considering the specific sleep disorders, pain characteristics, emotional state, and potential side effects. Further research is needed to optimize treatment strategies and improve outcomes for patients with chronic pain and sleep disorders.

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PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page and paragraph/ table #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both. **– MANDATÓRIO** - Página 1 (Título): "The Influence of Biological Mediators and Pharmacological Agents on Sleep in Chronic Pain Patients: A Systematic Review"	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. **– SEGUIR RECOMENDAÇÕES DA REVISTA** - Página 1 (Abstract): "Chronic pain and sleep disorders significantly impact the overall quality of life, triggering a cascade of physical, emotional, and social challenges. This systematic review synthesizes current evidence on the role of biological mediators and pharmacological agents influence on sleep and chronic pain. The main results demonstrated that various inflammatory cytokines and biological markers were elevated in patients with chronic pain conditions, showing associations with sleep disorders. Different drugs, including opioids, antidepressants, and antiepileptics, were commonly used and had varied effects on sleep stages, sleep quality, and pain management. While some drugs improved sleep efficiency and reduced pain intensity, others had mixed or negative effects on sleep quality and pain severity. The complexity pathophysiology of chronic pain and sleep disorders negatively affects the neurophysiology of the patient, being influenced by drugs and dependent on the inflammatory status of the patient."	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known. **– MANDATÓRIO** ***O rationale corresponde à justificação da importância da revisão sistemática*** - Página 1 (Parágrafo 1): "Chronic pain conditions have been linked to elevated levels of inflammatory cytokines, having an influential role in the central sensitization and heightened pain sensitivity (…) In a similar way, sleep disturbance is also linked with higher levels of biological markers (…) Cortisol, tau, β-amyloid 42, and fasting glucose are linked to both sleep disorders and chronic pain conditions, such as restless leg syndrome, fibromyalgia, and chronic low back pain [9-11]". - Página 2 (Paráfrago 2): "The bidirectional relationship between chronic pain and sleep involves multiple neurophysiological systems, including the opioid, monoaminergic, orexinergic, and immune systems, as well as the HPA axis and signaling molecules like melatonin, adenosine, and nitric oxide [24-27]" - Página 2 (Parágrafo 3): "Medications for managing pain and sleep disorders profoundly influence sleep pattern and	1, 2

		overall sleep quality, though their effects vary widely [34, 35]"	
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). **– MANDATÓRIO** - Página 2 (Parágrafo 4) "This systematic review aims to synthesize current evidence on the effects of various biological mediators and pharmacological agents on sleep in individuals with chronic pain." - Página 2 (Parágrafo 5): "The PICO question was: "How biological mediators and pharmacological agents affect the relation between chronic pain and sleep in human patients?""	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. **– FACULTATIVO** - Página 2 (Parágrafo 6): "The study protocol was registered in PROSPERO under the code CRD42024555479"	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. **– MANDATÓRIO** ***É altamente recomendado, de acordo com as boas práticas da Cochrane, que não sejam aplicados critérios de exclusão baseados na língua e/ou data de publicação dos estudos.*** - Página 2 (Parágrafo 7: "The inclusion criteria comprised articles written in English involving chronic pain patients, indicating drug involvement or measuring biological mediators, and analyzing perceived sleep quality and/or other sleep parameters as outcomes. The exclusion criteria included chronic pain of oncologic origin, other substances (E.g., alcohol and cannabis) and review articles."	2, 3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. **– MANDATÓRIO** ***Em consonância com as boas práticas da Cochrane, é mandatório que se verifique pesquisa em pelo menos duas bases de pesquisa bibliográfica (idealmente, deverão ser pesquisadas duas bases generalistas e uma específica da área). No caso de revisões sistemáticas de estudos experimentais/ensaios clínicos aleatorizados, é altamente recomendado que uma das bases pesquisadas corresponda à CENTRAL ou a bases de ensaios clínicos como a ClinicalTrials.gov.*** ***Estudos de revisão da literatura em que a pesquisa decorra numa única base de dados não serão classificados como revisões sistemáticas.*** - Página 2 (Parágrafo 6): "A search in three electronic bibliographic databases, Web of Science, PubMed, and Scopus, was carried out on 5 June 2024"	2
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. **– MANDATÓRIO** ***A query de pesquisa deve ser obrigatoriamente disponibilizada. A utilização de filtros de pesquisa da***	2

PRISMA 2009 Checklist

		InterTASC é altamente recomendada (https://sites.google.com/a/york.ac.uk/issg-search-filters-resource/home) - Página 2 (Parágrafo 6): "The search parameters were: "chronic pain AND sleep wake disorders" for Web of Science, "Chronic Pain"[Mesh]) AND "Sleep Wake Disorders"[Mesh for PubMed (] and "chronic AND pain AND sleep AND wake AND disorders" for Scopus)."	
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). **– MANDATÓRIO** ***As fases de selecção dos estudos primários devem ser descritas. Em consonância com as boas práticas da Cochrane, é mandatório que o processo de selecção envolva duas fases (fase de rastreio, em que os registos são seleccionados por título e abstract, e fase de inclusão, na qual se procede à leitura integral dos full texts). Em cada uma destas fases, o processo de selecção deve mandatoriamente envolver dois investigadores actuando de forma independente.*** - Página 2 (Parágrafo 7): "Titles and abstracts were screened by two authors using the Rayyan tool in blind mode to determine eligible studies. (…). In the second stage, the full-text articles of the eligible studies were examined for a more comprehensive understanding." - Página 3 (Parágrafo 4): "Search, selection, assessment (…) procedures were performed by two authors"	2, 3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. **– MANDATÓRIO** ***Trata-se de descrever de que forma se procedeu à extracção de dados dos estudos primários. Em consonância com as boas práticas da Cochrane, tal processo deverá envolver dois investigadores de forma independente.*** - Página 3 (Parágrafo 2): "Data extraction was manually performed" - Página 3 (Parágrafo 4): "data extraction procedures were performed by two authors"	3
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. **– MANDATÓRIO** ***Trata-se de descrever as variáveis para as quais foi obtida informação.*** - Página 3 (Parágrafo 2): "information sought included the type of study, population characteristics such as age and sex, type of chronic pain, sleep disturbance studied, drug involved or biological mediator measured, type and duration of intervention, main results, and complications observed"	3
Risk of bias in individual studies / Risk of bias across studies	12/ 15	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. **– MANDATÓRIO** ***Em todas as revisões sistemáticas, deverá existir um processo de avaliação da qualidade dos estudos primários. No caso de revisões sistemáticas de estudos experimentais/ensaios clínicos aleatorizados, a aplicação dos critérios de risco de viés (Risk of Bias) da Cochrane é altamente recomendada. No caso de***	3

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		revisões sistemáticas de estudos observacionais, poderão ser seguidos os critérios ROBINS ou os critérios dos National Institutes of Health (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools). - Página 3 (Parágrafo 5): "assessment of potential bias across individual studies was done by two authors utilizing appropriate tools in accordance with the study design. For randomized trials the revised Cochrane Risk of Bias tool (RoB 2) [58] and for non-randomized studies the Risk Of Bias In Non-randomized Studies – of Exposures (ROBINS-E) [59] or the Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) [60] if the study evaluated the effect of exposures or interventions. The final assessment for all studies was summarized using the Risk-of-bias VISualization tool (robvis) [61]."	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means). **– FACULTATIVO. APENAS NECESSÁRIO SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis. **– FACULTATIVO. APENAS NECESSÁRIO SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. **– FACULTATIVO. APLICÁVEL APENAS SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. **– MANDATÓRIO** - Página 4 (Parágrafo 1): "resulted in 1,279 publications (…) After duplicate removal, 1,007 studies were screened through reading the titles and abstracts and subsequently 43 were selected. (…) After conflict resolution (3 included and 3 excluded), 40 were retrieved for full text evaluation" - Página 4 (Parágrafo 1): "Subsequently, 1 manuscript was not retrieved, and 6 studies were excluded: one article in cannabinoids, one in alcohol, one article because it was not possible to find the full text; one was a review of literature; one did not include sleep parameters in results; one was a study protocol, and one was a preliminary study. Thus, 33 manuscripts were included in this systematic review. This process is summarized in Figure 1."	4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. **– MANDATÓRIO** - Página 5, 6 (Table 1): "Comparative Overview of Clinical Studies Involving Molecules on Sleep Disturbance and Chronic Pain"	5 - 13

		- Página 7 -13 (Table 2): “Comparative Overview of Clinical Studies Involving Drugs on Sleep Disturbance and Chronic Pain”	
Risk of bias within and across studies	19/ 22	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). – **MANDATÓRIO** - Página 15 (Parágrafo 4): “A total of six randomized trials were assessed using RoB 2 (Figure 3) concluding in an overall risk of bias of low in four and some concerns in two.” - Página 15 (Parágrafo 5): “Non-randomized studies were evaluated using ROBINS-E (Figure 4) or ROBINS-I (Figure 5) (…) The majority of studies raised concerns about potential bias due to confounding (…)Ten studies relied on questionnaires as the primary tool for measuring outcomes, raising concerns about potential bias”	15 - 17
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. – **FACULTATIVO. APLICÁVEL APENAS SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency. – **FACULTATIVO. MANDATÓRIO APENAS SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]). – **FACULTATIVO. APLICÁVEL APENAS SE FOR FEITA META-ANÁLISE** - Não aplicável, uma vez que não se realizou meta-análise.	
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). – **MANDATÓRIO** - Página 17 (Parágrafo 1): “elevated levels of inflammatory cytokines, particularly IL-6, play a crucial role in the pathophysiology of chronic pain and poor sleep quality” - Página 17 (Parágrafo 3): “while cytokines such as IL-6, IL-1β, and TNF-α can be present in peripheral sensitization and consequently central pain sensitivity [6, 7], hsCRP has contradictory influence in the specific context of chronic pain and sleep disorders” - Página 17 (Parágrafo 4): “Elevated morning cortisol and fasting glucose levels have been observed (…) indicating a potential link between chronic pain and metabolic dysregulation (…) elevated serum levels of tau and β-amyloid have been found in fibromyalgia (…) sleep disorders may contribute to the neurodegeneration in fibromyalgia” - Página 18 (Parágrafo 2): “higher DNA methylation scores correlates with increased insomnia severity, lower quality of life (functional and activity limitations) and has been linked to differential methylation in core circadian clock genes	17 - 20

		in individuals with high-impact chronic pain when compared to those without pain” - Página 18 (Parágrafo 4): “melatonin has been effective in improving sleep measures and alleviating pain in patients with conditions such as fibromyalgia and orofacial pain” - Página 18 (Parágrafo 5): “However, the benefits of melatonin are often transient, necessitating ongoing evaluation of its long-term efficacy and optimal dosing strategies” - Página 18 (Parágrafo 7): “Opioids can improve SE by reducing awakenings and the arousal index, particularly in older adults (…) However, chronic opioid use has been identified as a risk factor for central sleep apnea and ataxic breathing” - Página 19 (Parágrafo 1): “The impact of opioids on sleep is further complicated by their dose-dependent effects” - Página 19 (Parágrafo 3): “Mirtazapine improves sleep quality by increasing SWS and reducing WASO (…) Trazodone, commonly used off-label for insomnia, enhances sleep continuity and reduces SOL” - Página 19 (Parágrafo 4): “mirtazapine and trazodone, due to their sedative effects, can improve sleep quality in chronic pain patients” - Página 19 (Parágrafo 6): “Pregabalin has been shown to increase SWS and SE, reduce SOL and WASO, and enhance overall sleep quality [71]. These effects are particularly beneficial for patients experiencing both pain and sleep disorders” - Página 20 (Parágrafo 1): “effectively reduces SOL and increases TST, thereby improving sleep initiation and maintenance [73]. However, long-term use of benzodiazepines can lead to dependence and tolerance” - Página 20 (Parágrafo 2): “antipsychotics are also associated with adverse effects on sleep quality and respiratory function, such as increased risk of sleep apnea” - Página 20 (Parágrafo 3): “Orexin receptor antagonists such as suvorexant and lemborexant have shown efficacy in managing insomnia and improving sleep in chronic pain patients”	
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). **– MANDATÓRIO** - Página 20 (Parágrafo 4): “The division of results into biological mediators and drug effects highlights the heterogeneity of study designs and populations (…) seven studies were limited to women, potentially introducing sex bias, and most studies focused on unspecified chronic pain or included multiple etiologies, limiting applicability to broader chronic pain populations”	20
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research. **– MANDATÓRIO** - Página 20 (Parágrafo 6): “The choice of medication should be tailored to the individual patient's needs, considering the specific sleep disorders, pain characteristics, emotional state, and potential side effects” - Página 21 (Parágrafo 1): “Further research is needed to optimize treatment strategies and improve outcomes for patients with chronic pain and sleep disorders”	20, 21
FUNDING			

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Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. **– SEGUIR RECOMENDAÇÕES DA REVISTA** - Página 21 (Funding): “This study was performed within the scope of the grant “Cátedra de Medicina da Dor” from Fundação Grunenthal”	21

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

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- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as **EndNote**, **ReferenceManager** or **Zotero** to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for **Endnote** and **Zotero** are available.

References should be described as follows, depending on the type of work:

- Journal Articles:
  1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.
- Books and Book Chapters:
  2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
  3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
- Unpublished materials intended for publication:
  4. Author 1, A.B.; Author 2, C. Title of Unpublished Work (optional). Correspondence Affiliation, City, State, Country. year, *status* (*manuscript in preparation*; *to be submitted*).
  5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* year, *phrase indicating stage of publication* (*submitted*; *accepted*; *in press*).
- Unpublished materials not intended for publication:
  6. Author 1, A.B. (Affiliation, City, State, Country); Author 2, C. (Affiliation, City, State, Country). Phase describing the material, year. (phase: Personal communication; Private communication; Unpublished work; etc.)

- Conference Proceedings:
  7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).
- Thesis:
  8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.
- Websites:
  9. Title of Site. Available online: URL (accessed on Day Month Year).
  Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as **WebCite**. Archived websites should be cited using the link provided as follows:
  10. Title of Site. URL (archived on Day Month Year).

See the **Reference List and Citations Guide** for more detailed information.

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Preparing Figures, Schemes and Tables

- *IJMS* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.
- Images of cells and western blots should be large enough to see the relevant features. In addition, uncropped, untouched, full original images of western blots should be uploaded with the other figure files.

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Original Images Requirements

To ensure the integrity and scientific validity of electrophoretic blots, gels, and microscopy images, the Editorial Office reserves the right to request original, uncropped, and unadjusted images upon submission. Original images must be provided in a single zip archive as Supplementary Material files at a sufficiently high resolution (a minimum of 1000 pixels in width/height, or a resolution of 300 dpi or higher) or a link where original unprocessed images were deposited at the time of initial submission. Preferred formats for gels, blots, and microscopy images (light and electron microscopy e.g., TEM, SEM, Cryo-EM, etc.) are **TIFF**, PNG, GIF, and EPS. Failing to provide original images before the final decision might result in the rejection of the paper, and the issue may be raised with the authors' institutions.

Digital images within the manuscript submitted should be minimally processed. A certain degree of image processing is acceptable for publication, but the final image must correctly represent the original data and conform to community standards. Please note that electron microscopy images submitted with a manuscript should be presented in full, without cropping the image, and with all the relevant details clearly visible.

Where cropped images are shown in figures, a full scan of the entire original image(s) must be submitted as part of the Supplementary Material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique used must be declared in the methods section of the manuscript. Image grouping and splicing must be clearly stated in the manuscript and the figure text.

The use of software filters to improve image quality is not recommended. Slight adjustments to contrast settings, brightness, intensity, color, etc., are generally acceptable but must be applied equally to the entire image. Excessive image manipulations that are specific to one area of an image and are not performed on other areas are considered part of a non-ethical practice of image processing, as this emphasizes experimental data relative to the control.

Images gathered at different times or from different locations should not be combined into a single image unless it is stated that the resultant image is a product of time-averaged data or a time lapse sequence. If juxtaposing images are essential, the borders should be clearly demarcated in the figure and described in the legend.

Electrophoretic gels and blots

Please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel. When sample processing controls are run on different gels, this must be indicated in the figure legend. Cropped gels in the paper must retain all important bands.

- Molecular weight markers are included or indicated on the raw image, and any lanes not included in the final figure are marked with an “X” above the lane label on the original blot/gel image. All labeling and annotation should be performed without obscuring any data or background bands.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- Each original image is annotated, and corresponds to the figure in the main article or supplementary materials, and each lane or loading order is labeled.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

Spliced gels need a thin dividing line to be added to indicate the alteration. It is fine to remove a complete lane and splice the remaining lanes together, provided that the spliced fragments originate from the same original image. In such cases, the alteration must be indicated in the figure by a thin white or black line between the juxtaposed pieces and must be described in the figure legend. Images must contain the background “noise” they originally contained. The background should not appear as one uniform color.

It is not acceptable practice to “clean up” the background of images with rubberstamps, “wipe” tools to improve the aesthetic appearance or over-adjust the brightness or contrast to remove the background.

All experimental samples and controls used for one comparative analysis should be run on the same blot/gel image. For quantitative analyses, please provide the blots/gels for each independent biological replicate used in the analysis.

Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, the figure legend must state that the samples derive from the same experiment or parallel experiments and that gels/blots were processed in parallel.

We encourage the inclusion of the following with the final revised version of the manuscript for publication:

In the Methods section, specify the type of equipment (microscopes/objective lenses, cameras, detectors, filter model, and batch number), the acquisition software used, and the magnification or the scale bar in the figure caption. Although we appreciate that there is some variation between instruments, equipment settings for critical measurements should also be listed.

We encourage the deposition of unprocessed image files in a publicly available database (a **link** to the downloadable table from data availability instructions should be shared here) (including relevant metadata for acquisition information, including time and space resolution data (xyzt and pixel dimensions); image bit depth; experimental conditions such as temperature and imaging medium; and fluorochromes (excitation and emission wavelengths or ranges, filters, and dichroic beam splitters) if any).

Processing software should be named in the Methods section and any manipulations should be indicated in the relevant figure legends (such as type of deconvolution, three-dimensional reconstructions, surface and volume rendering, “gamma changes”, filtering, thresholding, and projection).

Comprehensive guidelines on data management and the ethical handling of digital images can obtained from The Office of Research Integrity: **http://ori.hhs.gov/images/ddblock/data.pdf**

MDPI is a member of the Committee on Publication Ethics and takes the responsibility to uphold strict ethical policies and standards very seriously.

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Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data including, but not limited to protocols, analytic methods, raw data, processed data, code, software, algorithms, and study material. The data should be FAIR – findable, accessible, interoperable, and reusable – so that other researchers can locate and use the data.

We recommend that data and code should be deposited in a trusted repository that will allow for maximum reuse (see the Data Preservation section below). If this is not possible, authors are encouraged to share the specific reason in the Data Availability Statement and make this material available upon request to interested researchers. In addition, research materials necessary to enable the reproduction of an experiment should be indicated in the Materials and Methods section. Individual journal guidelines can be found at the journal ‘Instructions for Authors’ page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by **TOP Guidelines**.

Where ethical, legal, or privacy issues are present, data should not be shared. The authors should clarify the availability status of the data upon submission and make any limitations or exceptions clear in the Data Availability Statement. Authors should ensure that the data shared is in accordance with consent provided by participants on the use of confidential data. Authors should ensure that the publication of such data does not compromise the anonymity of the participants or breach local data protection laws.

In situations where access is restricted to protect confidential or proprietary information, authors will be requested to clearly explain the restrictions on the dataset and make the data available upon request, with permission for the purposes of peer review.

MDPI recognizes that some institutions and funding agencies only require the retention of research data for a finite period after a project's completion or publication. However, there are no such limits specified within the MDPI Data Availability Policy and, therefore, we encourage the authors to archive their research data through appropriate data repositories or provide us with minimal datasets within Supplementary Material.

Data availability statements

Data availability statements are required for all articles published with MDPI. During the peer review and editorial decision process, authors can be asked to share existing datasets or raw data that have been analyzed in the manuscript, and whether they will be made available to other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analyzed in the manuscript.

Below are the recommended Data Availability Statements:

Data availability status	Recommended Data Availability Statement
Data available in a publicly accessible repository	The original data presented in the study are openly available in [repository name, e.g., FigShare] at [DOI/URL] or [reference/accession number].
Data available on request due to restrictions (e.g., privacy, legal or ethical reasons)	The data presented in this study are available on request from the corresponding author due to (specify the reason for the restriction).
3rd Party Data	Restrictions apply to the availability of these data. Data were obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
Embargo on data due to commercial restrictions	The data that support the findings will be available in [repository name] at [URL / DOI link] following an embargo from the date of publication to allow for commercialization of research findings.
Restrictions apply to the datasets	The datasets presented in this article are not readily available because [include reason, e.g., the data are part of an ongoing study or due to technical/ time limitations]. Requests to access the datasets should be directed to [text input].
Data derived from public domain resources	The data presented in this study are available in [repository name] at [URL/DOI], reference number [reference number]. These data were derived from the following resources available in the public domain: [list resources and URLs].
Data sharing is not applicable (only appropriate if no new data is generated or the article describes entirely theoretical research)	No new data were created or analyzed in this study. Data sharing is not applicable to this article.
Data is contained within the article or supplementary material	The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).
Dataset available on request from the authors	The raw data supporting the conclusions of this article will be made available by the authors on request.

Data preservation

MDPI acknowledges that researchers, institutions, journals, and data repositories have a shared responsibility to ensure long-term data preservation, and MDPI encourages authors to select data repositories with this goal in mind.

MDPI encourages authors to commit to preserving their datasets on their laboratory or institutional servers, for at least five years after publication. If, during that time, the repository to which the data were originally submitted disappears or experiences data loss, we may ask the authors to upload the data to another repository and publish a correction or update to the original publication.

If authors remove their data from the original public repository or change access criteria in a manner that is inconsistent with the publication, we may ask authors to notify the editorial office as soon as possible.

How to choose an appropriate data repository

MDPI encourages the submission of data to community-recognized data repositories where possible. We recommend the authors visit **re3data.org** or **fairsharing.org** to help identify registered and certified data repositories relevant to their subject area if no community resource is available. If the authors' institution has its generalist data repository this can be used to host authors' data as long as the repository can mint **DataCite DOIs**, and allows for data to be shared under open terms of use (for example the **CC0 waiver**).

Data repository criteria

The following criteria should be considered when selecting an appropriate repository, ensuring that platforms:

- Ensure long-term persistence and preservation of datasets in their published form;
- Provide stable identifiers for submitted datasets (DOIs in most cases);
- Allow public access to data without barriers, such as logins or paywalls;
- Support open licenses (CC0 and CC-BY, or their equivalents, are required in most cases);
- Provide confidential review of submitted datasets without the requirement for reviewers to provide identifying information.

Data citation

Authors are encouraged to formally cite any datasets stored in external repositories that are mentioned within their manuscript, including the main datasets that are the focus of the submission, as well as any other datasets that have been used in the work. For previously published datasets, authors should cite both the related research articles and the datasets themselves. Appropriate citation of data is checked and enforced by *Journal Editorial* staff before publication.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.

- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

Institutional Review Board Statement

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the **Declaration of Helsinki of 1975**, which was revised in 2013. According to point 23 of this declaration, approval from the local Institutional Review Board (IRB) or another appropriate ethics committee must be obtained before undertaking the research to confirm that the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in the 'Institutional Review Board Statement' Section of the article.

Example of an Institutional review board statement: "The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code) on [date of approval]."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed whether their anonymity is assured, why the research is being conducted, how their data will be used, and if there are any risks involved in participating. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or cite the local or national legislation that indicates ethics approval is not required for this type of study. When a study has been granted exemption, the name of the ethics committee that provided this should be stated in the 'Institutional Review Board Statement' Section with a full explanation for the rejection of ethical approval.

Informed Consent Statement

Manuscripts reporting studies involving human participants, human data, or human tissue must include a **statement of informed consent for participation** in research. Verbal informed consent to participate in a study can be acceptable under some circumstances (such as in ethnographic studies). The authors must explain the rationale for using this kind of consent in the "Informed Consent Statement" Section. For verbal informed consent, a copy of the script used must be provided during the submission stage.

For all manuscripts that include identifying patient/participant information (personal details, images, or videos relating to an individual person), written **informed consent for the publication** of these details must be obtained from patients/participants (or their relatives/guardians) before submitting to an MDPI journal. A blank version of the form used to obtain permission (without the patient/participant names or signature) should be provided upon submission. You may refer to our **template permission form** and provide an appropriate form after consulting with your affiliated institution.

For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients'/participants' and any other individuals' privacy, please do not send signed forms.

Private information identifying participants need not be included unless the identifiable materials are of relevance to the research (e.g., photographs of participants' faces that show a particular symptom). Patients'/participants' initials or other personal identifiers must not appear in any images. Patient/participant details must be anonymized as much as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. Steps necessary to protect privacy may include de-identifying data, adding noise, or blocking portions of the database. Editors reserve the right to reject any submission that does not meet these requirements.

The Editorial Office reserves the right to request further documentation when necessary. The submitted manuscript will be scrutinized by the Editorial Office, and upon request, documentary evidence (signed consent forms and any related discussion documents from the ethics board) must be provided.

Example of an Informed Consent Statement: "Informed consent for participation was obtained from all subjects involved in the study." OR "Informed consent for participation is not required as per local legislation [provide local legislation]." OR "Verbal informed consent was obtained from the participants. Verbal consent was obtained rather than written because [state the reason]", OR "Informed consent for publication was obtained from all identifiable human participants."

Requirements for Studies on Vulnerable Groups and Organ Transplants

If a study involves vulnerable groups, the manuscript will undergo an additional review by the editorial office. If requested, the author must provide documentary evidence, including blank consent forms and any related discussion documents from the ethics board or other relevant bodies. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., an explanation regarding why such categorization was needed must be clearly stated in the article.

Articles describing human organ transplantation studies are subject to all policies for research involving human subjects. Additionally, the authors must specify the institution(s), clinic(s), or department(s) from which the organs or tissues were sourced. MDPI does not accept manuscripts that report data on organs and/or other materials obtained from illegal commercial activity, executed prisoners, or other unethical practices relating to organ donations. Manuscripts addressing this practice, such as editorials or reports on its secondary consequences, may be considered at the discretion of the Editor-in-Chief but require a written appeal to the editorial office before submission. For further resources on organ transplantation, MDPI follows the glossary maintained by the Organ Procurement and Transplantation Network (**https://optn.transplant.hrsa.gov/patients/glossary/**).

Clinical Trials Registration

Registration

Clinical trials are subject to all policies regarding **Research Involving Human Subjects**. In addition, MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication. The ICMJE defines a clinical trial as any research project that prospectively assigns people or a group of people to an intervention, with or without concurrent comparison or control groups, to study the relationship between a health-related intervention and a health outcome. Therefore, 'clinical trial' not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refers to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors must pre-register clinical trials with an international clinical trials register. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation's **International Clinical Trials Registry Platform**. The name of the registry, trial registration number and date of registration should be included in the Institutional Reviewer Board statement or in the methods section.

Purely observational studies (e.g., cohort studies, cross-sectional studies, and case–control studies) do not require registration. Editors may consider exceptions to pre-trial registration requirements in some cases. If an exception is granted, authors must retroactively register the trial and clearly indicate the date and reasons for the retroactive registration in the methods section of the publication.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review.

Randomized Clinical Trial Reporting Guidelines

In addition to clinical trial registration, MDPI requires a completed **CONSORT 2010 checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized clinical trial. Checklist templates can be found on the **CONSORT website** which also describes several CONSORT checklist extensions for different designs and types of data beyond two-group parallel trials. At a minimum, clinical trial articles should report the content addressed by each item of the checklist.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/Author%20Checklist%20-%20Full.pdf**. The journal *IJMS* requires authors to submit the completed checklist at submission, and it will be made available to reviewers. Editors reserve the right to reject submissions that do not adhere to these guidelines based on ethical or animal welfare concerns, or if the procedure described does not appear to be justified by the value of the work presented.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.dpi.nsw.gov.au/dpi/animals/animal-ethics-infolink/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Dual Use Research of Concern

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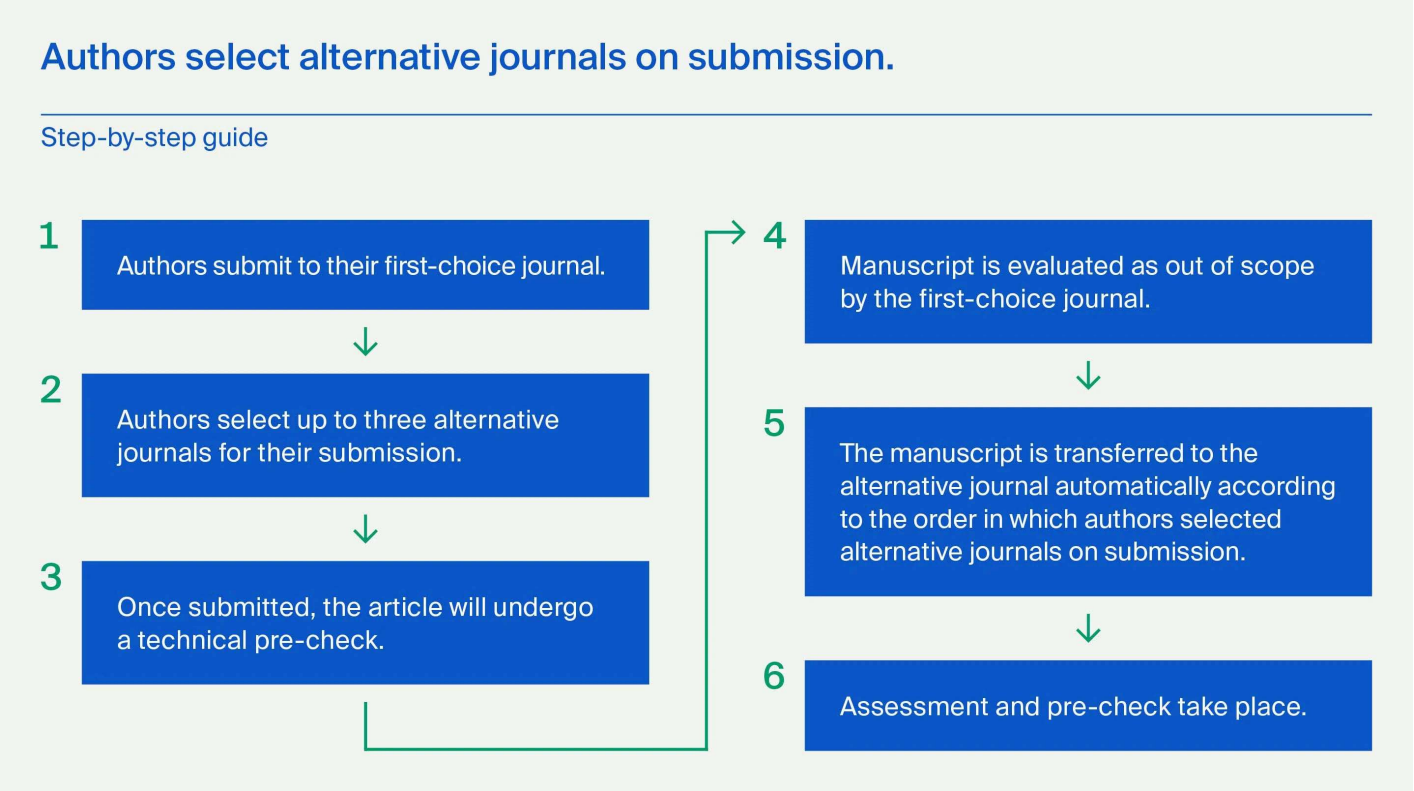

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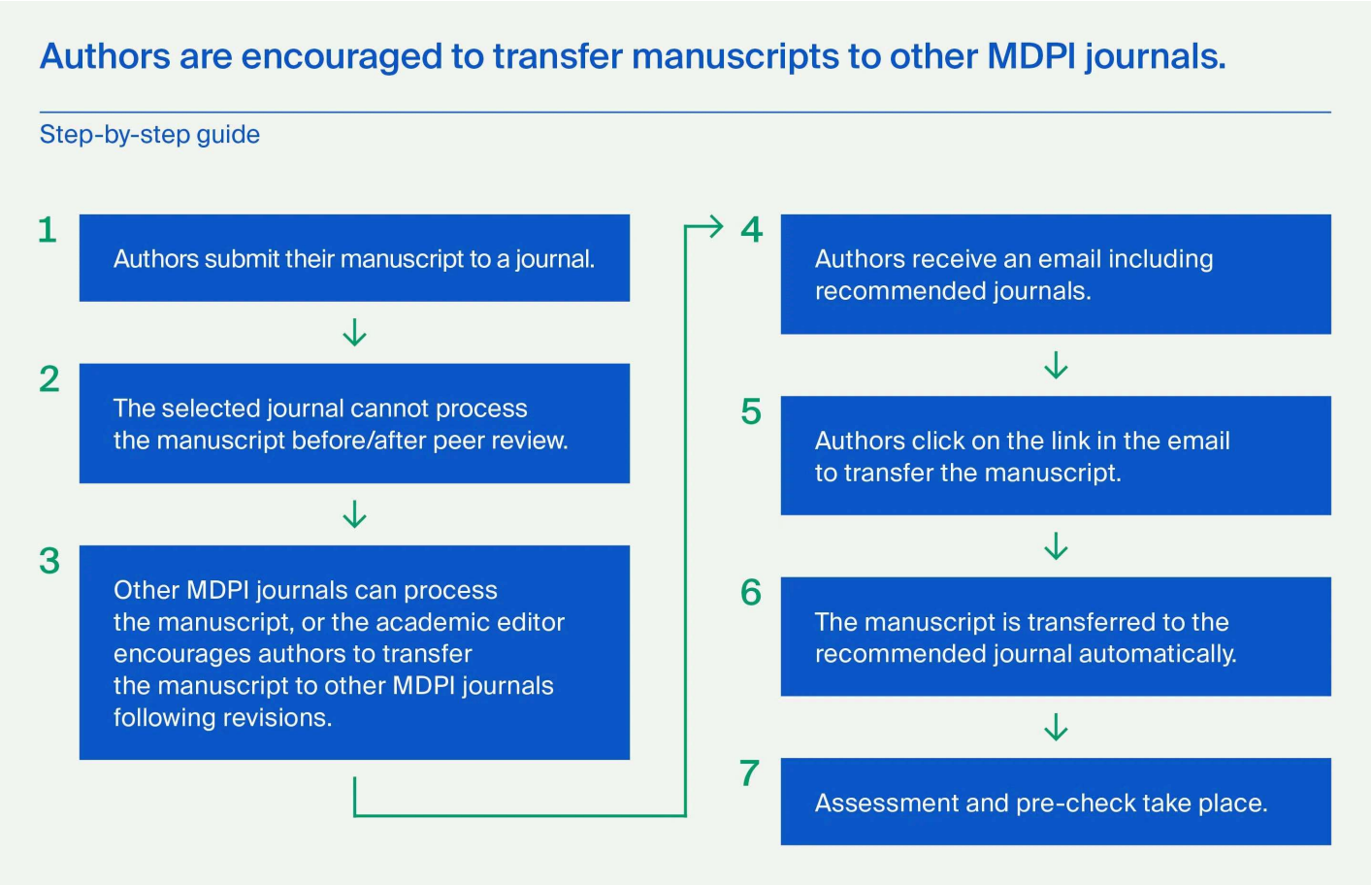

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