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Contents

Reviews

An underrated clinical tool: CBC-derived inflammation indices as a highly sensitive measure of systemic immune response and inflammation

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

This editorial explores the clinical potential of complete blood count-derived inflammation indices (CBC-DIIs) as sensitive and cost-effective measures of systemic inflammation and immune response.

Key words: immune response, systemic inflammation, complete blood count, CBC-derived indices, CBC parameters

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Highlights

- CBC-DIIs offer a sensitive and low-cost measure of systemic immune activation and suppression.
- The correlation of indices like SII, SIRI, and AISI extends beyond disease severity in cancer, cardiovascular, autoimmune, and neuropsychiatric conditions.
- CBC-DIIs demonstrate clinical utility in depression, trauma, periodontitis, diabetic complications, and other diseases and conditions.
- Current indices rely on linear algebraic expressions, which limit their specificity in complex or chronic disease states with an inflammatory background.
- Creating nonlinear CBC-DIIs equations and integrating these with genomic, imaging, and nonlinear computational models may enhance personalized diagnostics.

Introduction

Systemic inflammation is central to developing and progressing diverse diseases, including malignancies, cardiovascular diseases (CVD) and chronic inflammatory conditions. Traditionally, biochemical markers have been used to assess inflammation; however they often fail to capture the dynamic interplay of immune responses while incurring higher costs and complexity.

Several ratios and composite multiparametric indices of biochemical and hematological markers are used to assess inflammation, disease severity and prognosis in various conditions, like albumin-to-globulin ratio, C-reactive protein (CRP) to albumin ratio, comprehensive inflammation index, CRP to lactate dehydrogenase ratio, fibrinogen-to-albumin ratio (FAR), Glasgow prognostic score (GPS), monocyte-to-lymphocyte ratio (MLR), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), CD4/CD8 ratio, CD8/CD56 ratio, interleukin (IL)-1β/IL-10 ratio, IL-10/tumor necrosis factor alpha (TNF-α) ratio, etc.

The complete blood count (CBC), a routine and cost-effective test, offers an attractive alternative. Composite indices derived from standard CBC parameters enable a sensitive and rapid assessment of systemic immune activation. We focus on 3 such indices – the Systemic Immune-Inflammation Index (SII), Systemic Inflammation Response Index (SIRI) and Aggregate Index of Systemic Inflammation (AISI). These indices integrate measurements of neutrophils (NEU), platelets (PLT), monocytes (MON), and lymphocytes (LYM) to reflect the equilibrium between pro-inflammatory and anti-inflammatory processes. The discussion encompasses the biological mechanisms behind these indices, their clinical relevance as diagnostic and prognostic tools, and the limitations of their current linear models, setting the stage for future nonlinear and integrative diagnostic frameworks.

This editorial is intended for clinicians and biomedical researchers involved in diagnosing, prognosis and managing inflammation-related diseases. For clinicians, it highlights the practical and actionable potential of CBC-derived inflammation indices (CBC-DIIs), which are readily available, cost-effective and underutilized in routine practice. By highlighting their role in common diagnostic pathways and monitoring strategies, we aim to promote more confident clinical adoption. For researchers, we call attention to the field's methodological stagnation. Despite the increasing number of validation studies, a pressing need remains for innovation through systems biology, nonlinear modeling and integration with genomic and imaging data. These advances could transform CBC-DIIs into mechanistically insightful and computationally robust tools for personalized medicine.

The following sections discuss the biological mechanisms behind these indices, their clinical relevance and the limitations of their current linear models, setting the stage for an integrative diagnostic framework.

The mechanisms behind CBC-derived indices in inflammation assessment

The foundation of CBC-DIIs lies in the dynamic interplay between circulating cytokines,[1] hematopoiesis,[2] blood cell counts and their activation.[3,4] For clinicians, these shifts manifest as measurable changes in routine CBC parameters (Table 1,2), such as neutrophilia or thrombocytosis, providing an indirect but meaningful reflection of underlying immune activity.

Increased levels of pro-inflammatory cytokines can lead to cell activation, proliferation and increased counts (e.g., neutrophilia, thrombocytosis), while anti-inflammatory cytokines can reduce activation and maintain immune homeostasis. Each cytokine has distinct and overlapping effects, sometimes promoting or inhibiting the proliferation or activation of blood cells in response to specific immune challenges (Table 3,4). Understanding these mechanisms enhances clinicians' ability to interpret elevated indices in specific clinical contexts, such as distinguishing between infection and autoimmunity or assessing treatment response.

From a research standpoint, this mechanistic view provides fertile ground for biomarker development. Each cytokine exerts distinct effects on hematopoietic lineages, often in overlapping or opposing ways. Aggregating cell counts

Table 1. Cytokine receptors and cytokines secreted by immune cells commonly quantified in complete blood count (CBC) analyses

Cell type	Cytokine receptors	Cytokines secreted
B cells (B lymphocytes)	IL-4 receptor, IL-6 receptor, CD40 receptor, BAFF receptor, TNF receptors (TNFR1, TNFR2)	IL-4, IL-6, IL-10, TNF-α
BASO	IL-3 receptor, GM-CSF receptor, TNF Receptors (TNFR1, TNFR2)	IL-4, IL-13, histamine
EOS	IL-5 receptor, IL-3 receptor, GM-CSF Receptor, TNF receptors (TNFR1, TNFR2)	IL-5, GM-CSF, IL-3, TNF-α
LYM	IL-2 receptor (CD25), IL-7 receptor, CD40 receptor, TNF receptors (TNFR1, TNFR2), IL-4 receptor	IL-2, IFN- γ, IL-4, IL-10, IL-17
MON	toll-like receptors (TLR), CCR2, GM-CSF receptor, TNF receptors (TNFR1, TNFR2)	TNF-α, IL-6, IL-1, IL-10, TGF-β
NK (natural killer cells; part of LYM count)	IL-15 receptor, IL-2 receptor, IL-12 receptor, TNF receptors (TNFR1, TNFR2)	IFN- γ, TNF-α
NEU	IL-8 receptor (CXCR1, CXCR2), TNF receptors (TNFR1, TNFR2), IL-1R, GM-CSF receptor	TNF-α, IL-1, IL-8, GM-CSF, reactive oxygen species (ROS)
PLT	IL-1 receptor, TNF receptors (TNFR1, TNFR2), TGF-β receptor, GM-CSF receptor, thrombopoietin receptor (TPO), CXCR4, CXCR3 (chemokine receptors)	IL-1, TGF-β, platelet-derived growth factor (PDGF), thromboxane A2 CXCL4 (PF4), CXCL12 (SDF-1), IL-8 (CXCL8)
RBC	erythropoietin receptor (EpoR), IL-3 receptor, IL-6 receptor, GM-CSF receptor, TNF receptors (TNFR1, TNFR2)	IL-6, TGF-β, thrombospondin-1, erythropoietin (Epo)
T cells (T lymphocytes; part of LYM count)	IL-2 receptor, IL-7 receptor, CD40 receptor, IL-15 receptor, TNF receptors (TNFR1, TNFR2)	IL-2, IFN-γ, IL-4, IL-10, IL-17

Table 2. Cytokine receptors expressed by hematopoietic progenitor and stem cells

Cell type	Cytokine receptors	Cytokines secreted
CLP (common lymphoid progenitors)	IL-7, SCF, Flt-3, TLR ligands	IL-7, IL-15, IL-4, TLR ligands
CMP (common myeloid progenitors)	SCF, FL, IL-3, GM-CSF, TPO, IL-6, IL-1, IL-4	G-CSF, GM-CSF, IL-6, IL-3
EP (erythroid progenitor)	Epo, GM-CSF, SCF	EPO, GM-CSF, IL-3
GMP (granulocyte-macrophage progenitor)	GM-CSF, IL-3, IL-6, SCF, G-CSF, TLR ligands	G-CSF, IL-3, GM-CSF, IL-6
HSC (hematopoietic stem cells)	SCF (stem cell factor), FL (Flt-3 ligand), IL-3, GM-CSF, TPO (thrombopoietin)	SCF (stem cell factor), IL-3, TPO, GM-CSF, Flt-3 ligand, IL-6
MEP (megakaryocyte-erythroid progenitor)	Epo (erythropoietin), SCF, TPO, IL-3	EPO, TPO, IL-3, GM-CSF
MEP (monocyte-erythroid progenitor)	GM-CSF, SCF, IL-3, IL-5, TLR ligands	IL-3, IL-5, GM-CSF
MPPs (multipotent progenitors)	SCF, FL, IL-3, GM-CSF, TPO, IL-6	GM-CSF, IL-3, TPO, IL-6, IL-1, IL-4

into composite indices captures the net effect of these signals but lacks resolution in the specific pathways involved. Advanced studies that integrate CBC-DIIs with cytokine profiling or transcriptomic analyses could offer deeper insights into the biological mechanisms underlying immune imbalance and disease progression.

Composite indices based on CBC: SII, SIRI and AISI

Building on these mechanistic insights, 3 primary composite indices have been developed, each offering distinct value in assessing systemic inflammation. For clinicians, these shifts are reflected in measurable changes in routine CBC parameters (Table 1,2), such as neutrophilia or thrombocytosis, offering an indirect yet meaningful indication of underlying immune activity.

Systemic immune-inflammation index was developed by Hu et al.[5] in 2014. The SII incorporates the absolute counts of NEU, PLT and LYM using the following formula, with counts expressed as $\times 10^9$ cells/L:

$$SII = (NEU \times PLT)/LYM$$

Systemic immune-inflammation index captures the interplay between pro-inflammatory cells (NEU and PLT) and anti-inflammatory LYM. Elevated SII values typically indicate a predominance of pro-inflammatory activity, commonly observed in conditions such as cancer and CVD, where a high SII is associated with adverse outcomes.[6–9]

The SII was introduced by Qi et al.[10] The SIRI comprises NEU, MON and LYM, reflecting the involvement of innate and adaptive responses in inflammation. The formula, with counts expressed in $\times 10^9$ cells/L, is:

$$SIRI = (NEU \times MON)/LYM$$

The SIRI incorporates parameters that reflect the inflammatory response (such as NEU, MON and LYM count). The SIRI is particularly useful in diseases with

Table 3. Circulating cytokines and their impact on blood cell counts

Blood cell type	Impact on cell count
LYM	IL-2 stimulates T cell activation and proliferation, resulting in increased T cell count during immune responses. IL-7 is essential for T cell survival and memory T cell maintenance, contributing to T cell homeostasis. IFN-γ activates NK cells and macrophages (MΦ), enhancing immune responses and antigen presentation, contributing to LYM activation. IL-4 promotes Th2 differentiation, leading to B cell activation and antibody production. IL-5 stimulates eosinophil differentiation and activation, influencing the adaptive immune response. IL-10 is anti-inflammatory and helps regulate LYM activity, reducing LYM activation and inflammatory responses. IL-17 is released by Th17 cells, contributing to T cell activation and NEU recruitment during autoimmune and inflammatory diseases. IL-21 enhances B cell and T cell activation, promoting B cell proliferation and antibody production. TGF-β regulates T cell differentiation and suppresses autoimmune responses, aiding in immune tolerance and homeostasis. IL-12 enhances Th1 differentiation and NK cell activity, boosting the immune response to pathogens. IL-6 supports B cell activation and antibody production while influencing T cell differentiation. IL-15 supports NK cell survival and proliferation, playing a role in immune memory and response to viral infections.
MON	GM-CSF stimulates monocyte differentiation and proliferation, leading to an increased MON count during inflammation. TNF-α induces monocyte activation and increases MON migration to inflammation sites, contributing to monocytosis. IL-6 promotes MON differentiation into MΦ and enhances their inflammatory function. IL-1 is involved in MON recruitment to sites of infection and inflammation, driving monocytosis and activation. IL-10 suppresses MON activation, promoting immune tolerance and anti-inflammatory effects during chronic inflammation. TGF-β regulates MON and MΦ differentiation and plays a key role in immune regulation and wound healing. IL-8 is involved in MON chemotaxis, promoting their migration to inflammation sites. M-CSF enhances MON differentiation and survival, supporting their function in immune responses. CCL2 promotes MON recruitment to sites of inflammation, contributing to monocytosis and inflammatory responses.
NEU	IL-8 promotes NEU chemotaxis, leading to neutrophilia and neutrophil activation at sites of infection or inflammation. GM-CSF increases NEU production and survival in the bone marrow, leading to neutrophilia and enhanced neutrophil function. IL-1 enhances NEU release from the bone marrow and activation during inflammation. TNF-α induces neutrophil recruitment and activation at sites of infection and injury, leading to neutrophilia. IL-17 stimulates NEU recruitment and activation, contributing to neutrophil inflammation and response to infection. G-CSF stimulates NEU production and mobilization from the bone marrow, leading to neutrophilia. IL-6 indirectly supports NEU function during infection and inflammation. CXCL1 and CXCL2 act as chemotactic factors for NEUs, promoting NEU migration to the site of infection or injury. IL-4 reduces the inflammatory function of NEUs, contributing to resolution of inflammation. IL-10 reduces NEU activation during inflammatory processes, helping to prevent excessive inflammation.
PLT	IL-1 increases PLT activation and aggregation, leading to thrombocytosis and enhanced PLT function in inflammation or injury. TNF-α enhances PLT aggregation and increases PLT production during inflammation and immune responses. TGF-β regulates PLT aggregation and function during tissue repair and immune modulation. CXCL4 (PF4) promotes PLT aggregation and WBC cell recruitment, contributing to wound healing and inflammation. PDGF promotes tissue repair and platelet function in wound healing and tissue regeneration. IL-6 can influence PLT production during inflammation, leading to thrombocytosis. IL-8 can activate PLTs and enhance their ability to recruit immune cells during infection and inflammation. Thromboxane A2 enhances PLT aggregation and vasoconstriction, contributing to hemostasis and inflammation. IL-33 can activate platelets during allergic responses, leading to PLT degranulation and shift in their parameters. CXCL12 enhances PLT recruitment to sites of injury and inflammation. CXCL3 can attract PLTs and other immune cells to sites of injury, modulating immune responses.
RBC	Epo stimulates RBC production in response to hypoxia, leading to increased RBC count and improved oxygen-carrying capacity of the blood. IL-3 enhances the proliferation of erythroid progenitors, contributing to RBC production. IL-6 suppresses erythropoiesis during inflammatory responses, potentially leading to anemia of inflammation. TGF-β inhibits RBC differentiation during inflammation, reducing RBC production. IL-1 β a minor role in RBC production but can suppress erythropoiesis during stress and inflammation. GM-CSF has a minor effect on RBC production but mainly influences myeloid progenitors. Flt-3 Ligand (FL) supports hematopoietic stem cell (HSC) maintenance but does not directly affect RBC count.

Table 4. Circulating cytokines and their impact on blood cell parameters

Blood parameter	Impact on cell parameters
ALY (atypical lymphocytes)	IFN-γ, IL-2, IL-15, IL-17, IL-21 enhance T cell activation, contributing to an increase in ALY# and ALY% in immune activation states, such as infections, cancer, or autoimmune disorders.
ALY% (atypical lymphocyte percentage)	
IG (immature granulocytes)	TNF-α, IL-1, G-CSF, GM-CSF, CXCL1, CXCL2 (MIP-2) increase the release of IGs into circulation. Increase of IG%, is reflecting early NEU release during acute inflammation or infection.
IG% (immature granulocyte percentage)	
MCH (mean corpuscular hemoglobin)	IL-6, TNF-α, IL-1, TGF-β can reduce hemoglobin production, leading to low MCH during inflammation and anemia of chronic disease.
MCHC (mean corpuscular hemoglobin concentration)	IL-6, TNF-α, IL-1, TGF-β reduce MCHC, contributing to hypochromic anemia during systemic inflammation.
MCV (mean corpuscular volume)	IL-6, TNF-α, IL-1, Epo (Erythropoietin), Flt-3 Ligand (FL) influence RBC size. Increased Epo promotes larger RBCs, increasing MCV in hypoxia.
P-LCR (platelet large cell ratio)	IL-1, TNF-α, GM-CSF, IL-3, TPO, CXCL4 (PF4), PDGF, Thromboxane A2 increase PLT activation and the proportion of large PLTs.
PCT (plateletcrit)	IL-6, TNF-α, GM-CSF, TPO, CXCL4 (PF4), IL-33 increase PLT production and activation, leading to increased PCT during inflammation.
RDW-CV (red cell distribution width – coefficient of variation)	IL-6, TNF-α, IL-1, G-CSF, M-CSF increase RDW-CV, reflecting greater variation in RBC size due to inflammation or changes in erythropoiesis.
RDW-DV (red cell distribution width – distribution volume)	IL-6, TNF-α, G-CSF, M-CSF increase RDW-DV, indicating increased variation in RBC volume due to changes in erythropoiesis or inflammation.

a significant inflammatory component, including cancer, infections and trauma. Clinicians may find SIRI helpful in infectious and trauma settings, where rapid immune shifts are pronounced.

The AISI,[10] which combines the components of both SII and SIRI, integrates NEU, MON, PLT, and LYM into a single index with counts expressed as $\times 10^9$ cells/L:

$$\text{AISI} = (\text{NEU} \times \text{MON} \times \text{PLT})/\text{LYM}$$

The AISI combines these ratios and other inflammatory markers to give a more comprehensive measure of systemic inflammation. It is a robust tool for diagnosing and monitoring diseases with an inflammatory component. Its integration of multiple parameters may enhance specificity in complex cases.

In 2020, Fuca et al.[11] published the same equation, apparently specifically named pan-immune-inflammation value, to reflect its broad applicability across various disease states, particularly in oncology. Adhering to publication priority reasons, we use the AISI abbreviation to denote the index.

Despite their sensitivity, these indices rely on static, linear relationships that may oversimplify the inherently dynamic, nonlinear nature of immune interactions, leading to limitations in specificity.

From a research perspective, these indices provide a framework for capturing systemic immune status; however, their reliance on linear relationships constrains their application. This simplification overlooks threshold effects, feedback loops and synergistic interactions that are common in biological systems. As such, there is a growing need to transition toward nonlinear, model-driven approaches that more accurately reflect immune complexity.

The reciprocal diseases–systemic inflammation axis

Systemic inflammation and disease are interconnected in a reciprocal relationship. Self-amplifying cycle, where chronic pathological states fuel immune activation, and inflammation further exacerbates disease progression. For clinicians, this dynamic underscores the importance of monitoring inflammation as a consequence of disease and as a therapeutic target. Chronic conditions such as cancer, CVD and autoimmune disorders drive systemic inflammation, which in turn accelerates disease progression. Inflammation is not just a consequence of disease but a key mechanism that accelerates disease progression. Once systemic inflammation is established, it contributes to disease progression by disrupting signaling and metabolic pathways, leading to organ dysfunction and complications. Inflammation actively amplifies disease processes rather than merely reflecting them.

Clinical factors – such as comorbidities, infections, medications, stress, and environmental factors, also influence systemic inflammation. Although various clinical and external factors can influence inflammatory biomarkers – complicating their interpretation – these influences also offer important context for understanding the role of inflammation in disease. Incorporating such variables makes CBC-DIIs more applicable to real-world clinical settings, where ongoing interactions between disease processes and patient-specific factors are the norm.

The bidirectional relationship between disease and systemic inflammation underscores a critical gap in current research – the need for precise quantification of inflammatory activity and its feedback effects on disease progression. To accurately predict outcomes, it is imperative to quantify systemic inflammation in the context of both the underlying pathology and its external modulators. Composite indices – comprising diverse markers, such as cytokine panels, CBC parameter ratios, and metabolomic signatures – should be integrated into dynamic, nonlinear modeling frameworks and rigorously validated in longitudinal cohorts to provide a comprehensive view that augments prognostication and informs therapeutic decisions. Current models often overlook the cumulative and context-specific effects of inflammation over time. Consequently, future research should employ time-series data, systems modeling, and multimodal integrative diagnostics to elucidate these intricate interactions and provide a more comprehensive understanding of how immune dynamics influence clinical outcomes.

Clinical applications of CBC-derived indices

The CBC-DIIs such as the SII, SIRI and AISI have demonstrated utility across a diverse range of clinical conditions beyond oncology and CVD.[6,9,12–14] Elevated SII, SIRI and AISI values are correlated with disease risk, prevalence and severity in various conditions.[13,15–25] In oncology, elevated SII values are associated with a tumor microenvironment that facilitates immune evasion and tumor progression, and they are strongly correlated with poor prognosis.[6,7,9,26] Elevated SII levels have been associated with increased severity of depression, suggesting its potential as an auxiliary diagnostic indicator in depressive disorders.[21,27] Similarly, in patients undergoing maintenance hemodialysis, high SII has been identified as an independent risk factor for depression, highlighting its relevance in neuropsychiatric assessments within chronic illness populations.[28]

In dental health, higher SII values have been observed in patients with generalized stage III grade C periodontitis than in healthy individuals.[29] This association underscores the SII and SIRI utility in identifying systemic inflammation linked to periodontal disease.[22,30]

The Systemic Immune-Inflammation Index, SIRI and AISI have been found to correlate with disease activity in autoimmune disorders, such as psoriatic arthritis, ankylosing spondylitis, systemic lupus erythematosus, Sjögren's syndrome, and autoimmune encephalitis, indicating their potential as biomarkers for monitoring disease severity.[18,31–35]

Studies have demonstrated a positive association between elevated SII levels and the incidence of chronic

kidney disease (CKD) in adults, particularly in men. This suggests that SII could serve as a valuable marker for early identification and risk stratification in CKD.[36,37]

The SII has shown promise in differentiating between active and inactive disease states in infectious diseases, as evidenced by its effectiveness in distinguishing active pulmonary tuberculosis from non-tuberculous lung diseases.[38] In acute settings, such as sepsis,[24] serial measurements of these indices can provide real-time insights into the evolving inflammatory state, thereby guiding timely therapeutic interventions.

Elevated SII levels have been associated with an increased risk of type 2 diabetes mellitus (T2DM) and insulin resistance. Studies have found that higher SII levels are independently linked to elevated fasting plasma glucose, fasting insulin and HOMA-IR values, indicating a higher risk of developing T2DM and insulin resistance.[16,39] Furthermore, in patients with diabetic retinopathy, high SII values predicted both microvascular and macrovascular complications, as well as increased mortality risk within the first year, highlighting its potential as a prognostic marker in diabetic complications.[40]

In the context of acute trauma, particularly traumatic brain injury (TBI), SII has been identified as a valuable prognostic biomarker. A retrospective study involving 1,266 patients with severe TBI demonstrated that elevated SII levels at admission were independently associated with poorer outcomes, including higher mortality rates and unfavorable Glasgow Outcome Scores at 6 months post-injury.[41] Similarly, in pediatric TBI cases, significant differences in SII values were observed across mild, moderate and severe injury groups, suggesting its utility in assessing injury severity and guiding clinical management.[42]

These examples illustrate the broad applicability of CBC-DIIs in various clinical scenarios, providing clinicians with accessible tools for prognostication and aiding in the development of personalized patient management strategies.

While CBC-DIIs are invaluable in clinical practice, their interpretation should always be contextual, as elevated values may also occur in benign inflammatory responses. Consequently, they must be used in conjunction with other diagnostic tests and clinical assessments to ensure accuracy.

The future of advanced CBC-DIIs: Nonlinear interactions and advanced framework

Integrating routinely acquired imaging data – such as radiomic texture and histopathological classification (including architectural patterns, cytological variants, and grading) – with broad genomic signatures like transcriptomic profiles provides the complex, context-specific inputs that nonlinear models need to reveal threshold phenomena, feedback loops, and synergistic interactions driving immune and inflammatory responses – capabilities that linear CBC-based indices inherently lack.[43] Biological systems often exhibit threshold effects, feedback loops and context-dependent responses – phenomena that linear models cannot adequately represent. Small perturbations in one immune parameter can result in disproportionate downstream effects, a nuance that linear models are unequipped to handle.[44] This is frequently highlighted as a relationship that is roughly linear or a nonlinear relationship between CBC-DIIs, mortality risk, disease risk or severity, organ health, or other clinical indices.[20,22,36,45–53] Nonlinear modeling techniques offer a more flexible and biologically congruent framework, accommodating disproportionate, synergistic or antagonistic interactions among immune variables.

For instance, discrete shifts in blood cell counts and their parameters have varying impacts on different individuals. These nonlinearities can be captured using mathematical expressions that model each immune component as a distinct, interacting "factor," rather than assuming uniform additive or subtractive contributions. Factors can include immune activation states, regulatory capacity or systemic burden, each constructed from multi-variable sub-formulas that allow nonlinear responses to subtle changes in input data.

To operationalize such models, computational methods such as decision trees,[54] random forests[55] or gradient boosting[56] can be employed. These algorithms are particularly well-suited for identifying complex decision boundaries, such as distinguishing between immune activation and immune suppression in ambiguous or overlapping CBC profiles. More advanced approaches, such as support vector machines (SVMs)[57] or artificial neural networks,[58] can model high-dimensional interactions and uncover patterns that would otherwise remain obscured in linear frameworks. These techniques address multicollinearity and feature interaction and can be trained on real-world patient data to adapt their predictions to different clinical scenarios dynamically.

Moreover, incorporating temporal dimensions through recurrent neural networks (RNNs)[57] or long short-term memory networks (LSTM)[58] would enable the indices to account for how immune parameters evolve, thereby enhancing prognostic accuracy in chronic conditions or for monitoring treatment responses. Such time-aware models could flag abnormal trajectories even when snapshot values appear clinically acceptable.

Ultimately, nonlinear and machine learning-based frameworks offer the precision, adaptability and sensitivity needed to transform CBC-DIIs from static screening tools into dynamic, personalized biomarkers. They are especially valuable in contexts with immune suppression, comorbidity burden or fluctuating disease activity, where linear assumptions often fail. By embracing these advanced modeling strategies, future CBC-DIIs should more accurately reflect immune complexity, thereby supporting early diagnosis, risk stratification and tailored intervention.

Integration of CBC-derived indices with multimodal diagnostics

The integration of CBC-DIIs with other diagnostic modalities presents a promising area for enhancing personalized assessments of immune function. In accordance with the nonlinear, multimodal modeling framework outlined above, the following sections detail five domains – radiographic imaging and radiomics; genomic and transcriptomic profiling; biochemical laboratory markers; proteomic and metabolomic analyses; and bioelectrical impedance assessment – in which CBC-DIIs are integrated with established diagnostic modalities to improve the sensitivity and specificity of immunological personalized evaluations. Below, we present some illustrative examples of such integrations.

Radiographic imaging and radiomics: magnetic resonance imaging (MRI) and computed tomography (CT) scans offer detailed anatomical and functional insights, which, when combined with CBC-DIIs, enhance the evaluation of inflammatory and neoplastic conditions. For example, in multiple sclerosis, MRI improves the assessment of disease activity and progression.[59] Radiomics, which extracts quantitative features from medical images, further enhances this integration by correlating imaging phenotypes with hematological indices, thereby refining prognostic models in oncology and other fields.

Genomic and transcriptomic data: The incorporation of genomic and transcriptomic data with CBC-DIIs facilitates a deeper understanding of the molecular underpinnings of immune responses. Genetic variations influencing immune cell function can modulate blood cell phenotypes, and their integration can aid in identifying individuals at risk for autoimmune diseases or adverse drug reactions. Transcriptomic profiling, in conjunction with CBC-DIIs, can also assist in monitoring disease activity and therapeutic responses in conditions such as rheumatoid arthritis and systemic lupus erythematosus.

Biochemical laboratory results: Combining CBC-DIIs with biochemical markers, such as adipokines and bone metabolism markers, would enhance the evaluation of systemic inflammation, obesity and bone health. Such a multimodal approach improves the sensitivity and specificity of diagnostic algorithms by providing a more nuanced picture of the patient's inflammatory status.

Omics data integration: Integrating proteomics, metabolomics and other omics data with CBC-DIIs provides a holistic view of the immune system's status, potentially identifying novel biomarkers and pathways involved in disease processes. This comprehensive profiling can lead to personalized medicine approaches, e.g., metabolomic profiles combined with CBC-DIIs could improve the prediction of metabolic syndrome risk in high-risk populations.

Bioelectrical impedance analysis (BIA) provides noninvasive measurements of body composition, including fat mass, lean body mass and total body water, which are crucial for understanding metabolic and inflammatory status. Combined with CBC-DIIs, clinicians can gain a more comprehensive view of patients, especially those with systemic inflammation and conditions like obesity and sarcopenia.

While the integration of CBC-DIIs with other diagnostic modalities holds significant promise, challenges remain. Data standardization, interoperability and the development of robust analytical frameworks are essential for effective integration. Adopting standardized data formats and leveraging machine learning algorithms can facilitate the synthesis of diverse data types, leading to more accurate and individualized assessments of immune function. Without adherence to interoperability standards such as Fast Healthcare Interoperability Resources (HL7/FHIR; https://hl7.org/fhir/exchange-module.html), efforts to unify data across health systems may falter. Moreover, as models grow more complex, ensuring clinical interpretability becomes essential to avoid creating opaque decision tools that hinder, rather than support, frontline care.

Future directions

Future research should extend beyond CBC-DIIs dominating applications in oncology to fully harness the clinical and diagnostic potential of advanced multiparametric CBC-DIIs. Several underexplored domains offer opportunities for investigation.

In metabolic disorders such as obesity, where chronic low-grade inflammation is a hallmark,[52,60,61] The CBC-DIIs could aid in diagnosing and quantifying the systemic inflammatory burden linked to comorbidities,[48] as well as monitoring the efficacy of interventions. In depression, systemic immune activation is increasingly recognized as a contributing factor[62]; dedicated CBC-DIIs may provide objective markers to support diagnosis, predict treatment responsiveness and monitor residual inflammatory burden. In implant medicine, including dental implants[63] and orthopedic joint replacements,[64] specialized indices could serve as early indicators of immunosuppressive or inflammatory foreign body response, infection risk or long-term inflammatory complications. This would be especially valuable in enhancing pre- and post-operative surveillance. Allergic conditions may also benefit from adapted CBC-DIIs formulations that reflect acute and chronic immune activation.[64,65] Additionally, systemic oxidative stress often involves systemic inflammation and shifts in CBC counts and their parameters,[66,67] making a dedicated CBC-DII a potential tool for detecting and measuring the impact of oxidative stress on the immune system. In contexts of chronic immunosuppression, such as organ transplantation or chronic inflammatory diseases, dedicated CBC-DIIs could help identify atypical immune profiles. Likewise, nuanced index behavior may reveal

suppressed or dysregulated immune response patterns indicative of emerging complications in primary or acquired immunodeficiencies.

A common limitation of currently available CBC-DSIIs is their lack of specificity, despite their sensitivity. These indices rely on static, linear relationships that may oversimplify the inherently dynamic and nonlinear nature of immune interactions, resulting in limitations in specificity. However, for clinical and research applications, new advanced CD-SIIs with enhanced condition or disease specificity may offer a superior advantage. For instance, in depression, the objective would be to identify specific blood cell ratio patterns associated with depression severity. In contrast, in obesity, the focus would be on identifying specific patterns of adipose tissue inflammation. Due to shared biological mechanisms of depression and obesity,[68] research suggests that obesity is a causal risk factor for elevated risk of depression and increases the risk of depression,[69,70] and observational studies have provided some evidence for a bidirectional association, indicating that psychological distress may also contribute to an increase in body mass index (BMI).[71] As a result, the development of distinct CD-SIIs specific to adipose tissue inflammation and depression would provide a significantly deeper understanding of the obesity–depression correlation. This would enable the creation of tools to quantify correlated systemic inflammation levels and monitor treatment responses.

Conclusions

The CBC-DIIs offer a robust and cost-effective method for assessing systemic inflammation by utilizing routine CBC tests. These indices provide sensitive measures of immune activation, aiding in the diagnosis, prognosis and monitoring of diseases ranging from cancer and CVD to autoimmune conditions. Despite their promise, the current linear models underpinning these indices do not fully capture the complexity of immune–inflammatory interactions, particularly in patients with chronic conditions or those who are immunocompromised.

Advancing these tools will require a shift toward nonlinear frameworks that reflect the dynamic, non-proportional nature of biological systems. Integrating these models with multi-modal data, such as imaging, genomics and longitudinal patient records, can dramatically improve diagnostic precision and personalization. However, such integration is not without challenges. Data from different sources vary in format, scale and clinical context, making harmonization and interpretation difficult without adherence to interoperability standards

For clinicians, future indices must remain accessible, interpretable and grounded in routine practice. For researchers, the imperative is to drive innovation in data modeling, integration and validation, ensuring that these tools are both biologically insightful and practically deployable. By embracing multi-disciplinary approaches and addressing these technical and clinical challenges, CBC-DIIs may evolve into truly personalized biomarkers of precision diagnostics in systemic inflammation.

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Editorial

Author guidelines in medical journals: Essential rules and rationale

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Instructions for authors issues by editorial offices of scientific journals require periodical critical analysis in order to maintain their clarity and understandability. In this editorial, selected aspects of such guidelines published issued by *Advances in Clinical and Experimental Medicine* were reappraised by the editors of this journal – regulations concerning financial disclosure and conflict of interest, as well as acknowledgements, equal contribution of 2 or more authors, tables, figures, and references were discussed. Reasons for chosen rules were provided – those which (based on editors' experience and expertise) may not seem obvious to authors – e.g., why equal contribution is permitted, while co-first authorship is not. Multiple examples of papers fulfilling the analyzed rules in a copybook fashion were provided. In Conclusions, it was briefly discussed whether some of the rules specified in instructions for authors could be enforced only after acceptance for publication (e.g., when numeration of tables and figures is concerned or rules regarding acknowledgements). In this section, it was also explained why other rules listed above should be fulfilled before the peer review commences, for 3 reasons: 1) information about funding sources and conflict of interest is crucial for the ethical integrity of the whole work and cannot be added at a later stage; 2) Satisfactory quality of tables and figures is a prerequisite for peer review; 3) Resolving many issues after acceptance would be cumbersome (e.g., reducing the number of tables or figures) or at least significantly extend the time required for editing.

Key words: editor, scientific journal, instructions for authors, scientific publishing, co-authorship

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Highlights

- Manuscript-submission hurdles: Non-standardized author guidelines across scientific journals create formatting errors, resubmissions, and lost review time.
- Guideline compliance averts desk rejection: Precise adherence to journal instructions speeds editorial decisions and reduces costly delays.
- Ethics & transparency at stake: Missing conflict-of-interest disclosures, funding statements, or correctly formatted tables, figures, and references can derail peer-review acceptance.
- Journal spotlight – *Advances in Clinical and Experimental Medicine*: Offers a concise, step-by-step author checklist that streamlines initial screening and copy-editing.
- Feedback fuels improvement: Continuous input from authors and readers refines guidelines and aligns editorial expectations with best publishing practices.

Introduction

There are no universal standards regarding how a scientific paper should be prepared when submitted to a journal, not even within respective fields of knowledge. Differences include the stipulated spelling (British English or US English; nowadays, many periodicals do not state any particular preference), rules concerning tables and figures (their number form and other characteristics), reference standard (Harvard or different types of Vancouver referencing), or usage of abbreviations and acronyms. Various approaches to disclosing conflict of interest and funding sources and diverse policies on data sharing can also be found. Many editorial offices refer to guidelines from entities like the Committee on Publication Ethics (COPE) or the International Committee of Medical Journal Editors Recommendations (ICMJE). Still, these suggestions are far from being universally endorsed. Therefore, authors planning to submit their manuscript to a specific journal should carefully read instructions for authors available on its website and closely follow their tenets; a substantial number of scientific periodicals accept for further consideration (i.e., for peer review) only materials which are in agreement with rules in force in each journal.

The issue of authors' insufficient compliance with the provided guidance during the initial submission has already been discussed in the literature, albeit not extensively and mainly in opinion pieces. Nathan Efron, editor of *Clinical and Experimental Optometry*, stated in his editorial[1] that in his personal experience, most of the manuscripts submitted to this journal are proof that authors either do not read or disregard the instructions for authors (we refrain from any speculation regarding the reasons for this problem). In turn, Nevzat Karabulut,[2] editor-in-chief of *Diagnostic and Interventional Radiology*, elaborated on often observed shortcomings in submitted manuscripts, particularly concerning conflict of interest disclosure, scientific integrity, and authorship. An important aspect was pointed out by Jawaid and Jawaid[3] – manuscripts that are not adequately prepared are sent for adjustments, which extends the time the authors have to wait for the editors' decision about publication. Nonadherence to some rules can result in desk rejection because not all errors can be rectified – a good example is the maximal number of authors, which Teixeira Da Silva[4] discussed; it seems rather improbable that in such a situation, one or more authors would just agree to their removal.

However, preparing instructions for authors in major medical journals is also far from perfect. Schriger et al.[5] analyzed the findings of 2 observational studies on the contents of such guidelines and found that only 13% of journals commented on the content and style of data tables and figures, and that instructions, with few exceptions, focused on formatting issues. Their study was published in 2006, but this problem is still far from solved, as pointed out in 2020 by Lang,[6] who in his editorial provided a subjective but highly informative list of requirements he found in journals' instructions for author, divided into "unsupported" (i.e., baseless in his opinion), "unclear", "unusually specific", and "unusually demanding" ones, and offered several inspiring recommendations for editors. Also, shortcomings of instructions for authors in specific areas are reported – Sims et al.[7] found that of 27 analyzed emergency medicine journals, 11 (11/27, 40.7%) did not mention a single guideline.

Instructions for authors in our journal – *Advances in Clinical and Experimental Medicine* (*Adv Clin Exp Med*) – are a result of our editors' professional experience, following the example of the most prestigious medical journals, and implementing guidelines published by COPE and ICMJE. We strive to make them simultaneously in-depth and easily comprehensible – their main goal is to shorten both the initial verification stage (during which newly submitted manuscripts are assessed regarding their adherence to our guidelines) and editing in case they are accepted for publication.

Objectives

This editorial aims to present selected aspects of instructions for authors currently (May 2025) in force in *Adv Clin Exp Med* – the rule discussed below are those to which

model adherence can be presented using the examples of papers already published in this journal, as well as 2 other journals – *Dental and Medical Problems* and *Polimery w Medycynie – Polymers in Medicine* – owned by the same entity (Wroclaw Medical University, Wrocław, Poland) and adhering to very similar policies. When applicable, reasons for rules that may not seem obvious were also provided. The objective of the present editorial is also to inspire discussion around our instructions – as Lang[6] stated, editors should periodically review their guidelines to ensure their expectations are reasonable; such review is impossible without proper feedback from both our authors and readers.

Financial disclosure

This information should describe sources of funding that have supported the work. The statement appears in the "Funding sources" section on the 1st page in a published paper. All financial and material support for the research and work must be identified, including listing of support that might constitute or give the appearance of influencing the findings.

The statement should include:

– specific grant numbers;
– initials of authors who received each award;
– full names of commercial companies that funded the study or authors;
– initials of authors who received salary or other funding from commercial companies.

It should also be stated whether any sponsors or funders (other than the named authors) played any role in:

– study design;
– data collection and analysis;
– decision to publish;
– preparation of the manuscript.

Information about funding sources should be reported while submitting the manuscript in a dedicated section of the submission procedure, not in the Acknowledgements section or in the paper itself.

An example of correct financial information disclosure is an editorial by Di Gregorio and Battaglia,[8] where 2 grants were presented with appropriate details. In a study by Chachaj et al.,[9] apart from the name and number of grant, the following disclaimer was included: The funder accepted the study protocol and had no role in the collection, analysis, interpretation, writing of the manuscript, or the decision to submit the manuscript for publication. It is an open question whether such statements should be a required element of financial disclosure in scientific journals.

Conflict of interest

A conflict of interest may have financial or non-financial character – the former in the case of accepting, e.g., lecture fees or providing paid consultation, the latter often when there are professional or personal (albeit not financial) ties between one or more of the authors and external entities or individuals which may have influenced the course of research and/or publication. A scoping review of non-financial conflicts of interest in health-related journals was published by Wiersma et al.,[10] showing how diverse this issue may be. A review of current practices, biases, and the role of public registries in improving transparency has been provided by Dunn et al.,[11] while Fontanarosa and Bauchner[12] discussed specifically the policies adopted in *Journal of American Medical Association (JAMA)*. Separate guidelines were also issued by ICMJE,[13] World Association of Medical Journals (WAME)[14,15] and *New England Journal of Medicine (NEJM)*.[16] Editorials presenting a broader view were published by Charlton[17] and Rahman-Shepherd et al.[18]; the book *Conflict of Interest in Medical Research, Education, and Practice*[19] remains the most exhaustive source of knowledge on this problem.

In *Adv Clin Exp Med*, all authors must provide disclosures before submission for inclusion in the "Conflict of Interest" statement. A conflict of interest exists when one's professional judgment about the execution of the research and/or the presentation of the content is, or could reasonably be perceived to be, influenced by other interests.

Items included in the disclosure statement should cover: consulting fees or paid advisory boards (for the past 3 years or the known future), equity ownership/stock options (publicly or privately traded firms, excluding mutual funds), lecture fees when speaking at the invitation of a commercial sponsor (for the past 3 years or the known future), employment by the commercial entity that sponsored the study, grant support from industry, patents and/or royalties, expert witness, and other activities performed for a commercial sponsor.

Financial conflicts of interest include, but are not limited to:

– awarded, planned, or pending patents, including individual applications or those belonging to the institution to which the authors are affiliated and from which the authors may benefit;
– ownership of stocks, shares, or stock options, even if not publicly traded;
– paid employment or consultancy;
– received payment for serving as a member of an advisory committee/board for any entity engaged in activity related to the subject matter of this contribution.

Non-financial conflicts of interest that could impact the research reported here include, but are not limited to:

– acting as an expert witness;
– member of a government or other advisory board;
– relationship (paid or unpaid) with organizations and funding bodies, including nongovernmental organizations, research institutions, or charities;
– membership in lobbying or advocacy organizations;
– writing or consulting for a company whose activities might impact the objectivity of this paper;

– personal relationships (e.g., friend, spouse/partner, family member, mentor, adversary) that could affect objectivity;

– personal beliefs (political, religious, ideological, or other) related to a paper's topic that might interfere with an unbiased publication process (at the stage of authorship, peer review, editorial decision-making, or publication).

Conflict of interest is rarely reported – it is debatable whether it only seldom occurs, or the authors are unaware of less obvious situations in which it arises. Zielonka and Jamroziak[20] reported such conflict for the 2nd author (research support from AbbVie, Janssen; consulting role for AbbVie, AstraZeneca, Janssen, Beigene), while Chen and Nakagawa[21] pointed out that the 1st author is also the author of a work extensively cited and appraised in their paper – such sensitivity regarding possible conflict of interest should be singled out for praise. In a paper by Kosowski et al.,[22] one of the co-authors – John A. Todd – disclosed that he is an employee (Sr. Vice President & Chief Scientific Officer) and a Singulex, Inc., Alameda, USA stockholder.

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In this section, those who contributed to the work but do not meet our authorship criteria should be listed with a description of the contribution. Individuals or companies that have assisted with the study should be mentioned, including advisors, administrative support, and suppliers who may have donated or given materials used in the study. A similar definition of acknowledgements in scientific papers is included in the instructions for authors of journals from the Springer Nature group.[23]

Common misconceptions regarding this section include:

– describing funding sources in it;

– mentioning the funding institutions once more (apart from the disclosure in the Funding sources section), this time to thank them (while there is no need to duplicate this information);

– disclosing conflict of interest in this section.

Paul-Hus and Desrochers[24] also pointed out that information about financing relatively frequently appears in Acknowledgements; however, many journals they analyzed did not have a dedicated "Funding sources" (or similar) section, at least when the survey was conducted (2019). They also showed examples of information about conflict of interest (or potential such conflict) appearing there and instances they called "crossovers", when disclosures regarding funding sources and conflict of interest are mixed with one another and/or with acknowledgements understood as defined above. Such confusion seems a result of both the lack of a specific section for disclosing other information within the published article in several journals and the relaxed enforcement of journals' policies by editors. Paul-Hus and Desrochers[24] also discuss the type of acknowledgements implemented in *Adv Clin Exp Med*, naming it "authorial voice" and calling for a more personal space where the authors can speak for themselves, in their name, on matters they judge worth mentioning. We agree with this view and we are open to authors' proposals, should they deem necessary to express any more personal message to the readers in the Acknowledgements section.

Examples of such usage of this section can be found in an editorial by Kurkinen,[25] who stated in Acknowledgements that the views and opinions expressed in the paper are his only and do not reflect those of his employers, as well as thanked 5 other researchers for their "interest, understanding and continued support". It is worth noting that these thanks pertain all Prof. Kurkinen's research activities, not only the editorial in which they appear and can be therefore viewed as more personal ones – the author wanted to emphasize that those people are essential for him in general (they were addressed again in the same vein in his other editorial[26]).

More conventional acknowledgements were published in the paper by Akdeniz,[27] who thanked one person for helping with recruiting participants and another for statistical analysis, in a study by Repczyńska et al.[28] (who thanked the families for their participation in the research and for their consent to publish the data), and in the article by Edebal and Doğan,[29] who acknowledged their English-language editor. Authors also often acknowledge institutions that made specific research possible, as in Alzamanan et al.[30]

Equal contribution

The sequence of author names on a publication indicates the allocation of credit and accountability. When not listed alphabetically, it is generally understood that the first author has made the most significant contribution. In many cases, only being the first author (sometimes also the corresponding author) fulfills the requirements for promotion or stipulations of the entity financing the research of a given author. Therefore, authors of some papers expect that 2 (rarely more) of them will be considered co-first or co-corresponding authors. Such practice has already been introduced in several scientific medical journals. Efron in his editorial[31] summarized arguments for and against this solution and concluded that it will be retained in *Clinical and Experimental Optometry*, although some editors of this journal have reservations. Already in 2013 Conte at al.[32] called for recognition of co-first authorship in biomedical and clinical publications. Huang et al.[33] described this phenomenon in the pharmacy and anesthesia journals, while Khoshpouri et al.[34] discussed it on an example of radiology journals. This problem was also analyzed in the context of gender equity by Rose-Clarke and Fellmeth[35] and Aakhus et al.,[36] while Lapidow and Scudder[37] offered a librarian's perspective. An important aspect was signaled by Else,[38] who pointed out that it is not yet clear whether co-first authors are perceived as equal;

similar doubts and concerns were expressed by Owens et al.[39] The more exhaustive research so far on this issue was conducted by Hosseini and Bruton,[40] who called for clearer policies both in journals allowing co-first authorship and refraining from such practice.

O'Sullivan[41] proposed some questions that the editors of scientific journals should ask themselves regarding the possible consequences of allowing co-first authorship. One result is that the citation will always be how the journal lists the article – many authors confront the situation of co-first authorship, and debate over whose name should be first. In other words: For purely technical reasons, on the 1st page of the paper, only 1 of the authors is the first. Some journals resolve this issue using asterisks by the names of 2 authors and a byline or footnote explaining that they are co-first authors. In our opinion, such practice is confusing because in citations in other publications, there is no option of marking that a given papers has 2 co-first authors. If they were listed in the order they are on the 1st page, without any footnotes (e.g., X, Y, Z et al., and in the text as X et al.). Wiley's editorial staff has also noted this problem.[42] Therefore, in *Adv Clin Exp Med*, there can be only 1 first author and 1 corresponding author for each manuscript (both functions can also be held by the same person). We do not permit co-first authorship and co-corresponding authors for any reason. However, we offer to mark the names of 2 chosen authors on the first page of the paper with asterisks [*] and to place a disclaimer [X and Y contributed equally to this work] on the same page. Many authors accept such a solution, and examples of its implementation are papers by Najar Nobari et al.[43] and Bębenek and Godlewski.[44]

Tables and figures

Many types of scientific findings can be conveyed only using tables and figures; others could also be presented as plain text, but figures and/or tables are much more informative and allow for comprehension at a single glance instead of painstakingly reconstructing the information from long, convoluted passages of text. However, correctly preparing these elements of a scientific article is not always intuitive. *Scientific Writing and Publishing: A Comprehensive Manual for Authors* by Denys Wheatley[45] includes long chapters only about tables and charts, graphs and other types of graphical data representation. Useful guidelines regarding tables and figures were also offered by Divecha et al.,[46] Vickers et al.[47] and Tuncel and Atan.[48] Advice regarding tables in papers on cardiology can be found in an article by Boers[49]. At the same time, Slutsky,[50] Liu[51] and Inskip et al.[52] tackled this issue in the context of medical publications in general. Hmejlak and Hooper[53] proved that in medicine using figures stems from the "show and tell", not "show, don't tell" mentality – an ideal situation in the convergence of images and text. Jansen and Tyler[54] discussed key points regarding figures on an example of gastrointestinal research, but their remarks are pertinent in all fields of medicine.

Instructions regarding figure preparation have been released by Elsevier,[55] DovePress,[56] Wiley,[57] Nature,[58] *PLoS Medicine*,[59,] while guidelines about tables by Elsevier,[55] Taylor & Francis,[60] *PLoS Medicine*,[61] DovePress,[56] and *Journal of Clinical Investigation*.[62]

Advances in Clinical and Experimental Medicine accept for consideration manuscripts with up to 10 tables and 10 figures (2 tables and 2 figures in research letters) – papers with multiple tables and/or figure are challenging to read in PDF format because the tables and figures dominate over the text and the reader must skip several pages filled only with tables/figures to follow the discourse. In HTML format, the tables and figures are published at the end of the paper, and the text contains only links, but in PDF format, such a solution is not feasible. Therefore, if authors of a given manuscript must present more than 10 figures and/or tables, the additional ones should be made available as shared data.

Tables and figures should be placed in separate files, not pasted into the main body of the text. Tables should be submitted in an editable format (not inserted as pictures in *jpg, *tif or *pdf format). There cannot be empty cells in the table – if no information is given, a cell within the table should be filled with a hyphen (–); it signifies that a given cell contains no value intentionally and not by mistake.

References to tables and figures should be placed according to the sequence of citing them in the manuscript. The text should include references to all tables and figures. All tables and figures must be consecutively numbered. Separate tables and figures must have separate numbers (i.e., Table 1, Table 2, Table 3, not Table 3A, Table 3B, etc., or Table 3.1, Table 3.2, etc.) when separate tables or figures are considered.

All abbreviations used in a given table/figure should be explained below it or in the caption, even if a given abbreviation has already been described in the text – the reader should not be forced to search the text to understand abbreviations, mainly that some readers scan the abstract, tables and figures – and only if they seem interesting, read the whole text.

A single figure can have no more than 6 panels (A–F). One panel means one chart – multiple charts cannot form one panel. All elements of each figure must be legible when viewed on an A4 page in a PDF file in full-screen mode, without zooming; overtly complex figures with multiple charts/graphs combined into single panels cannot fulfill this requirement and are therefore unfit for publication in PDF format.

Each figure should have a concise, self-explanatory caption describing accurately what the figure depicts. When applicable, be sure that both the figure captions and the figures contain corresponding labels for multiple panels (A, B, C, etc.). If any magnification is used in the photographs, it should be indicated using scale bars within the figures.

Conveying information using tables can be done in different ways. Vázquez-Rodríguez et al.[63] proved that large tables can also be clear and easy to comprehend, while Kiliś-Pstrusińska et al.[64] employed smaller tables, which allowed for the precise presentation of different aspects of their results. In many reviews and met-analyses, large tables are unavoidable – as in Martins et al.[65] or Sokołowska et al.[66] Finally, there are papers in which tables complement figures, providing additional data, as in Wang et al.[67] – visual composition of the whole paper is of paramount importance in such cases.

Examples of correct figure preparation are papers by Ding et al.,[68] Li et al.,[69] Yan et al.,[70] and Lin et al.[71] All 4 teams of authors employed diverse types of graphs, charts and microscopic images in a single paper, provided them in good quality and complemented them with appropriate captions.

References

References should be limited only to the most recent positions directly connected to the presented topic; older papers and other materials should be cited only when necessary (e.g., when a definition is mentioned).

The following sources should not appear on the reference list:

– Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., "unpublished work" or "data not shown"). Instead, such data should be deposited in a publicly available database (e.g., as a preprint if it is a complete but yet unpublished manuscript). Articles that are accepted for publication but not yet published should be cited as [in press].

– Retracted papers – they cannot be cited, even as retracted articles. If the retraction occurs between the submission of the manuscript and its acceptance, editors ask authors to remove the retracted papers from the reference list or replace it by other, non-retracted publication.

– Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list).

The references should be consecutively numbered, not prepared in Harvard style and not marked in the text using solely links to the reference list (without numbers) or DOIs pasted into the text. The reference list should be in the order of works cited in the text, not alphabetical. References should be identified by Arabic numerals (without parentheses) in superscript and numbered consecutively in the order in which they are first mentioned in the manuscript. All references from the reference list should be mentioned in the text, consecutively and with no gaps (e.g., Reference 16 cannot appear until reference 15 is mentioned at least once). No references should be included in the abstract or headings/subheadings.

This also includes references in tables – they should fit into the consecutive numeration of the references in a place where a given table is first mentioned. Citations in the tables should be numbered and included in the reference list. The references in the tables should be in the order they appear on the reference list. References in tables should fit into the consecutive numeration of the references in a place where a given table is first mentioned. Any distortions regarding the order of references in the tables can be detrimental to the comprehensibility of the paper. Sometimes the authors provide a separate reference list for a table or tables, but a manuscript can have only one reference list.

All positions on the reference list should follow the American Medical Association (AMA) citation style, according to the 10th edition of the *AMA Manual of Style*.[72] Abbreviations for journal names should be provided according to Index Medicus. If a journal is not listed in Index Medicus, its full title should be given. The abbreviation et al. should be applied when there are more than 6 authors, but only the first 3 authors should be listed in such situations (e.g., Hu X, Chang J, Mao M, et al.).

DOIs should be provided for all positions on the Reference list for whom a DOI has been assigned. If a cited position lacks the DOI, PMID should be provided. If there is also no PMID, a direct URL to the published paper (not to a database) should be provided. These data are required when the reference list is entered into reference managing software; many authors can provide an EndNot, Mendeley or JSON file, but this is not a norm – not all authors use reference managers and we cannot expect all of them to do it.

Among papers whose references fulfilled all the above list, 3 are especially good examples: 2 editorials by Daly et al.,[73] and Kurkinen and Daly,[74] in which several sources without DOIs and PMIDs were correctly cited, and another editorial by Staicu,[75] who appropriately references books and other documents not available online. Jain et al.[76] in a study published in *Polimery w Medycynie – Polymers in Medicine* (a journal adhering to similar rules) correctly cited several nonstandard reference positions – among them a handful of patents from different countries. Examples of correct references in tables can be found in Frąckiewicz et al.,[77] Shao et al.[78] and Song et al.,[79] as well as in Yacoub et al.[80] and Nucci et al.[81] A paper by Misiak and Kurpas[82] about preprint policy of *Adv Clin Exp Med* is also worth mentioning due to 8 preprints released using various preprint servers, cited in this article according to AMA rules.

Conclusions

Instructions for authors in a scientific journal should never be regarded as having a definite form – always some issues could be clarified, others presented more concisely, while yet another complemented with examples. Comparing instructions currently in force in *Adv Clin Exp Med* with similar guidelines prepared by editorial offices

of other medical periodicals is definitely outside the scope of this paper; however, our editors follow changes in such documents provided by leading scientific journals to seek inspiration. Equally important is the feedback we receive from both authors and section editors with whom the authors communicate – the main issue is usually whether certain aspects of a manuscript could be addressed not during the initial verification (i.e., before the peer review) but only following the acceptance for publication, during linguistic editing. Among the problems discussed above, this is the case only when numeration of tables and figures is concerned (provided that there are less than 10 tables and less than 10 figures, the way they are numbered can be corrected later) and regarding acknowledgements (information provided there – albeit important from authors' point of view) is usually not paramount for manuscript assessment). Other rules listed above should be fulfilled before the peer review commences, for 3 reasons. First, information about funding sources and conflict of interest is crucial for the ethical integrity of the whole work and cannot be added at a later stage. Second, satisfactory quality of tables and figures is a prerequisite for in-depth peer review – the reviewers must be able to assess their contents. Lastly, several issues theoretically could be resolved after the acceptance. Still, it would be cumbersome (e.g., reducing the number of tables or figures) or at least significantly prolong the editing. First, the authors would be asked to complement the reference list with DOIs or change the reference system from Harvard style to the one used in *Adv Clin Exp Med*, and only then could the linguistic correction start. Therefore, we aim to make our instructions for authors more concise and comprehensible, but not relaxed. If we are to sustain the results of development our journal experienced in recent years (as summarized in an editorial by Misiak and Kurpas[83]), we must simultaneously be open for authors' needs and streamline the editorial process without compromising either the scientific integrity or linguistic accuracy and correctness of papers published in *Adv Clin Exp Med*.

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Assessing the efficacy of psychological interventions in enhancing the quality of life of patients diagnosed with cancer and psychiatric disorders: An umbrella analysis

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Psychosocial interventions encompass psychotherapy and psychological education that explicitly target psychosocial adaptation. These interventions have been shown to have a substantial positive effect on reducing anxiety and depression, as well as improving overall quality of life (QoL). Nevertheless, there is still no consensus concerning the therapeutic effectiveness of these interventions.

Objectives. An umbrella review of systematic reviews and meta-analyses was conducted to determine the efficacy of psychological interventions in improving the QoL and psychological wellbeing of patients with cancer and mental illness.

Materials and methods. A comprehensive literature search was conducted across multiple databases, namely PubMed, Embase, Scopus, and Cochrane Library. Assessing the Methodological Quality of Systematic Reviews 2 (AMSTAR-2) was used to evaluate research methodological rigor.

Results. The 12 papers analyzed in this umbrella review explored psychological therapy for cancer and psychiatric patients. The included reviews covered in total 8,198 studies. The AMSTAR-2 rated 8 of 12 studies as high-quality and 4 as intermediate. A total of 369 studies examined cancer, 166 schizophrenia and 165 psychoses. Psychological therapy improved the QoL for cancer, schizophrenia and psychosis by 1.87, 1.48 and 1.61, respectively. Psychiatric and cancer patients have anxiety, sleep issues and a lower QoL. This umbrella study showed that psychological interventions improved QoL in both groups of patients.

Conclusions. Psychological therapy appears to improve cancer and mental illness patients' QoL and wellbeing. Most evidence is from high- and middle-income nations. Therefore, further high-quality research that covers a larger geographical area and rigorous systematic reviews with complete meta-analyses is needed to gain useful insights in this field.

Key words: mental health, cancer, quality of life, psychosis, psychological interventions

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Introduction

The concept of quality of life (QoL) seeks to encompass the overall welfare of an individual or a population, taking into account both favorable and unfavorable aspects of their lives at a particular moment in time.[1] Quality of life assessments must encompass a minimum of 5 discrete dimensions: physical health, mental health, participation in social and role activities on a daily basis, and overall perceptions of wellbeing.[2,3] The prevalence of cancer is increasing worldwide, and the individuals afflicted with cancer are susceptible to a multitude of psychological disorders, which significantly impair their overall QoL.[4] A significant decline in the overall QoL was noted in both the physical and psychological aspects of cancer patients. They exhibit a diverse range of symptoms, and the insufficient control of symptoms can impede an individual's ability to carry out their everyday tasks effectively. Increased symptom burden has been linked to elevated levels of mental distress and impaired physical and social functioning, as well as a reduced overall QoL. In addition to pain, sleep disturbances and fatigue, they endure substandard economic conditions.[5,6]

Similarly, there has been an increase in the prevalence of mental health disorders, including anxiety, phobias, obsessive-compulsive disorder, and panic disorder, across the globe.[7] Psychiatric disorder patients also experience a lowered QoL compared to the general population. This is due to the challenges posed by their mental illness, which hinders their ability to carry out daily activities, leading to a decreased level of independence. Primarily due to demographic shifts, the prevalence of mental health disorders has increased by 13%.[8] Those suffering from severe mental illnesses tend to exhibit a reduction in their QoL compared to the general population. This is largely attributed to their impaired functional capacity, diminished autonomy, and the presence of low self-assurance and self-esteem.[9]

Psychological interventions include several approaches that positively impact the patient's QoL by potentially alleviating the suffering arising from both the diagnosis and medical therapy.[10] Psychosocial interventions include psychotherapy and psychological education aimed specifically at psychosocial adaptation. Psychosocial therapies, such as cognitive behavioral therapy (CBT) and acceptance and commitment therapy (ACT), are frequently provided by psychologists, social workers, mental health counselors, and community health professionals. These evidence-based therapies are delivered both individually and in groups.[11,12] Scientific evidence validated the efficacy of all of these treatments, demonstrating significant positive impacts in lowering anxiety and depression while also enhancing QoL. Furthermore, these benefits persisted in both short- and long-term follow-ups.[13,14] Despite the examination of this topic in several systematic reviews and meta-analyses,[15–19] the most beneficial intervention for improving the QoL of cancer and mental illness patients remains unclear. Nonetheless, research on the efficacy of psychological therapies is characterized by a heterogeneity of strategies (e.g., individual or group therapy) and causes (e.g., improving personal skills or fostering emotional wellness).

Typically, individual studies and reviews have focused on a limited range of psychological outcomes. Collecting evidence on multiple outcomes is essential for assessing the effectiveness of an intervention on patient psychological wellbeing and for designing more effective treatments. Therefore, this study conducted an umbrella review of selected systematic reviews and meta-analyses[20–31] to determine the efficacy of psychological interventions in improving the QoL and psychological wellbeing of cancer and psychiatric patients.

Objectives

This study aimed to evaluate the effectiveness of various psychological interventions in enhancing the QoL and psychological wellbeing of cancer and psychiatric patients by conducting an umbrella review of selected systematic reviews and meta-analyses on the subject.

Materials and methods

An umbrella review was conducted in accordance with recently published guidelines.[32] Regarding reporting, we followed the Preferred Reporting Items for Overviews of Reviews (PRIOR) protocol.[33] Assessing the Methodological Quality of Systematic Reviews (AMSTAR) guidelines were applied to the evaluation of study quality.

Inclusion and exclusion criteria

For studies to be considered, they had to meet all of the subsequent criteria. The following criteria were used to evaluate the effectiveness of psychological interventions in improving the QoL of patients with cancer and mental illnesses: 1) systematic reviews that incorporated individual participant meta-analyses or meta-analyses of meta-analyses; 2) included individuals who had been diagnosed with any type of cancer or any mental illness; 3) reported efficiency of psychological interventions for improving the QoL of cancer and psychiatric patients: and 4) examined any of the outcomes listed below: a) change in QoL scores; b) improvement in psychological wellbeing; c) change in functioning capabilities; d) behavioral changes; and e) symptom reductions. Bibliographic references that were obsolete, anecdotal or solely relied on expert assessments were excluded from the assessment process. Furthermore, studies that were dependent on animal experiments or trials were excluded, along with those in which the authors lacked access to the primary

data and critical information. Furthermore, non-research publications, qualitative studies, studies involving patients with human immunodeficiency virus (HIV) infection and other systemic diseases, and papers published in languages other than English were likewise omitted.

Literature search strategy

A literature search was performed using various databases, including PubMed, Embase, Scopus, Web of Science, and the Cochrane Library. The search covered the years 2000–2023 and utilized specific key words such as "psychological interventions", "cancer"; "mental health"; "schizophrenia"; "psychosis"; "quality of life"; "QoL"; "positive psychology"; "wellbeing"; "positive psychiatry"; "depression"; "nursing"; "schizophrenia-spectrum disorders"; "cognitive behavioral therapy"; "psychoeducational therapy"; "supportive expressive therapy"; "anxiety"; "depression"; "mood"; "meta-analysis"; "systematic review and meta-analysis"; and "systematic review". The key words were identified and verified for consistency in both the MEDLINE and Embase databases, in accordance with the PICOS framework.

Methodology for study selection

The aforementioned key words were entered into the Title (ti), Abstract (abs) and Keyword (key) fields in the Scopus search. Cochrane search terms included "psychological interventions" and "quality of life". The PICO structure was applied to establish specific criteria for selection. "P" in this context represented patients diagnosed with either cancer or mental illnesses; "I" denoted the psychological intervention for improving the QoL, "C" represented a control, and "O" comprised the clinical outcomes, specifically the change in QoL scores and psychological wellbeing. The research design incorporated in this study was limited to the implementation of systematic reviews and meta-analyses. The inclusion criteria stipulated that only publications written in English would be considered. The identification of relevant studies was conducted through an unbiased and thorough examination of the related literature by 2 researchers: H.G. and Y.Y. Additional relevant papers were identified by carefully screening the references listed in the research selected for final analysis.

Screening and study selection

Following a preliminary screening of the titles and abstracts of the acquired articles, full texts of potentially eligible references were examined. Two evaluators conducted the screening process; in the event of any inconsistencies, the inclusion or exclusion of the material was resolved through deliberation.

Data extraction and critical appraisal of the included studies

Data extraction was conducted by one of the authors, and the information extracted was subsequently verified by 2 other authors. Any discrepancies were resolved by discussion. If an eligible publication had data on multiple disorders, we selectively collected information on QoL and psychological wellbeing outcomes that were relevant to our study. Initially, we gathered pertinent details from the eligible reviews, such as author information, publication year, journal of publication, study type, number of included studies, participants' health conditions (cancer or mental illnesses), participants' age, primary outcomes, statistical parameters analyzed, study conclusions, and methods of analysis and heterogeneity (if available). To encompass the full geographic range of evidence, we collected data regarding the specific locations of the individual studies that were included in the relevant reviews. This involved gathering information about the countries where the research was carried out. Regarding methodological rigor, we collected data on whether the authors utilized a prevalidated tool or an additional set of extracted questions to evaluate the methodological rigor of the studies included in each systematic review. If the answer was affirmative, we have documented the specific tool employed and the primary findings of the evaluation were classified into 3 broad groups: studies exhibiting weak methodological rigor, studies demonstrating a high level of methodological rigor, and studies displaying intermediate or mixed patterns from the 2 groups. The methodological rigor of the included systematic reviews was evaluated by 2 authors using the AMSTAR-2 program. Any differences were resolved with the assistance of a 3rd author. AMSTAR-2 utilizes a checklist consisting of 16 items or domains, of which 7 are deemed crucial for ensuring the overall validity of a review. The essential domains to be taken into account are as follows: 1) ensuring protocol registration prior to commencing the review; 2) conducting a thorough and comprehensive literature search; 3) providing a rationale for excluding specific studies; 4) assessing the risk of bias in the included studies; 5) employing suitable statistical techniques for conducting a meta-analysis; 6) considering the influence of bias when interpreting the findings; and 7) evaluating the existence and consequences of publication bias. Finally, utilizing abstracts and full-text analyses, we retrieved pertinent information regarding the primary conclusions drawn from each of the reviews included. Furthermore, if the review involved multiple disease areas, we only selected the primary outcomes from the individual studies concerning how the psychological interventions improved QoL.

Statistical analysis of primary outcomes of the included studies

Due to high heterogeneity in the designs, study questions, outcomes, and metrics, a descriptive analysis was performed. Separate tables were created for the characteristic information, methodological assessment, and summary estimates, 95% confidence intervals (95% CIs) and heterogeneity estimates of each systematic review and meta-analysis. All of the included systematic reviews and meta-analyses suggested that psychological interventions were efficient in improving QoL. However, because the strategies used were so different, the overall odds ratios (ORs) of the studies included were also calculated to evaluate how strongly the psychological intervention was linked to QoL. The participant's health conditions served as the basis for grouping the studies. An OR value higher than 1 was considered statistically significant and shows that psychological intervention is highly effective in improving the QoL of people with mental illnesses and cancer.

Results

Study selection

The process of selecting studies, in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines,[34] is presented in Fig. 1. An electronic scanning technique was employed to perform an exhaustive search across multiple databases, leading to the identification of 254 studies that satisfied the inclusion criteria specified in the PICOS framework.[35] Prior to screening, 6 duplicate documents were eliminated, bringing the total number of papers screened to 248. A total of 164 papers were subsequently excluded for having invalid titles and abstracts, and 84 records were requested for retrieval. Twelve records were not retrieved in their entirety; the eligibility of the remaining 72 reports was evaluated. Upon implementation of the inclusion-exclusion criteria, 60 studies were determined to be ineligible and were subsequently excluded. The principal determinants that led to the exclusion of research studies were their failure to provide essential outcome measures, non-access to complete text papers, unsuitable study designs, and unavailability in the English language. In conclusion, 12 systematic reviews and meta-analyses that satisfied the predetermined inclusion criteria and spanned a time period from 2000 to 2023 were incorporated into this umbrella review.

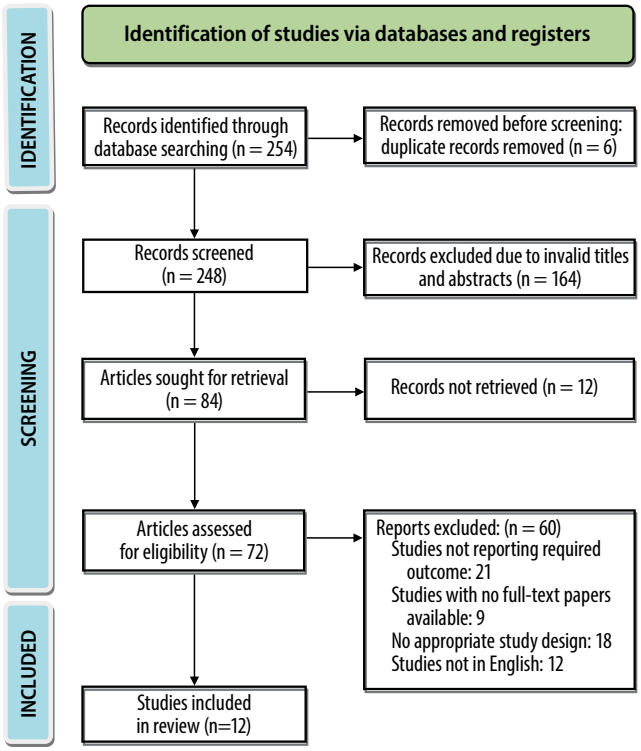

Fig. 1. Flowchart for selection of included systematic reviews and meta-analyses

Characteristics of included reviews

The included reviews were published between 2013 and 2023. Included articles were obtained from Scopus (n = 53), PubMed (n = 114), Embase (n = 29), and the Cochrane Library (n = 48). Out of the 12 included reviews, 6 reported the efficacy of psychological interventions for improving the QoL of cancer patients. On the other hand, 6 evaluations analyzed the effectiveness of psychological therapies in improving the QoL of patients with mental diseases. Four of these reviews focused on breast cancer patients.[20–23] The article by De La Torre-Luque et al.[24] described an improvement in QoL among patients with multiple cancers, whereas the article by Son et al.[25] described an improvement in QoL among patients with colorectal cancer. Three articles, Jagan et al.,[27] Pina et al.[28] and Valiente et al.,[30] provided findings regarding QoL improvements in schizophrenia patients. Turner et al.[29] and Wood et al.[31] provided findings regarding QoL improvement in patients with psychosis. The article by McGlanaghy et al.[26] describes the efficiency of psychological interventions in improving the QoL of both psychosis and schizophrenia patients. All included reviews were considered articles without any geographical restriction. The included evaluations comprised a variety of study counts, spanning from 8 to 198. Each of the 12 included reviews incorporated data pooling and meta-analyses in addition to qualitative analyses. Table 1 provides an overview of the characteristics of the reviews.

Methodological quality

The frequency of each AMSTAR-2 rating for each domain across evaluations is summarized in Fig. 2. In addition, the domain-specific methodological quality evaluations for each review are provided in Table 2. Twelve of the included

Table 1. Characteristics of the included studies (all 12 were systematic reviews and meta-analyses)

Study ID	Year of publication	Journal of publication	Country of study	Number of included studies	Title of the study	Health condition	Age of participants	Primary outcome	Statistical parameters analyzed	Conclusions
Faller et al.[20]	2013	*Journal of Clinical Oncology*	Germany	198	Effects of psycho-oncologic interventions on emotional distress and quality of life in adult patients with cancer	cancer patients	>18 years	QL-index, QLQ-C30	SMD with 95% CI	Psycho-oncologic interventions are associated with significant, small-to-medium effects on emotional distress and quality of life.
Guarino et al.[21]	2020	*Journal of Clinical Medicine*	Italy	45	The effectiveness of psychological treatments in women with breast cancer	cancer patients	>18 years	ABS, BSI, POMS, EORTC-C30, MCS-12, QoL scale, WHO-5	SMD with 95% CI	Psychological interventions have significant efficacy of improving mood and quality of life in women with breast cancer.
Hwang et al.[22]	2023	*Clinical Psychopharmacology and Neuroscience*	South Korea	28	Effects of psychosocial interventions for patients with breast cancer	cancer patients	>18 years	QL-index, QLQ-C30	SMD with 95% CI	Psychological intervention support enhancing quality of life in patients with breast cancer.
Li et al.[23]	2022	*Gland Surgery*	China	12	Efficacy and safety of psychological intervention nursing on the quality of life of breast cancer patients	cancer patients	>18 years	CARES-SF, SF-36, QL-index, FACT, FLIC and QLQ-C30	MD, SMD with 95% CI	Psychological nursing intervention can significantly improve the quality of life of patients.
De La Torre-Luque et al.[24]	2016	*International Journal of Clinical and Health Psychology*	Spain	78	Psychological treatments to improve quality of life in cancer patients	cancer patients	>18 years	QL-index, QLQ-C30	Hedges' g with 95% CI	Psychological treatments should be considered as crucial for the patient's health in cancer contexts.
Son et al.[25]	2018	*Health and Quality of Life Outcomes*	South Korea	8	Effect of psychosocial interventions on the quality of life of patients with colorectal cancer	cancer patients	>18 years	QL-index, QLQ-C30	Hedges' g with 95% CI	Psychosocial interventions have beneficial effect on the quality of life of colorectal cancer patients.
McGlanaghy et al.[26]	2021	*Schizophrenia Research*	UK	94	Psychological interventions for schizophrenia and psychosis	schizophrenia and psychosis	>18 years	QLS, PANSS, BPRS	SMD with 95% CI	Psychological interventions could potentially be effective for schizophrenia and psychosis.
Jagan et al.[27]	2023	*International Kournal of Environmental Research and Public Health*	Malaysia	27	Effectiveness of psychological interventions for internalized stigma among adults with schizophrenia spectrum disorders	schizophrenia	>18 years	PPI, PPT, PWB, QLS	SMD with 95% CI	Psychological interventions are successful in lowering levels of internalized stigma and improving quality of life.
Pina et al.[28]	2021	*Brazilian Journal of Psychiatry*	Brazil	9	Positive psychology interventions to improve wellbeing and symptoms in people on the schizophrenia spectrum	schizophrenia	>18 years	PPI, PPT, PWB, QLS	SMD with 95% CI	Positive Psychology Inventory appears to be a promising resource for schizophrenia spectrum, with possible effects on wellbeing.
Turner et al.[29]	2014	*American Journal of Psychiatry*	the Netherlands	48	Psychological interventions for psychosis	psychosis	>18 years	QLS, PANSS, BPRS	Hedges' g with 95% CI	Psychological interventions have reliable efficacy for psychosis.

Table 1. Characteristics of the included studies (all 12 were systematic reviews and meta-analyses) – cont.

Study ID	Year of publication	Journal of publication	Country of study	Number of included studies	Title of the study	Health condition	Age of participants	Primary outcome	Statistical parameters analyzed	Conclusions
Valiente et al.[30]	2019	*Schizophrenia Research*	Spain	36	Effect of psychological interventions on quality of life in schizophrenia	schizophrenia	>18 years	QL-index, QLQ-C30	SMD with 95% CI	Psychological interventions have significant moderating effect on wellbeing and quality of life in schizophrenia.
Wood et al.[31]	2020	*Schizophrenia Research*	Ireland	23	Cognitive behavioral informed psychological interventions for psychiatric in-patients with psychosis	psychosis	>16 years	QL-index, QLQ-C30, PWB, BPRS	SMD with 95% CI	Psychological interventions have potential to be effective for psychiatric inpatient care.

MD – mean difference; SMD – standardized mean difference; 95% CI – 95% confidence interval; ABS – Affect Balance Scale; BSI – Brief Symptom Inventory; POMS – Profile of Mood States; EORTC-C30 – European Organization for Research and the Treatment of Cancer Core Quality of Life Questionnaire; MCS-12 – Medical Outcomes Studies Short-Form General Health Survey; QoL scale – Quality of Life Scale; WHO-5 – World Health Organization-5 Wellbeing Index; CARES-SF – Cancer Rehabilitation Evaluation System-Short Form; SF-36 – Short Form 36 Questionnaire; QL-Index – Quality of Life Index questionnaire; FACT – Functional Assessment of Cancer Therapy; FLIC – Functional Living Index-Cancer; QLQ-C30 – Quality of Life Core Questionnaire; PedsQL – Pediatric Quality of Life; DQoLY – Diabetes Quality of Life Measure for Youths; CHQ-CF87 – Child Health Questionnaire-Child Form 87 items Mental Health; PPI – Positive Psychology Inventory; PPT – positive psychotherapy; PWB – Psychological Well-Being Scale; QLS – Quality of Life Scale; PANSS – Positive and Negative Syndrome Scale; BPRS – Brief Psychiatric Rating Scale.

studies[20–31] addressed the review questions using the PICO elements, provided an explanation of their study design selection, compiled a list of excluded studies, evaluated their conclusions, employed appropriate statistical methods, assessed the potential impact of bias risk in individual studies, and conducted quantitative synthesis. Eight reviews (Faller et al.,[20] Li et al.,[23] De La Torre-Luque et al.,[24] Son et al.,[25] McGlanaghy et al.,[26] Jagan et al.,[27] Pina et al.,[28] Valiente et al.,[30] and Wood et al.[31]) stated that the review methods were established prior to use and provided justification for substantial deviations from the protocol. While articles by Guarino et al.[21] and Hwang et al.[22] partially met this criterion, article by Turner et al.[29] failed to do so. With the exception of article Li et al.,[23] which could benefit from a more exhaustive search strategy, every article employed a comprehensive literature search. With the exception of the papers by Hwang et al.[22] and Son et al.,[25] all the other studies used double study selection; however, the methodology in the study by Turner et al.[29] was ambiguous, so it received a partial yes. Likewise, apart from the studies by Faller et al.[20] and Wood et al.,[31] all remaining studies extracted data in duplicate; however, study by Son et al.[25] was assessed as a partial affirmation. With the exception of the study by Valiente et al.,[30] all remaining studies provide sufficient descriptions of the included studies. Except the studies by Faller et al.,[20] Li et al.,[23] De La Torre-Luque et al.,[24] Pina et al.,[28] and Turner et al.,[29] all remaining studies disclosed the funding sources for the aforementioned studies. Excluding the study by Son et al.[25], all other studies adequately investigated publication bias, and with the exception of study by Wood et al.,[31] all other studies accounted for the risk of bias in the individual studies when interpreting or discussing the results. Furthermore, all 12 included studies did not disclose any potential sources in conflicts of interest. Based on the aforementioned evaluations, 8 of the 12 studies[21–24,26–28,30] received a high overall assessment, whereas the remaining 4[20,25,29,31] were deemed to be of moderate quality.

Extraction results

The extracted populations of the ncluded randomized clinical trials (RCTs) were from different countries, with the majority of studies conducted in high-income or middle-income countries, including Germany, Italy, Spain, South Korea, the UK, Malaysia, Brazil, the Netherlands, Spain, and Ireland. The health conditions of the participants included in the studies were as follows: breast cancer for studies by Faller et al., Guarino et al., Hwang et al., and Li et al.,[20–23], colorectal cancer for the study by Son et al.,[25], and lung, breast or prostate cancer for the paper by De La Torre-Luque et al.[24] Patients with schizophrenia were the subject of research in articles by Jagan et al., Pina et al. and Valiente et al.,[27,28,30] whereas patients with psychosis were the focus of studies by Turner et al. and Wood et al.[29,31] Patients with schizophrenia and psychosis

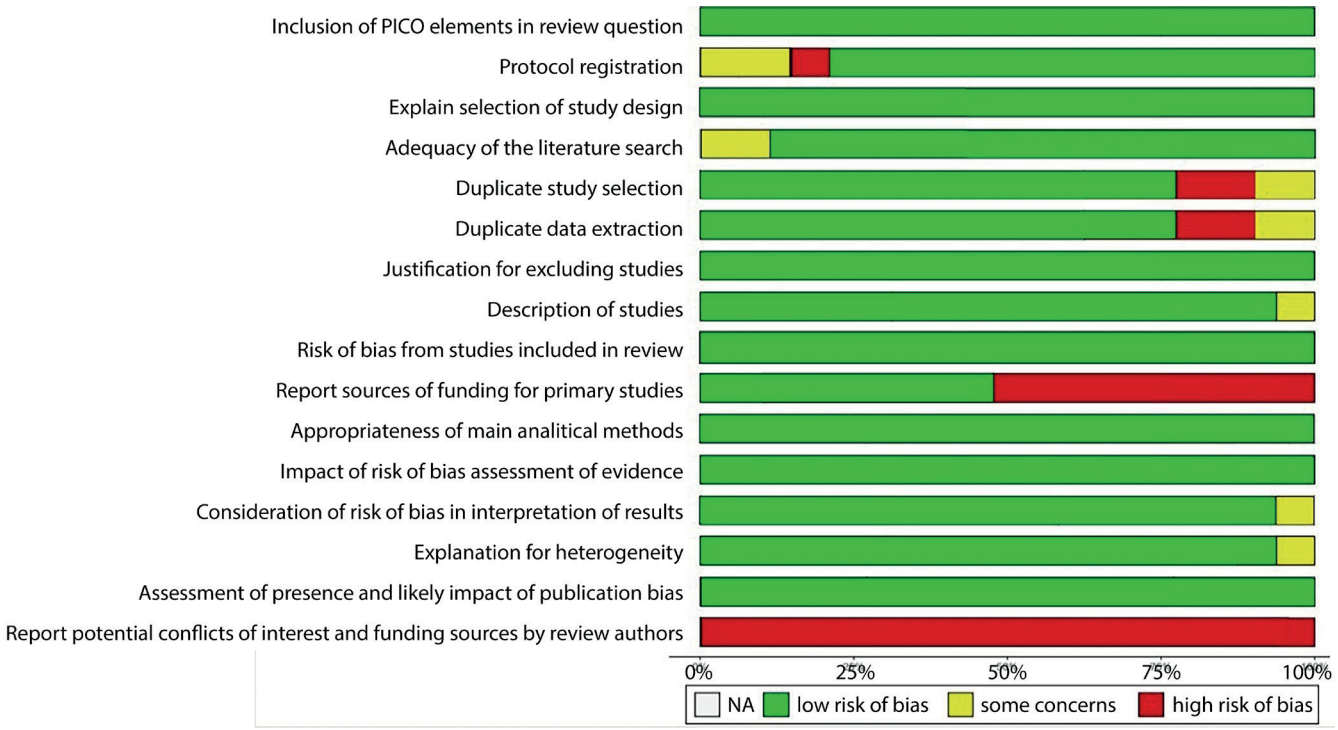

Fig. 2. Frequency of risk of bias using the Assessing the Methodological Quality of Systematic Reviews 2 (AMSTAR-2) parameters

were both covered in the paper by Mc Glanaghy et al.[26] The effectiveness of psychological interventions in enhancing QoL was demonstrated across all the included studies through the assessment of the QL-index and QLQ-C30 (which were evaluated in studies by Faller et al.,[20] Hwang et al.,[22] Li et al.,[23] De La Torre-Luque et al.,[24] Son et al.,[25] Valiente et al.,[30] and Wood et al.[31]), World Health Organization-5 Well-Being Index (WHO-5; Guarino et al.[23]), Agitated Behavior Scale (ABS), Brief Symptom Inventory (BSI), Profile of Mood States (POMS), European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30), Mental Component Summary (MCS-12), Cancer Rehabilitation Evaluation System-Short Form (CARES-SF), Short Form 36 Health Survey (SF-36), Functional Assessment of Cancer Therapy (FACT), Functional Living Index-Cancer (FLIC) (Guarino et al.[23]), Quality of Life (QoL) Scale (QLS) (Li et al.[26]), Positive and Negative Syndrome Scale (PANSS; Li et al.,[23] Glanaghav et al.[26] and Turner et al.[29]), Brief Psychiatric Rating Scale (BPRS; Glanaghav et al.,[26] Turner et al.[29] and Wood et al.[31]), patient and public involvement (PPI), positive psychotherapy (PPT) (Jagan et al.[27] and Pina et al.[28]), and Psychological Wellbeing Scale (PWB; Jagan et al.,[27] Pina et al.[28] and Wood et al.[31]). Table 3 displays the summary estimates of these factors for the included reviews, searched using the specific key words shown in Table 4.

Faller et al.[20] retrieved 198 studies reporting 218 treatment-control comparisons, encompassing 22,238 patients. The researchers found notable short- to medium-term effects of individual and group psychotherapy, as well as psychoeducation, that persisted in both the medium-term (≤6 months) and long-term (>6 months). It was noted that relaxation training exhibited noticeable short-term effects, whereas studies that preselected participants based on heightened distress observed substantial post-treatment effects. Guarino et al.[21] utilized 45 studies with high methodological heterogeneity that satisfied all inclusion criteria. The researchers documented a moderate effect size for CBT and psychoeducational interventions as a whole. Notwithstanding certain constraints, these findings provide partial confirmation of the efficacy of psychoeducational and CBT in enhancing the wellbeing of women diagnosed with breast cancer. Eight investigations with 33 datasets were utilized in the meta-analysis by Hwang et al.[22] Cognitive interventions, meditation and psychological education programs were anticipated to aid in the reduction of negative emotions and improvements in the QoL of breast cancer patients, according to an assessment using the Jadad scale.

In their meta-analysis, Li et al.[23] incorporated a total of 12 articles for evaluation, employing the EORTC OLQ-C30. Significant statistical differences between the intervention and control groups (mean difference (MD) = 12.74, 95% CI: 6.34–19.14, $p < 0.001$) suggested that psychological nursing interventions can substantially enhance the QoL for patients diagnosed with breast cancer, despite the high heterogeneity among the studies ($I^2 = 92\%$). De La Torre-Luque et al.[24] conducted a meta-analysis including 78 investigations. A moderate level of methodological quality was demonstrated by the majority of the studies (60.2% of the sample), as opposed to strong and weak levels (24%). This finding provides support for the notion that offering psychological treatments

Table 2. Methodological assessment of the included studies-AMSTAR-2 evaluation

Study ID	Faller et al.[20]	Guarino et al.[21]	Hwang et al.[22]	Li et al.[23]	De La Torre-Luque et al.[24]	Son et al.[25]	McGlanaghy et al.[26]	Jagan et al.[27]	Pina et al.[28]	Turner et al.[29]	Valiente et al.[30]	Wood et al.[31]
Q1	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q2	Y	PY	PY	Y	Y	Y	Y	Y	Y	N	Y	Y
Q3	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q4	PY	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q5	Y	Y	N	Y	Y	N	Y	Y	Y	PY	Y	Y
Q6	N	Y	Y	Y	Y	PY	Y	Y	Y	Y	Y	N
Q7	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q8	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	PY	Y
Q9	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q10	N	Y	Y	N	N	Y	Y	Y	N	N	Y	Y
Q11	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q12	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q13	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	PY
Q14	Y	Y	Y	Y	Y	PY	Y	Y	Y	Y	Y	Y
Q15	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Q16	N	N	N	N	N	N	N	N	N	N	N	N
Overall assessment	moderate	high	high	high	high	moderate	high	high	high	moderate	high	moderate

N – no; PY – partial yes; Y – yes.
AMSTAR-2 overall assessment rating
1. High – the review accurately and comprehensively summarizes the studies on the topic.
2. Moderate – the review has multiple problems but no major ones. It may accurately summarize study outcomes.
3. Low – the review has a critical flaw and may not provide an accurate and comprehensive summary of the studies that address the question of interest.
4. Critically low – the review has multiple critical flaws and should not be relied on.
Questions:
– Q1: Did the research questions and inclusion criteria for the review include the components of PICO?
– Q2: Did the report of the review contain an explicit statement that the review methods were established prior to the conduct of the review and did the report justify any significant deviations from the protocol?
– Q3: Did the review authors explain their selection of the study designs for inclusion in the review?
– Q4: Did the review authors use a comprehensive literature search strategy?
– Q5: Did the review authors perform study selection in duplicate?
– Q6: Did the review authors perform data extraction in duplicate?
– Q7: Did the review authors provide a list of excluded studies and justify the exclusions?
– Q8: Did the review authors describe the included studies in adequate detail?
– Q9: Did the review authors use a satisfactory technique for assessing the risk of bias (RoB) in individual studies that were included in the review?
– Q10: Did the review authors report on the sources of funding for the studies included in the review?
– Q11: If meta-analysis was performed did the review authors use appropriate methods for statistical combination of results?
– Q12: If meta-analysis was performed, did the review authors assess the potential impact of RoB in individual studies on the results of the meta-analysis or other evidence synthesis?
– Q13: Did the review authors account for RoB in individual studies when interpreting/discussing the results of the review?
– Q14: Did the review authors provide a satisfactory explanation for, and discussion of, any heterogeneity observed in the results of the review?
– Q15: If they performed quantitative synthesis did the review authors carry out an adequate investigation of publication bias (small study bias) and discuss its likely impact on the results of the review?
– Q16: Did the review authors report any potential sources of conflict of interest, including any funding they received for conducting the review?

should be essential for the health of cancer patients. Son et al.[26] conducted a meta-analysis comprising 8 studies, none of which yielded a statistically significant impact on QoL. However, the overall result of their analysis provided evidence for the positive effect of face-to-face psychosocial interventions on the QoL of colorectal cancer patients during the post-intervention period.

Ninety RCTs involving 8,440 randomized participants divided into 24 psychological intervention and control groups were utilized by McGlanaghy et al.[26] They compared direct and indirect evidence for the effectiveness of each psychological intervention using network meta-analysis and a frequentist approach. They concluded that psychological interventions were more probable than control groups to reduce total symptoms, with 1 intervention, mindfulness-based psychoeducation, consistently ranked as the most likely to completely reduce symptoms. In their meta-analysis, Jagan et al.[27] utilized extractable data from

Table 3. Summary estimates of the included studies

Study ID	Outcome	Number of studies	MD/SMD	95% CI	I^2 (%)	p-heterogeneity
Faller et al.[20]	distress anxiety depression QoL	198 198 198 198	SMD: 0.15 SMD: 0.37 SMD: 0.43 SMD: 0.10	–0.09–1.28 –0.75–0.83 –0.68–1.42 –0.19–0.16	94.7 57.2 78.1 0	0.091 0.911 0.490 0.883
Guarino et al.[21]	anxiety depression mood quality of life	10 12 10 13	SMD: –0.39 SMD: –0.35 SMD: –0.18 SMD: 0.39	–0.92–0.14 –0.80–0.10 –0.42–0.06 –0.07–0.84	98 98 99 99	<0.012 <0.011 <0.011 <0.011
Hwang et al.[22]	quality of life	33	SMD: 1.21	0.95–1.48	69	<0.011
Li et al.[23]	FACT scores SF-36 QLQ-C30	3 2 5	MD: 12.74 MD: 6.12 SMD: 0.5	6.34–19.14 5.17–7.06 –0.11–1.27	83 0 92	0.003 0.406 <0.001
De La Torre-Luque et al.[24]	QoL	78	Hedges' g: 0.35	0.25–0.45	–	<0.012
Son et al.[25]	QoL	8	Hedges' g: 0.112	0.002–0.221	0	0.045
McGlanaghy et al.[26]	PE SC SST WB	14 4 13 12	SMD: –0.70 SMD: –0.32 SMD: –0.46 SMD: –0.59	–0.99–-0.41 –0.61–-0.03 –0.68–-0.23 –1.29–0.11	–	–
Jagan et al.[27]	GP NECT efficacy of the overall interventions	4 3 18	SMD: –0.51 SMD: –0.44 SMD: –0.69	–1.15–0.13 –0.70–-0.19 –1.15–-0.24	85 0 94	<0.001 0.934 <0.001
Pina et al.[28]	QoL and wellbeing	4	SMD: 0.93	–0.01–1.87	93	<0.001
Turner et al.[29]	CBT	6	Hedges' g: 0.42	0.15–0.69	28.61	–
Valiente et al.[30]	QoL and wellbeing SR F RW	36 8 16 12	SMD: 0.22 SMD: –0.07 SMD: 0.09 SMD: 0.51	0.10–0.35 –0.22–0.08 –0.03–0.20 0.26–0.77	64 0 0 79	<0.001 0.695 0.487 <0.001
Wood et al.[31]	CBTp	8	SMD: –0.238	–0.624–0.148	–	0.227

MD – mean difference; SMD – standard mean difference; 95% CI – 95% confidence interval; FACT – Functional Assessment of Cancer Therapy; SF-36 – Short Form 36 Questionnaire; QLQ-C 30 – Quality of Life Questionnaire Core 30; QoL – quality of life; SR – symptoms reduction; F – functioning; RW – related to wellbeing; GP – group psychoeducation; NECT – narrative enhancement and cognitive therapy; PE – psychoeducation; SC – supportive counselling; SST – social skills training; WB – wellbeing; CBTp – cognitive behavioral intervention on positive symptoms.

Table 4. Database search strategy

Database	Search strategy
Scopus	#1 "Psychological interventions" OR "Cancer" OR "Mental health" OR "Schizophrenia" OR "Psychosis" OR "Quality of life" OR "QoL" OR "Positive psychology" OR "Wellbeing" OR "Positive psychiatry" OR "Depression" OR "Schizophrenia spectrum disorders". #2 "Nursing" OR "Cognitive behavioral therapy" OR "Psycho-educational therapy" OR "Supportive expressive therapy" OR "Anxiety" OR "Mood" OR "Meta-analysis" OR "Systematic review and meta-analysis" OR "Systematic review". #3 #1 AND #2
PubMed	#1 "Cancer" OR "Mental health" OR "Psychological interventions" [MeSH Terms] OR "Schizophrenia" OR "Psychosis" [All Fields] OR "Positive psychology" OR "Wellbeing" OR "Quality of life" OR "QoL" [All Fields] OR "Positive psychiatry" OR "Schizophrenia spectrum disorders" [All Fields] OR "Depression" [All Fields] #2 "Nursing," OR "Cognitive behavioral therapy," [MeSH Terms] OR "Psycho-educational therapy," OR "Supportive expressive therapy," OR "Anxiety," [All Fields] OR "Mood", OR "Meta-analysis", OR "Systematic review and meta-analysis" [All Fields], OR "Systematic review" [All Fields]. #3 #1 AND #2
Embase	#1 "Cancer"/exp$ OR "Mental health"/exp OR "Psychological interventions"/exp OR "Schizophrenia"/exp OR "Psychosis"/exp OR "Positive psychology"/exp OR "Wellbeing"/exp OR "Quality of life"/OR "QoL"/exp OR "Positive psychiatry"? exp OR "Schizophrenia spectrum disorders"/exp OR "Depression"/exp #2 "Nursing"/exp OR "Cognitive behavioral therapy"/exp OR "Psycho-educational therapy"/exp OR "Supportive expressive therapy"/exp OR "Anxiety"/exp OR "Mood"/exp OR "Meta-analysis"/exp OR "Systematic review and Meta-analysis"/exp OR "Systematic review"/exp #3 #1 AND #2
Cochrane Library	#1 (Cancer): ti, ab, kw@ OR (Mental health): ti, ab, kw OR (Psychological interventions): ti, ab, kw OR (Schizophrenia): ti, ab, kw OR (Psychosis): ti, ab, kw OR (Positive psychology): ti, ab, kwOR (Wellbeing): ti, ab, kw OR (Quality of life) OR (QoL): ti, ab, kw OR (Positive psychiatry):OR (Schizophrenia spectrum disorders): ti, ab, kw OR (Depression): ti, ab, kw (Word variations have been searched) #2 (Nursing): ti, ab, kw OR (Cognitive behavioral therapy): ti, ab, kw OR (Psycho-educational therapy): ti, ab, kw OR (Supportive expressive therapy): ti, ab, kw OR (Anxiety): ti, ab, kw OR (Mood): ti, ab, kw OR (Meta-analysis):ti, ab, kw OR (Systematic review and Meta-analysis): ti, ab, kw OR (Systematic review) (Word variations have been searched) #3 #1 AND #2

#MeSH terms – Medical Subject Headings; $exp – explosion in Emtree (searching of selected subject terms and related subjects); @ ti, ab, kw – either title or abstract or keyword fields.

18 studies. The results indicated a significant overall effect (Z = 3.00; p = 0.003; 95% CI: 0.69 (1.15–0.24); n = 1,633). However, there was substantial heterogeneity in the findings ($Tau^2 = 0.89$; $\chi^2 = 303.62$, degrees of freedom (df) = 17; $p < 0.001$; $I^2 = 94\%$). The authors concluded that the majority of psychological interventions effectively reduced the levels of internalized stigma.

In their meta-analysis, Pina et al.[28] utilized 9 studies and identified a significant effect (p = 0.042) for wellbeing enhancement (Z = 2.01). They documented psychological intrusions as a potentially beneficial resource for patients on the schizophrenia spectrum, with symptom reduction and improved wellbeing as potential outcomes. Turner et al.[29] compared psychological interventions for psychosis with 3,295 participants across 48 outcome trials. It was noted that CBT exhibited a considerably higher efficacy in reducing positive symptoms (g = 0.16) compared to other interventions combined. Likewise, the effectiveness of social skills training in mitigating negative symptoms was found to be significantly higher (g = 0.27).

In their analysis, Valiente et al.[30] incorporated a total of 36 articles and assessed the impact of psychological interventions on the QoL experienced by individuals with schizophrenia. The results of their study indicated a modest but noteworthy impact of the intervention on wellbeing outcomes, as well as a substantial moderating effect when wellbeing was the principal objective. Psychological interventions specifically aimed at enhancing wellbeing are suggested as a supplementary approach to the treatment and promotion of mental health. Seventeen trials employing interventions including cognitive behavioral intervention on positive symptoms (CBTp), ACT and metacognitive therapy (MCT) were analyzed by Wood et al.[31] through 22 studies. It was determined that psychological interventions informed by cognitive behavioral principles yielded substantial positive outcomes. Specifically, cognitive behavioral interventions exhibited a noteworthy positive impact on negative symptoms following therapy, total symptoms during both therapy and follow-up, functioning during therapy and follow-up, and readmission during follow-up. It was proposed that psychological interventions may prove efficacious for individuals who are admitted to psychiatric inpatient care or experiencing an acute crisis.

Statistical analyses

Using the results extracted on changes in QoL scores, the overall OR of the included studies was calculated to assess how strongly psychological interventions were associated with QoL. The studies were grouped based on the health conditions of the participants. A total of 369 studies were found concerning cancer, 166 to schizophrenia and 165 to psychosis. Figure 3 displays the OR forest plot. The OR for the likelihood of psychological therapies to increase QoL was 1.87 (95% CI: 1.35–2.54, $I^2 = 76\%$) for cancer, 1.48 (95% CI 1.12–1.86, $I^2 = 61\%$) for schizophrenia and 1.61 (95% CI: 1.29–1.94, $I^2 = 51\%$) for psychosis. Since all OR values were greater than 1, there is a strong correlation between the 2 parameters and a better chance that psychological intervention will improve the QoL of people with mental illnesses and cancer. Furthermore, the symmetrical funnel plot in Fig. 3 and the statistically insignificant Begg's test (p = 0.714), which is higher than the predetermined significance threshold of 0.05, demonstrates a lack of publication bias. This shows that psychological interventions are very successful in improving the QoL in cancer and psychiatric patients.

Discussion

As the medical paradigm has been revised, there has been a growing acknowledgment that disease management must encompass psychological therapy in addition

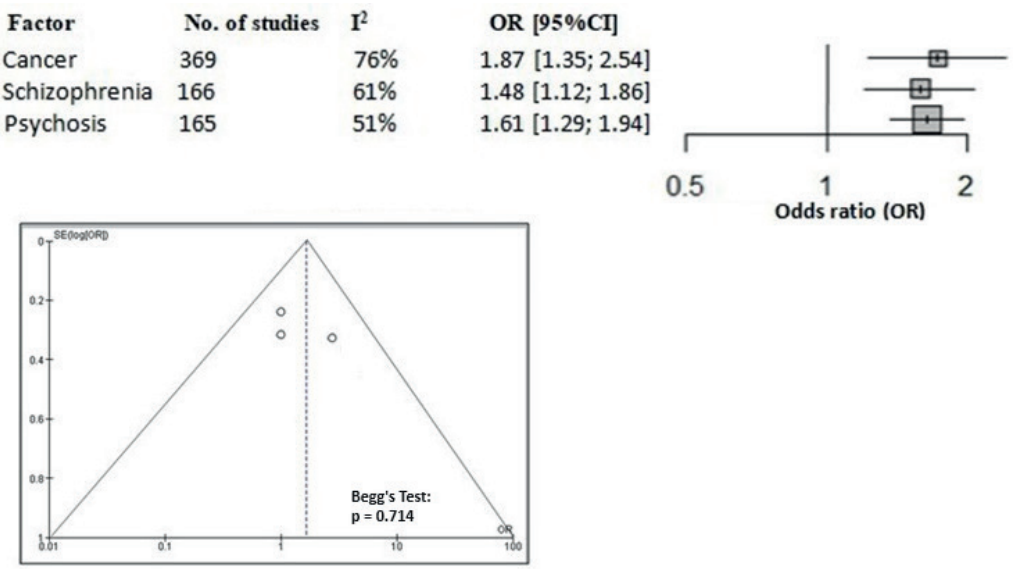

Fig. 3. Forest plot of odds ratio (OR) and funnel plot for the efficacy of psychological interventions in improving quality of life (QoL)

to physiological interventions.[36] Additionally, QoL should consider both mental and physical health. Psychological interventions primarily pertain to the utilization of psychological activities involving patients to facilitate their recovery from illness and promote positive physiological and biochemical changes.[37,38] Treatment procedures are often accompanied by persistent anxiety, depression, dread, and other adverse affective states among cancer and psychiatric patients.[37,38] These emotions not only have a detrimental effect on the patient's personal wellbeing but also an emotional and happiness-reducing effect on their families.[39,40] Patients would be able to accurately and thoroughly comprehend the disease if the nursing staff could comprehend the psychological activities of postoperative patients and promptly implement individualized psychological treatments in accordance with the patient's psychological capacities and differences. By doing so, individuals can confront the disease, relax their emotions and accept the physical changes that occur during and after treatment. As a result, their treatment adherence will be enhanced.[41–43] Positive psychological nursing interventions can alleviate physical symptoms, stimulate the immune system, impede the progression of cancer, and enhance the patient's prognoses while aiding in the diagnosis and treatment of mental illness and cancer, as well as improving a patient's treatment compliance.[44,45]

A multitude of investigations assessed the QoL in cancer patients who underwent long-term psychological interventions in nursing, including cognitive and pain management, health education and psychological counseling. Significant improvement in QoL was more prevalent in the study groups than in the control groups, according to these findings ($p < 0.05$).[46–48] Several studies, however, have concluded that psychological nursing interventions do not significantly impact a cancer patient's QoL.[49,50] Numerous studies have examined the QoL and mental health of cancer patients through the use of psychological nursing interventions in recent years.[51–55] The incidence of cancer is significantly higher, and research indicates that patients exhibit disrupted emotional functioning as a result of lower survival rates compared to other types of cancer. Both direct and indirect psychological therapies are essential to enhance the result of cancer treatment. The combination of cognitive interventions with progressive muscle relaxation techniques has been found to be highly efficient in enhancing the QoL of patients, while also being cost-effective. Early psychological assistance during the cancer treatment process and before surgery shows the potential to enhance psychological wellbeing and results.[56–58] However, a consensus regarding the therapeutic efficacy of these interventions has yet to be reached. Therefore, the primary objective of this umbrella review was to evaluate the clinical effectiveness of various psychological interventions, namely CBT, psychoeducational interventions and supportive-expressive therapy, in relation to distinct psychological outcomes encompassing mood, anxiety, depression, and QoL among patients diagnosed with cancer and mental illnesses.

The present umbrella review demonstrates that psychological therapies have a broad and substantial impact on the QoL and physiological wellbeing of patients with cancer and mental diseases (Fig. 4). A noticeable enhancement was noticed in their QoL score, psychological wellbeing, functional capacity, and behavior, coupled with a considerable reduction in symptoms. Figure 5 displays a flowchart elucidating the purpose, synthesis and main outcomes of this umbrella review.

However, the preponderance of information is derived from affluent and moderate-income nations, as well as low-quality research. Hence, it is crucial to undertake well-executed investigations that encompass a broader geographic scope and carry out meticulous systematic reviews with rigorous meta-analyses to get vital insights into this domain.

Limitations

There are certain limitations to this study. First, although we followed recent recommendations regarding the optimal databases to search for umbrella reviews while conducting the search, it is still possible that we overlooked additional pertinent systematic reviews. This umbrella review consists mainly of systematic reviews that utilized studies with a moderate-to-high risk of bias. Furthermore, the conclusions drawn from these reviews were primarily based on retrospective observational, cross-sectional and case-series research designs, which are prone to residual confounding and are insufficient for establishing temporal associations. Risk of bias assessments revealed substantial variation in the quality of the studies, with notable disparities in the quality observed among particular interventions. Selection biases can also affect likelihood estimates. Furthermore, it is imperative to recognize the possible presence of selection bias in our study, as a considerable number of papers were omitted. Lastly, the majority of the results were gathered in high-income nations, which consequently restricts their applicability to countries with lower and middle income. Therefore, this emphasizes the need for additional research to gain a more comprehensive understanding of the effectiveness of psychological interventions in enhancing QoL and their long-term effects.

Conclusions

Compelling data indicates that psychological therapies have a wide range and significant effect on the QoL and physiological wellbeing of individuals with cancer and mental illnesses. A significant improvement was observed in their QoL scores, psychological wellbeing, functional capacity, and conduct, accompanied by a significant decrease

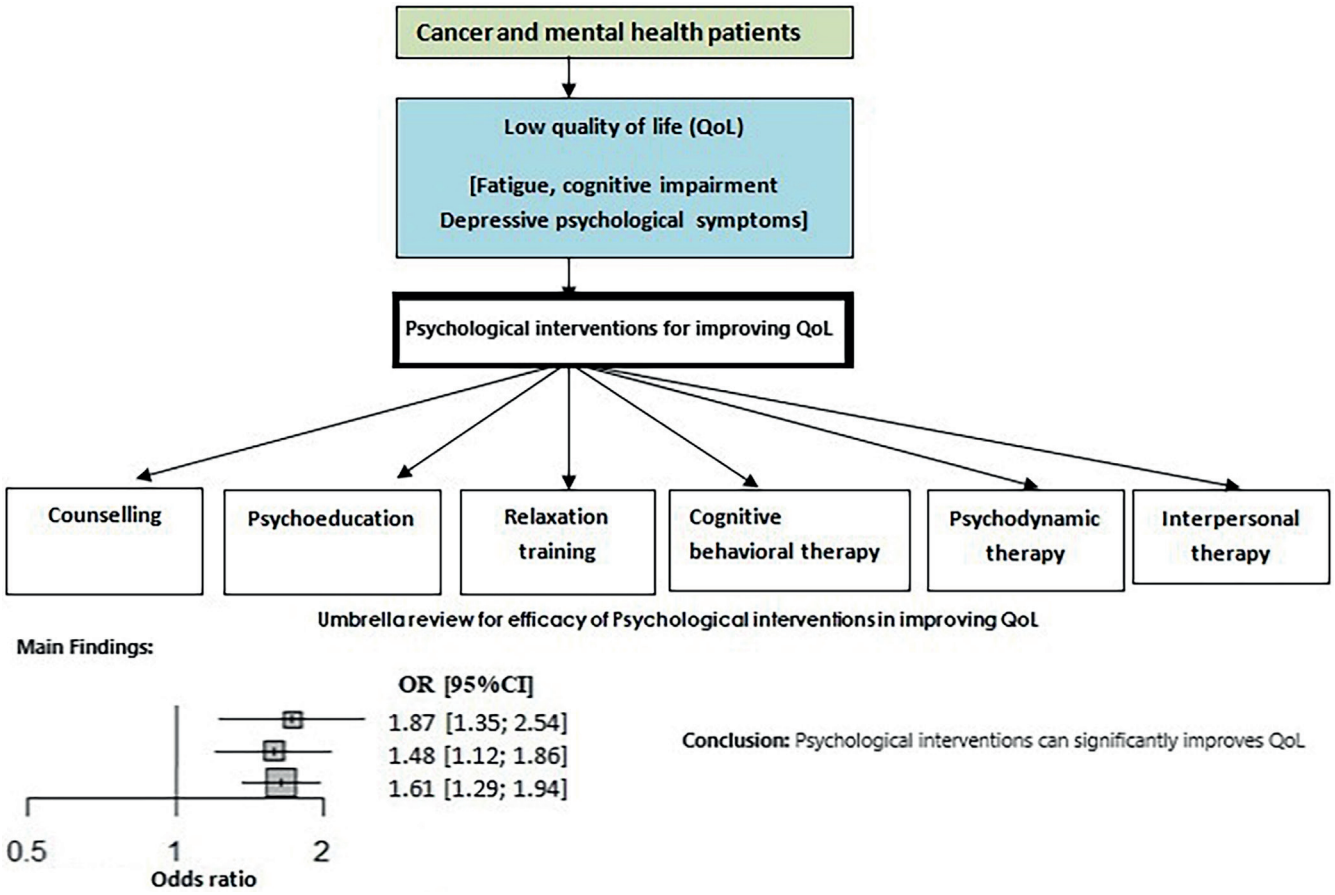

Fig. 4. Visual summary of the umbrella review concerning the impact of psychological interventions quality of life (QoL)

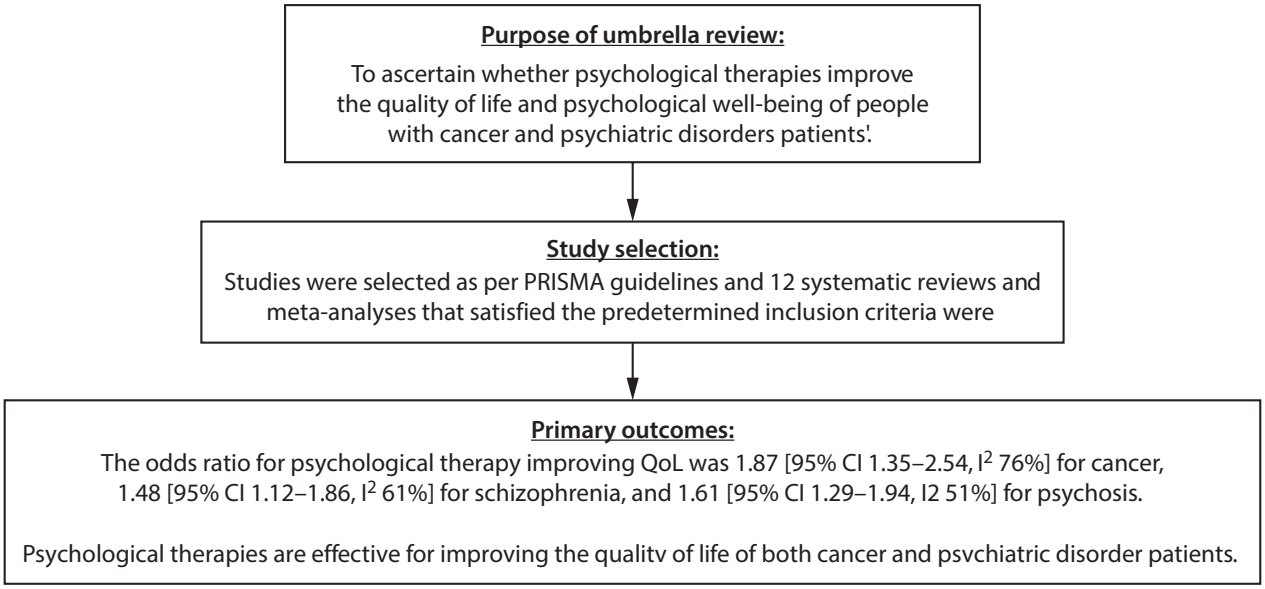

Fig. 5. Flowchart elucidating the purpose, synthesis and primary outcomes of this umbrella review

in symptoms. Nevertheless, the majority of evidence comes from high- and middle-income countries and low-quality studies. Therefore, it is important to conduct future high-quality studies that cover a larger geographical area and perform rigorous systematic reviews with careful meta-analyses to gain valuable insights into this field.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.12792916. The package includes the following files:

Supplementary Table 1. Quality of Life Questionnaire.

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Iodine deficiency and its association with periodontitis: A randomized controlled triple-blinded clinical study

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Abstract

Background. Numerous research studies have explored the impact of micronutrients, including dietary minerals such as iron, zinc, selenium, copper, and vitamins A, B complex, C, D, and E, on periodontitis. However, there is no literature investigating the role of iodine in periodontal health.

Objectives. This study aimed to examine the potential influence of iodine, a trace element, on periodontal health, an area that has not yet been researched.

Materials and methods. The study recruited a total of 73 participants, including 33 periodontally healthy control subjects and 40 patients with stage III periodontitis. Iodine levels in urine samples were measured using a spectrophotometric method, and the results were expressed in µg/L.

Results. Lower iodine levels were observed in patients with periodontitis. Individuals with low iodine levels were found to be 1.04 times more likely to develop periodontitis than those with high iodine levels. The study found that if a person's urine iodine value is below 76.93 µg/L, the probability of having periodontitis is 72.5%; if it is above this value, the probability of not having periodontitis is 90.9%.

Conclusions. These findings suggest that urinary iodine levels could be a valuable metric for future research, as indicated by the variance in mean urinary iodide levels. Further extensive studies could establish urinary iodine levels as a useful biomarker for the diagnosis, prognosis and treatment plan of periodontitis.

Key words: periodontitis, risk, periodontal health, iodine deficiency, urine iodine level

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Background

According to the World Health Organization (WHO), micronutrients are essential in very small quantities (measured in micrograms or milligrams per day) and act as "magical tools" that support the production of enzymes, hormones and other necessary substances for healthy growth and development of the body.[1] Micronutrients, including essential vitamins and minerals, are crucial for promoting proper metabolic function, facilitating wound healing, and preventing illnesses and infections.[2] In vitro studies have demonstrated that vitamins and trace elements have a significant impact on every aspect of immune function, and deficiencies in these nutrients can compromise immune function.[3]

Major minerals such as sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), and sulfur (S) are required in amounts greater than 100 mg/day, while trace minerals such as iron (Fe), zinc (Zn), selenium (Se), copper (Cu), iodine, fluoride (F), cobalt (Co), chromium (Cr), manganese (Mn), and molybdenum (Mo) are required in amounts less than 100 mg/day.[4] Reports indicate that deficiencies in micronutrients have been described as a "silent epidemic" affecting approx. 2 billion people globally, regardless of age or gender.[5] The WHO has noted micronutrient deficiencies in several Middle Eastern countries, especially among children and women of childbearing age, involving Ca, Fe, Zn, vitamin A, vitamin D, and folic acid. Iron deficiency is prevalent among a majority of women in these countries, with over 13.2 million preschool children having retinol serum levels below 0.7 μmol/L, leading to approx. 800,000 cases of night blindness.[6]

Nutrients are crucial for maintaining the health of the human body and all metabolically active tissues. They also play critical roles in the regeneration process, managing oxidative stress and maintaining adequate immunity.[7] While substantial data support the significance of vitamins and major minerals in periodontal and overall health, there is limited evidence supporting the functions of trace minerals. Inadequate intake of trace minerals could increase the risk of periodontal disease, either by exacerbating infections caused by periodontopathogens or by influencing biological gradients and host hemostasis.[8]

Periodontitis is a multifactorial disease resulting from complex interactions between the dental plaque biofilm and the host's immunological inflammatory response. Furthermore, several environmental and host-related risk factors significantly contribute to the disease development, altering the host's response and disease progression.[9] Several approaches have been employed to slow the progression of periodontitis. Alongside conventional periodontal therapies, host-modulating agents such as dietary components (e.g., omega-3 fatty acids), dietary minerals (e.g., Fe, Zn, Se, Cu) and vitamins (e.g., A, B complex, C, D, and E) with immunomodulatory properties have been utilized to elicit favorable host responses. Furthermore, trace minerals impact periodontal health by exerting local effects on hard and soft tissues and systemic effects on immune-inflammatory mechanisms.[10]

Iodine, with an atomic weight of 126.9 g/mol, is a crucial component of thyroid gland hormones, which are essential in mammals. Despite its widespread presence in the environment, iodine is unevenly distributed, with oceans containing the highest iodide levels (approx. 50 μg/L). Iodide ions in seawater oxidize into elemental iodine, which is volatile and evaporates into the atmosphere, returning to the soil through rainfall, thus completing the cycle. However, in many regions, the iodine cycle is slow and incomplete, resulting in iodine-deficient soils and groundwater. Crops grown in these soils have low iodine concentrations, contributing to iodine deficiency in humans and animals that consume these crops.[11]

Studies have shown that iodine functions as an antioxidant in various tissues, including the breasts.[12–14] When exposed to hydrogen peroxide, iodide acts as an electron donor, which may reduce the damage caused by oxygen free radicals.[15] Iodide efficiently eliminates reactive oxygen species (ROS) within human blood cells.[14] In addition, iodine has been found to interact with acute-phase proteins and is utilized by immune system cells and liver cells.[16,17] Besides its antioxidant properties, iodine has anti-inflammatory effects. Although levels of C-reactive protein (CRP) and interleukin 6 (IL-6) remain stable after administering different doses of iodide, negative correlations are observed between CRP and free tetraiodothyronine (FT4), as well as between urinary iodine and free triiodothyronine (FT3) and IL-6. This suggests a connection between iodide, thyroid hormone production and inflammatory status assessed with CRP and IL-6.[18]

Objectives

While several studies have explored the impact of trace elements, such as Fe, Zn, Se, and Cu, on periodontal health, the current study is unique in its emphasis on the significance of iodine, a trace element, in relation to periodontal health. The hypothesis of the study and the primary outcome suggest that the urinary iodine content differs between patients with periodontitis and periodontally healthy individuals.

Materials and methods

Study design

The present cross-sectional study included a total of 73 participants referred by the Department of Periodontology, Faculty of Dentistry, Atatürk University (Erzurum, Turkey), in 2022. Participants were systemically healthy, non-smoking individuals comprising 33 periodontally healthy control subjects (H) and 40 patients with stage III

periodontitis (P). All participants were provided with information about the study and signed an informed consent form. The study protocol was approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Atatürk University (approval No. 2021/11), and registered under Clinical Trials ID NCT05724251. The study was conducted in accordance with the Declaration of Helsinki.

Settings and division of patients into groups

To be eligible, individuals had to be free of systemic illnesses or conditions such as hepatic or renal dysfunction, diabetes, organ transplantation or cancer therapy, cardiovascular disease, pregnancy or lactation, goiter, hyper- or hypothyroidism, or unbalanced thyroid-stimulating hormone levels, and had to be in good systemic health. Those previously treated for periodontal disease requiring antibiotics for infective endocarditis prophylaxis during dental procedures were excluded. Additionally, subjects who had taken antibiotics, immunosuppressive drugs or nutritional supplements (especially iodine) in the past 6 months were excluded. Participants were asked about their dietary habits regarding the use of iodine-containing salt in daily nutrition or avoiding salt at meals, and only those with similar dietary patterns were enrolled in the study. Vegetarians or participants with dietary habits potentially affecting iodine intake were excluded (Fig. 1).

Clinical periodontal status was assessed through intraoral examination and by referring to panoramic X-ray records. The control group (H) was defined as having a probing depth (PD) ≤3 mm, no clinical signs of gingival inflammation (<10% of sites with bleeding upon probing and absence of gingival redness/edema), no radiographic evidence of alveolar crestal bone loss, and no tooth loss due to periodontitis (each subject had at least 5 teeth per jaw section and 20–22 teeth in total).[19]

Patients classified with stage III periodontitis met criteria including interdental clinical attachment level (CAL) ≥5 mm, tooth loss ≤4 teeth due to periodontitis (at least 5 teeth per jaw section and 22 teeth per patient), PD ≥ 6 mm, vertical bone loss ≥3 mm, and Class II or III furcation involvement, as per the 2017 World Workshop criteria for periodontitis classification.[20]

Blinding

Physicians at the periodontology clinic categorized patients into periodontally healthy and periodontitis groups without disclosing group assignments to the patients before urine sample collection. Urine samples were collected by physicians unaware of group assignments. Biochemical analyses were performed by physicians blinded to participant groupings. Data results were recorded and forwarded to the author for statistical analysis without mentioning the group identifiers.

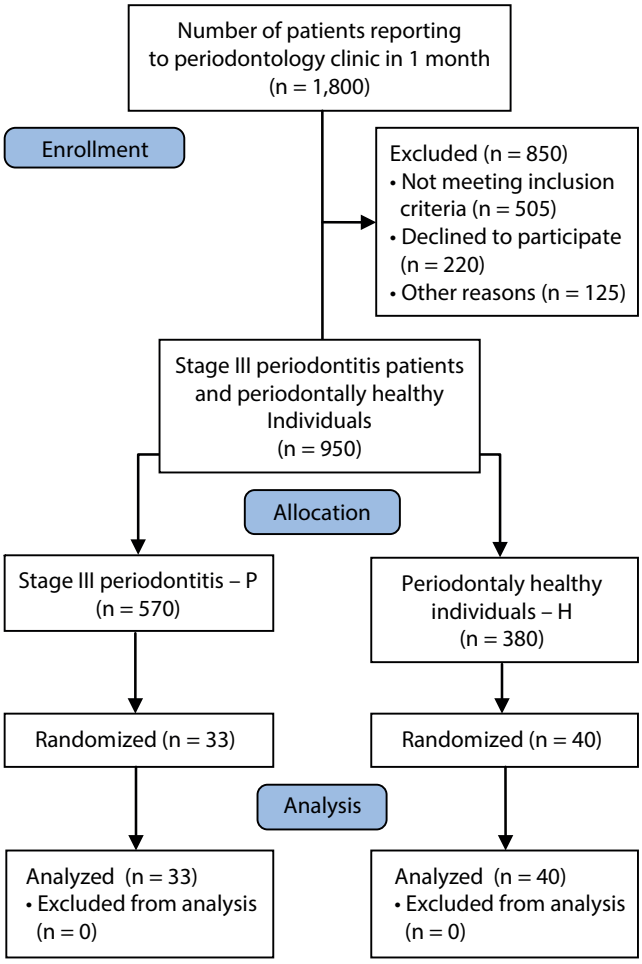

Fig. 1. Study flow diagram

Randomization

Randomization was performed using computer-generated random number allocation. After diagnosis, patients with periodontitis and periodontally healthy individuals were randomly assigned to groups (Fig. 1).

Collection and storage of urine samples

Study participants provided spot urine samples via normal micturition at the Department of Periodontology, Faculty of Dentistry, Atatürk University, before any surgical intervention or drug treatment. Samples were centrifuged at 2,000 rpm for 5 min at 4°C. The resulting supernatant was collected using an automatic pipette and divided into aliquots. Samples were then stored at –80°C until the day of analysis. There were no periods of recruitment or follow-up sessions for urine sample collection.

Analysis of urine iodine levels

Urine iodine levels were measured using a spectrophotometric method with the LTA IODIDE IN URINE (LTA s.r.l., Milan, Italy) kit in accordance with the manufacturer's

instructions. Results were reported in µg/L within the following categories: severe deficiency: 0–19 µg/L; moderate deficiency: 20–49 µg/L; mild deficiency: 50–99 µg/L; optimal nutritional value: 100–199 µg/L; adequate nutritional value: 200–299 µg/L; excessive nutritional value: >300 µg/L.

According to the WHO, most iodine absorbed by the body eventually appears in the urine. Therefore, measuring urinary iodine concentration (UIC) in casual urine specimens is recommended for monitoring iodine status. The UIC is highly sensitive to recent changes in iodine intake, as up to 90% of iodine is absorbed and excreted in the urine. Renal clearance of iodine, in the range 30–50 mL plasma/min, primarily depends on glomerular filtration in humans, with no reported tubular secretion or active transport mechanisms.[21,22]

Statistical analyses

For the difference (effect size: 0.71) between the 2 groups to be significant, the G*Power (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower) program calculated that there should be at least 33 subjects in each group at 80% power and 95% confidence level. A total of 73 subjects were included in the study, with 33 having periodontitis and 40 being healthy.

Statistical analyses were conducted using IBM SPSS v. 20 (IBM Corp., Armonk, USA). Descriptive statistics such as the mean, standard deviation (SD; for normally distributed variables) or median, 1st quartile and 3rd quartile (Q1 and Q3; for non-normally distributed variables or insufficient sample size to determine normality), percentage, and number were used to summarize the data. The normal distribution of continuous variables was assessed using various methods, including the Shapiro–Wilk test, Kolmogorov–Smirnov test, Q–Q plot, skewness, and kurtosis. The Mann–Whitney U test was employed to compare 2 independent groups when normality assumptions were not met. Iodine concentration and age variables were compared using the Mann–Whitney U test due to their non-normal distribution in the periodontitis and healthy groups. Gender and periodontitis/healthy status were analyzed using a 2×2 contingency table, with the appropriate χ^2 test selected based on expected values: Pearson's χ^2 test for values greater than 5. The Fisher–Freeman–Halton test was used to compare iodine levels and periodontitis/healthy status, given the expected value was less than 5. Logistic regression analysis was conducted to model the relationship between age, gender, iodine concentration, and periodontitis/healthy status. Results from the logistic regression model were expressed as odds ratios (ORs) with a 95% confidence interval (95% CI). Analysis of covariance (ANCOVA) was employed to determine the effect of age on the comparison of periodontitis/healthy status and iodine concentration. The statistical analysis in this study was exploratory and did not incorporate correction for multiple comparisons. Therefore, caution is advised in interpreting the results due to the potential for uncontrolled type I errors, which could affect the validity of future meta-analyses. This exploratory analysis provides a foundation for future research in the field. Receiver operating characteristic (ROC) analysis was used to determine whether iodine concentration could be used in the diagnosis of periodontitis. Sensitivity and specificity were calculated to assess the validity of the diagnostic test results. In addition, the Youden index was used to determine the cutoff point. Statistical significance was set at $p < 0.05$.

Results

Table 1,2 shows that there were significant differences in sex, age, and iodine levels between patients and controls ($p = 0.029$, $p < 0.001$ and $p < 0.001$, respectively; Fig. 2). After adjusting for sex and age, the difference in iodine levels remained statistically significant ($p < 0.001$). Iodine levels were markedly lower in the patient group compared to the control group, and this difference was unaffected by sex or age. Table 3 shows no significant difference in sex and iodine levels among the study subjects ($p = 0.073$; Fig. 2).

Table 1. Sex comparison of periodontitis patients and periodontally healthy individuals

Sex	H n (%)	P n (%)	χ^2	p-value
M	24 (72.7)	19 (47.5)	4.754	0.029*
F	9 (27.3)	21 (52.5)		

M – male; F – female; H – periodontally healthy individuals; P – stage III periodontitis patients; *: $p < 0.05$ statistically significant.

Table 2. Age and iodine comparison in periodontitis patients and periodontally healthy individuals

Variable	H		P		Z-value	p-value	Age ANCOVA p-value	Sex adjusted
	mean ±SD	median (Q1–Q3)	mean ±SD	median (Q1–Q3)				
Age [years]	27.76 ±4.72	27 (25–30)	39.83 ±7.77	38.5 (35–45)	–6.102	<0.001*	–	–
Iodine (effect size: 1.67)	153.93 ±67.36	167,93 (98.01–210.61)	56.88 ±47.05	43.5 (25.68–75.26)	–5.619	<0.001*	<0.001*	<0.001*

H – periodontally healthy individuals; P – stage III periodontitis patients; SD – standart deviation; Q1 – 1st quartile; Q3 – 3rd quartile; Z-value was calculated using Mann–Whitney U test; *: $p < 0.05$ statistically significant; ANCOVA – analysis of covariance.

Table 3. Comparison of sex and iodine levels

Sex	Iodine levels		Z-value	p-value
	Mean ±SD	median (Q1–Q3)		
M	110.48 ±72.84	93.11 (47.05–182.05)	−1.794	0.073
F	86.80 ±76.36	54.05 (28.25–147.58)		

M – male; F – female; Q1 – 1st quartile; Q3 = 3rd quartile; SD – standard deviation; Z-value was calculated using Mann–Whitney U test.

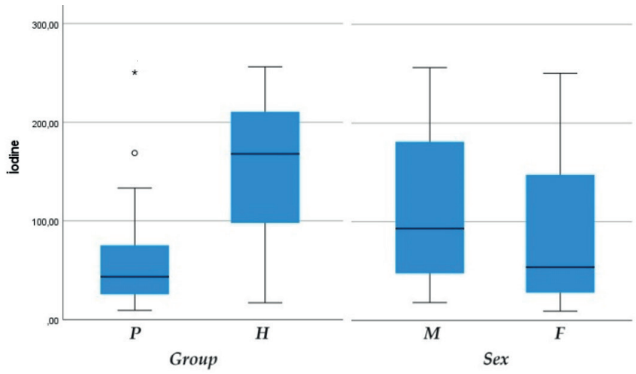

Fig. 2. Iodine comparison in periodontitis patients (P) and periodontally healthy individuals (H), and between male and female participants

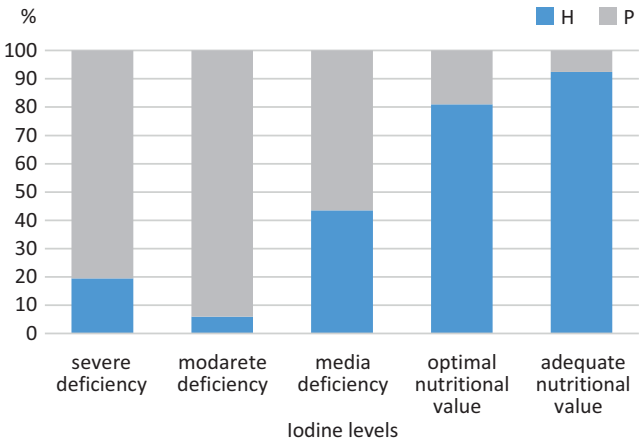

Fig. 3. Comparison of iodine levels between periodontitis patients (P) and periodontally healthy individuals (H)

We found a significant difference in urinary iodine levels between the patient and control groups ($p < 0.001$). Urinary iodine levels in the control group were within the optimal and adequate range, whereas those in the patient group were classified as severely to moderately deficient (Table 4, Fig. 3). Logistic regression analysis further indicated that age, sex and iodine levels were statistically significant between the patient and control groups ($p < 0.001$). The analysis revealed that the patient group had lower iodine levels than the control group. Individuals with low iodine levels were 1.04 times more likely to develop periodontitis than those with higher iodine levels (Table 5).

According to the results of the ROC analysis, the presence or absence of periodontitis can be predicted by examining the iodine values of the patients (0.884 ±0.042; $p < 0.001$). When individuals' iodine levels were below

Table 4. Comparison of iodine levels in periodontitis patients and periodontally healthy individuals

Iodine level	H		P		Test value	p-value
	n	%	n	%		
Severe deficiency	1	3.0%	5	12.5%	34.183	<0.001*
Moderate deficiency	1	3.0%	19	47.5%		
Medium deficiency	7	21.2%	11	27.5%		
Optimal nutritional value	14	42.4%	4	10.0%		
Adequate nutritional value	10	30.3%	1	25%		

Test value: Fisher–Freeman–Halton test; *: $p < 0.05$ statistically significant; H – periodontally healthy individuals; P – stage III periodontitis patients.

Table 5. Logistic regression analysis – variables in the equation

Variable	B ±SE	p-value	OR	95% CI for OR	
				lower	upper
Age	0.478 ±0.150	0.001*	1.614	1.203	2.166
Gender	2.737 ±1.263	0.030*	15.447	1.300	183.489
Iodine	−0.039 ±0.012	0.001*	0.961	0.938	0.984
Constant	−15.252 ±5.042	0.002	0.000	–	–

*: $p < 0.05$ statistically significant; B – correlation coefficient; SE – standard error; OR – odds ratio; 95% CI – 95% confidence interval.
Model summary: –2 Log likelihood = 25.054; Cox–Snell $R_2 = 0.644$; Nagelkerke $R_2 = 0.862$. Variable(s) entered on step 1: age, gender, iodine level.

Table 6. Receiver operating characteristic (ROC) analysis for iodine in the between periodontitis patients (P) and periodontally healthy individuals (H)

Group	Iodine level		AUC					
	mean ±SD	median (Q1–Q3)	area ±SE	95% CI for area lower–upper	cutoff	sensitivity	specificity	p-value
P	56.88 ±47.05	43.5 (25.68–75.26)	0.884 ±0.042	0.802–0.966	76.930	0.725	0.909	<0.001*
H	153.93 ±67.36	167.93 (98.01–210.61)						

* – statistical significance ($p < 0.05$); Q1 – 1st quartile; Q3 – 3rd quartile; M – male; F – female; H – periodontally healthy individuals; P – stage III periodontitis patients; SD – standard deviation; SE – standard error; 95% CI – 95% confidence interval; AUC – area under the ROC curve.

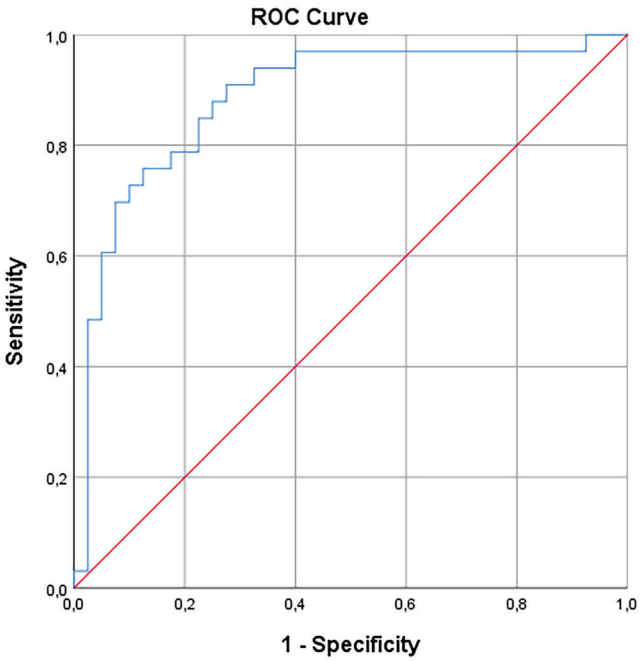

Fig. 4. Receiver operating characteristic (ROC) curve

76.93, the likelihood of periodontitis was 72.5%; when above this value, the probability of not having periodontitis was 90.9% (Table 6, Fig. 4).

Discussion

Periodontitis is a condition characterized by immune-inflammatory responses that result from the accumulation of dental plaque biofilm, leading to inflammation of the periodontal ligament and alveolar bone. Some experts suggest that a periodontal lesion can be considered a type of "wound" that requires an environment conducive to healing.[23]

The consensus across all studies is that maintaining optimal levels of micronutrients is crucial for periodontal health. Nutritional deficiencies can negatively affect the immune response and contribute to the progression of periodontal disease. Maintaining balanced levels of trace elements, including Fe, Zn, Se, and Cu, is vital for preventing the advancement of chronic diseases such as periodontitis.[8] Therefore, this study aimed to investigate the potential effects of iodine deficiency on periodontitis.

The findings of this study revealed a significant reduction in urinary iodine levels among individuals with periodontitis ($p < 0.001$). No prior studies have reported similar results. Previous research has predominantly relied on serum or saliva samples to measure micronutrient levels.[8]

Iodine is an essential micronutrient that depends mainly on the level of dietary iodine intake. Adequate food and micronutrient supply is crucial for an effective and rapid immune response in inflammatory processes.[24] The main dietary sources of iodine in the human population are iodized salt, seafood containing iodine from seawater, and cereals grown in iodine-rich soils. However, recurrent floods and soil erosion in mountainous regions cause iodine deficiency in the soil, resulting in inadequate iodine content in water and food. As a result, humans and animals consuming food grown in these lands may also experience iodine deficiency.[25] Nutritional deficiencies in certain food components can lead to dental defects, oral mucosal problems and periodontal issues affecting oral health.[26] In this study, participants in the periodontitis group had an iodine level of 51.63 ±4.98, which was below the normal range (with a median deficiency of 50–99 µg/L), whereas the iodine level in the healthy group (160.30 ±11.90) was within the normal range (with an optimal nutritional value of 100–199 µg/L).

Iodine deficiency has a geographical distribution affecting 40% of the world's population.[27] It is characterized by iodine-deficient soil washed away by glaciers, heavy rains or floods, often seen in mountainous regions such as the Himalayas, the Andes and large mountain ranges in China. Iodine deficiency also occurs in Central Asia, Central Africa and Europe. Literature indicates that iodine deficiency is prevalent among adults in the geographical region where the participants of the study reside.[28,29] A study in the same region found that the extent of iodine deficiency in subjects was classified as severe (15.6%), moderate (22%) and mild (26.8%).[28] Another study in the same region investigating the relationship between iodine deficiency and dental caries found lower urinary iodine levels in patients with dental caries compared to healthy persons.[30] Our study identified iodine deficiency levels in periodontitis patients as severe (12.5%), moderate (47.5%) and mild (27.5%). This finding among participants may be attributed to their geographical region and corresponding dietary habits that lack sufficient iodine.

Iodine is considered the most electron-rich essential element for animal and human nutrition and plays a crucial

role in early human development. Iodine deficiency disorders encompass a range of consequences, from endemic goiter (thyroid gland enlargement in areas with low iodine content in soils or water) to growth retardation, central nervous system development impairment in children (cretinism), intellectual impairment, neonatal hypothyroidism (insufficient newborn thyroid function), pregnancy loss, and infant mortality.[31]

Its inorganic form, iodide, acts as an antioxidant by counteracting the damaging effects of hydrogen peroxide (H_20_2) on human cells.[32] Reactive oxygen species cause oxidative damage and can be categorized into free oxygen radicals and non-radical molecules. Non-radical ROS, such as H_2O_2, can react with cellular macromolecules, including DNA, leading to damage. Reactive oxygen species can cause severe DNA damage in periodontal tissue cells, contributing to periodontal tissue destruction.[33] The significant reduction in iodine levels observed in patients with periodontitis underscores the potential importance of iodine in maintaining periodontal health. The antioxidant properties of iodide suggest it may act as an antimicrobial agent in saliva. The presence of an H_2O_2/peroxidase system in salivary glands supports the idea that iodide may have a bactericidal or bacteriostatic effect.[34]

Soriguer et al.[35] conducted a study in which healthy volunteers were administered a diet program with a specific daily amount of iodine for 6 months. They observed a negative correlation between urinary iodine levels and CRP, an inflammation marker, and a positive correlation with glutathione peroxidase enzymes, which have antioxidant effects. These findings support the hypothesis that iodine possesses anti-inflammatory and antioxidant properties. The precise mechanism of iodine action remains unclear; however, it is known to penetrate microorganisms and target specific molecules such as amino acids (including cysteine and methionine), nucleotides and fatty acids, resulting in cell death. Iodine also has antiviral properties, but its effectiveness varies among different virus types. It is less effective against viruses lacking lipid envelopes and parvoviruses. Iodine may target the surface proteins of enveloped viruses and destabilize membrane fatty acids by reacting with unsaturated carbon bonds.[36]

Daily intake of trace elements, including Fe, Zn, Se, Cu, iodine, and Mn, ranging from 50 μg to 18 mg, is recommended for normal body functions. In patients with chronic renal failure, trace element levels may decrease due to inadequate food intake, increased dialysis and catabolic reactions. Maintaining optimal trace element levels has been shown to enhance immune function – reducing infection susceptibility and improving quality of life in these patients. Studies indicate that imbalances in trace element homeostasis correlate with mortality in end-stage renal disease patients.[37] A healthy diet is essential for maintaining good health and a strong immune system, and it is justifiable to correct any micronutrient deficiencies. This can be achieved through dietary modifications or dietary supplements. Salt iodization is considered the most effective method to combat iodine deficiency, and it is a cost-effective way to contribute to economic and social development in almost all countries. Because only a small portion of the diet consists of micronutrients, maintaining balance sometimes requires the administration of dietary supplements.[38] Fortified foods and supplements may offer a practical solution, especially when dietary modification is difficult. In cases where salt iodization is not feasible, vulnerable groups may benefit from iodine supplements or iodophor antiseptics.[39,40] However, there is still controversy surrounding the cost–benefit and widespread use of multimicronutrient supplements, and this issue has yet to be resolved.[8] Further investigation is needed to assess the efficacy of supplement administration in periodontitis patients through randomized controlled clinical trials (RCTs).

Limitations

The study only examined a limited number of potential risk factors and did not consider other factors that might contribute to the development of periodontitis, such as smoking or genetics. Another limitation is that the study only included patients and controls from a specific geographic region, which could restrict the generalizability of the findings to other populations. The study population consisted of patients with stage III periodontitis. From a clinical perspective, investigating the levels of iodine trace elements in patients with early-stage periodontitis could be more interesting. Lastly, the study only assessed urinary iodine levels as a proxy for iodine status, which may not accurately reflect iodine levels in the body.

Conclusions

The findings of this study suggest that iodine levels in the body can indicate the ongoing destruction process in periodontitis. Differences in average urine iodide levels could be a useful measure for future investigations. As more extensive studies are conducted, iodine levels may become a useful biomarker for the diagnosis, prognosis and treatment plan of periodontitis. However, the research results presented in this paper are preliminary and should be treated as such. For generalizable results that can be applied broadly, they should undergo more extensive analysis.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.11609037. The package includes the following files:

Supplementary Table 1. ANCOVA age analysis.
Supplementary Table 2. ANCOVA sex analysis.
Supplementary Table 3. Logistic regression analysis.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Comparative study of quality of life after aortic valve replacement through partial upper ministernotomy versus full median sternotomy

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Abstract

Background. Upper ministernotomy for sutureless aortic prosthesis implantation provides an attractive opportunity compared to conventional access. Although in the last decade, the former has gained popularity, data comparing quality of life (QoL) following these procedures are scarce.

Objectives. The purpose of this study was to assess the patient's QoL after aortic valve replacement (AVR) using a ministernotomy approach compared to a full sternotomy.

Materials and methods. One hundred fifteen AVR patients were operated on using either minimally invasive access with sutureless valve implantation through an upper median ministernotomy (group I; n = 58) or through a full sternotomy (group II; n = 57) with either biological Edwards Perimount Magna™ (Edwards Lifescience, Irvine, USA) (n = 30) or mechanical On-X™ (Carbomedics, Austin, USA) (n = 27) aortic valve prostheses implantation by 1 experienced surgeon. At the end of the follow-up period, QoL was assessed using the EQ-5D-5L scale telephone survey.

Results. In group I, there were significantly fewer problems with mobility, pain and usual activities than in group II ($p < 0.05$). Moreover, the visual analogue scale (VAS) and Health Index (HI) scores were more favorable for patients treated with ministernotomy. Additionally, group II participants provided comments beyond the survey questions, such as tiredness, dyspnea or pain. These kinds of remarks were not reported in group I. Ultimately, the EQ-5D-5L Index Score (IS) was consistent with the variables and more beneficial for group I subjects. Each group was compatible with the benefits for patients in group I.

Conclusions. Cardiac surgical procedures for severe aortic stenosis through minimally invasive access are associated with improved QoL parameters.

Key words: quality of life, ministernotomy, EQ-5D-5L, aortic valve disease

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Background

Minimally invasive aortic valve replacement (miniAVR) was first described in 1993 and popularized between 1996 and 1997.[1] Less invasive access due to shorter skin incisions may be recommended for a group of young, particularly female, patients for whom cosmetic outcome is important. Additionally, such procedures are associated with a low bleeding rate, reduced ventilation time and shortened stay in the postoperative intensive care unit (PICU) as well as in the hospital,[2] which may also be an attractive solution for the elderly and those with comorbidities who are not qualified for transcatheter aortic valve implantation (TAVI) procedures.

The early outcomes of minimally invasive approaches for severely diseased aortic valves are unequivocal. On the one hand, they enable reduced blood loss and probability of wound infection, but on the other, they are linked to longer cross-clamping (also called ischemic) and cardiopulmonary bypass times.[3]

Despite the common application of miniAVR around the world, there are still confusing data comparing the mini- to full sternotomy approaches. Of note, there are even fewer studies estimating any differences between the impact of surgical access and quality of life (QoL).

Objectives

The purpose of this study was to assess the patient's QoL after AVR from a ministernotomy approach compared to a full sternotomy.

Materials and methods

This study involved 115 consecutive patients who underwent AVR procedures. Group I (n = 58) had a minimally invasive J-shape upper ministernotomy with implanted sutureless aortic valve prostheses, while group II (n = 57) had full sternotomy access. Group II was divided into 2 subgroups: group II A (n = 25) had implantation with biological Edwards Perimount Magna™ (Edwards Lifescience, Irvine, USA), and group II B (n = 32) had implantation with mechanical prosthesis On-X™ (Carbomedics, Austin, USA) aortic valve prostheses. All procedures were conducted in a single cardiac surgical department between 2018 and 2023. To avoid false answers in the survey, we assessed the study patients' after a min of 3 months after surgery. All individuals were operated on electively by 1 experienced consultant who is particularly interested in aortic valve surgery. The patients' demographics and basic baseline clinical characteristics, including comorbidities, were comparable between the studied groups (Table 1).

According to the rules of the Local Bioethical Committee of Poznan University of Medical Sciences, the Statement of Ethics Approval is not required for retrospective data analysis of patients treated with the use of standard methods.

Table 1. Patients' parameters: Age, body mass index (BMI), EuroScore II, and comorbidities

Patients	Group I (n = 58) (Q1; Q3)	Group II (n = 57) (Q1; Q3)	p-value
Age [years]	67 (31; 81)	67 (52; 79)	0.441
BMI [kg/m^2]	27 (20; 37)	28 (19; 41)	0.447
EuroScore II [%]	0.86 (0.50; 3.83)	1.20 (0.56; 4.51)	0.582
Comorbidities, n (%)			
Diabetes	11 (19%)	20 (35%)	0.065
Hypertension	32 (55%)	41 (72%)	0.500
Chronic lung disease	6 (10%)	0	0.347
Poor mobility	1 (1%)	1 (1,75%)	1.000
Smoking	10 (17%)	17 (29%)	0.136

Q1 – 1st quartile; Q3 – 3rd quartile.

At the end of follow-up, we conducted the EQ-5D-5L scale telephone survey to assess QoL. We used the EQ-5D-5L questionnaire to collect the data. To obtain the most accurate and objective results, the interviewer was not informed of which subject group he was calling. Each EQ-5D instrument comprises a short descriptive system questionnaire and a visual analogue scale (EQ-VAS) that are cognitively undemanding and take only a few minutes to complete. Patients were asked to rate their general health on the day they complete the questionnaire using the EQ-VAS, a 0–100 scale. Data are presented as medians with ranges (min–max). Categorical data are expressed as numbers (n) with percentages (%). The EQ-5D-5L scale is used to evaluate QoL. It consists of 5 questions with 5 answer options for responding to each. It evaluates mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. The valuation research using the EQ-VT was undertaken with available data prepared for Poland.[4] The Severity Index (SI) is obtained by adding the digits that correspond to the levels of the 5 dimensions in each state of health, subtracting 5 and multiplying by 5, which produces a new index (0–100), where 0 indicates a total absence of health problems and 100 is the highest degree of severity. Subtracting the SI from 100 provides the Health Index (HI).

Continuous variables are presented as medians with ranges (min–max), whereas categorical variables are expressed as numbers (n) with percentages (%).

Surgical details

Patients in group I (n = 58) underwent surgery through a partial (upper) J-shape ministernotomy from the jugular notch of the sternum up to the third intercostal space, whereas group II subjects underwent a complete

sternotomy (n = 57). The hearts were arrested after connecting the patients to standard cardiopulmonary bypass (right atrium and ascending aorta) by means of cold cardioplegic solutions administered directly into the coronary ostia. Following the removal of the original valve, sutureless or biological Edwards Perimount Magna™ (n = 25) or mechanical On-X™ (n = 32) valve prostheses were implanted. All knots in the conventional mechanical and biological valve implantations were tied manually in the normal fashion. The later steps of the procedures followed standard protocol, and the chest was always closed in the usual fashion, placing 4 sternal wires for ministernotomy closure or 8 sternal wires for full sternotomy closure. At the end of the AVR procedure through minimally invasive access (group I), our routine practice was to open the right pleural cavity and leave 2 drains (1 in the pleura and 1 in the pericardium).

Data management and analysis

The data analysis was performed anonymously. First, the quantitative variables were checked for normality using the Shapiro–Wilk test, and because they did not satisfy the criteria of normal distribution, they were presented as medians with ranges (min–max) and compared with the Mann–Whitney U test. Categorical data were expressed as numbers (n) with percentages (%). For the EQ-5D-5L, VAS and HI, the t-test was used, whereas, for the estimated regression coefficient, Pearson's correlation coefficient was used. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistica v. 13.3 software package (TIBCO Software Inc., Palo Alto, USA).

Results

EQ-5D-5L index

At the end of follow-up, group I patients reported fewer problems with mobility, usual activities and pain/discomfort than group II patients overall. Out of the 5 categories of possible inconveniences, self-care problems were at least reported in both groups (in less than 10%), with comparable rates (Table 2).

When comparing the mean EQ-5D-5L Index scores of the 2 groups, we found no statistically significant differences in the results; however, the mean index values in group I were marginally higher. Furthermore, there was no statistically significant difference in index scores between genders. The mean index was lower in group I for female patients, while it was lower for males in groups II A and II B. Overall, there were no statistically significant differences between group I and groups II A and II B or between group II A and group II B (Table 3).

EQ-5D-5L visual acuity scale

In group I, 72% of respondents rated their health as good or very good. The rest rated their health status as average or poor. Overall, of the 57 patients in group II, only 40% reported their health status as good or very good. In subgroup II A, 40% of patients rated their health status as good or very good, and in subgroup II B, 37% of patients rated their health as good or very good. The rest of the respondents reported their health status as average or poor. There was a statistically significant difference between reports from respondents in the subgroups (groups II A and II B)

Table 2. Reported problems after different surgery approaches

Reported problems after different surgery approaches	Group I (n = 58)	Group II (n = 57)		p-value		
		group II A (n = 25)	group II B (n = 32)	group I vs II A	group I vs II B	overall group I vs II
Mobility n (%)	7 (12)	7 (28)	13 (41)	0.217	0.217	**0.012**
Self-care n (%)	4 (7)	4 (16)	3 (9)	0.260	0.460	0.482
Usual activities n (%)	9 (15)	6 (24)	17 (30)	0.548	0.012	**0.019**
Pain/discomfort n (%)	11 (19)	11 (44)	13 (41)	0.127	0.106	**0.004**
Anxiety/depression n (%)	13 (22)	8 (32)	14 (44)	0.600	0.177	0.174

Values in bold are statistically significant.

Table 3. Comparing EQ-5D-5L index score in both groups

Patients	Group I (n = 58)	Group II (n = 57)		p-value		
		group II A (n = 25)	group II B (n = 32)	group I vs II A	group I vs II B	overall group I vs II
Mean						
Index score	1	0.939	0.939	0.771	0.928	0.833
Gender						
Female	0.982	0.965	0.943	0.781	0.936	0.872
Male	1	0.939	0.938	0.968	0.880	0.904

Table 4. Visual analogue scale (VAS)

VAS	Group I (n = 58)	Group II (n = 57)	
		group II A (n = 25	group II B (n = 32)
Min	30	10	40
Mean	80	70	70
Max	100	100	100
p-value			
Overall group I vs II	<0.05	group I vs II A	group I vs II B
		<0.05	<0.05

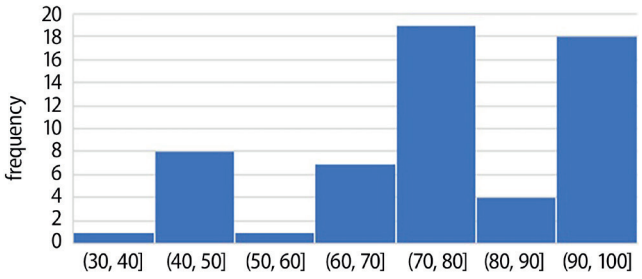

Fig. 1. Visual analogue scale (VAS) frequency distribution for ministernotomy

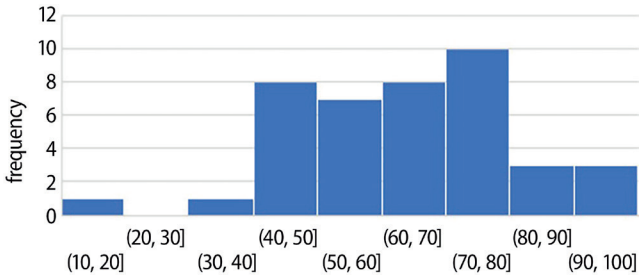

Fig. 2. Visual analogue scale (VAS) frequency distribution for full sternotomy

and those in group I that favored group I. We compiled the main VAS data in Table 4 and Fig. 1,2.

Patients' remarks

Although the results of the EQ-5D-5L index score and VAS are borderline, there was a significant difference when patients' remarks were compared. These comments were given spontaneously outside the survey.

According to the patients' remarks, it can be observed that patients who underwent full sternotomy reported problems with tiredness, dyspnea and pain. Some patients rated their health status as worse than before surgery. Some of the comments concerned patients who had undergone surgery 2 years earlier. A summary of the reported remarks is included in Table 5.

Severity and health index

Using Pearson's correlation coefficient, we estimated the regression coefficient between the VAS and the HI. The regression coefficient was statistically significant in both groups.

Discussion

The increasing popularity of less invasive procedures and the increasing experience of surgeons allow them to perform complex cardiac surgical interventions with the same quality but through smaller skin incisions. According to data from the Polish National Registry of Cardiac Surgery Procedures (Krajowy Rejestr Operacji Kardiochirurgicznych (KROK)), there has been a systematic increase in the rate of less invasive operations for isolated aortic valve disease. It has been reported that in Poland in 2022, almost the same number of isolated AVR procedures were performed via full sternotomy (n = 1,346) as through minimally invasive access (n = 1,344). The results are presented in Fig. 3.

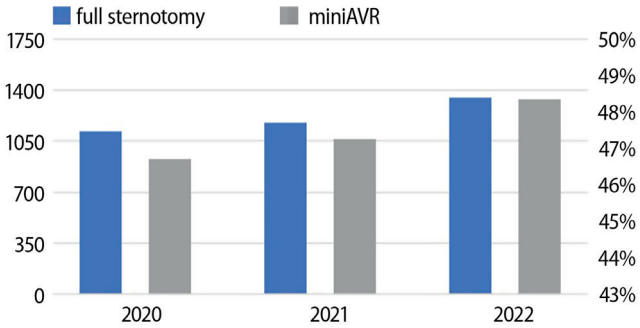

Fig. 3. Comparison of the number of operations depending on access in isolated aortic valve repair (AVR) surgery in Poland by years

Despite the obvious improvement in surgical techniques, technologies and anesthetic management, there is a debate about the clear benefits of less invasive AVR procedures.[5] Some reports have failed to confirm significant differences

Table 5. Summary of reported remarks

Remarks	Tiredness	Dyspnea	Discomfort/pain	Inability to work	Health worse than before surgery
Full sternotomy	11	9	7	1	3
Ministernotomy	0	0	0	0	0
p-value	**0.0003**	**0.001**	**0.01**	0.495	0.118

Values in bold are statistically significant.

between the 2 approaches.[2] Currently, ministernotomy for AVR is not the gold standard of surgical management, and globally, most of these procedures are still performed using the full sternotomy approach.[6,7] Although surgeries for aortic valve disease are commonly performed worldwide, information comparing the differences in patients' QoL between mini- and full sternotomy is very scarce.

EQ-5D-5L index score

In our assessment of both groups of patients, there was no statistically significant difference between the mean index scores in both groups (p = 0.833). However, in all of the analyzed parameters, the mean index score was lower in the full sternotomy approach group. A similar trend was presented by Rodriguez et al. in their study, where the ministernotomy group had a lower index than the full sternotomy approach group.[7]

Visual analogue scale

Group I patients reported a better state of health than group II individuals, which was confirmed by means of EQ-VAS analysis. Of interest, comparing the average value of health self-perspective to the entire Polish population (73.7 points), we showed that patients after ministernotomy reported better (median: 80 points) health, whereas those after standard full surgical access reported a worse (median: 70 points) state of health.[4] The time after the operation had no effect on the results.

We believe the current assessment is reliable because the HI calculated from the EQ-5D-5L questionnaire correlates with the VAS and presents statistically significant favorable results for group I subjects. There were no statistically significant differences in the QoL results between the different types of implanted aortic prostheses.

Differences between patients' self-reported problems

Mobility, self-care and usual activities

It is well known that cardiac surgeries performed via complete median sternotomy elicit a clinical spectrum of systemic effects, including changes in patient body structure and function, activity level and participation in activities of daily life.[8]

This study found that patients' mobility and daily activities differed markedly between the groups in favor of group I. In our opinion, this may be due to relieved discomfort in the early postoperative period, which can lead to quicker recovery following ministernotomy. This fact can be of significance in respect to daily activities and self-care. According to Claessens et al., improvement in physical functioning was more prominent in minimally invasive patients, and the pain scores of patients undergoing complete sternotomy improved significantly more slowly.

Overall, the general health and energy scores improved in both groups after surgery. However, the minimally invasive cardiac surgery patients had an earlier improvement in their general health and indicated that they had significantly more energy than the conventional surgery patients.[9]

Pain/discomfort

Fewer participants in the ministernotomy group experienced severe pain soon after their procedures, which was the opposite of patients who underwent the full sternotomy approach. This difference may result from limited stretching of the sternum during a partial sternotomy; in addition, the presence or absence of sternal fractures may be another important contributor to the early postoperative pain level.[2]

According to Huang et al., chronic pain after heart surgery may become a real problem. In a study of 244 patients after cardiac surgery by sternotomy, persistent pain (defined as pain persisting for more than 2 months after surgery) was seen in almost 30% of the patients. The cause of persistent pain after sternotomy is multifactorial and includes tissue destruction, intercostal nerve trauma, scar formation, rib fractures, sternal infection, stainless steel sutures, and/or costochondral avulsion.[10]

Anxiety/depression

According to Horne et al.,[11] up to 40% of patients are depressed after cardiac surgery. Preoperative depression and postoperative stressful events were the strongest independent associations postoperatively. Physical inactivity was associated with preoperative depression and new depression 6 months postoperatively. In the current study, there was no discernible difference between the 2 groups' levels of depression or anxiety.

Other remarks

Some of the study participants provided additional comments outside of the survey questions. From our perspective, these "off-topic" comments further demonstrate the difference in patient's QoL following AVR carried out through the 2 different approaches. Group II patients were much more eager to share inconveniences, and approx. 40% of them usually included negative remarks in their questionnaires. Most respondents complained of dyspnea, tiredness and chest pain. Unfortunately, some of them claimed that their health was even worse than it was before surgery. On the contrary, not only did group I subjects not mention any postoperative discomfort, but several of them even practiced sports or frequented the gym.

Limitations

We are aware of the numerous flaws in this study. First, the study's retrospective, non-randomized methodology and the observation of a small number of patients at a single institution diminish its statistical power. Second, even though we believe the EQ-5D-5L is an excellent tool for evaluating QoL, we are conscious that certain results cannot be completely objective due to the differences in years following surgery and the respondents' subjective emotions.

Conclusions

Cardiac surgical procedures for severe aortic stenosis through minimally invasive access are associated with improved QoL parameters.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Squamous and basal skin cancers in 17,207 solid organ transplant recipients: Real-world data from national health insurance database in Poland

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Conflict of interest
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Abstract

Background. Immunosuppressive therapy in organ transplant ensures proper graft function for many years, but it is burdened with a negative impact on the development of skin cancer in them.

Objectives. To characterize the impact of immunosuppressive therapy in transplant recipients on the development of non-melanoma skin cancers (NMSC).

Materials and methods. A total of 17,207 Polish patients who underwent liver, heart or kidney transplants between 2010 and 2022 and were on immunosuppression were included in the study. Immunosuppression was most commonly achieved using a regimen of tacrolimus (TAC) or cyclosporine A (CsA) combined with mycophenolic acid (MPA) and glucocorticosteroids (GS). Data on NMSC incidence from the National Health Fund in this population were analyzed and compared against incidence of NMSC in general Polish population in the same period.

Results. Renal transplant recipients demonstrated a significantly elevated risk of NMSC compared to the general population, with a 1-year cumulative incidence of 0.09% vs 0.04% ($p < 0.001$), a 5-year incidence of 1.21% vs 0.18% ($p < 0.001$) and a 10-year incidence of 4.18% vs 0.36% ($p < 0.001$). Liver transplant recipients exhibited an elevated risk for the development of NMSC, which persisted and increased over time (incidence of 0.09% vs 0.04% at 1 year ($p < 0.001$), 0.83% vs 0.18% at 5 years ($p < 0.001$) and 2.65% vs 0.36% at 10 years ($p < 0.001$)). Heart transplant recipients also showed a significantly higher cumulative incidence of NMSC at 1 year (0.09% vs 0.04%, $p < 0.001$), 5 years (0.89% vs 0.18%, $p < 0.001$) and 10 years (4.06% vs. 0.36%, $p < 0.001$) post-transplantation.

Conclusions. Organ transplant recipients have an 2 times at 1 year, 4,5 times after 5 years and 9 times after 10 years increased risk of NMSC on average as opposed to general Polish population in the same period.

Key words: transplantation, skin cancer, transplant recipients, non-melanoma skin cancer

Background

A total of 1,910 organ transplants, including 1,055 kidney transplants, 550 liver transplants and 178 heart transplants, were carried out in Poland in 2023, according to the National Health Fund.[1] This substantially outnumbers earlier figures of transplantation (e.g. 1,608 organ transplants in 2012).[2] Currently, organ transplantations are common worldwide, with the numbers of these procedures performed each year globally reaching hundreds of thousands.

Hailed as the only long-term curative treatment for end-stage liver, heart or kidney disease, organ transplantation involves recipients in extensive, lifelong care. Recipients of the lifelong immunosuppressive therapy required for proper graft function are susceptible to numerous illnesses, and cancer is one of the most common in this group of patients.

Non-melanoma skin cancers (NMSCs), comprising mainly of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), are the most common tumors developing after organ transplantation and both constitute over 90% of all skin tumors that appear in individuals after organ transplantation.[3]

The outcomes of various studies have receive transplant recipients experience an increased risk of skin malignancy, ranging from 2 to 250 times higher compared to the general population, with the exact risk depending on specific population under investigation.[4–9] The highest increase in this particular risk was observed in Australia (a 250 times higher risk compared to the general population). In this country, the cumulative incidence of skin cancer progressively rises from 7% after 1 year of immunosuppression to 45% after 11 years, eventually reaching 70% after 20 years of immunosuppression.[5] Authors from Scandinavian countries report a 20–100-fold increase in the risk of developing NMSCs.[6,7] In the Netherlands, the risk of skin cancer was observed to be 3% after 5 years, 24% after 15 years and even 40% after 20 years from organ transplantation.[8] In one of the Italian studies, the cumulative risk after 5 years was 7.5%, and after 15 years, it was 28.8%.[9] The risk of developing skin cancer steadily rises in all the presented data as more time passes since the transplantation. However, the numerical values significantly vary among them, even in the European countries sharing similar remaining NMSC risk factors, like long life expectancy, large percentage of elderly in the demographical structure, UV exposure, and cancer awareness within society.[6–9]

Despite sharing a similar geographical latitude, findings from European studies also reveal considerable variation in the risk of neoplasia. This indicates that there may be other risk factors to cancer development, including the effects of administered treatments, genetic predispositions, or the prevalence and influence of HPV infection.[10–13] Regrettably, despite a lot of publications, epidemiological studies with a sizable patient population are lacking; such studies could facilitate an accurate evaluation of the actual risk of skin cancer among transplant recipients in Poland.

Objectives

In this study, we performed a large-scale investigation regarding the specific risk faced by transplant recipients by focusing on the real-world incidence of NMSC in Poland over the last 13 years. The data on skin cancer episodes involving all Polish residents were sourced from the National Health Fund dataset, a governmental public health institution. This is the largest analysis conducted on this topic in Poland to date, aiming to shed light on the frequency and risk factors associated with NMSC among organ transplant recipients.

Materials and methods

The National Health Fund provided information on skin cancer incidence in patients who had received a kidney, heart or liver transplant. The International Classification of Diseases, 10th Revision, Clinical Modification (ICD-10-CM) code for skin cancer (C44.0–C44.9) was used to identify a cohort of patients with a diagnosis of skin cancer based on any inpatient or outpatient claim associated with the dataset. Code C44.0–C44.9 is used by hospitals for reporting to the National Health Fund the diagnosis of NMSC regardless of histopathology (SCC and BCC are reported using the same code). Patients who underwent renal, heart or liver transplantation between 2010 and 2022 are included in the dataset and were identified by specific codes used for transplantation reimbursements by the National Health Fund. A history of prior organ transplantation as well as the transplantation of more than 1 of the specified organs were considered exclusion criteria. Basic demographic characteristics of the patients included in the analysis is presented in the Table 1.

Statistical analyses

The primary objective of this analysis was to compare the cumulative incidence rates of skin malignancies, excluding melanoma, in patients who have undergone organ transplantation (kidney, heart or liver) with those in the general population. An additional aim was to determine whether organ transplantation is a significant risk factor for the development of skin cancers when compared to the general population. The studied hypotheses were formulated as follows:

H_0: The cumulative incidence rate of skin cancer is the same in both the specific post-transplant (Tx) cohort and the general population at each time point (1, 5 and 10 years).

H_1: The cumulative incidence rate of skin cancer differs between the specific Tx cohort and the general population at each time point.

The cumulative incidence rate at specific time point t is calculated as the sum of the incidence rates for each year up to that point using Equation 1:

Table 1. Clinical and demographic characteristics of the solid organ transplants recipients (Tx)

Characteristic	Kindey Tx	Liver Tx	Heart Tx
Mean age at time of organ transplantation [years]	47.97	44.36	46.87
Median age at time of organ transplantation [years]	50.5	50	52
Mean age at the time of NMSC diagnosis [years]	65.36	62.71	62.19
Median age at the time of NMSC diagnosis [years]	68	64	65.75
Mean time to diagnosis [years]	5.02	5.33	6.68
Median time to diagnosis [years]	5.25	5.5	6

NMSC – non-melanoma skin cancer.

$$Cumulative\ incidence\ rate = \sum_{i=0}^{t} \frac{New\ events_i}{At\ risk_i} \quad (1)$$

We compared cumulative incidence rates of skin cancer between the Tx cohort and the general population at 1 year, 5 years and 10 years using a Z-test for 2 proportions.

The proportion of patients who developed skin cancer by the specified time point (1, 5 or 10 years) was calculated for both the Tx cohort and the general population.

The pooled incidence rate was calculated with Equation 2:

$$\hat{p} = \frac{n_{Tx} \cdot \hat{p}_{Tx} + n_{Pop} \cdot \hat{p}_{Pop}}{n_{Tx} + n_{Pop}} \quad (2)$$

where n_{Txn} and n_{Pop} represent the number of patients at risk in the Tx cohort and general population, respectively; $\hat{P}_{Tx}$– cumulative incidence rate in the Tx cohort; and $\hat{P}_{Pop}$– cumulative incidence rate in general population.

The Z-statistic for comparing the cumulative incidence rates between the cohort of patients after transplantation and overall population was calculated using Equation 3:

$$Z = \frac{\hat{p}_{Tx} - \hat{p}_{Pop}}{\sqrt{\hat{p}\left(1-\hat{p}\right) \cdot \left(\frac{1}{n_{Tx}} + \frac{1}{n_{Pop}}\right)}} \quad (3)$$

This Z-statistic was compared to the standard normal distribution to obtain the p-value.

When comparing the Tx cohort to the general population, the Tx cohort is not excluded from the population dataset. Due to the very small proportion of Tx patients, their inclusion does not impact the observed differences.

An alpha level $\alpha = 0.05$ was set as the criterion for determining statistical significance. A $p < 0.05$ was considered statistically significant, indicating a difference in cumulative incidence rates between the 2 groups at the respective follow-up periods.

Moreover, to establish a transplantation as a risk factor, we estimated risk ratio (RR) comparing the risk of skin cancer in the transplantation cohort to the risk in the general population using Equation 4:

$$RR = \frac{Incidence\ rate\ in\ Tx\ cohort}{Incidence\ rate\ in\ General\ population} \quad (4)$$

To compute the 95% confidence interval (95% CI) for RR, we applied an approximation for the standard error (SE) of the logarithm of the RR using Equation 5:

$$SE_{logRR} = \sqrt{\frac{1}{e_{Tx}} + \frac{1}{e_{Pop}}} \quad (5)$$

where e_{Tx}, e_{Po} is the number of skin cancer events in the Tx cohort and overall population respectively.

The 95% CI for RR was calculated using Equations 6,7.

$$Cl_{lower} = e^{log(RR) - 1.96 \times SE_{logRR}} \quad (6)$$

$$CI_{upper} = e^{log(RR) + 1.96 \times SE_{logRR}} \quad (7)$$

The RR is significantly greater than 1.0, which suggested that the transplantation is a risk factor for skin cancer.

Analyses were conducted using the R statistical language (v. 4.3.3; R Core Team, 2024; R Foundation for Statistical Computing, Vienna, Austria) on Windows 11 Pro 64 (build 22631).

We computed the cumulative incidence rate of NMSC, coded as C44, among recipients of organ transplants (Tx) in comparison to a control group over a 1-, 5- and 10-year follow-up period.

The Polish National Cancer Registry provided the information used to build the dataset for this time period (2010–2022).[14]

Results

Population of patients with non-melanoma skin cancer (C44)

The population of Poland during the period from 2013 to 2022, according to data from the World Bank,[15] ranged from 38.04 million at the beginning of the analyzed timeframe to 36.1 million by the final year of assessment. Incidence data for the respective years within the study period were derived based on the findings of the reports from the Polish National Cancer Registry.[16] The search criteria were focused on identifying newly reported cases of incidence associated with NMSC (C44) across the entirety of the Polish region, encompassing both genders and all age groups.

The cumulative incidence rates were calculated in accordance with established epidemiological methodologies, taking into account the annual variations in population size as well as the yearly incidence of new cancer cases reported by the Polish National Cancer Registry. These results, as detailed in Table 2, provide insight into the burden of skin cancer in the Polish population over a 10-year

Table 2. Risk table of non-melanoma skin cancer (NMSC) events (C44) with follow-up over 10 years (2013–2022) in patients' Polish population

Year	Follow-up [years]	Patients at risk	NMSC (C44)	
			new events	cumulative incidence rate [%]
2013	1	38,040,196	13,516	0.0355
2014	2	38,011,735	14,470	0.0736
2015	3	37,986,412	13,267	0.1085
2016	4	37,970,087	12,506	0.1413
2017	5	37,974,826	13,731	0.1774
2018	6	37,974,750	14,502	0.2155
2019	7	37,965,475	14,687	0.2542
2020	8	37,899,070	10,810	0.2826
2021	9	37,747,124	13,633	0.3185
2022	10	36,821,749	15,716	0.3597

Table 3. Risk table of non-melanoma skin cancer (NMSC) events (C44) with follow-up over 10 years in patients after kidney transplantation

Follow-up [years]	Patients at risk	NMSC (C44)	
		new events	cumulative incidence rate [%]
0	12,205	0	0.0000
1	11,673	10	0.0857
2	10,676	19	0.2636
3	9,788	24	0.5088
4	8,895	30	0.8461
5	7,870	29	1.2145
6	6,893	35	1.7227
7	5,868	28	2.1993
8	4,859	27	2.7550
9	3,920	22	3.3162
10	2,998	26	4.1835

follow-up period. The results demonstrate a steady increase in the cumulative incidence rates for NMSC (C44) over the 10-year follow-up period in the Polish population.

The incidence of NMSC (C44) is significantly higher from the outset, with a cumulative incidence rate starting at 0.0355% in 2013 and increasing to 0.3597% by 2022. The consistently higher annual incidence, ranging from 10,810 to 15,716 cases, suggests that NMSC represent a more substantial and increasing risk to the population.

Cohort of patients with kidney transplantation

The number of patients at risk for developing skin malignancies following kidney transplantation, over the period from 2010 to 2022, was approx. 12,205, as detailed in Supplementary Table 1. This cohort represents individuals who underwent kidney transplantation and were subsequently exposed to long-term immunosuppressive therapy, which is well-documented to increase the risk of certain malignancies, particularly skin cancers.[5–9]

Table 3 provides a detailed summary of the cumulative incidence of non-melanoma skin cancer (NMCS) (C44) over a 10-year follow-up period post-transplantation.

Notably, the cumulative incidence rate for other skin malignancies is significantly higher, rising from 0.0857% at 1 year to 4.1835% after 10 years.

Results of comparing incidents rate between the cohort of patients after kidney transplantation and overall population are reported in Table 4.

The results in Table 4 indicate that kidney transplant recipients have a significantly higher risk of developing NMSC (C44) compared to the general population. The risk ratio for NMSC (C44) at 1 year, the cumulative incidence rate for transplant recipients is 0.0857%, with an RR = 2.41, indicating more than double the risk compared to the general population. By 5 years, the incidence rate rises dramatically to 1.2145%, with an RR = 6.84, and by 10 years it reaches 4.1835%, with an RR = 11.63. These findings demonstrate that kidney transplant recipients are at a significantly elevated risk of developing NMSC over time, with the risk increasing substantially as the follow-up period lengthens.

Table 4. Results of Z-test and RR for cumulative incidence of NMSC (C44) in kidney transplant recipients compared to the general population

Event	FU	Cumulative incidence rate [%]		z	p-value	RR	95% CI
		Tx	population				
NMSC (C44)	1Y	0.0857	0.0355	29.30	<0.001	2.41	1.29–4.48
	5Y	1.2145	0.1774	240.71	<0.001	6.84	5.69–8.24
	10Y	4.1835	0.3597	436.16	<0.001	11.63	10.27–13.17

RR – risk ratio; 95% CI – 95% confidence interval.

The Z-test results for NMSC is highly significant ($p < 0.001$) at all time points, showing that the observed differences between the transplant cohort and the general population are not due to random chance. This strongly supports the conclusion that organ transplantation, specifically kidney transplantation, is a major risk factor for the development of NMSC.

Liver transplant recipients vs general population

The data from Table 5 indicate that patients who undergo liver transplantation are at an elevated risk for NMSC (C44) over a 10-year period. By the end of the first year, 3 new cases of NMSC are observed, resulting in a cumulative incidence rate of 0.0931%. This rate continues to rise. By year 5, the cumulative incidence of NMSC reaches 0.8311%, and by year 10, it escalates to 2.6462%. The consistent appearance of new cases throughout the follow-up period, especially between years 5 and 10, highlights the prolonged and progressive risk of NMSC in liver transplant recipients.

The results from Table 6 show that liver transplant recipients are at a significantly higher risk of developing NMSC (C44) compared to the general population. The risk for other skin malignancies (C44) is pronounced. At 1 year, the cumulative incidence rate in liver transplant recipients is 0.0931%, with an RR = 2.61 (95% CI: 0.84–8.12), suggesting a moderately increased risk, though with some uncertainty due to the wide confidence interval. By 5 years, the cumulative incidence rises sharply to 0.8311%, with an RR = 4.68 (95% CI: 2.99–7.35), reflecting a significantly elevated and more precisely estimated risk. At 10 years, the cumulative incidence for NMSC reaches 2.6462%, with an RR = 7.35 (95% CI: 6.77–7.99), indicating that

Table 5. Risk table of non-melanoma skin cancer (NMSC) events (C44) with follow-up over 10 years in patients after liver transplantation

Follow-up [years]	Patients at risk	NMSC (C44)	
		new events	cumulative incidence rate [%]
0	3,584	0	0.0000
1	3,224	3	0.0931
2	2,819	3	0.1995
3	2,499	4	0.3595
4	2,200	1	0.4050
5	1,878	8	0.8311
6	1,609	2	0.9555
7	1,316	5	1.3355
8	1,059	0	1.3355
9	777	6	2.1076
10	557	3	2.6462

Table 6. Results of Z-test and RR for cumulative incidence of non-melanoma skin-cancers (NMSC)in liver transplant recipients compared to the general population

Event	FU	Cumulative incidence rate [%]		z	p-value	RR	95% CI
		Tx	population				
NMSC (C44)	1Y	0.0931	0.0355	17.67	<0.001	2.61	0.84–8.12
	5Y	0.8311	0.1774	74.15	<0.001	4.68	2.99–7.35
	10Y	2.6462	0.3597	112.44	<0.001	7.35	6.77–7.99

RR – risk ratio; 95% CI – 95% confidence interval.

liver transplant recipients are more than 7 times as likely to develop NMSC compared to the general population. This long-term risk is highly significant and consistent.

The Z-test results are significant ($p < 0.001$) for NMSC at all time points, confirming that the differences in cumulative incidence rates between liver transplant recipients and the general population are statistically significant. The data clearly established liver transplantation as a major risk factor for NMSC, where the risk increases markedly over time.

These findings highlight the critical need for regular dermatologic screening and preventive strategies in liver transplant recipients, with a specific focus on NMSC, which pose a substantial and growing risk in this population.

Heart transplant (Tx) recipients vs general population

The following Table 7 presents the risk of developing NMSC over a 10-year period in a cohort of heart transplant recipients. The data highlight both the cumulative incidence of these cancers and the number of new events at each follow-up interval, providing valuable insights into the timing and progression of skin cancer risk in this vulnerable population.

The data in Table 7 highlight the increasing cumulative incidence of non-melanoma skin cancer (NMSC) (C44) over a 10-year follow-up period in heart transplant recipients. The incidence of NMSC (C44) shows a steady increase over time, with new events occurring consistently throughout the follow-up period. By the 10th year, the cumulative incidence rate of NMSC reaches 4.0609%, indicating a high risk. The data suggest that heart transplant recipients are at significantly greater risk for NMSC, with the risk progressively increasing as follow-up extends.

The data in Table 8 show a distinct pattern of non-melanoma skin cancer (NMSC) risk in heart transplant recipients compared to the general population. The risk of NMSC (C44) is high and clearly defined. At 1 year, the cumulative incidence rate in heart transplant recipients is 0.0909%, with an RR = 2.56 compared to the general population. Although this indicates more than double the risk, the confidence interval (95% CI: 0.37–18.18) suggests a lack of precision, likely due to the smaller number of cases early on. By 5 years, the cumulative incidence rate increases sharply to 0.8871%, with an RR = 5.00 and a much narrower confidence interval (95% CI: 2.24–11.13), demonstrating a significantly elevated risk. At 10 years, the cumulative incidence reaches 4.0609%, with an RR = 11.29, and the confidence interval (95% CI: 9.64–13.22) indicates a highly significant and reliable increase in the risk of NMSC in heart transplant recipients.

The Z-test results across all time points for NMSC is significant ($p < 0.001$), confirming that the differences in cumulative incidence rates between heart transplant recipients and the general population are not due to chance.

Table 7. Risk table of NMSC events (C44) with follow-up over 10 years in patients after heart transplantation

Follow-up [years]	Patients at risk	NMSC (C44)	
		new events	cumulative incidence rate [%]
0	1,418	0	0.0000
1	1,099	1	0.0909
2	918	1	0.1998
3	740	1	0.3349
4	616	1	0.4972
5	513	2	0.8871
6	418	1	1.1263
7	341	1	1.4195
8	276	1	1.7818
9	204	2	2.7622
10	154	2	4.0609

Table 8. Results of Z-test and RR for cumulative incidence of NMSC (C44) in heart transplant recipients compared to the general population

Event	FU	Cumulative incidence rate [%]		z	p-value	RR	95% CI
		Tx	population				
Other skin malignancies (C44)	1Y	0.0909	0.0355	9.92	<0.001	2.56	0.37–18.18
	5Y	0.8871	0.1774	42.08	<0.001	5.00	2.24–11.13
	10Y	4.0609	0.3597	95.71	<0.001	11.29	9.64–13.22

RR – risk ratio; 95% CI – 95% confidence interval.

Discussion

Following organ donation, NMSC are frequent and significantly increase mortality.[13,17] Numerous research studies have looked at the risk of NMSC following transplantation, showing a wide range of the reported rise in incidence. This raises many questions regarding the scale of this phenomenon in Poland. The studies that are available so far for the Polish population either focus on a small number of participants, describe individual cases, or relate to earlier periods when immunosuppressive medication was frequently administered differently than it is today.[16–19] The purpose of our research was to reexamine the relationship between Poland's organ transplant recipients and the incidence of SCC or BCC in comparison with the corresponding incidence observed over the same time span across the general Polish population. After analyzing data from the National Health Fund, we discovered that abundant NMSC cases were present in the 3 most numerous post-transplant patient groups: those who had received a kidney, liver or heart transplant.

Upon comparing data from the National Health Found to population data published by the National Cancer Registry,[14] the risk of NMSC occurrence in renal transplant recipients was nearly 12 times higher after 10 years of transplantation compared to the population risk at the same time (cumulative risk 4.18% vs 0.36%). The escalating incidence in the renal transplant recipient cohort over time is probably indicative of a persistent and compounding risk factor for NMSC attributable to long-term immunosuppressive treatment, which may not only enhance the survival of malignant cells but also promote the accumulation of oncogenic mutations and inhibit the body's natural antitumor immune defenses. This impact of immunosuppression seems to be independent from other well-known risk factors, like UV exposure, as other risk factors are evenly affecting general population in Poland as equally as they affect post-transplant patients.

The 10-year data also show an increase in the cumulative risk to 2.65% among liver transplant patients, compared to 0.36% in the general population (2010–2022). This enduring elevation in NMSC incidence underscores the chronic risk associated with long-term immunosuppression and possible additional hepatic comorbidities that may further compromise immune surveillance against skin cancer.

Patients who have undergone heart transplants in Poland also statistically more frequently received a NMSC diagnosis over the course of follow-up. Cumulative incidence climbs to 4.06%, compared to 0.36% within the general population during the same time. This trend suggests a continued increased risk of NMSC associated with long-term immunosuppression, and possibly other factors unique to heart transplant recipients, such as the increased incidence of viral infections that can contribute to skin cancer risk. In conclusion, the cumulative risk of NMSC compared to the general population is also significantly higher in all 3 mentioned groups of patients.

Limitations

It should be noted that our investigation is subject to various limitations. Firstly, information from the National Health Fund database on the matter is limited to the period after 2010. Furthermore, we tried to reduce the possibility of overdiagnosis by only taking into account hospital and clinical data related to the diagnoses (i.e., we eliminated ICD codes entered into the national online reporting system at primary care facilities without prior histopathological confirmation). Additionally, since SCC and BCC were not given distinct ICD-10 codes, it is not possible to conduct a separate analyses on the incidence of these 2 skin malignancies. Fortunately, since 2022, all Polish pathology reports have been entered into nationwide online reporting system, which in future will enable pathology diagnosis-directed, rather than code-based, analysis.

Additionally, another weakness of this study is the well-known phenomenon of underreporting of skin cancers like SCC and BCC.[20] On the other hand, the underreporting of the C44 code applies to the same extent both to transplant recipients and the general population, thus allowing for valid comparison.

There are many well-established factors influencing the increased incidence of skin cancers, such as the patient's age, UV exposure or HPV infection. However exposure to these factors is no different in general population and its subpopulation of transplant recipients in Poland. It might be even speculated, that transplant recipients are younger as opposed to general population at the time of NMSC diagnosis – we are currently exploring that issue in the ongoing further epidemiological study. Therefore, we believe that they do not have a significant impact on the final result.

Additionally, the type of immunosuppression used and the duration of its application also affect the incidence of cancers. There are many drugs and their combinations used in organ recipients to prevent the rejection of the transplanted organ. However the most commonly used immunosuppressive treatment regimen for solid organ transplant recipients in our dataset was: tacrolimus (TAC) or cyclosporine A (CsA) combined with mycophenolic acid (MPA) and glucocorticosteroids (GS). We are intending to perform subgroups analyses on strictly and narrowly defined groups receiving specific immunosuppression.

Nevertheless, the strength of the report derives from a rigorous examination of the available data, stringent inclusion criteria, and their integration to produce a clinically meaningful consensus. Utilizing data from one of the most trustworthy medical information repositories for transplant recipients in Poland, this study represents, to the best of our knowledge, the first extensive analysis of such a large group of organ transplant recipients in Poland and presumably in Europe as well. According to our research, organ transplantation and subsequent immunosuppression exposes transplant recipients to a much

higher risk of NMSC. This underlines the need to comprehensibly inform the patients on the risk and educate them how to perform self-examinations.[21] It was acknowledged in recently published Polish Oncological Society guidelines for melanoma and NMSC post-treatment surveillance – 5 years following transplantation, guidelines advise that patients who have undergone transplantation should have their skin evaluated for cancer at least twice a year.[22–24]

Conclusions

The risk of NMSC in patients who have long-term survived renal, heart and liver transplants has been steadily rising over time since transplantation in Poland. Based on >17,000 transplant recipients, we concluded that the risk of developing NMSC after transplantation was higher than the general population risk. The 10-year cumulative incidence of NMSC increased in renal (4.18% vs 0.36, $p < 0.001$), liver (2.65% vs 0.36, $p < 0.001$) and heart recipients (4.06% vs 0.36, $p < 0.001$) vs general Polish population. The overall NMSC risk was continuously rising since transplantation staring from on average 2 times higher risk at 1 year to 12 times higher risk after 10 years compared to the risk of NMSC in general Polish population.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.14606095. The package includes the following files:

Patient population at risk following kidney, heart, and liver transplantation in 2010–2022.

Supplementary Table 1. Number of patients at risk following kidney transplantation by year of transplantation with 10-year follow-up.

Supplementary Table 2. Number of patients at risk following heart transplantation by year of transplantation with 10-year follow-up.

Supplementary Table 3. Number of patients at risk following liver transplantation by year of transplantation with 10-year follow-up.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Human umbilical cord mesenchymal stem cell-derived exosomes combined with mouse nerve growth factor can more effectively ameliorate the motor disorder and brain pathological injury in mice with cerebral palsy

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Abstract

Background. Cerebral palsy (CP) is a neurodevelopmental disorder and motor disorder syndrome. It has been confirmed that mesenchymal stem cells (MSCs) and mouse nerve growth factor (mNGF) can repair brain tissue damage and nerve injury; however, exosomes derived from healthy cells may have a comparable therapeutic potential as the cells themselves.

Objectives. The purpose of this study was to explore the improvement effect of human umbilical cord mesenchymal stem cell (hUC-MSCs)-derived exosomes on a CP model and determine whether there is a synergistic effect when combined with mNGF.

Materials and methods. Exosomes were isolated from hUC-MSCs and examined using transmission electron microscopy (TEM), particle size and western blot (WB). A total of 38 BALB/c mice (male, postnatal day 6 (PND6)) were randomly divided into 5 groups: sham group, CP group, CP-exo group, CP-mNGF group, and CP-exo-mNGF group. Hypoxic induction after unilateral common carotid artery ligation combined with lipopolysaccharide (LPS) infection was used to construct the CP model. Pathological damage to neuron tissue and synaptic structures in the hippocampus was confirmed using light microscopy after hematoxylin–eosin (H&E) staining and TEM, respectively. Survival of neurons was evaluated using Nissl staining. Western blot was applied to monitor PSD-95 and synaptophysin (SYN) protein levels.

Results. This study indicated that exosomes released by hUC-MSCs ameliorated brain damage and synaptic structure destruction in CP mice induced by hypoxic ischemia and LPS infection. When combined with mNGF, there was more effective improvement. In the CP group, neuronal function was severely impaired; however, hUC-MSCs-derived exosomes and mNGF improved it. PSD-95 and SYN proteins were presynaptic and postsynaptic proteins, respectively. Interestingly, the PSD-95 and SYN protein levels were significantly lower in the CP mice, but with the addition of hUC-MSCs-exosomes or mNGF, they increased significantly, especially in the CP-exo-mNGF group.

Conclusions. The nerve function injury in CP can be improved the most when hUC-MSCs-derived exosomes are combined with mNGF through intraperitoneal (ip.) administration.

Key words: exosome, human umbilical cord mesenchymal stem cells, cerebral palsy, mouse nerve growth factor

Background

Cerebral palsy (CP) is a nonprogressive and permanent neurodevelopmental disorder syndrome caused by various risk factors. It occurs in 2–3 out of 1,000 live births and has multiple etiologies that result in brain injury, affecting movement, posture and balance.[1] Additionally, some symptoms become more obvious over time. Although no definite method exists to date that cures CP, supportive treatment and drug therapy may alleviate CP symptoms to a certain extent and improve the patient's motor skills and functional ability. The primary treatment for CP is rehabilitation training, including intramuscular onabotulinum toxin A injections, systemic and intrathecal muscle relaxants, selective dorsal rhizotomy, and physical and occupational therapies.[1,2] However, all these treatment measures have the drawbacks of slow action and possible regression after the intervention is discontinued.[1,2] Therefore, alternative or complementary therapeutic measures are urgently needed. Studies have confirmed that cerebral ischemia–hypoxia in the perinatal period or up to 1-year postpartum is the main cause of CP. Inflammation is now believed to be a key component in the development of CP.[3] In this study, a neonatal mouse model of CP was established using combined hypoxia–ischemia and inflammation to create an injury that better mimicked the neurodegeneration seen in human CP.

Mesenchymal stem cells (MSCs) are a type of multipotent stem cell originating from the mesenchyme that possess various crucial biological characteristics. Their main features include multipotency, self-renewal, immunomodulation, and secretion of factors.[4] Additionally, the antiapoptotic, paracrine and multidirectional ability of MSCs to differentiate has driven their current evaluation in translational research and clinical trials for treating many common diseases, including neurological disorders involving central nervous system (CNS) structures, such as stroke, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's and Parkinson's diseases, multiple sclerosis, and spinal cord injury.[5] Mesenchymal stem cells can exert their effects through neuroprotection, neural regeneration, anti-inflammatory effects, promotion of angiogenesis and vasculogenesis, and immunomodulation.[5,6] Human umbilical cord MSCs (hUC-MSCs) are somatic stem cells derived from umbilical cord blood that have self-renewal and multidirectional differentiation potential. They can be used as a cell source for treating many diseases and repairing tissue damage.[7,8] Compared with other MSCs, hUC-MSCs have gained the attention of researchers for tissue injury repair because of their low cost, minimal invasiveness, convenient isolation, large cell content, high gene transfection efficiency, low immunogenicity,[9] and lack of ethical issues. Therefore, in recent years, hUC-MSCs have gradually become the focus of somatic stem cell research. Increasing evidence shows that hUC-MSCs exert their therapeutic effects mainly through extracellular vesicles (EVs) produced by paracrine actions. Extracellular vesicles have emerged as a new promising alternative to whole-cell therapy because of their lower immunogenicity and tumorigenicity and easier management.[10] The MSC-derived exosomes (MSC-exos) have been used as a therapeutic approach in treating several diseases, such as neurological, cardiovascular, liver, kidney, and bone diseases, as well as cutaneous wounds, inflammatory bowel disease, cancers, infertility, and other disorders. Furthermore, besides treating CNS diseases, MSC-exos play a role in mediating anti-inflammation, increasing neuronal growth, maintaining the number of neurons, and promoting neurite remodeling.[11–14] Their clinical use might provide substantial advantages compared with live cells due to their potential to reduce undesirable side effects after application, including infusional toxicity, uncontrolled cell growth and possible tumor formation.[15] However, the role of hUC-MSC-exosomes (hUC-MSC-exos) in CP is unclear. Hence, this study hypothesized that increasing neuronal growth and alleviating white matter damage using hUC-MSC-exos may be responsible for this effect.

Nerve growth factor (NGF) in mice is synthesized through a multistep process within cells. Initially, the NGF gene, encoded in the mouse genome, undergoes transcription in the cell nucleus, resulting in the production of messenger RNA (mRNA). This mRNA is then translated into the precursor protein of NGF. The precursor protein undergoes post-translational modifications, such as cleavage and glycosylation, ultimately yielding mature NGF protein. Once synthesized, NGF is secreted from cells into the extracellular space.[16,17] It promotes neuronal survival and growth, supports nerve regeneration, regulates synaptic plasticity, and modulates pain perception.[18] These roles highlight NGF's importance in maintaining the structure and function of the nervous system in mice. The mNGF is commonly used in nerve injury treatment.[19] Some researchers have reported that mNGF was effective in treating CNS injury and developmental disorders in children.[20] Feng et al. found exogenous NGF have great potential for post-traumatic stress disorder (PTSD) treatment, preventing the impaired hippocampal cytoarchitectures.[21]

Currently, treatment options for CP are limited, and the therapeutic effects of hUC-MSC-exos in CP remain to be explored. It has yet to be investigated whether combination therapy with mNGF could yield better therapeutic outcomes.

Objectives

This study aimed to investigate the effects of hUC-MSC-exos on mice with CP and attempted to determine whether the combination of hUC-MSC-exos and mNGF has a better therapeutic effect for mice with CP.

Materials and methods

Cell culture and exosome preparation

Exosomes were isolated from hUC-MSCs, collected by supercentrifugation[22] and examined using transmission electron microscopy (TEM), particle size and western blot (WB) analysis. Frozen hUC-MSCs (#CP-CL11; Procell, Wuhan, China) underwent cell resuscitation and were incubated in a culture medium at 37°C. The cells were then washed with phosphate-buffered saline (PBS) and digested with trypsin. Next, they were cultured with Dulbecco's modified Eagle's medium (Gibco, Waltham, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco). When the cell confluency reached 80%, the conditioned media were harvested and centrifuged at 3,000 g for 15 min. The supernatant was filtered using a 0.45-μm filter membrane (Gibco, Carlsbad, USA), and the filtrate was collected. The filtrate was moved to a new centrifugal tube, an overspeed rotor was selected, and the filtrate was centrifuged at 100,000 g at 4°C for 70 min. The supernatant was removed and resuspended in 10 mL of precooled 1 × PBS. The overspeed rotor was selected and centrifuged again at 4°C at 100,000 g for 70 min. The pellet was resuspended in PBS and used for further experiments. Western blot analysis was performed to detect the expression levels of the known exosomal markers CD9 (Abcam, Cambridge, USA) and CD81 (Abcam), as well as tumor susceptibility gene 101 (TSG101; Abcam). The exosome morphology was observed using a transmission electron microscope (Hitachi HT-7700; Hitachi Tokyo, Japan), and the exosome particle size analysis was conducted using a nanoparticle tracking analyzer (NTA; Particle Metrix GmbH, Inning am Ammersee, Germany). The exosomes were either stored at −80°C or used immediately for subsequent experiments.

Animals and groupings

The study was carried out in accordance with the Chinese National Research Council's Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Gansu Province People's Hospital (Lanzhou, China; approval No. 2022-007). A total of 38 male BALB/c mice on postnatal day 6 (PND6), along with their mothers, were obtained from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China) and housed in a specific pathogen-free (SPF) laboratory at the Lilei Biomedicine Experiment Center (Chengdu, China). The mice were kept on a normal chow diet under pathogen-free conditions with a 12-h light/dark cycle, at a temperature of 22°C ±1°C and a humidity of 40–60%.

The mice were randomly divided into 2 groups: the sham group (n = 6) and the CP group (n = 32). The experimental CP model was induced as described in a previous study[23] by hypoxic conditions following unilateral common carotid artery ligation combined with lipopolysaccharide (LPS) infection. After the induction, the mice were randomly divided into 4 subgroups, each containing 8 mice: the CP group; the CP-exosomes (CP-exo) group, in which the mice were intraperitoneally (ip.) injected with 125 μg of exosomes; the CP-mNGF group, in which the mice received ip. injections of mNGF at a dose of 5 μg/kg/day for 14 consecutive days; and the CP-exosome-mNGF (CP-exo-mNGF) group, in which the mice were ip. injected with both 125 μg of exosomes and 5 μg/kg/day of mNGF for 14 days. This protocol was based on the research by Sun et al. and Gao et al.[24,25]

The health and behavior of the mice were monitored weekly. During the experiment, 2 mice, 1 from both the CP group and the CP-mNGF group, died. After 14 days of intervention (P23), all surviving mice had gained weight and undergone a neurological deficit assessment. The mice were then deeply anesthetized with an ip. injection of 0.3% sodium pentobarbital (50 mg/kg) and sacrificed by decapitation; their brains were then rapidly removed. The hippocampus tissues were dissected on ice, immediately frozen on dry ice and stored at −80°C for subsequent experiments.

Behavioral test

The behavioral evaluation was performed on the 14th day after the intervention using a horizontal grid test and pole-climbing test.[26,27] The horizontal grid test was designed to evaluate the grasping ability of the mice's limbs. The pole-climbing experiment was employed to evaluate the motor coordination ability of the mice.

A double-blind method was employed in the behavioral experiments to ensure objectivity. The frequency of falls during the horizontal grid test and the results of the pole-climbing test were assessed by a third party who was unaware of the purpose of the experiment. Subsequently, statistical analysis was conducted on these measurements.

Evaluation of brain histological pathology using hematoxylin–eosin staining

The fixed tissue was dehydrated using an automatic dehydrator, embedded and sectionalized. All the aforementioned actions were performed according to the standard operating procedure of pathological examination (dehydration, trimming, embedding, slicing, dyeing, sealing, etc., and finally microscopic examination). A panoramic 250 digital section scanner (3DHISTECH, Budapest, Hungary) was used for image collection. Specific lesions were obtained at different magnification factors (×40, ×100 and ×400).

Survival of neurons examined using Nissl staining

The preparation of tissues for Nissl staining was based on the methods described by Xu et al.[28] Transverse slices were stained with 1% cresol purple for Nissl staining, according to the manufacturer's specifications. Cells positive for Nissl in the anterior horns were identified as motor neurons, following previously established methods.[29]

Protein detection in mouse brain tissue

The brain samples were taken out and put in 2-mL grinding tubes, and then 3-mm steel balls and radioimmunoprecipitation assay (RIPA) buffer were added to each tube and placed in a high-speed and low-temperature tissue-grinding instrument (–20°C, grinding 4 times, 60 s each time). The samples were taken out and placed in a refrigerator at 4°C for 30 min for cracking. After 30 min, the samples were taken out and centrifuged at 6,761.66 g for 10 min at 4°C. After centrifugation, the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, USA). Western blot assay was used to detect proteins. Equal amounts of protein extracts (50 μg) were separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked and incubated overnight with rabbit anti-PSD-95 (1:2,000, #AF5283; Affinity, Cincinnati, USA), rabbit anti-SYN (1:2,000, #AF0257; Affinity) and rabbit anti-β-actin (1:100,000, #AC026; ABclonal, Wuhan, China) antibodies. The following day, the membranes were incubated with anti-rabbit immunoglobulin G (IgG) (H + L) secondary antibody (1:5,000, #s0001; Affinity) for 2 h at room temperature. The proteins were visualized using enhanced chemiluminescence (ECL) reagents purchased from Millipore (Billerica, USA). Analysis of the results was performed using ImageJ software (National Intitutes of Health (NIH), Bethesda, USA). All experiments were conducted in triplicate.

Statistical analyses

The data were analyzed using SPSS v. 17.0 software (SPSS Inc., Chicago, USA). All data are presented as medians and min–max range. Due to concerns about the reliability of using one-way analysis of variance (ANOVA) with a sample size smaller than 10, non-parametric tests were employed to analyze the data. The Kruskal–Wallis test was used to compare differences among multiple groups, followed by Dunn's post hoc test with Bonferroni correction for pairwise comparisons between groups. Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate. A p-value of less than 0.05 was considered statistically significant.

Results

Isolation and identification of hUC-MSCs-exos

Previous studies have suggested that hUC-MSCs could repair damaged tissues.[30] The purchased cells were cultured and passed on (Fig. 1A), and hUC-MSCs-exos were obtained using supercentrifugation. Transmission electron microscopy demonstrated that the exosomes had a typical bilayer membrane vesicle structure (Fig. 1B). The NTA results showed a diameter distribution average of 123.8 nm (Fig. 1C). The highly studied specific markers TSG101 and CD81 expressed positively (Fig. 1D). These results suggest that the isolated substances could be identified as exosomes.

hUC-MSCs-exos ameliorated motor function in mice with CP

In this study, the balance and motor functions of mice with CP were tested using pole-climbing experiments and the horizontal network test. Following experimental modeling, except for the sham group, most animals in the model group moved autonomously but could not extend their left 2 limbs. Some animals exhibited weak movement in the left limbs and moved in a sideways circle. The frequency of limbs falling into the mesh or the scores from the pole-climbing experiments in mice with CP were significantly higher than those in the sham group (Fig. 2, Tables 1–4, Kruskal–Wallis test, all $p < 0.001$), indicating that there was successful construction of the CP model. The horizontal grid test showed a gradual decrease in the frequency of limbs falling into the mesh in the CP-exo, CP-mNGF and CP-exo-mNGF groups compared to the CP group, with a significant improvement noted in the CP-exo-mNGF group ($p = 0.02$, Table 3); however, there was no statistical significance after being adjusted with Bonferroni correction. This suggests that the combined treatment of hUC-MSC-exos and mNGF could significantly improve motor disorders in mice with CP. The weight of the experimental animals decreased, except in the sham group, with the least weight loss observed in the CP-exo-mNGF group. Furthermore, the weight gain of the mice in the CP-exo-mNGF group was significantly different from that in the CP group.

Mouse nerve growth factor combined with hUC-MSC-exos treatment alleviated the pathological damage to the mouse brain

The pia mater was found to be intact using a light microscope (Olympus BX53; Olympus Corp., Tokyo, Japan), with no obvious inflammatory exudation in any group. As shown in Fig. 3, compared with the sham group, the pathological changes in the brains of the mice in the CP group, including patchy necrosis, nerve fiber

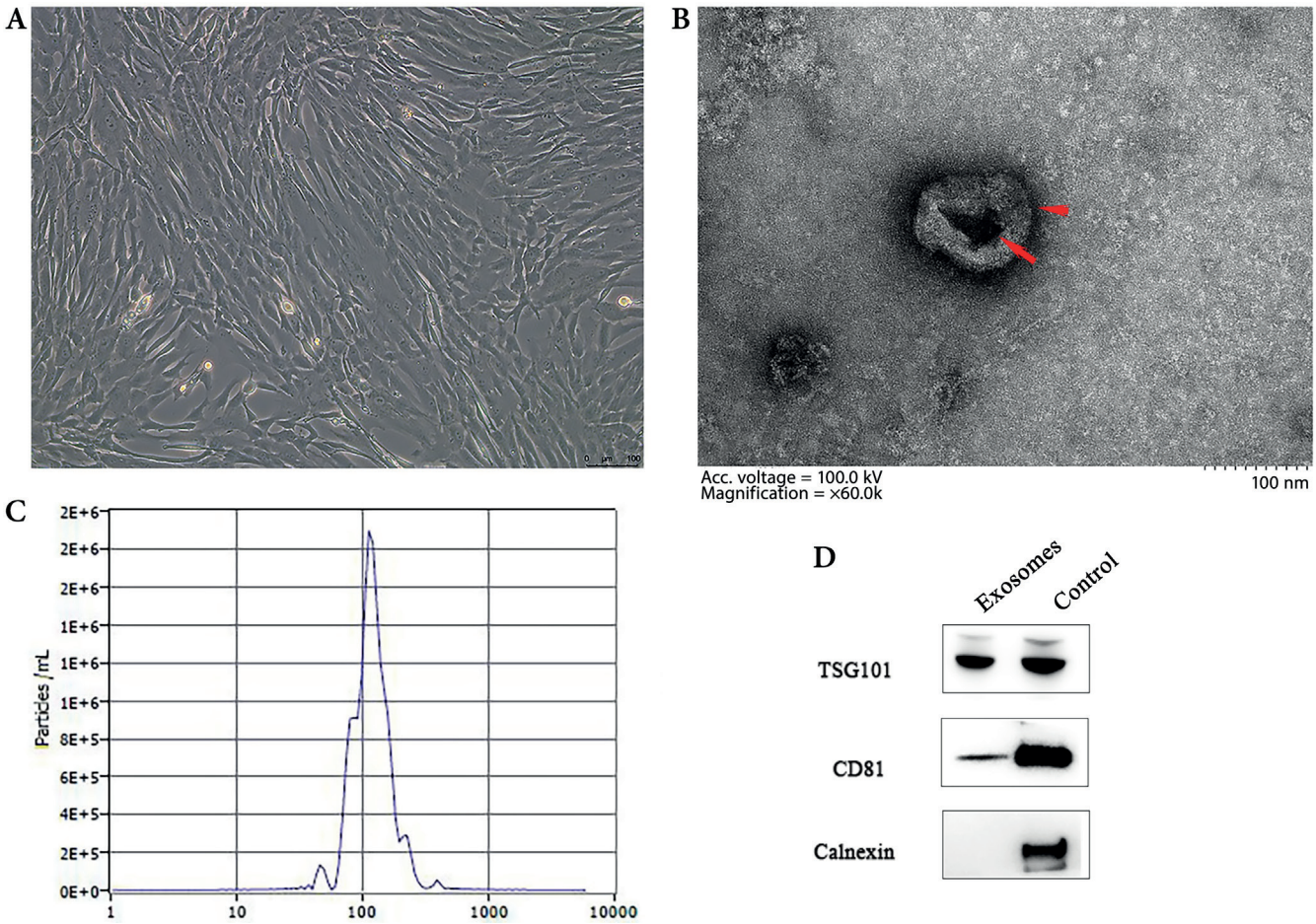

Fig. 1. Identification of human umbilical cord mesenchymal stem cell exosomes (hUC-MSCs-exos). A. Culture of hUC-MSCs; B. Morphological characteristics of exosomes viewed under an transmission electron microscope; C. Nanoparticle tracking analysis (NTA); D. Western blot analysis showing the expression levels of exosomal surface markers TSG101 and CD81

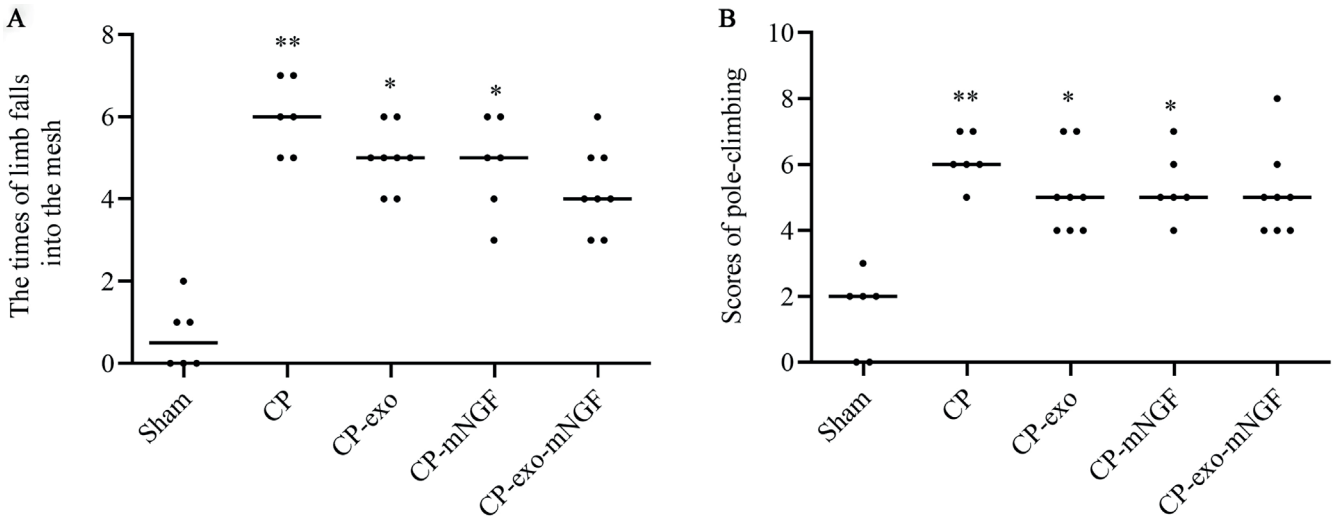

Fig. 2. Results of horizontal network test and pole-climbing experiments. The Kruskal–Wallis test was employed for statistical analysis. A. Frequency of falls during the horizontal grid test; B Scores of pole-climbing. Significance as compared with the sham group (*$p < 0.05$, **$p < 0.01$) and the cerebral palsy (CP) group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

demyelination, glial cell proliferation in the white matter area, and pyramidal cell necrosis in the hippocampus area, were consistent with the characteristic pathological changes in a brain tissue ischemia and hypoxia model, suggesting that the experimental modeling was successful. However, the degree of pathological changes was different in the brain tissues of mice in the CP, CP-exo, CP-mNGF, and CP-exo-mNGF groups. Patchy necrosis

Table 1. Statistics of the horizontal network test and the pole-climbing experiments

Groups	n	The frequency of limb slips into the mesh	Pole-climbing test results
Sham	6	0.5 (0.0, 2.0)	2.00 (0.00,3.00)
CP	6	6.0 (5.0, 7.0)**	6.00 (5.00,7.00)**
CP-exo	8	5.0 (4.0, 6.0)*	4.50 (4.00,5.00)*
CP-mNGF	6	5.0 (3.0, 6.0)*	5.00 (4.00,7.00)*
CP-exo-mNGF	8	4.0 (3.0, 6.0)	5.00 (4.00, 8.00)

n – number; CP – cerebral palsy; exo-hUC – MSC-exosome; mNGF – mouse nerve growth factor. Significance as compared with sham group (*p < 0.05, **p < 0.01) and CP group ($^{\#}$p < 0.05, $^{\#\#}$p < 0.01).

was only found in the CP group (83.3%; 5/6) but not in other groups (χ^2 = 27.356, p < 0.001). However, demyelination of nerve fibers in the white matter region was identified in the CP, CP-exo and CP-mNGF groups (83% (5/6), 1/8 (12.5%) and 3/6 (50%), respectively). This phenomenon was not found in the CP-exo-mNGF group (χ^2 = 17.516, p = 0.02). Glial cell proliferation between nerve fibers was observed in the CP, CP-exo, CP-mNGF, and CP-exo-mNGF groups (83% (5/6), 87.5% (7/8), 83.3% (5/6), and 75% (6/8), respectively), but no differences were found between groups (χ^2 = 0.446, p > 0.05). The decreased proportion of pyramidal cell necrosis observed in the hippocampus area in the CP-mNGF and CP-exo-mNGF groups was greater than in the CP group (50% (3/6) and 50% (4/8) vs 100% (6/6), χ^2 = 4.00, p = 0.046; χ^2 = 4.20, p = 0.040). Compared with the CP group, there was less pathological damage in the CP-mNGF and CP-exo-mNGF groups, and significantly fewer pathological changes were found in the CP-exo-mNGF group. This suggests that mNGF and mNGF-exos could improve pathological damage in CP, while mNGF-exos could improve it more significantly.

Table 2. Results of Kruskal–Wallis test as presented in pole-climbing experiments and the horizontal network test

Variables	n	Median	H	df	p-value
Horizontal grid test	34	5	20.427	4	<0.001
Pole climbing test	34	5	18.026	4	<0.001

n – number; H – Kruskal–Wallis H value; df – degrees of freedom.

Table 3. Pairwise comparisons of groups (Fig. 2A)

Group 1/Group 2	Test statistic	SE	Standard test statistic	p-value	Adjusted p-value[a]
Sham-CP-exo-mNGF	–11.938	5.268	–2.266	0.023	0.234
Sham-CP-mNGF	–16.417	5.631	–2.915	0.004	0.036
Sham-CP-exo	–17.125	5.268	–3.251	0.001	0.012
Sham-CP	–24.167	5.631	–4.291	<0.001	0.000
CP-exo-mNGF-CP-mNGF	4.479	5.268	0.850	0.395	1.000
CP-exo-mNGF-CP-exo	5.188	4.877	1.064	0.287	1.000
CP-exo-mNGF-CP	12.229	5.268	2.322	0.020	0.203
CP-mNGF-CP-exo	0.708	5.268	0.134	0.893	1.000
CP-mNGF-CP	7.750	5.631	1.376	0.169	1.000
CP-exo-CP	7.042	5.268	1.337	0.181	1.000

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests; SE – standard error; CP – cerebral palsy group; exo – exosome; mNGF – mouse nerve growth factor; sham – sham group; CP-exo – CP exosomes group; CP-mNGF – CP-mNGF group; CP-exo-mNGF – CP-exosome-mNGF group.

Table 4. Pairwise comparisons of groups (Fig. 2 B)

Group 1/Group 2	Test statistic	SE	Standard test statistic	p-value	Adjusted p-value[a]
CP-CP-exo	–11.375	5.822	–1.954	0.051	0.507
CP-CP-mNGF	–12.625	5.822	–2.168	0.030	0.301
CP-CP-exo-mNGF	–24.000	5.822	–4.122	<0.001	0.000
CP-sham	32.000	5.822	5.496	<0.001	0.000
CP-exo-CP-mNGF	–1.250	5.822	–.215	0.830	1.000
CP-exo-CP-exo-mNGF	–12.625	5.822	–2.168	0.030	0.301
CP-exo-sham	20.625	5.822	3.543	<0.001	0.004
CP-mNGF-CP-exo-mNGF	–11.375	5.822	–1.954	0.051	0.507
CP-mNGF-sham	19.375	5.822	3.328	<0.001	0.009
CP-exo-mNGF-sham	8.000	5.822	1.374	0.169	1.000

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests; SE – standard error; CP – cerebral palsy group; exo-exosome; mNGF – mouse nerve growth factor; sham – sham group; CP-exo – CP exosomes group; CP-mNGF – CP-mNGF group; CP-exo-mNGF – CP-exosome-mNGF group.

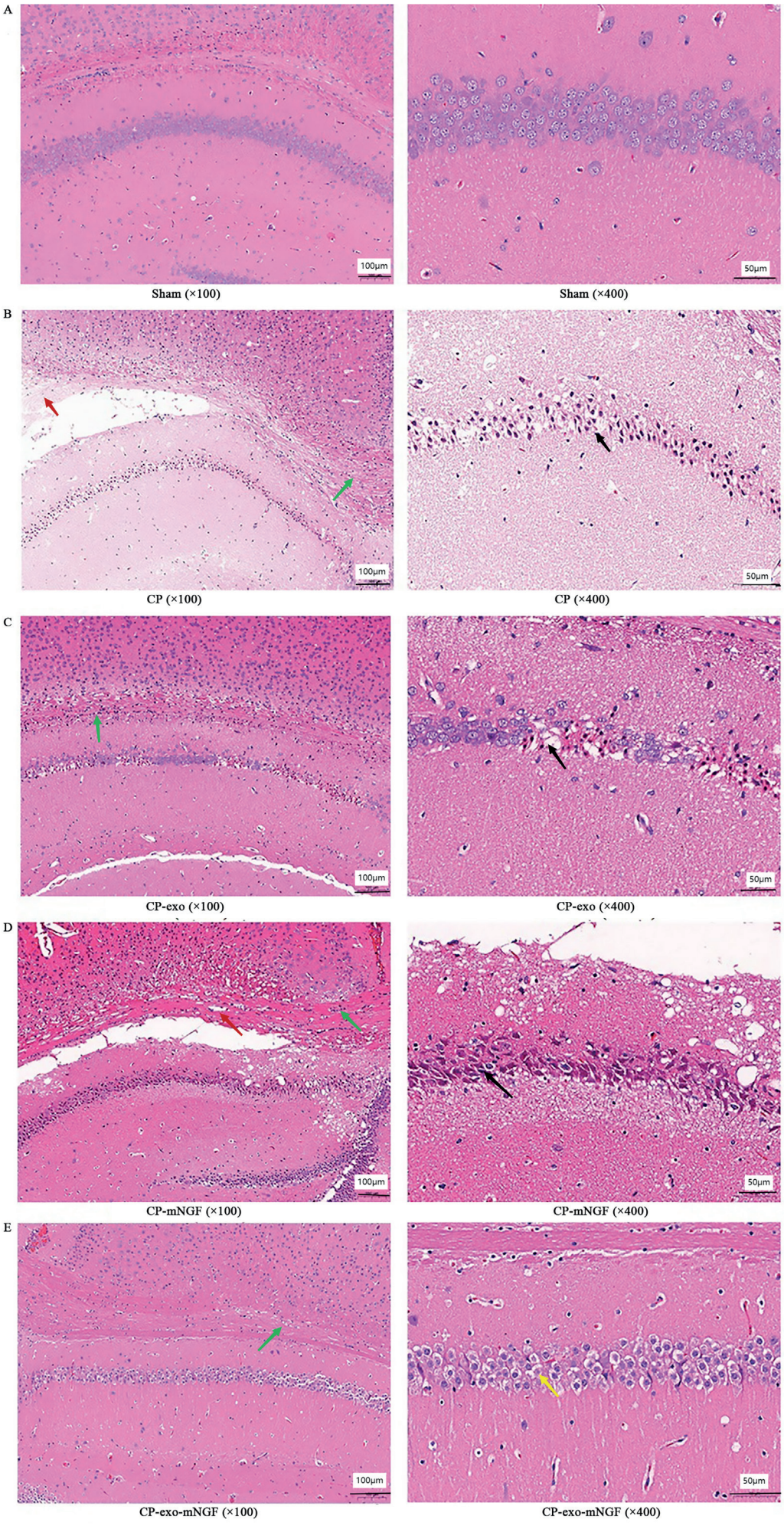

Fig. 3. Representative images of hematoxylin and eosin (H&E) staining in the mouse brain. A. sham group; B. CP group; C. CP-exo group; D. CP-mNGF group; E. CP-exo-mNGF group. Red arrows indicate nerve fiber demyelination, green arrows indicate hyperplasia of glial cells in the white matter area, black arrows indicate pyramidal cell necrosis, and yellow arrows indicate vacuolar degeneration of pyramidal cells. All images were taken at ×100 and ×400 magnification; scale bar = 500 μm (n = 6–8 rats per group)

CP – cerebral palsy; exo – exosome; mNGF – mouse nerve growth factor.

mNGF combined with hUC-MSCs-exos treatment improved the synaptic structure in mice with CP

Transmission electron microscopy revealed that the synaptic structures, comprising presynaptic membranes, postsynaptic membranes and synaptic clefts, were well preserved and abundant in the sham group. In contrast, the CP group exhibited significant dissolution of brain tissue structure and blurring of ultrastructures, with few remaining synapses. In the CP-exo and CP-mNGF groups, some regions showed dissolution, but the synaptic structures remained abundant and clear. The CP-exo-mNGF group displayed synaptic structures similar to those of the sham group (Fig. 4), highlighting the beneficial effects of the combined treatment on synaptic integrity in the CP models. Neuronal function was severely impaired in mice with CP but could be recovered using hUC-MSCs-exos, and the effect was better when hUC-MSCs-exos were combined with mNGF.

Nissl bodies, a marker of the functional state of neurons, were counted in images at ×400 magnification (Fig. 5A). Compared to the sham group, the number of Nissl bodies in the hippocampal pyramidal cells of the mice in the CP group was significantly lower (67.67 (44.00, 76.67) vs 1.33 (0.33, 3.33), Kruskal–Wallis test, $H = 4.815$, $p < 0.001$ (Table 5)). In the CP-exo-mNGF group, the number of Nissl bodies was significantly higher than in the CP group, indicating recovery of neuronal function (32.66 (18.33, 50.67) vs 1.33 (0.33, 3.33), Kruskal–Wallis test, $H = -3.442$, $p < 0.001$, adjusted $p = 0.006$ (Table 5)).

PSD-95 and SYN protein levels were upregulated after hUC-MSCs-exos or mNGF treatment

The expression levels of PSD-95 and SYN proteins in the brain tissues were assessed using WB analysis. The expression levels in the CP group were significantly lower than in the sham group (Kruskal–Wallis test, PSD 95: CP vs sham, $H = 32.000$, $p = 0.000$; SYN: CP vs sham, $H = 32.000$, $p = 0.000$ Fig. 6B,C, Tables 6–9), but they were significantly higher in the CP-exo-mNGF groups compared to the CP group (PSD-95, 0.70 (0.065, 0.88) vs 0.16 (0.15, 0.27); SYN, 0.83 (0.70, 0.94) vs 0.32 (0.23, 0.43), all $p < 0.001$, Tables 8,9).

Discussion

Cerebral palsy is a permanent motor disorder that results from brain injury and neuroinflammation during the perinatal period. Current therapeutic options for CP are limited. The MSC-exos have demonstrated potential as therapeutics, not only due to their intrinsic properties but also because they are good at crossing epithelial barriers, similar to their parent cells.[31] As CP occurs within 1 month before or after birth, neonatal pups were selected as subjects in this study. To investigate the mechanism of CP, a compound CP model of hypoxic induction after unilateral common carotid artery ligation combined with LPS infection was adopted to simulate the pathological

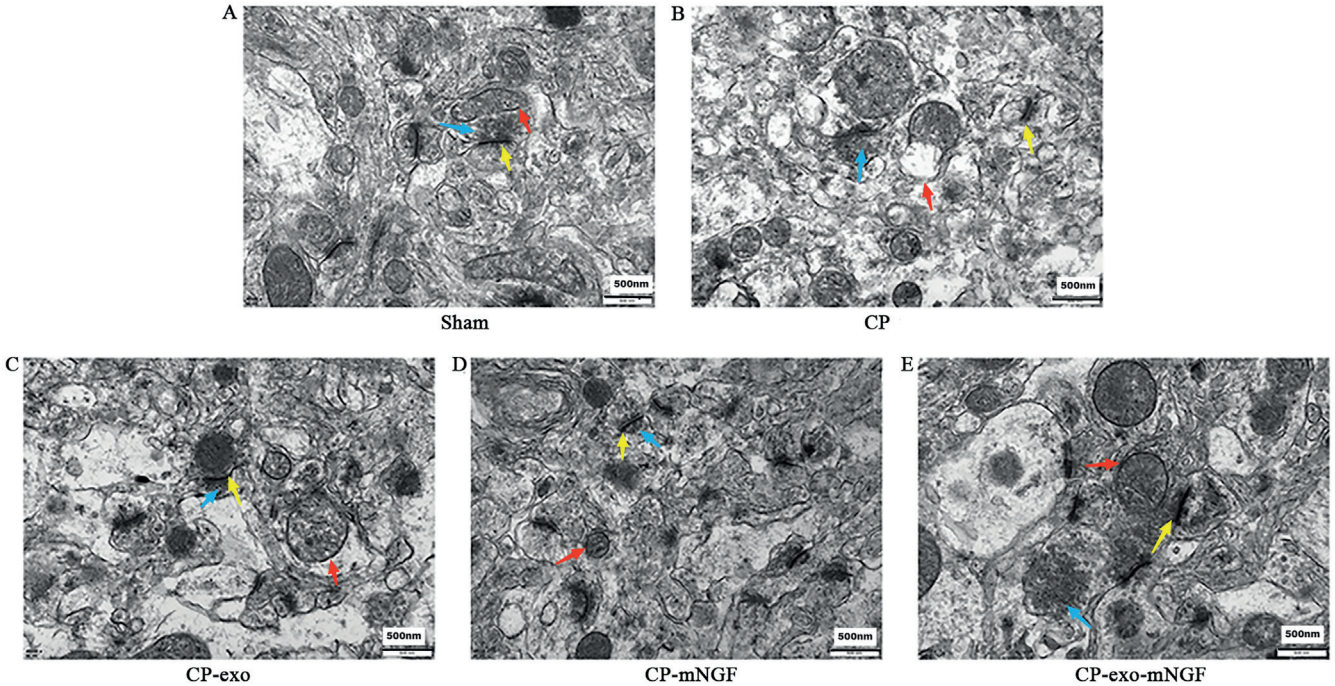

Fig. 4. Transmission electron microscopy (TEM) results in different mouse brains. A. Sham mice; B. Mice with CP; C. Mice with CP-exo; D. Mice with CP-mNGF; E. Mice with CP-exo-mNGF. Red arrows indicate mitochondria in the presynaptic membrane, blue arrows indicate aggregation of synaptic vesicles, and yellow arrows indicate postsynaptic membrane compact. All images were taken at ×40,000 magnification

CP – cerebral palsy; exo – exosome; mNGF – mouse nerve growth factor.

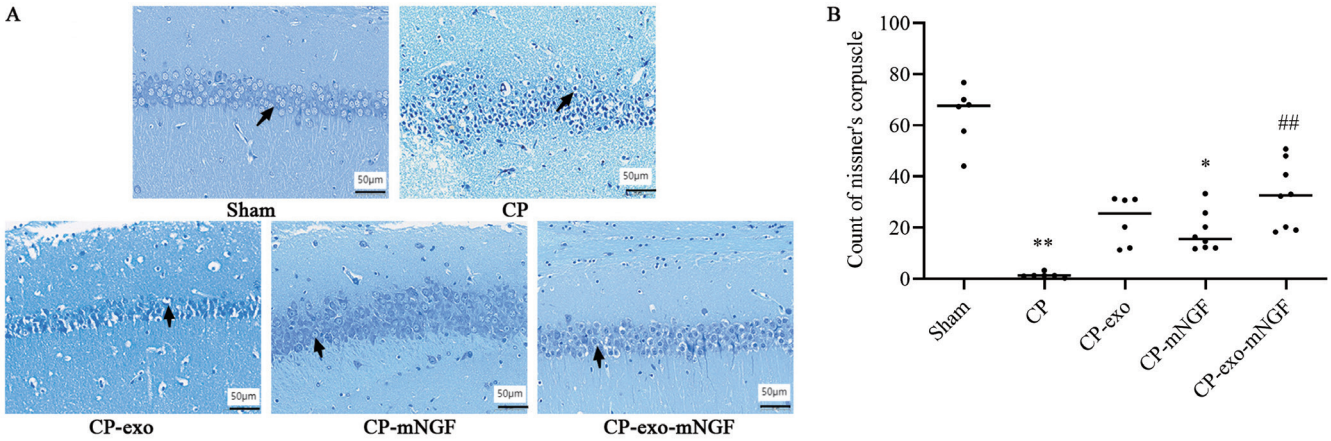

Fig. 5. Nissl body counts are reduced in the hippocampus following hypoxic–ischemic and lipopolysaccharide-induced brain injury and improved by hUC-MSCs-exos and mNGF treatment (Kruskal–Wallis test). A. Statistical analysis of Nissl body counts in the hippocampus; B. Representative images of Nissl body staining in the hippocampus. All images were taken at ×400 magnification; scale bar = 500 μm (n = 6–8 mice per group). Significance as compared with the sham group (*$p < 0.05$, **$p < 0.01$) and the CP group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

hUC-MSCs – human umbilical cord mesenchymal stem cells; CP – cerebral palsy; exo – exosome; mNGF – mouse nerve growth factor.

Table 5. Statistics for Fig. 5

Variables		Min	Q1	Median	Q3	Max
Nissl bodies	sham	44.00	54.25	67.67	71.66	76.67
	CP	0.33	0.83	1.33	1.83	3.33
	CP-exo	11.33	11.83	25.50	31.08	31.33
	CP-mNGF	13.5	12.08	11.67	24.33	33.33
	CP-exo-mNGF	18.33	19.33	32.66	46.18	50.67

min – minimal value; max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; CP – cerebral palsy; exo – exosome; mNGF – mouse nerve growth factor.

Table 6. Results of Kruskal–Wallis test as presented in Fig. 6

Variables	n	Median	H	df	p-value
PSD-95	40	0.53	35.919	4	<0.001
SYN	40	0.45	35.919	4	<0.001

SYN – synaptophysin protein; n – number; H – Kruskal–Wallis H value; df – degrees of freedom..

changes in the brain after CP.[23] In this study, the horizontal grid test and pole-climbing test results indicated that hUC-MSC-exos, especially combined with mNGF, were beneficial for the recovery of motor function, irrespective of the ability of limb grasp or motor coordination. White matter injury is a structural injury that leads

Table 7. Statistics for PSD-95 and SYN protein expression levels in groups (Fig. 6B and Fig. 6C)

Variables		Min	Q1	Median	Q3	Max
PSD95	sham	1.00	1.00	1.00	1.00	1.00
	CP	0.14	0.15	0.18	0.27	0.31
	CP-exo	0.38	0.42	0.45	0.53	0.61
	CP-mNGF	0.36	0.43	0.47	0.54	0.59
	CP-exo-mNGF	0.65	0.67	0.71	0.83	0.88
SYN	sham	1.00	1.00	1.00	1.0	1.00
	CP	0.23	0.28	0.32	0.36	0.43
	CP-exo	0.45	0.54	0.56	0.65	0.95
	CP-mNGF	0.43	0.53	0.55	0.65	0.95
	CP-exo-mNGF	0.70	0.77	0.83	0.92	0.94

min – minimal value; max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; SYN – synaptophysin protein; CP – cerebral palsy; exo – exosome; mNGF – mouse nerve growth factor.

Table 8. PSD-95 protein expression levels of pairwise comparisons between groups

Group 1/Group 2	Test statistic	SE	Standard test statistic	p-value	Adjusted p-value[a]
CP-CP-exo	–11.375	5.822	–1.954	0.051	0.507
CP-CP-mNGF	–12.625	5.822	–2.168	0.030	0.301
CP-CP-exo-mNGF	–24.000	5.822	–4.122	<0.001	0.000
CP-sham	32.000	5.822	5.496	<0.001	0.000
CP-exo-CP-mNGF	–1.250	5.822	–.215	0.830	1.000
CP-exo-CP-exo-mNGF	–12.625	5.822	–2.168	0.030	0.301
CP-exo-sham	20.625	5.822	3.543	<0.001	0.004
CP-mNGF-CP-exo-mNGF	–11.375	5.822	–1.954	0.051	0.507
CP-mNGF-sham	19.375	5.822	3.328	<0.001	0.009
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[a] Significance values have been adjusted by the Bonferroni correction for multiple tests; SE – standard error; CP – cerebral palsy group; exo-exosome; mNGF – mouse nerve growth factor; sham – sham group; CP-exo – CP exosomes group; CP-mNGF – CP-mNGF group; CP-exo-mNGF – CP-exosome-mNGF group.

Table 9. SYN protein expression levels of pairwise comparisons between groups

Group 1/Group 2	Test statistic	SE	Standard test statistic	p-value	Adjusted p-value[a]
CP-CP-mNGF	–11.375	5.822	–1.954	0.051	0.507
CP-CP-exo	–12.625	5.822	–2.168	0.030	0.301
CP-CP-exo-mNGF	–24.000	5.822	–4.122	<0.001	0.000
CP-sham	32.000	5.822	5.496	<0.001	0.000
CP-mNGF-CP-exo	1.250	5.822	0.215	0.830	1.000
CP-mNGF-CP-exo-mNGF	–12.625	5.822	–2.168	0.030	0.301
CP-mNGF-sham	20.625	5.822	3.543	<0.001	0.004
CP-exo-CP-exo-mNGF	–11.375	5.822	–1.954	0.051	0.507
CP-exo-sham	19.375	5.822	3.328	<0.001	0.009
CP-exo-mNGF-sham	8.000	5.822	1.374	0.169	1.000

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests; SE – standard error; CP – cerebral palsy group; exo-exosome; mNGF – mouse nerve growth factor; sham – sham group; CP-exo – CP exosomes group; CP-mNGF – CP-mNGF group; CP-exo-mNGF – CP-exosome-mNGF group.

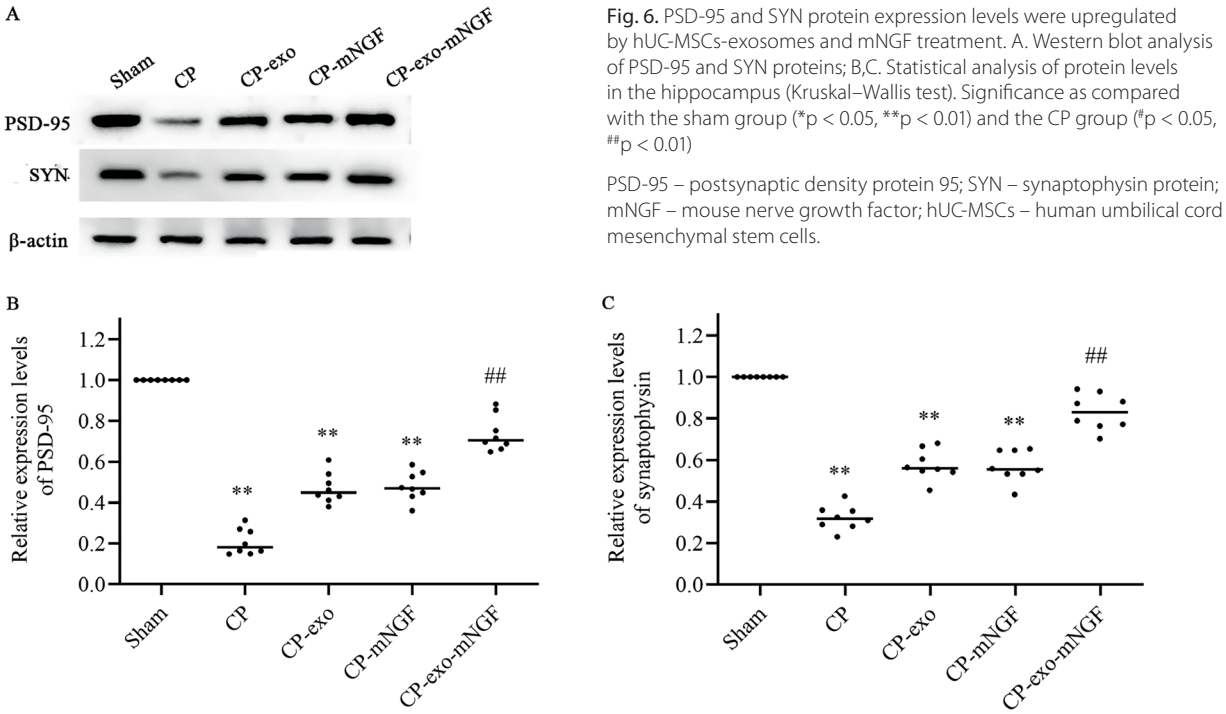

Fig. 6. PSD-95 and SYN protein expression levels were upregulated by hUC-MSCs-exosomes and mNGF treatment. A. Western blot analysis of PSD-95 and SYN proteins; B,C. Statistical analysis of protein levels in the hippocampus (Kruskal–Wallis test). Significance as compared with the sham group (*$p < 0.05$, **$p < 0.01$) and the CP group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

PSD-95 – postsynaptic density protein 95; SYN – synaptophysin protein; mNGF – mouse nerve growth factor; hUC-MSCs – human umbilical cord mesenchymal stem cells.

to a slowing down or interruption of nerve conduction velocity and damage to cortical and subcortical neurons, resulting in cognitive deficits and intellectual development disorders.[32,33] In this study, the ability of hUC-MSC-exos to improve cerebral injury in mice with CP was assessed, and pathological evidence was obtained.

Treatment with hUC-MSC-exos alone or in combination with mNGF significantly reduced the demyelination of nerve fibers and necrosis of pyramidal cells in the hippocampus area. Demyelination and cell necrosis are common neuropathological features in patients with CP and are closely associated with motor impairments and other clinical symptoms. This finding suggests that hUC-MSC-exos, either alone or combined with mNGF, may exert therapeutic effects through neuroprotective actions or by providing anti-inflammatory and antioxidative effects. The hippocampus plays a crucial role in memory processing, converting short-term to long-term memories, so neuroprotective therapies targeting this region are essential. McDonald et al. confirmed that the hippocampus was particularly vulnerable to neonatal hypoxic–ischemic brain injury, mediated through significant neuroinflammation.[34] However, clinically, frequently used therapeutic hypothermia did not protect the hippocampus following neonatal hypoxic ischemia in a murine model.[30] Nissl bodies were used as a marker of neuronal functional state in this study. The results showed that neuronal function was severely impaired in mice with CP, but it could be recovered using hUC-MSC-exos, and a better effect was observed with ip. injection of hUC-MSC-exos combined with mNGF. This method may be used in the future to alleviate symptoms caused by hippocampal damage.

Neonatal hypoxic–ischemic encephalopathy (HIE) causes permanent motor deficit (CP) and may result in significant disability and death. The brain damage process, the "HIE cascade," is divided into 6 stages[35]: 1) energy depletion, 2) impairment of microglia, 3) inflammation, 4) excitotoxicity, 5) oxidative stress, and 6) apoptosis in capillaries, glia, synapses, and/or neurons. Synapses are functional connections between neurons and key parts of information transmission.[36] In line with a previous study,[35] the results of this study indicate that the ultrastructure was blurred and the synaptic structure was obviously damaged in mice with CP. However, a combination of hUC-MSC-exos and mNGF treatment could promote the recovery of synaptic function and improve nerve conduction function to improve motor function in mice.

Synaptophysin and PSD-95 mainly regulate synaptic formation and synaptic plasticity. Synaptophysin is a synaptic vesicle glycoprotein that is expressed in neuroendocrine cells and in most neurons in the CNS.[37] It is a hallmark of synaptic vesicle maturation, and it is also considered an indirect marker of synaptogenesis in the developing brain.[38] Likewise, PSD-95 is involved in the maturation of excitatory synapses.[39] Evidence has shown that PSD-95 is a promising target for ischemic stroke therapy, as well as for other CNS disorders, as it protected synapses from β-amyloid,[40] which were downregulated in an ischemia model.[41] A recent study showed that the levels of presynaptic SYN and postsynaptic PSD-95 decreased in the hippocampus of LPS-exposed mice, which might contribute to the impairment of synaptic plasticity and the decline in learning and memory observed in behavioral tests, suggesting that impairments in synaptic plasticity might be responsible for LPS-induced cognitive deficits.[42] Interestingly, this study also found that SYN and PSD-95 proteins were downregulated in the hippocampus of mice with CP, suggesting synaptic formation and dysfunction of neurotransmitters in mice with CP. This phenomenon supports the results of the TEM in this study. However, after mice with CP were injected with hUC-MSC-exos and mNGF, the levels of synapse-associated proteins SYN and postsynaptic PSD-95 both increased significantly, suggesting that hUC-MSCs-exos combined with mNGF could promote the reconstruction of contact structures and improve synaptic conduction and neural information transmission. Therefore, the modulation of these proteins by hUC-MSCs-exos or mNGF could lead to the long-term modulation of synaptic transmission, affecting motor control.

This study demonstrated the considerable efficacy of combining human umbilical cord MSC (hUC-MSC)-derived exosomes with mNGF in alleviating motor disorders and brain pathological injuries in mice with CP. These treatments may promote neuroregeneration and repair, enhancing synaptic stability and function, thereby improving neurological impairments in CP. Additionally, they could reduce inflammation and oxidative stress, protecting neurons from further damage and facilitating brain injury repair. These findings lay a critical theoretical foundation for developing innovative therapeutic strategies for CP. The successful outcomes of combined exosome and nerve growth factor therapy underscore the potential of stem cell-derived biologics to enhance neurological function in CP. This combination could significantly enhance therapeutic efficacy, potentially establishing a pivotal approach for future CP treatments. Moreover, this study provides essential theoretical support for the clinical application of this combined therapy. Further clinical studies are necessary to validate the safety and efficacy of this therapy, potentially offering new treatment options for patients with CP. Additional research should also determine the optimal dosages, administration routes and timing for this therapy to customize treatment strategies and maximize therapeutic benefits.

Limitations

Immunohistochemical analysis could not be conducted in this study due to financial constraints; however, it will be addressed in future research. Definitive evidence confirming the role and mechanism of action of hUC-MSC-exos combined with mNGF in CP models is still lacking. Detailed mechanisms of action still need to be clarified.

Conclusions

Combined therapy using exosomes derived from hUC-MSCs and mNGF shows promising mechanisms and translational implications, which is relevant to clinical practice in managing motor disorders and brain injuries in CP.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.14212279. The package includes the following files:

Supplementary Fig. 1. The research design.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Enhancement of human native skin fibroblast proliferation in natural salso-bromo-iodic mineral water added to in vitro culture

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Abstract

Background. The favorable regenerative effects of some mineral waters on wound healing have long been empirically demonstrated.

Objectives. The aim of this experimental study is to investigate the effects of an Italian salso-bromo-iodic mineral water (Rivanazzano, Italy) on an in vitro human native fibroblast culture model to identify any potential regenerative actions.

Materials and methods. Human native fibroblasts were cultured under different experimental conditions:
– Dulbecco's modified Eagle's medium (DMEM) reconstituted with distilled water (control);
– DMEM reconstituted with filtered mineral water collected from the spring;
– DMEM reconstituted with filtered mineral water collected at the balneotherapy facility;
– DMEM reconstituted with filtered, heated mineral water collected at the balneotherapy facility;
– DMEM partially replaced with filtered mineral water collected from the spring at different concentrations (10%, 20%, 30%, 40%, 50%);
– DMEM partially replaced with filtered, heated mineral water collected at the balneotherapy facility at different concentrations (10%, 20%, 30%, 40%, 50%);
– DMEM partially replaced with filtered mineral water collected at the balneotherapy facility at different concentrations (10%, 20%, 30%, 40%, 50%).

Cell proliferation and viability were evaluated using spectrophotometric analysis following staining with the XTT Microculture Tetrazolium Assay. Statistical analyses were performed for each experimental condition at 24, 48 and 72 h.

Results. The best outcomes were observed in fibroblasts cultured with DMEM partially replaced with filtered mineral water collected from the spring, within the range of 20–50%.

Conclusions. Our research results showed that Rivanazzano salso-bromo-iodic mineral water has a stimulating effect on in vitro human native fibroblast cultures. This activity was most pronounced with water collected from the spring, and it decreased with water collected at the balneotherapy facilities. These findings could form the basis for clinical applications in wound healing and balneotherapy.

Key words: human cell culture, fibroblast, microbiota, mineral water, wound healing

Background

Natural mineral water, according to EU Directive 2009/54/EC, is defined as "microbiologically wholesome water, originating in an underground water table or deposit and emerging from a spring at one or more natural or bore exits".[1,2] The demonstrated effects of natural mineral waters on the skin include anti-inflammatory, antipruritic and anti-oxidative properties.[3–6] Furthermore, they improve skin hydration through the repair of the epidermal–dermal barrier.[3–6]

The favorable regenerative effects of some mineral waters on wound healing have long been empirically demonstrated.[7–11] Our research group previously investigated the biological properties of a calcium magnesium bicarbonate-based mineral water (Comano, Italy) and demonstrated the regenerative effect of its native mineral water non-pathogenic microflora on experimental in vitro cell and ex vivo tissue culture models.[12–17]

Among the many natural mineral water springs in our region, the Rivanazzano (Italy) salso-bromo-iodic natural mineral water (Table 1) is noted for its anti-inflammatory properties, mucosal antiseptic effects and stimulation of tissue blood circulation, and is traditionally recommended for the treatment of skin and mucosal inflammatory diseases, capillary fragility, water tissue retention, and edematous fibrosclerotic panniculopathy (cellulite). This water is also used for the preparation and topical skin application of therapeutic anti-inflammatory thermal muds.[18]

We aimed to investigate its biological properties using the same experimental methods as in our previous research on Comano calcium magnesium bicarbonate-based natural mineral water.[14]

Rivanazzano mineral water is delivered from the natural spring source to 2 different destinations within the thermal baths via a dedicated pipeline. At the balneotherapy facilities, water is kept at the natural spring temperature, whereas at the 2nd location, it is heated to 70°C for the preparation of thermal muds.

Objectives

The general aim of this experimental study is to examine the effects of Rivanazzano salso-bromo-iodic mineral water, collected at different sites of the Rivanazzano spa resort, on an in vitro human native fibroblast culture model.[14,16]

Two specific aims were investigated:

1) The effect on native human skin fibroblasts cultured in Dulbecco's modified Eagle's medium (DMEM) reconstituted with salso-bromo-iodic water collected at different sites and DMEM reconstituted with distilled water (control) after 24, 48 and 72 h was assessed (Experiment 1).

Table 1. Physical-chemical composition of the Rivanazzano salso-bromo-iodic mineral water

Parameter	Value
Temperature at the spring	13.9°C
pH	7.7
Conductivity at 20°C	8,100 µS/cm[7]
Fixed residue at 180°C	12,400 mg/L
Oxidability	48.0 mg/L (O_2)
Silica (SiO_2)	50.5 mg/L
Bicarbonates (HCO_3)	814 mg/L
Chlorides (Cl)	7,300 mg/L
Sulphates (SO_4)	35.7 mg/L
Sodium (Na)	4,490 mg/L
Potassium (K)	24.5 mg/L
Calcium (Ca)	143 mg/L
Magnesium (Mg)	78.5 mg/L
Dissolved iron (Fe)	3.37 mg/L
Ammonium ion (NH_4)	22.4 mg/L
Total phosphorus (P)	0.13 mg/L
Sulphide level (H_2S)	not detectable
Strontium (Sr)	15.8 mg/L
Lithium (Li)	0.417 mg/L
Aluminum (Al)	<0.01 mg/L
Bromides (Br)	37.1 mg/L
Iodides (I)	13.9 mg/L
Antimony (Sb)	<0.0005 mg/L
Arsenic (As)	0.001 mg/L
Barium (Ba)	3.6 mg/L
Boron (B)	54.1 mg/L
Cadmium (Cd)	<0.0001 mg/L
Chromium (Cr)	0.001 mg/L
Copper (Cu)	<0.1 mg/L
Cyanides (CN)	<0.001 mg/L
Fluorides (F)	1.4 mg/L
Lead (Pb)	<0.001 mg/L
Manganese (Mn)	0.06 mg/L
Mercury (Hg)	<0.0001 mg/L
Nickel (Ni)	<0.001 mg/L
Nitrates (NO_3)	<1.0 mg/L
Nitrites (NO_2)	<0.002 mg/L
Selenium (Se)	0.005 mg/L
Anionic surfactant agents	<50 µg/L
Mineral oils – dissolved or emulsified hydrocarbons	41 µg/L
Benzene	<0.1 µg/L
Polycyclic aromatic hydrocarbons	not detectable
Organohalogen compounds	not detectable
Total hardness	59.6°F

2) The effect on native human skin fibroblasts cultured in DMEM reconstituted with distilled water and partially replaced with different percentages of filtered salso-bromo-iodic water collected at different sites compared to DMEM reconstituted with distilled water (control) after 24, 48 and 72 h was described (Experiment 2).

Materials and methods

The experimental in vitro study was conducted in cooperation with the Plastic and Reconstructive Surgery Unit of the Department of Clinical-Surgical, Diagnostic, and Pediatric Sciences; the Advanced Technologies for Regenerative Medicine and Inductive Surgery Research Center; the Immunology and General Pathology Laboratory of the Department of Molecular Medicine of the University of Pavia (Italy); the Unit of Biostatistics and Clinical Epidemiology of the Department of Public Health, Experimental, and Forensic Medicine of the University of Pavia; and the Plastic and Reconstructive Surgery Unit, Department of Surgery of the Istituti Clinici Scientifici (ICS) Maugeri SB SpA IRCCS in Pavia. The experiments were carried out from September 2018 to February 2020. The study was approved by the Ethics Committee of the ICS Maugeri SB SpA IRCCS in Pavia (project identification code 2064) on September 26, 2016. The study conformed to the 1975 Declaration of Helsinki, and informed written and signed consent was obtained from all the patients.

Human skin specimen collection and processing

Human skin samples were collected in the operating rooms of the ICS Maugeri. The samples were obtained from anatomical specimens harvested during reduction mammoplasty or abdominoplasty sessions performed on 4 healthy female patients aged 43–60 years. The specimens were processed in the Immunology and General Pathology Laboratory of the Department of Molecular Medicine of the University of Pavia using the same methods as in previous studies conducted by our research group on other natural mineral waters.[14–17,19,20]

Mineral water collection

The natural source of Rivanazzano salso-bromo-iodic water is connected to the thermal baths by a dedicated piping system. The mineral water samples were collected from December 2019 to February 2020 by a single individual using an aseptic procedure. The sample gatherer, wearing sterile surgical gloves, collected 250 mL of the water in sterile bottles from 3 sites: the natural spring source; the balneotherapy facility in the thermal baths at the end of the dedicated pipeline where the water had been heated to 70°C (HRWB); and the balneotherapy facility in the thermal baths at the end of the dedicated pipeline, which preserved the natural spring temperature (RWB). The samples were then transported under isothermal conditions (mean temperature 13.2°C) to the Immunology and General Pathology Laboratory of the Department of Molecular Medicine of the University of Pavia.

Fibroblast cell cultures

The human native skin fibroblasts were extracted and processed using the same methods as in previous studies.[14,16]

To reduce potential sources of bias, a cell suspension of human native skin fibroblasts was seeded in triplicate using a multichannel pipette into 198 wells within a 96-multiwell plate at a concentration of 2×10^3 cells per well. The cells were maintained in DMEM with 4,500 mg/L glucose, 0.584 g/L L-glutamine and 0.11 g/L sodium pyruvate (Sigma-Aldrich, St. Louis, USA; Merck KGaA, Darmstadt, Germany), reconstituted with distilled water (Milli-Q, Merck-Millipore, Darmstadt, Germany) and enriched with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA). The cultures were incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C for 24 h to allow cell adhesion within the wells. After this period, the culture media were removed from all wells, and a gentle rinse was performed with phosphate-buffered saline (PBS; Sigma-Aldrich; Merck KGaA). Each well was then refilled with 100 µL of different culture media according to the experimental conditions. The culture media were changed on the 2nd day. All samples were incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C for 24, 48 and 72 h.

The endpoint

Quantification of cell proliferation and viability (endpoint) was evaluated using spectrophotometric analysis to measure absorbance at 475 nm after cell staining with the XTT Microculture Tetrazolium Assay, using the SPECTROstar® Omega microplate reader (BMG Labtech-Euroclone; Pero, Milano, Italy).[16,21]

Experimental design

The experimental design was based on a previous study on human native skin fibroblast in vitro cultures.[14] Two different experimental plans were implemented to achieve the specific aims.

In both experimental plans, 3 wells within a 96-multiwell plate without cells were filled with 100 µL of DMEM to provide a baseline "white" absorbance assessment. The XTT solution was prepared by mixing 5 mL of XTT labeling reagent with 0.1 mL of electron coupling reagent. In each well with cells, the medium was removed, and a gentle rinse was performed with PBS. After removing

the PBS, each well was refilled with 100 µL of DMEM. A volume of 50 µL of XTT solution was then added to each well and incubated in a humidified atmosphere of 95% air with 5% CO_2 at 37°C for 4 h.

Spectrophotometric analyses were conducted at 24 and 48 h after the addition of different culture media, along with the white absorbance assessment. Subsequently, at 48 h, the culture media were removed from the 3rd multiwell. A gentle rinse was performed with PBS, and each well was refilled with 100 µL of the specific culture medium according to the experimental conditions. The XTT staining procedure was then repeated at 72 h.

Experiment 1: Human native skin fibroblasts cultured with DMEM reconstituted with distilled water (control) vs DMEM reconstituted with filtered salso-bromo-iodic water collected at different sites at 24, 48 and 72 h

Four different experimental conditions were compared:

A. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with distilled water and enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA) (control).

B. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with filtered RWS enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA).

C. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with filtered HRWB enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA).

D. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with filtered RWB enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA).

Experiment 2: Human native skin fibroblasts cultured with DMEM reconstituted with distilled water (control) vs DMEM reconstituted with distilled water and partially replaced with different amounts of filtered salso-bromo-iodic water collected at different sites at 24, 48 and 72 h

A total of 16 different experimental conditions were compared:

A. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with distilled water and enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA) (control).

B. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with distilled water and enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA), partially replaced with filtered RWS at 5 different percentages (10%, 20%, 30%, 40%, and 50%).

C. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with distilled water and enriched with 3.7 g/L sodium bicarbonate, 10% FBS and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA), partially replaced with filtered HRWB at 5 different percentages (10%, 20%, 30%, 40%, and 50%).

D. DMEM without $NaHCO_3$ with 1.0 g/L D-glucose reconstituted with distilled water and enriched with 3.7 g/L sodium bicarbonate, 10% FBS, and 1% (10,000 U/mL) penicillin and streptomycin (10 mg/mL) (all from Sigma-Aldrich; Merck KGaA), partially replaced with filtered RWB at 5 different percentages (10%, 20%, 30%, 40%, and 50%).

Statistical analyses

The quantitative variables were expressed as the mean with standard deviation (±SD) in the different experimental conditions at different times. To examine the effect of time and sites of water collection, a 2-way analysis of variance (ANOVA) for repeated measures (RM-ANOVA) was used after checking the assumptions. According to Verma, if the epsilon of Greenhouse and Geisser (ε-GG) was more than 0.750, the p-value with the correction of Huynh–Feldt (ε-HF) was reported.[22]

Finally, if the ANOVA proved significant, all comparisons among the sites of water collection per time were tested using a post hoc test with the Bonferroni approach. To describe the effect of different water partial replacement of the cell culture media at different times per site of water collection, the average level of absorbance with SD was used. The level of statistical significance was set at 0.05. All the analyses were made with STATA® v14 (https://doi.org/10.5281/zenodo.12762630).

Results

Experiment 1: Human native skin fibroblast cultures with DMEM reconstituted with distilled water (control) vs DMEM reconstituted with filtered salso-bromo-iodic water collected at different sites

The evaluation indicated that the normality assumption was not met ($p < 0.05$); however, with ε-HF = 1 it was possible to consider RM-ANOVA robust to non-normality, as reported by Blanca et al.[23] The cultures with DMEM reconstituted with filtered RWS displayed a slightly higher mean absorbance value compared to the controls at all observation times (Fig. 1, Table 2). The cultures with DMEM reconstituted with filtered RWB, in contrast, displayed lower absorbance values than the controls at all times. The cultures with DMEM reconstituted with filtered HRWB displayed even lower values, with the lowest values observed at 24, 48 and 72 h.

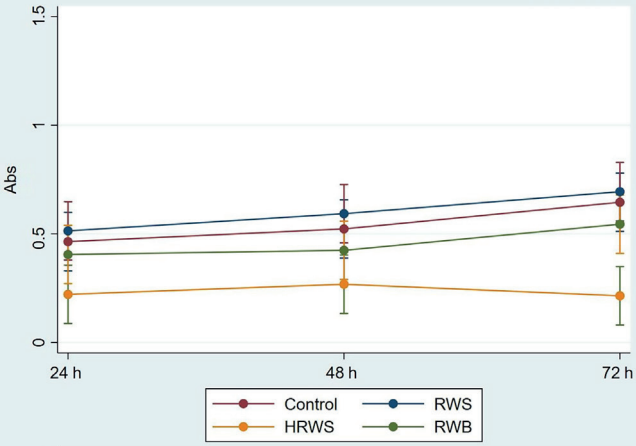

Fig. 1. Trend of mean absorbance values in cultures with Dulbecco's modified Eagle's medium (DMEM) compared to DMEM reconstituted with filtered salso-bromo-iodic water collected at different sites

Control – DMEM reconstituted with distilled water; RWS – DMEM reconstituted with filtered salso-bromo-iodic water collected from the spring; HRWB – DMEM reconstituted with filtered heated salso-bromo-iodic water collected at the balneotherapy facility; RWB – DMEM reconstituted with filtered salso-bromo-iodic water collected at the balneotherapy facility.

The interaction between time and site of water collection was not statistically significant (F = 0.56, degrees of freedom (df) = 6, p = 0.758). However, both the main effects of time and site of water collection were statistically significant (F = 3.34, df = 2, p with Huynh–Feldt correction = 0.038 and F = 16.72, df = 3, p < 0.001, respectively).

More precisely, there was a growing trend in vitality over time for DMEM, RWS and RWB. The trend for HRWB reached a plateau at 48 h and then decreased (Fig. 1). The multiple comparisons revealed that, for the main effect of time, there was a statistical difference only between 24 and 72 h (t = 2.57, df = 118, p = 0.033), but not for comparisons at 48 h (24 h vs 48 h t = 1.06, df = 118, p = 0.874; and 48 h vs 72 h t = 1.51, df = 118, p = 0.397) (Table 2). For the main effect of having 2 separate collection sites, there was a difference only with HRWB compared to the other sites of water collection (control vs HRWB t = −5.57, df = 88, p < 0.001; RWS vs HRWB t = −6.57, df = 88, p < 0.001; RWB vs HRWB t = 4.02, df = 88, p = 0.001) (Table 2).

Experiment 2: Human native skin fibroblast cultures with DMEM reconstituted with distilled water (control) compared to DMEM reconstituted with distilled water partially replaced with different amounts of filtered salso-bromo-iodic water collected at different sites

The results of the cultures with DMEM compared to DMEM partially replaced with filtered salso-bromo-iodic water collected at different sites are summarized in Table 3.

A. Human native skin fibroblast cultures with DMEM reconstituted with distilled water (control) compared to DMEM reconstituted with distilled water partially replaced with different amounts of filtered salso-bromo-iodic water collected at the spring

At 24 h, all experimental conditions with Rivanazzano mineral water showed a mean absorbance value higher than the controls. The absolute highest value was observed in the cultures with DMEM partially replaced with 20% filtered mineral water collected from the spring.

At 48 h, all experimental conditions with Rivanazzano mineral water showed a mean absorbance value higher than the controls. The cultures with DMEM partially replaced with 20%, 30% and 40% filtered mineral water collected from the spring showed the highest absorbance values. At 72 h, the cultures with DMEM partially replaced

Table 2. Mean absorbance values with standard deviation in the cultures with DMEM reconstituted with distilled water (Control) vs DMEM reconstituted with filtered salso-bromo-iodic water collected at different sites and at different times.

Culture conditions	Two-way ANOVA				p-value		
	24 h n = 15 (mean ±SD)	48 h n = 15 (mean ±SD)	72 h n = 15 (mean ±SD)	Total n=45 (mean ±SD)	vs RWS (B)	vs HRWB (C)	vs RWB (D)
Control	0.464 ±0.165	0.523 ±0.155	0.646 ±0.171	0.544 ±0.178 (A)	F =16.72, df = 3, p ≤ 0.001		
RWS	0.514 ±0.299	0.593 ±0.354	0.694 ±0.411	0.600 ±0.357 (B)	>0.90 A vs B	<0.001 A vs C	0.736 A vs D
HRWB	0.221 ±0.195	0.268 ±0.237	0.215 ±0.231	0.235 ±0.218		<0.001 B vs C	0.068 B vs D
RWB	0.405 ±0.237	0.424 ±0.260	0.545 ±0.314	0.458 ±0.273 (D)			0.001 C vs D
Total n = 60	0.401 ±0.250 (I)	0.452 ±0.282 (II)	0.525 ±0.344 (III)	F 3.34, df = 2, p-value = 0.038	0.874 I vs II	0.033 I vs III	0.397 II vs III

mean ±SD – mean value ± standard deviation; ANOVA – analysis of variance; df – degrees of freedom; control – DMEM reconstituted with distilled water; RWS – DMEM reconstituted with filtered salso-bromo-iodic water collected at the spring; HRWB – DMEM reconstituted with filtered heated salso-bromo-iodic water collected at the balneotherapy facility; RWB – DMEM reconstituted with filtered salso-bromo-iodic water collected at the balneotherapy facility. The sphericity was tested according to the Mauchly's test of sphericity; if there was a violation, the p-value was reported using the Greenhouse–Geisser or Huynh-Feldt correction.

Table 3. Mean absorbance values with standard deviation in the cultures with DMEM reconstituted with distilled water (control) compared with DMEM reconstituted with distilled water partially replaced with filtered mineral water collected at the different sites at five different percentages (10%, 20%, 30%, 40% and 50%).

Water collection site	Absorbance values	100 µL DMEM (control)			90 µL DMEM + 10 µL filtered mineral water			80 µL DMEM + 20 µL filtered mineral water			70 µL DMEM + 30 µL filtered mineral water			60 µL DMEM + 40 µL filtered mineral water			50 µL DMEM + 50 µL filtered mineral water		
		24 h n = 15	48 h n = 15	72 h n = 15	24 h n = 15	48 h n = 15	72 h n = 15	24 h n = 15	48 h n = 15	72 h n = 15	24 h n = 15	48 h n = 15	72 h n = 15	24 h n = 15	48 h n = 15	72 h n = 15	24 h n = 15	48 h n = 15	72 h n = 15
RWS	mean	0.464	0.523	0.645	0.545	0.654	0.625	0.646	0.812	0.857	0.617	0.779	0.910	0.642	0.750	0.997	0.626	0.710	0.941
	SD	0.166	0.154	0.171	0.156	0.142	0.267	0.110	0.164	0.242	0.206	0.201	0.158	0.256	0.197	0.260	0.197	0.280	0.318
HRWB	mean	0.464	0.523	0.645	0.594	0.562	0.473	0.637	0.485	0.474	0.524	0.453	0.476	0.559	0.436	0.366	0.552	0.359	0.348
	SD	0.166	0.154	0.171	0.214	0.356	0.315	0.268	0.308	0.270	0.186	0.310	0.301	0.253	0.310	0.110	0.322	0.233	0.156
RWB	mean	0.464	0.523	0.645	0.636	0.883	0.868	0.673	0.806	0.719	0.610	0.796	0.733	0.606	0.663	0.910	0.543	0.512	0.627
	SD	0.166	0.154	0.171	0.113	0.108	0.251	0.136	0.118	0.275	0.183	0.106	0.246	0.148	0.174	0.318	0.212	0.079	0.127

DMEM – Dulbecco's modified Eagle's medium powder reconstituted with distilled water; RWS – filtered salso-bromo-iodic water collected at the spring; HRWB – filtered heated salso-bromo-iodic water collected at the balneotherapy facility; RWB – filtered salso-bromo-iodic water collected at the balneotherapy facility; mean – mean absorbance; SD – standard deviation.

with 20%, 30%, 40%, and 50% filtered salso-bromo-iodic water collected from the spring showed higher values than the controls. However, the mean absorbance value in the cultures with DMEM partially replaced with 10% filtered salso-bromo-iodic water was lower than in the controls.

B. Human native skin fibroblast cultures with DMEM reconstituted with distilled water (control) vs DMEM reconstituted with distilled water partially replaced with different amounts of filtered HRWB

At 24 h, all experimental conditions with filtered HRWB showed a mean absorbance value higher than the controls. Specifically, the highest absorbance values were found in the cultures with DMEM partially replaced with 20% and 10% filtered HRWB.

At 48 h, only the cultures with DMEM partially replaced with 10% filtered HRWB showed a mean absorbance value higher than the controls. All other replacement percentages had absorbance values lower than in the controls.

At 72 h, all experimental conditions with filtered HRWB showed a mean absorbance value lower than the controls.

C. Human native skin fibroblast cultures with DMEM reconstituted with distilled water (control) vs DMEM reconstituted with distilled water partially replaced with different amounts of filtered RWB

At 24 h, all experimental conditions with filtered RWB showed a mean absorbance value higher than the controls. The highest absorbance values were observed in the cultures with DMEM partially replaced with 20% and 10% filtered RWB.

At 48 h, all experimental conditions with filtered RWB also showed a mean absorbance value higher than the controls. Within this group, the highest values were observed in the cultures with DMEM partially replaced with 10% filtered RWB.

At 72 h, the absorbance values in the cultures with DMEM partially replaced with 10%, 20%, 30%, and 40% filtered RWB were higher than in the controls. Under these experimental conditions, the highest values were observed in the cultures with DMEM partially replaced with 40% filtered RWB. In the cultures with DMEM partially replaced with 50% filtered RWB, the values at 72 h were almost equal to those in the controls.

Discussion

Spring water's connection to life has been relevant in human history, health, work, culture, and religion since ancient times. In Roman culture, the use of mineral water was part of everyday life, both for hygienic and cosmetic purposes. The therapeutic properties of some mineral waters on wound healing have long been empirically demonstrated.[7–11] These favorable effects are traditionally

attributed to their chemical-physical composition, unique to every spring. Recently, increased evidence has suggested that the therapeutic properties of natural mineral waters might also be related to their biological components, complementing the chemical-physical ones.[24,25] The role of some natural mineral waters' native non-pathogenic bacterial microflora and bacterial extracts in actively enhancing both cell function and a complex skin regeneration process has already been reported.[16,26–31] Within this context, the mineral waters' native microbial population and chemical-physical constitution are likely to mutually interact in promoting the water-specific therapeutic effects.[32–35]

The medically relevant properties of natural mineral waters are anti-inflammatory, immune-modulating and regenerative. Within the wound healing process, an inversely proportional relationship between the magnitude of the inflammatory reaction and the effectiveness of the proliferative phase has been presented.[36] Topical treatments with natural mineral waters have demonstrated favorable effects both on skin irritations[37] and cutaneous re-structuring after aggressive cosmetic skin procedures.[38,39]

The regenerative properties of calcium magnesium bicarbonate-based mineral water have been investigated and showed over the last decade by our research group using both animal in vivo experimental models and in vitro and ex vivo human models.[12–17]

Our study focused on salso-bromo-iodic natural mineral water, whose therapeutic effects have long been empirically related to anti-inflammatory, antiseptic and angio-trophic properties.[18]

The mineral water replacement of the cell culture medium, at any percentage, with filtered mineral water allowed for a significant enhancement of fibroblast proliferation in the cultures with water collected from the spring. The strongest stimulation of human native skin fibroblast proliferation was found in the cultures with DMEM partially replaced with 40% Rivanazzano salso-bromo-iodic natural mineral water collected from the spring. The same result was observed with the unheated mineral water collected at the balneotherapy facility, although to a lesser degree. The heated mineral water, however, demonstrated poor results compared to both the replacements with the mineral water collected at other sites and the controls, except at 24 h with the lower replacement percentages. These outcomes might suggest that the favorable effects of Rivanazzano mineral water could be related to active substances influenced by environmental factors, such as the delivery in the pipeline system and thermal exposure.

The DMEM reconstitution with filtered mineral water collected from the spring did not demonstrate a substantial advantage in fibroblast proliferation compared to the controls, thus showing only a non-toxic effect.

All these data suggest that the partial replacement of the culture medium with filtered mineral water collected from the spring represents a potentially favorable combination for fibroblast in vitro proliferation, although the mechanism remains unexplained. Conversely, the reconstitution of DMEM with the same mineral water collected at the end of the dedicated pipeline in the balneotherapy facility showed substantial inhibition of in vitro cell proliferation. This effect was even worse with the DMEM reconstitution with heated mineral water. These effects might be related to cell-inhibiting substances produced during water delivery through the pipeline and, more significantly, after thermal conditioning at 70°C. It is unknown whether the fibroblast proliferation inhibiting effect was due to catabolites from the biological component of the mineral water or to irreversible damage within the water's inorganic composition. However, the general picture from our study suggests that in the Rivanazzano mineral water collected from the spring, an active role in fibroblast proliferation and viability might be played by thermolabile, non-pathogenic microbial flora in harmonious combination with the water's inorganic substrate.

Limitations

In this study, we deliberately used native skin-derived human fibroblasts. This option has both limitations and advantages. Only a limited number of replication cycles are allowed with these cells. They are far more delicate than immortalized cells because they have higher trophic requirements and display greater sensitivity to the contact inhibition process. Furthermore, they exhibit some inconsistency in their functional features according to different donors.[40] However, native skin-derived human fibroblasts can be considered the closest approximation to in vivo ones because they share the same morphology, physiology and genetic features. Therefore, results from studies on native skin-derived human fibroblasts might best approximate the condition of fibroblasts in the in vivo context.

The results from this study should be considered just a preliminary step in investigating any stimulating effects of Rivanazzano salso-bromo-iodic water on human skin because fibroblasts represent only a single cell component within the complex integrated human skin environment. Therefore, we aim to further investigate the cell proliferative stimulating effect of Rivanazzano natural mineral water by repeating the same experimental sequence previously performed on Comano mineral water:

1) Identification of Rivanazzano mineral water's native non-pathogenic bacterial microflora;

2) Assessment of the water's effects on a human ex vivo skin experimental wound healing model.

Conclusions

The results from our research demonstrate some favorable effects of Rivanazzano salso-bromo-iodic mineral water on in vitro human native skin fibroblast cultures. This study could be the first step in exploring the potential

regenerative properties of Rivanazzano salso-bromo-iodic natural mineral water on human skin, potentially expanding its therapeutic applications to the field of wound healing.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.12762630. The package includes the following file:

Supplementary File 1. STATA® v14 output.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Nerolidol inhibits proliferation and triggers ROS-facilitated apoptosis in lung carcinoma cells via the suppression of MAPK/STAT3/NF-κB and P13K/AKT pathways

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Abstract

Background. Lung cancer (LC) is the leading cause of malignancy-related mortalities globally, and the existing treatment interventions are associated with harmful side effects. In the current study, we evaluated the anti-tumor efficiency of nerolidol (NRD) on human non-small cell lung cancer (NSCLC) cells.

Objectives. Nerolidol is a sesquiterpene alcohol extracted from the essential oils of aromatic flora with known anti-cancer activities.

Materials and methods. The latent action of NRD on antiproliferative and apoptotic effects in A549 cells is uncertain. Thus, our work is designed to explore the antiproliferative and apoptotic actions of NRD (20 and 25 μM/mL) against A549 cells. The activity of NRD on A549 cell cytotoxicity, intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), apoptosis, anti-apoptotic proteins, and MAPK/TAT3/NF-κB and P13K/AKT signaling pathways were assessed using MTT tests, dichlorodihydrofluorescein diacetate (DCFH-DA), dual acridine orange/ethidium bromide (AO/EB), DAPI, Rh-123, reverse transciption polymerase chain reaction (RT-PCR), and western blot analyses.

Results. We found that NRD could inhibit NSCLC cell viability through elevated intracellular ROS and MMP loss and elicited apoptosis in a quantity-dependent manner. Similarly, NRD can reduce inflammatory cytokines and anti-apoptotic elements, as well as trigger apoptotic signaling pathways.

Conclusions. Our data established that NRD decreases A549 cell proliferation through ROS-mediated apoptosis, triggering the MAPK/STAT3/NF-κB and P13K/AKT pathways, suggesting that NRD is a possible protective remedy for NSCLC.

Key words: apoptosis, lung cancer, proliferation, nerolidol, MAPK/STAT3/NF-κB

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Background

Lung cancer (LC) is the 2nd most common tumor. Its incidence and fatality rates are rising at alarming rates because of long-term heavy smoking, air pollution and dietary practices.[1–3] Nearly 228,150 new LC cases were diagnosed worldwide in 2019.[4] Meanwhile, LC is more prevalent in men than women, with a low rate of survival (i.e., 5 years).[5] The LC categories include small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer is a rare, fast-growing LC. It can affect anyone, but typically affects people who have a long history of smoking tobacco. Most of these subtypes (i.e., approx. 80%) belong to the NSCLC.[6] Recently, chemotherapy, radiation and surgical resection have proven to be less effective as cancer cells migrate and metastasize.[7,8] Chemotherapy is a significant treatment for advanced NSCLC and SCLC; however, it often fails clinically owing to drug resistance.[9] As a result, there is an urgent need for effective new drugs with low toxicity for the comprehensive treatment of LC.

The primary method currently used by many bioactive components to exert anti-tumor actions is to cause malignant cells to signal for apoptosis, which leads to programmed cell death.[10] Natural products have been found to trigger apoptosis in tumor cells by activating reactive oxygen species (ROS).[11] Reactive oxygen species prompt oxidative DNA destruction and, subsequently, a leakage of cytochrome-c and further initiates the caspase cascade.[12] Several reports have revealed that numerous signaling paths tend to trigger apoptosis of malignant cells, comprised of signal transducer and activator of transcription-3 (STAT3), mitogen-activated protein kinase (MAPK), and nuclear factor kappa-B (NF-κB) cascades, which play a crucial role in programmed cell death due to stimulation or inhibition of ROS.[13,14] Reactive oxygen species, mainly generated by the mitochondria, play a significant role in several signaling pathways.[15] P53 and MAPK are 2 key downstream molecules controlled by ROS and are contained in cancer cell relocation, incursion, apoptosis, and halt of the cell cycle.[16,17] The phosphatidylinositol 3-kinase (PI3K) signal is firmly linked to the regulation of propagation, differentiation and apoptosis.[18] The PI3K signaling pathway and its downstream protein kinase B (Akt) are known to contribute to the occurrence and progression of cancer.[19] Inhibitors that target apoptotic signaling pathways and cell cycle regulators serve as a potential remedy for LC.[20] Medications from natural derivatives are also beneficial in treating LC with fewer harmful side effects.[21]

Abundant natural ingredients and phytochemicals that act as anti-cancer agents have been found to have therapeutic properties that affect the propagation, incursion, metastasis, and apoptosis of various carcinoma cells.[22] Nerolidol (NRD) is a famous sesquiterpene alcohol isolated from aromatic floras as an essential oil from plants such as neroli, ginger, lemongrass, lavender, and tea.[23] It possesses numerous medicinal properties: anti-oxidative, anti-cancer, apoptotic, and anti-inflammatory.[24] According to recent reports, NRD has been found to alleviate inflammation, oxidative stress and apoptosis in cardiac injury stimulated by cyclophosphamide.[25] It has also been shown to inhibit the inflammatory response in LPS-induced acute lung injury (ALI) via inducing antioxidants and AMPK/Nrf-2/(HO)-1 signaling.[26] Moreover, NRD has demonstrated its worth as an anti-tumor agent due to its ability to control the existence and spread of cell fragments and act as a chemosensitizer in malignancies.[27–29] It has been demonstrated that NRD increases the effectiveness of doxorubicin (DOX) in lymphoblast and ovarian cancer cells, as well as its utility in breast carcinogenesis.[30]

Objectives

However, to the best of our knowledge, the anti-cancer, anti-inflammatory and apoptotic actions of NRD on LC human cells have yet to be explored. Hence, this research report sheds light on the efficacy of NRD in A549 NSCLC and investigates its hidden molecular mechanisms.

Materials and methods

Chemicals

Nerolidol (<90% purity), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), antibiotics, phosphate-buffered saline, MTT assay, acridine orange/ethidium bromide (AO/EB), DCFH-DA 2′– 7′- dichlorodihydrofluorescein diacetate, Rh-123, DAPI (4′,6-diamidino-2-phenylindole), and sodium dodecyl sulfate (SDS) were purchased from ExCell (Shanghai, China). For western blot analysis, the antibodies were procured from Biosharp (Hefei, China).

Culturing of NSCLC

Adenocarcinoma NSCLC human cell line A549 was acquired from Procell (Wuhan, China). A549 cells were cultured in DMEM along with FBS (10%), 1% antibiotics and 5% CO_2 at 37°C and 95% humidity.

Non-small cell lung cancer proliferation assay

Non-small cell lung cancer cell viability was determined using the MTT test.[31] The A549 cells were seeded into 96 wells (1×10^5 cells/well) and cultured at 37°C in an incubator having 5% CO_2. After instant preservation, these A549 cells were sustained with diverse quantities of NRD (5–35 μM/mL) for 24 h. Afterward, MTT (10 μL) was added to NRD-administered A549 cells and retained for an additional 4 h to allow the transformation of MTT into

formazan crystals due to the enzymatic action of mitochondrial dehydrogenase. The insoluble formazan was dissolved using dimethyl sulfoxide (DMSO) (150 µL). Cell propagation was estimated at 490 nm and expressed as a viability ratio vs A549 cells (100%). The IC_{50} was calculated according to the method given below:

Inhibition of cell viability (%) = (control optical density (OD) – test OD) × 100.

Intracellular ROS assay

Non-small cell lung cancer was grown on 6-well plates for 24 h, then treated with varying concentrations of NRD (control and 20 and 25 µM/mL). The treated and control cells were stained using DCFH-DA (10 µM/mL) and were conserved for 30 min at 37°C. Thereafter, they were washed in ice-cold PBS, twice to get rid of any leftover dye. Fluorescence was determined at the excitation (485 ±10 nm) and emission (530 ±12.50 nm) stages simultaneously via a multimode reader.[32]

Assessment of apoptosis via dual staining

Non-small cell lung cancer A549 cells were treated with NRD (control and 20 and 25 µM/mL), which were later analyzed with AO/EB staining.[33] The NSCLC cells were exposed to 20 and 25 µM/mL of NRD for 24 h. A mixture of dyes containing AO/EB was administered to all the groups. The treated cells were left in the dark for 20 min at room temperature. To make sure that the unbinding dye was removed, PBS was added, and the cells were observed using an Olympus fluorescence microscope (Nikon Eclipse TS100; Nikon Corp., Tokyo, Japan).

Examination of mitochondrial apoptosis

Using DAPI and Rh-123 staining, the apoptotic value of NRD on A549 cells was determined.[34] Human NSCLC were sowed in well plates and conserved at 37°C with CO_2 (5%) for 1 day. Following the administration of NRD at varying concentrations (control and 20 and 25 µM/mL), the cells were twice submerged in PBS, fixed with 4% paraformaldehyde, cleaned, stained with DAPI, and incubated for 20 min. A549 cells with DAPI were stained with Rh-123 for 30 min at 37°C. The treated cells were washed twice with methanol to remove extra stains, followed by washing with PBS; ultimately, the matrix metalloproteinase (MMP) distinction was eliminated.

Western blot analysis

Human NSCLC A549 cells were cultured with NRD (control and 20 and 25 µM/mL) for 1 day. The cell lysates were prepared by cooling the lysis buffer to ensure the presence of protease inhibitors before performing the western blot experiment for analysis of proliferating cell nuclear antigen (PCNA), p53, caspase-9, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), and interleukin-6 (IL-6), and P13K/Akt protein levels. The total protein concentration was then measured using a Protein Assay Kit (MilliporeSigma, St. Louis, USA). The proteins were electrophoretically separated and transferred to a polyvinylidene difluoride (PVDF) film. The probe was then used to block the film for 60 min before administering primary antibodies in 1:1,000 dilutions, which were isolated overnight at 4°C. Next, the secondary antibodies (1:5,000) were added. The protein bands were then stained for identification and quantified using ImageJ (National Institutes of Health (NIH), Bethesda, USA) software's densitometry function.

mRNA expression assay

Whole RNA was extracted from NSCLC A549 cells following the kit's protocol and using the TRIzol® reagent (Abcam, Cambridge, USA). The isolated RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Abcam) following the manufacturer's instructions. Then, using the Fast Start SYBR Green Master mix (Abcam), it was examined how the cDNAs were rendered in accordance with the manufacturer's instructions. Band strength was assessed, and electrophoresis was performed using ImageJ software.

Statistical analyses

The data from each group were analyzed statistically using GraphPad Prism v. 8.0.2 (GraphPad Software, San Diego, USA) and IBM SPSS software v. 25 (IBM Corp., Armonk, USA). The measurement data were reported as medians (Q1 and Q3). The normality of the distribution was tested using the Kolmogorov–Smirnov test. Since all the distributions were normal, the Brown–Forsythe test was used to establish the equality of variances, and then significant differences between multiple groups were analyzed using the Kruskal–Wallis test and Dunn's post hoc test. If the p-value was <0.05, the data divergence was statistically notable. All tests in this study were bilateral.

Results

The results of the Kruskal–Wallis test and Dunn's post hoc test are presented in Table 1–3.

Cytotoxic effects of NRD on human NSCLC cells

The MTT was used to assess the cytotoxicity of NSCLC A549 cells at varying concentrations (5–35 µM/mL) of NRD.

Table 1 and Fig. 1 show how NRD lowered the cytotoxic activity and proliferation of A549 cells in an amount-dependent way. Nerolidol <10 μM/mL has no impact on the viability of A549 cells. On the other hand, it was found that A549 cell growth was inhibited ($p < 0.05$) at high NRD doses of 15, 20, 25, 30, and 35 μM/mL. The MTT test showed that the NRD's IC_{50} for A549 cells was 20 μM/mL. Based on the data on the inhibitory concentration value, more experiments were carried out using NRD (20 and 25 μM/mL).

Nerolidol enhances ROS in human NSCLC cells

Intracellular ROS have been related to a variety of triggers, such as apoptosis and termination of the cell cycle. After supplementing A549 cells with NRD (20 and 25 μM/mL), it was noted that ROS increased. Fluorescently labeled cells were used to directly examine the ROS assembly. At 25 μM/mL of NRD, the fluorescence intensity was substantially higher ($p < 0.05$) than in untreated A549 control cells (Table 2,3, Fig. 2).

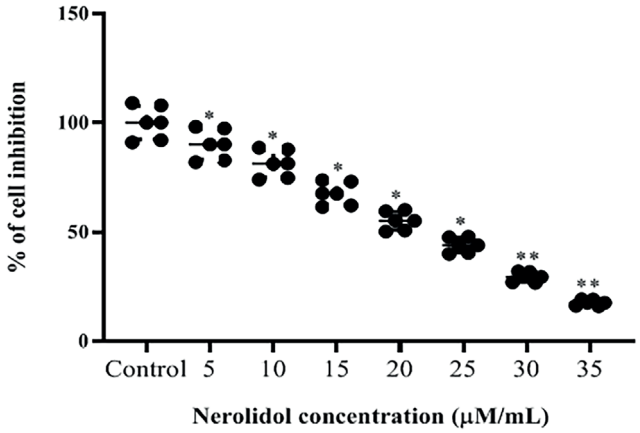

Fig. 1. Nerolidol inhibits human non-small cell lung cancer (NSCLC) cell proliferation. Human NSCLC A549 cells were preserved with diverse dosages (5–35 μM/mL) of nerolidol (NRD) for 24 h. Cell viability was estimated using MTT assay. The data are presented as dots. The horizontal lines show the medians. *,**$p < 0.05$ vs untreated controls were considered statistically significant

Table 1. Groups compared with each other

Variables	Control	5 μM	10 μM	15 μM	20 μM	25 μM	30 μM	35 μM	Test value (H)**	p-value*
MTT	100.03 (91.73–108.23)	90.07 (82.60–97.59)	81.28 (74.53–87.94)	67.58 (61.9–73.22)	55.13 (50.55–59.64)	43.94 (40.30–47.60)	29.36 (26.92–31.77)	17.61 (16.14–19.07)	45.82	0.01522

Data were presented as median (Q1 and Q3); *p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; **degrees of freedom (df) is equal to 7.

Table 2. Groups compared with each other

Variables	Control (n = 6)	20 μM (n = 6)	25 μM (n = 6)	Test value (H)**	p-value*
ROS	5.20 (4.76–5.63)	39.23 (35.97–42.50)	51.51 (47.24–55.73)	15.15	0.001
AO/EB	4.20 (3.85–4.55)	40.07 (36.75–43.41)	52.12 (47.80–56.40)	15.17	0.001
PCNA	1.00 (0.91–1.08)	0.75 (0.68–0.81)	0.34 (0.31–0.37)	15.23	<0.001
p53	1.00 (0.91–1.08)	1.26 (1.15–1.36)	1.80 (1.65–1.94)	15.20	<0.001
Caspase-9	1.00 (0.91–1.08)	1.25 (1.14–1.35)	1.97 (1.80–2.13)	15.20	<0.001
TNF-α	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.38 (0.35–0.41)	15.23	<0.001
NF-κB	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.38 (0.35–0.41)	15.23	<0.001
COX-2	1.00 (0.91–1.08)	0.84 (0.76–0.91)	0.52 (0.47–0.56)	14.43	0.001
iNOS	1.00 (0.91–1.08)	0.70 (0.64–0.76)	0.49 (0.45–0.53)	14.43	<0.001
IL-6	1.00 (0.91–1.08)	0.85 (0.77–0.92)	0.61 (0.56–0.66)	13.91	0.001
Pin1	1.00 (0.91–1.08)	0.58 (0.53–0.63)	0.31 (0.28–0.33)	15.23	<0.001
STAT3	1.00 (0.91–1.08)	0.82 (0.75–0.89)	0.61 (0.56–0.66)	15.26	<0.001
P38	1.00 (0.91–1.08)	0.76 (0.69–0.82)	0.50 (0.46–0.54)	15.22	<0.001
JNK	1.00 (0.91–1.08)	0.71 (0.65–0.77)	0.46 (0.42–0.50)	15.26	<0.001
P65	1.00 (0.91–1.08)	0.63 (0.57–0.68)	0.40 (0.36–0.43)	15.20	<0.001
PI3K/pPI3K	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.45 (0.41–0.49)	15.23	<0.001
AKT/p-AKT	1.00 (0.91–1.08)	0.81 (0.74–0.88)	0.48 (0.44–0.52)	15.25	<0.001

ROS – reactive oxygen species; AO/EB – acridine orange/ethidium bromide; PCNA – proliferating cell nuclear antigen; p53 – tumor protein p53; TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa B; COX-2 – cyclooxygenase-2; iNOS – inducible nitric oxide synthase; IL-6 – interleukin-6; Pin 1 – peptidylprolyl cis/trans isomerase, NIMA-Interacting 1; STAT-3 – signal transducer and activator of transcription 3; p38 – P38 mitogen-activated protein kinases; JNK – c-Jun N-terminal kinase; p65 – transcription factor RELA; p-PI3K – phosphatidylinositol 3-kinase (PI3K)/phosphorylated PI3K; p-AKT – protein kinase B (AKT)/phosphorylated AKT. Data were presented as median (Q1 and Q3); *p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; **degrees of freedom is equal to 2.

Table 3. The results of the Dunn's post hoc test

Figure	Explained variable	C vs 20 µM	C vs 25 µM	20 µM vs 25 µM
Fig. 2	ROS	0.155	<0.001	0.155
Fig. 3	AO/EB	0.154	<0.001	0.154
Fig. 5	PCNA	0.153	<0.001	0.153
Fig. 5	p53	0.154	<0.001	0.154
Fig. 5	Caspase-9	0.154	<0.001	0.154
Fig. 6	TNF-α	0.153	<0.001	0.153
Fig. 6	NF-κB	0.153	<0.001	0.153
Fig. 6	COX-2	0.118	<0.001	0.243
Fig. 6	iNOS	0.152	<0.001	0.152
Fig. 6	IL-6	0.096	0.001	0.347
Fig. 7	Pin1	0.153	<0.001	0.153
Fig. 7	STAT3	0.152	<0.001	0.152
Fig. 7	P38	0.153	<0.001	0.153
Fig. 7	JNK	0.152	<0.001	0.152
Fig. 7	P65	0.154	<0.001	0.154
Fig. 8	PI3K/pPI3K	0.153	<0.001	0.153
Fig. 8	AKT/p-AKT	0.153	<0.001	0.153

ROS – reactive oxygen species; AO/EB – acridine orange/ethidium bromide; PCNA – proliferating cell nuclear antigen; p53 – tumor protein p53; TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa B; COX-2 – cyclooxygenase-2; iNOS – inducible nitric oxide synthase; IL-6 – interleukin-6; Pin 1 – peptidylprolyl cis/trans isomerase, NIMA-Interacting 1; STAT-3 – signal transducer and activator of transcription 3; p38 – P38 mitogen-activated protein kinases; JNK – c-Jun N-terminal kinase; p65 – transcription factor RELA; p-PI3K – phosphatidylinositol 3-kinase (PI3K)/phosphorylated PI3K; p-AKT – protein kinase B (AKT)/phosphorylated AKT.

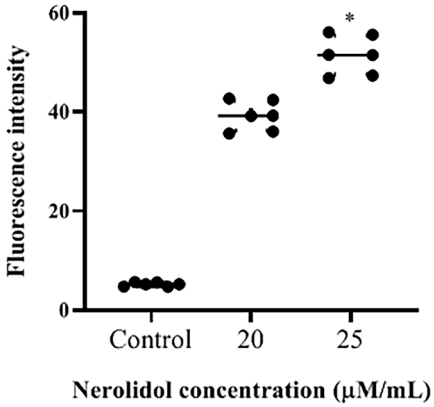

Fig. 2. Nerolidol enhances the accumulation of ROS in human non-small cell lung cancer (NSCLC). Human A549 cells were supplemented with nerolidol (NRD) (20 and 25 µM/mL) for 1 day. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs controls was considered statistically significant

Nerolidol-stimulated apoptosis on NSCLC

The characteristic morphological changes caused by the AO/EB labeling were visible in apoptotic cells. Supplementing NRD (20 and 25 µM/mL) demonstrated quantity-dependently deeper cell apoptosis. In the primary 20 µM/mL NRD treatment, chromatin condensation and membrane blebbing were visible as light greenish and yellow spots. The ethidium bromide co-stain indicated that the membrane integrity had been weakened when

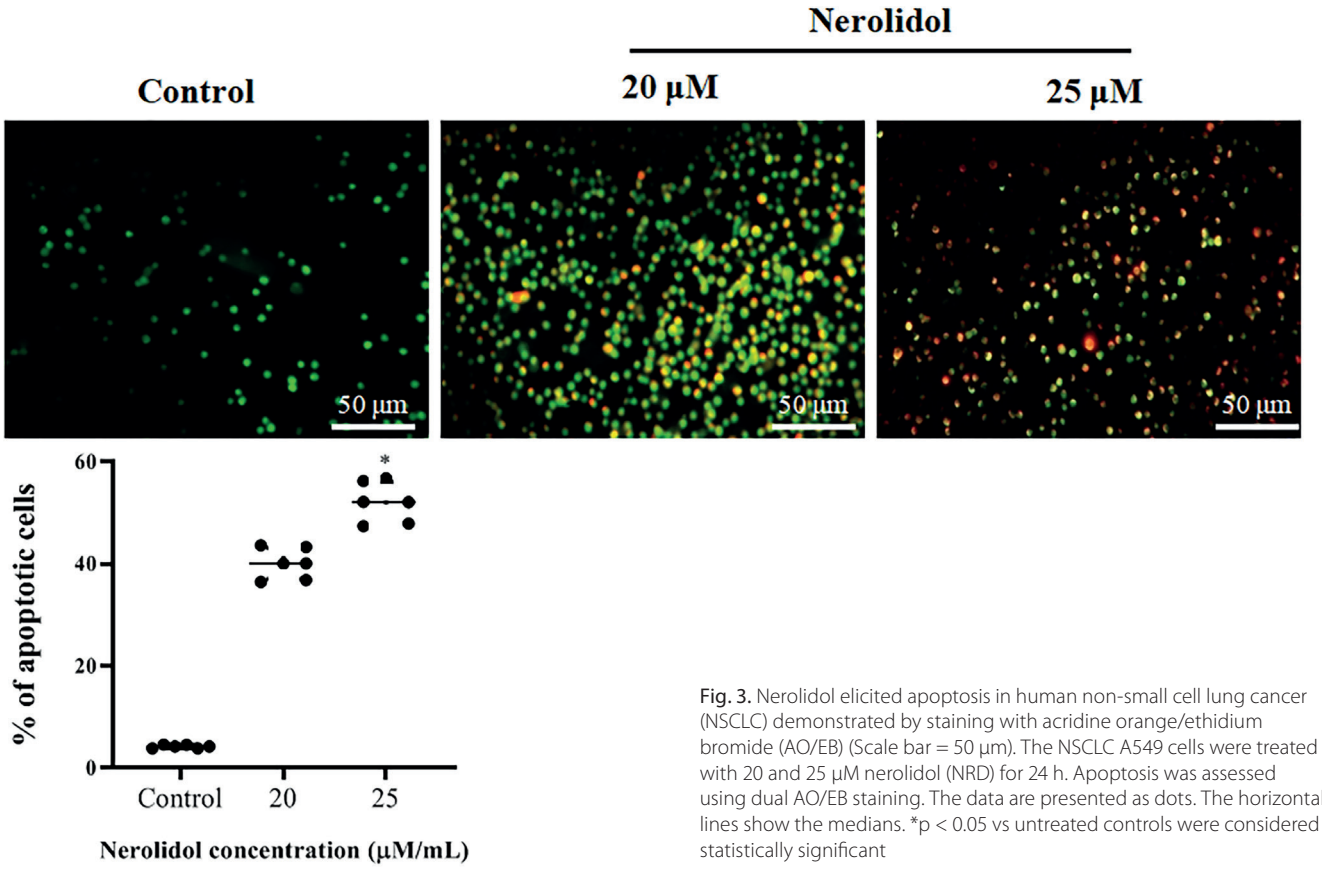

Fig. 3. Nerolidol elicited apoptosis in human non-small cell lung cancer (NSCLC) demonstrated by staining with acridine orange/ethidium bromide (AO/EB) (Scale bar = 50 µm). The NSCLC A549 cells were treated with 20 and 25 µM nerolidol (NRD) for 24 h. Apoptosis was assessed using dual AO/EB staining. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs untreated controls were considered statistically significant

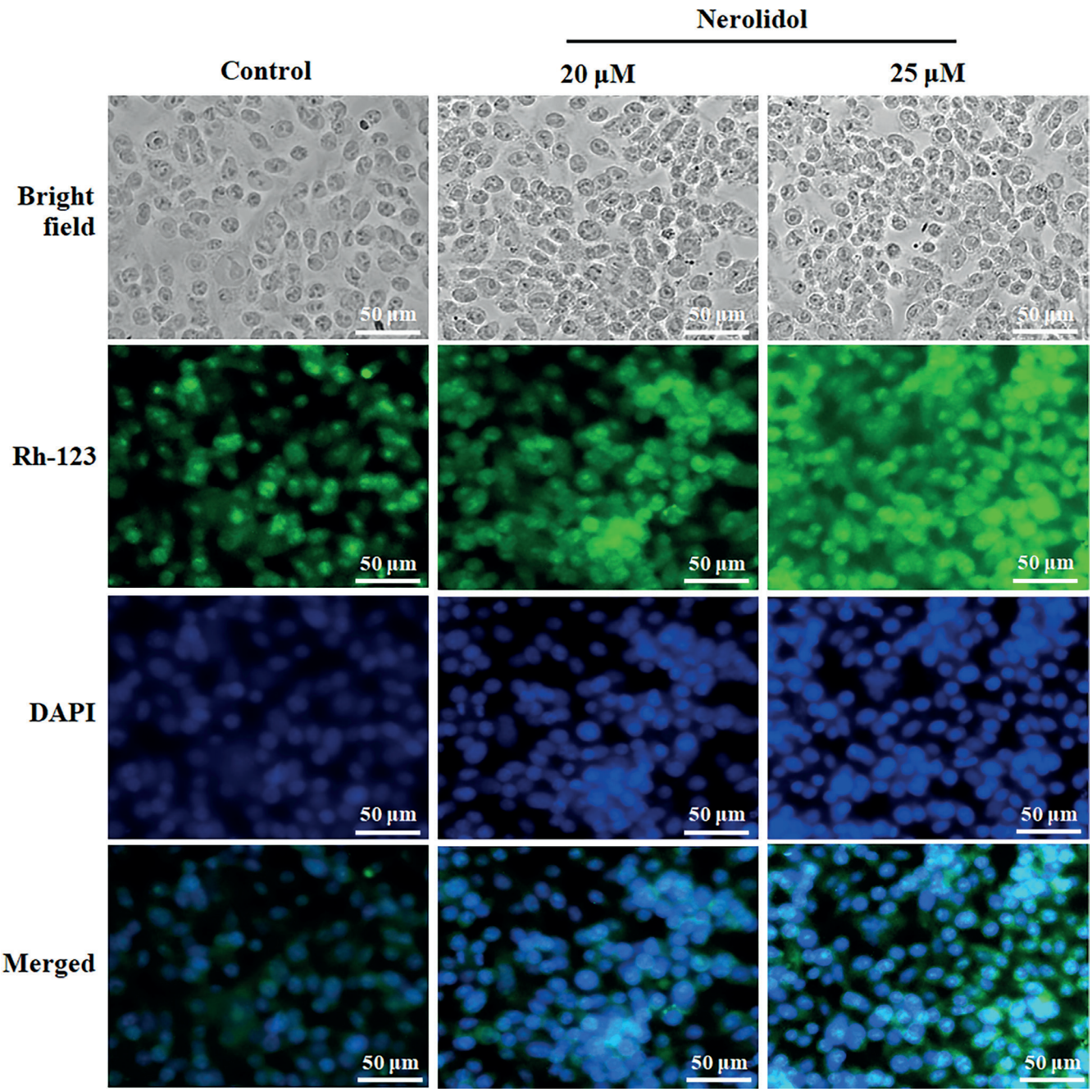

Fig. 4. NRD stimulates apoptosis in human non-small cell lung cancer (NSCLC). NSCLC A549 cells were supplemented with NRD (20 and 25 µM/mL) for 24 h (Scale bar = 50 µm). DAPI (4',6-diamidino-2-phenylindole)and Rh-123 staining uptake were used to explore NSCLC apoptosis. These images were taken using a fluorescence microscope after the A549 cells had been stained with a combination of Rh-123 and DAPI and left for 24 h

25 µM/mL of NRD was used, which is why the apoptotic cells were orange in color. The A549 cells treated with 25 µM/mL NRD showed signs of membrane blebbing, constricted chromatin, irregular nuclei, and late-phase apoptotic impacts (Table 2,3, Fig. 3).

Nerolidol triggered mitochondrial-mediated apoptosis in human NSCLC cells

The apoptotic structures of A549 cells were examined using DAPI after NRD (20 and 25 µM/mL) was assessed. DAPI labeling can be used to detect it by looking for the development of adducts on dual-stranded DNA (Fig. 4). In contrast to the A549 control, the NRD-induced apoptosis on A549 cells was characterized by severely condensed nuclei, nuclear body collapse, and degradation of membrane integrity in a quantity-dependent way. Results showed that NRD acts against A549 cells in an antiproliferative and apoptotic manner.

Depolarization of the mitochondrial membrane following Rh-123 labeling was expected to cause the initial cells to die. The presence of complexly close and under-control mitochondria with an elevated MMP was confirmed with the fluorescent dye Rh-123. Extremely intense fluorescence was observed

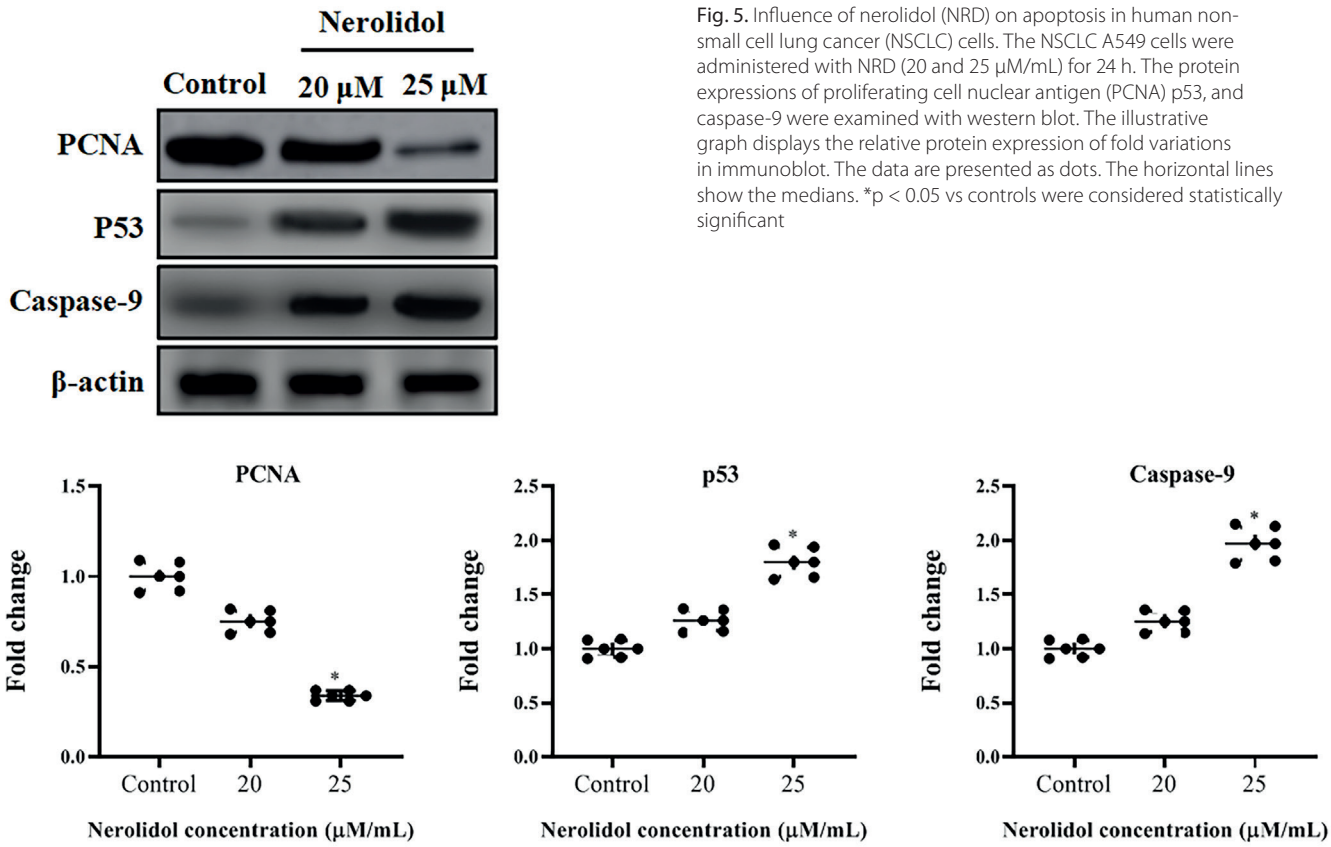

Fig. 5. Influence of nerolidol (NRD) on apoptosis in human non-small cell lung cancer (NSCLC) cells. The NSCLC A549 cells were administered with NRD (20 and 25 μM/mL) for 24 h. The protein expressions of proliferating cell nuclear antigen (PCNA) p53, and caspase-9 were examined with western blot. The illustrative graph displays the relative protein expression of fold variations in immunoblot. The data are presented as dots. The horizontal lines show the medians. *$p < 0.05$ vs controls were considered statistically significant

in the A549 control, which increased MMP. The resulting yellow and green clusters were caused by the fluorescence of Rh-123. Rh-123 fluorescence assembly in NRD declined in a quantity-dependent way. These results are distinct because A549 cells had decreased MMP and caused mitochondria-mediated apoptosis from the NRD (Fig. 4).

Nerolidol triggered apoptosis in human NSCLC cells

Nerolidol-treated A549 cells showed an increase in caspase-9 protein levels but a decrease in PCNA and p53 protein levels. In A549 cells, NRD (20 and 25 μM/mL) elevated caspase-9, increased p53 protein expression and reduced PCNA in a dose-dependent manner (Table 2,3, Fig. 5).

Impact of NRD on the protein cascades of anti-apoptotic inflammatory cytokines

The expression of anti-apoptotic pro-inflammatory marker proteins on A549 cells was assessed to clarify the molecular processes involving NRD. The protein levels of iNOS, COX-2, TNF-α, NF-κB, and IL-6 were shown to be elevated in untreated A549 cells, while these levels decreased in a quantity-dependent manner in A549 cells that were supplied NRD at 20 and 25 μM/mL. The aforementioned effects indicated that NF-κB inhibition is a component of the NRD-induced suppression of A549 cell growth (Table 2,3, Fig. 6).

Nerolidol suppresses NSCLC mRNA

Nerolidol-added A549 showed reduced levels of Pin-1, STAT-3, p38, JNK, and p65 mRNA in comparison to controls. A549 cells supplemented with NRD (20 and 25 μM/mL) showed a concentration-dependent reduction in apoptotic mRNA levels (Table 2,3, Fig. 7).

Nerolidol attenuates the P13K/Akt pathway in NSCLC

P13K/Akt particularly affects the development of NSCLC through the apoptotic signaling pathway. P13K and Akt protein expression was upregulated in A549 cells, but P13K/Akt expression was downregulated in A549 cells exposed to NRD (20 and 25 μM/mL). P13K/Akt protein levels were lowered by NRD in NSCLC cells A549 in a quantity-dependent manner (Table 2,3, Fig. 8).

Discussion

One of the most significant factors contributing to global mortality rates remains LC.[1,2] Many approaches, including chemotherapy, surgical resection and novel molecular targets, have been tried in an attempt to identify and treat LC as soon as possible; however, but the outcomes are still not up to date.[7–9] Therefore, it is essential to investigate the most effective treatment modalities or to develop

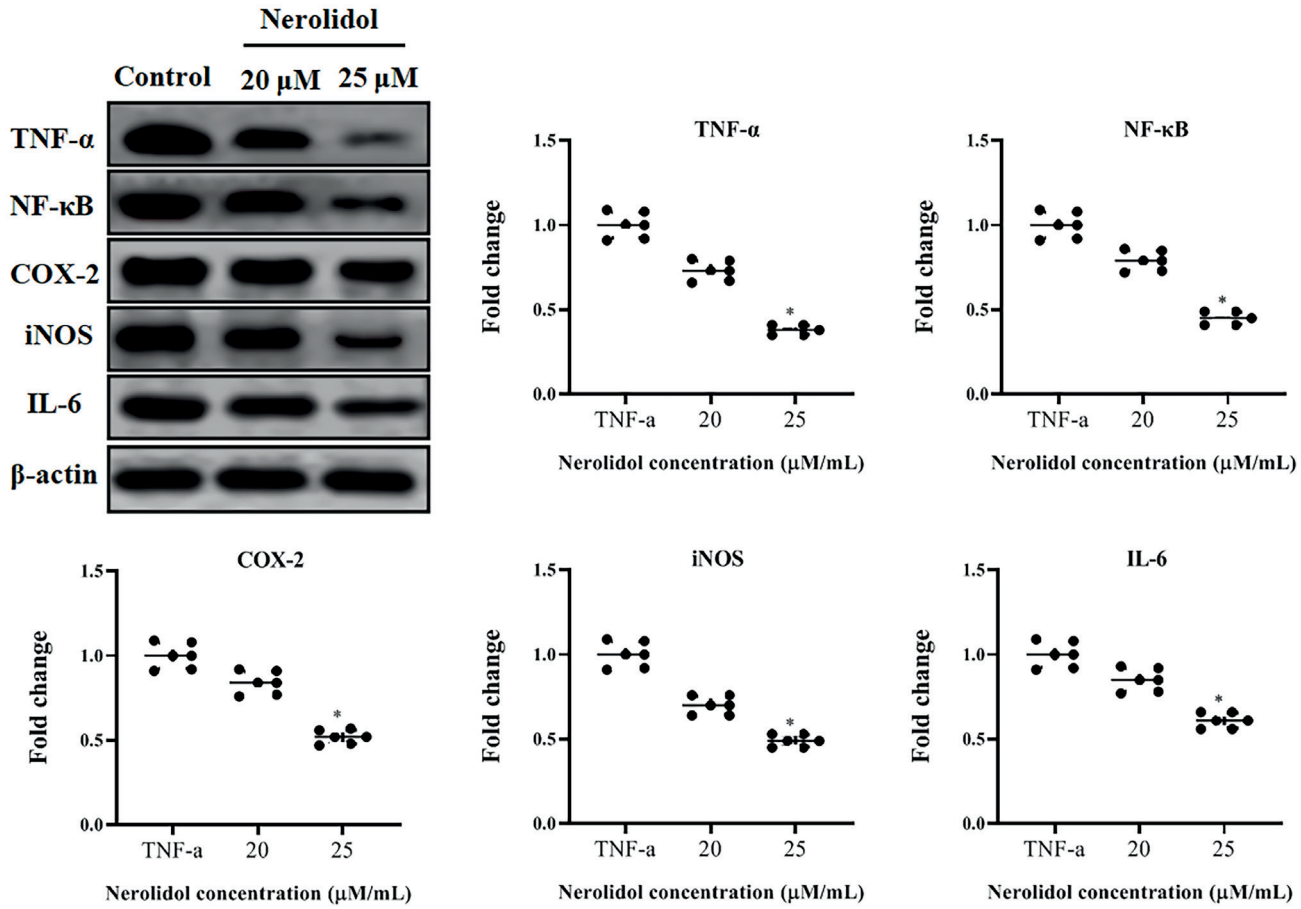

Fig. 6. Effect of nerolidol (NRD) on the protein levels of cytokines. Proliferating cell nuclear antigen A549 cells were supplemented with 20 and 25 μM/mL of NRD for 24 h. Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), and interleukin-6 (IL-6) protein levels in A549 cells were assessed with western blot. The relative protein expression of immunoblot fold variations is shown in the illustrative graph. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs controls was considered statistically significant

cutting-edge corrective measures. In recent times, there has been increased awareness of the anti-cancer properties of natural bioactive substances.[10,11] Comprehensive research has been done on the antiproliferative and apoptotic effects of NRD in a number of cancer types, including oral cancer, breast cancer, ovarian cancer, hepatocellular carcinomas, osteosarcoma, etc.[35,36] Cucurbitacin E (Cu E), a triterpene of cucurbitacins commonly found in culinary plants of the *Cucurbitaceae* family, was examined by Hsu et al.[37] Cucurbitacin E dramatically reduced the viability and proliferation of A549 cells that carried KRAS and wild-type EGFR mutations. In the present investigation, we showcased the anti-tumor impacts of NRD along with its anti-inflammatory, antiproliferative and apoptotic properties. According to our findings, NRD effectively and quantity-dependently reduced the growth, inflammation and activation of apoptosis in NSCLC A549 cells.

We investigated the underlying mechanisms of NRD in NSCLC A549 cells by examining varying concentrations of the compound (5–35 μM/mL). The MTT test results showed that NRD significantly inhibited the proliferation of A549 cells and changed proliferation-associated proteins in a concentration-dependent manner. Furthermore, we used the western blot test to assess levels of p53, PCNA and caspase-9 in A549 cells. In A549 cells, we discovered that NRD increased p53 and the caspase-9 response while suppressing the amount of PCNA. These results demonstrate the antiproliferative action of NRD on NSCLC cells, indicating its efficacy in the treatment of LC. For DNA replication to occur and for rapidly proliferating cells to maintain genomic integrity, PCNA is necessary.[38] Given its function in the proliferation of malignant cells, PCNA has been widely used as a tumor marker. Liuxin et al.[39] recently confirmed that NSCLC cells exhibited high levels of PCNA expression. However, the role of PCNA in NSCLC and its latent molecular mechanisms are not fully understood. For the first time, the present study revealed that PCNA was suppressed due to NRD treatment. Proliferating cell nuclear antigen has both proliferative and anti-apoptotic properties.[40] Therefore, it is expected that the NRD downregulation of PCNA and upregulation of p53 will exhibit antiproliferative effects and may even aid in p53-mediated apoptosis.

Apoptosis is a dynamic homeostatic mechanism, which stabilizes cell division and cell death.[41] To further elucidate the roles of NRD in NSCLC, NRD (20 and 25 μM/mL) was

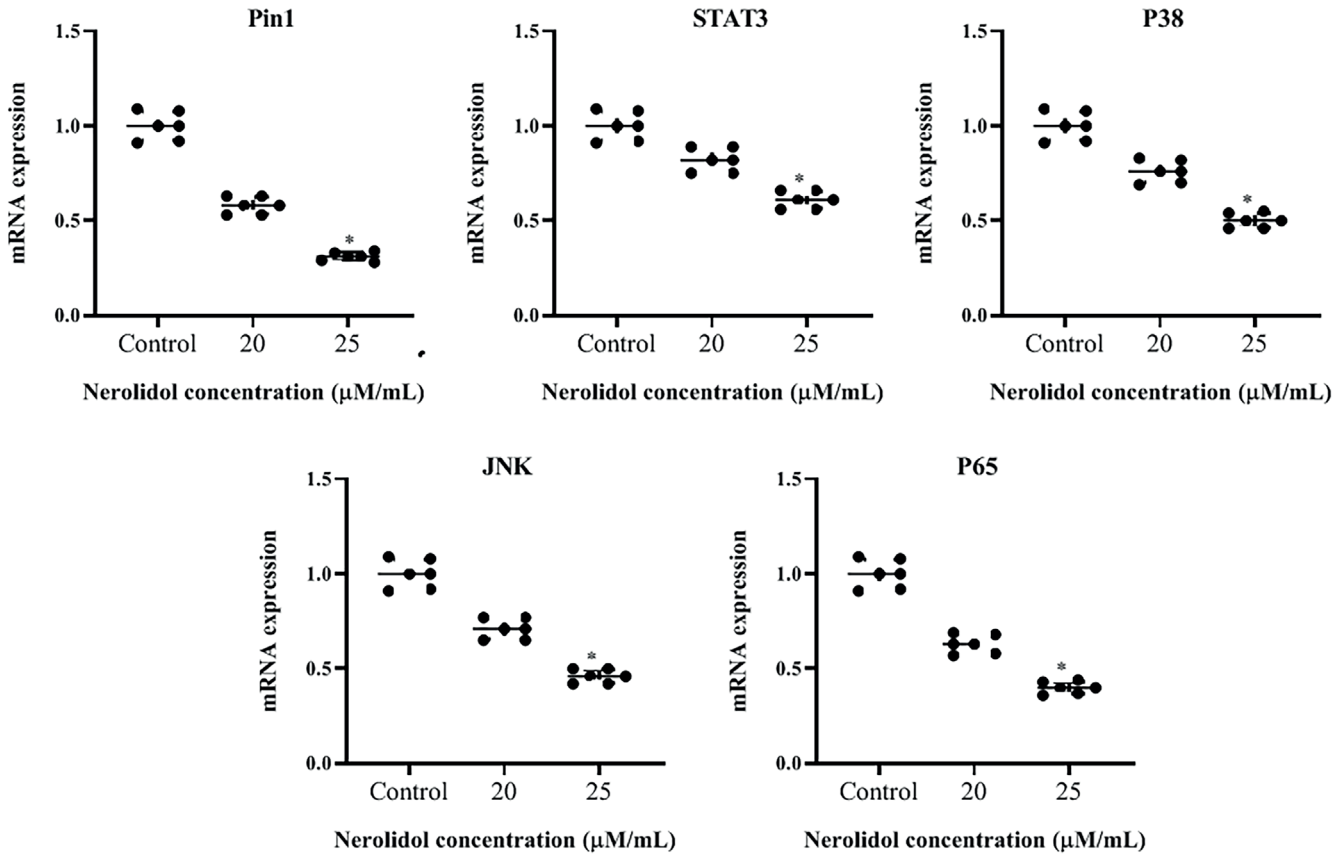

Fig. 7. Impact of nerolidol (NRD) on the mRNA levels of NSCLC cells. Human human non-small cell lung cancer (NSCLC) A549 cells were treated with 20 and 25 μM of NRD for 24 h. The mRNA levels of Pin-1, STAT-3, p38, JNK, and p65 were assessed with reverse transciption polymerase chain reaction (RT-PCR). The data are presented as dots. The horizontal lines show the medians. *p < 0.05 against untreated controls were considered statistically significant

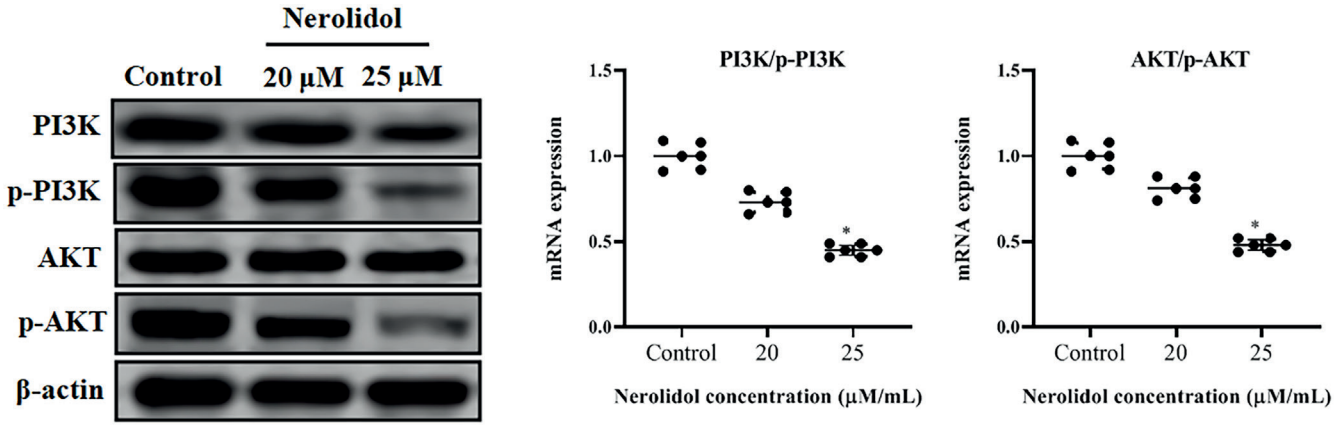

Fig. 8. Nerolidol attenuates the P13K/AKT signaling pathway on human non-small cell lung cancer (NSCLC). Human NSCLC A549 cells were treated with 20 and 25 μM of nerolidol (NRD) for 24 h. p-P13K, P13K, p-AKT, AKT, and β-actin proteins were determined using western blot assay. The illustrative graph displays the relative protein expression of fold variations in immunoblot. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs untreated controls were considered statistically significant

administered to A549 cells. By using AO/EB dual labeling, cell morphological changes of NRD-driven apoptosis were observed. Apoptosis is a typical process of cell death characterized by a variety of morphological changes. Reactive oxygen species play a crucial role in intracellular apoptosis. According to extensive research, an increase in ROS levels triggers the oxidation of intracellular particles, disrupts the mitochondrial membrane potential (MMP), and eventually leads to pathogenic processes. Reactive oxygen species can also be employed as a signaling molecule to transduce extracellular stimulus signals, directly stimulating apoptosis or indirectly contributing to intracellular signal transduction.[42] According to Lin et al.,[43] ROS-mediated MAPK, STAT3, NF-κB, and TGF-β1 signaling pathways caused cell cycle arrest and death in A549 human LC cells. Therefore, 10-HDA may be

a potential therapy for human LC. Similarly, in the current study, NRD prompted the formation of ROS in A549 cells and reduced MMP in a quantity-dependent way. Reactive oxygen species facilitated the signaling of STAT3, MAPK and NF-κB pathways to restrict A549 cell propagation and retard the tumor and metastasis of cells, thus exhibiting anti-cancer properties.

Previous research has revealed that apoptosis is controlled via numerous pathways, including STAT3, MAPK and NF-κB.[44,45] Mitogen-activated protein kinases are crucial for the transduction of signals from the cell surface to the nucleus. The nucleus of both typical and tumor cells contained Pin-1, but its nuclear-cytoplasmic dissemination can be altered due to phosphorylation by kinases JNK and p38 in the MAPK family, which have profound actions on inflammation, growth, apoptosis, and differentiation.[46,47] The STAT protein family is contained inside the cytoplasm and is capable of translocating to the nucleus and getting attached to DNA following stimulation.[48] Among the members of the STAT family, STAT3 contributes to signal transduction and is strongly associated with malignancies involving several cytokines. Inflammation is a fundamental element that is associated with neoplasm incursion, metastasis and death.[49] Pro-inflammatory cytokines trigger cell signaling pathways that stimulate cancer growth, targeting the prevention of these signaling pathways is essential for triggering apoptosis and hindering invasion and metastasis.[50] The NF-κB is an important transcription nuclear factor that regulates the tumor cell cycle to induce cell death.[51] Based on our data, it appears that NRD regulates the Pin-1/MAPK/STAT3/NF-κB-p65 pathways, while MAPK functions as a precursor to STAT3 signaling.

As it promotes the proliferation and survival of cancerous cells, PI3K/Akt signaling is important in the development of cancer and can stimulate the growth and survival of malignant cells.[52] One important cytoprotective pathway linked to a number of diseases is the PI3K/AKT pathway. Increased activation of the PI3K/AKT pathway is associated with multiple tumor hallmarks and is an intriguing target for novel anti-cancer treatments. The PI3K/AKT pathway has been implicated in both carcinogenesis and the development of NSCLC.[53] It has been found that glycogen phosphorylase B initiates NSCLC cell propagation and migration through PI3K/AKT signaling.[54] An earlier study reported that farnesol mitigates LC invasion and relocation by restraining the PI3K/AKT pathway.[55] Hence, we postulated that NRD is likely to hinder the progression of LC by modifying the PI3K/AKT signaling pathway. Our findings specified that the PI3K/AKT pathway might be one of the regulatory mechanisms involved in the antiproliferative and apoptotic effects of A549 cells by NRD. Arunachalam et al.[56] examined rats treated with NERO (50 mg/kg, orally) for 5 days. Doxorubicin-injected rats showed elevated levels of cardiac marker enzymes and enhanced oxidative stress markers along with alterations in the Nrf2/Keap1/HO-1 signaling pathways. Doxorubicin administration also induced the activation of NF-κB/MAPK signaling and increased the levels and expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) as well as the expression of inflammatory mediators (iNOS and COX-2) in the heart. Doxorubicin also triggered DNA damage and apoptotic cell death in the myocardium. Additionally, histological studies revealed structural alterations in the myocardium. Treatment with NERO was protective against the harmful effects of DOX on the myocardium, as demonstrated by the recovery of changed biochemical markers and reduced levels of inflammation, apoptosis and oxidative stress. These studies may provide compelling evidence for the originality of NRD in the current investigation.

These studies strongly supported and highlighted the novelty of NRD in the current study. According to Isik et al.,[57] 51 patients treated for cholangitis due to incomplete or inappropriate non-operative biliary interventions were evaluated retrospectively between 2005–2016. Işık et al.[58] conducted a bibliometric investigation on the 100 most cited articles on abdominal wall hernias in Turkey. It is a very interesting, valuable, and encouraging study for abdominal wall hernias, particularly for inguinal hernias.

Limitations

Our findings revealed that NRD retards the growth of A549 cells by ROS-mediated apoptosis, which activates the MAPK/STAT3/NF-κB and P13K/AKT pathways and suggests that NRD may be a protective treatment for NSCLC. Even with the advanced cellular model and clinical model, we still need to demonstrate molecular-level expressions.

Conclusions

The current study found that NRD reduced proliferation, inflammation and ROS-induced apoptosis in A549 cells by inhibiting MAPK/STAT3/NF-κB and P13K/AKT pathways. The potential novel insight that NRD may offer into LC treatment may contribute to its beneficial efficacy in treating NSCLC. This study may be the first to explain the fundamental processes by which NRD inhibits the growth of NSCLC tumors. Nerolidol may, therefore, be utilized as a chemopreventive drug for LC.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.12054552. The package contains the following files:

Supplementary Fig. 1. Results of Kruskal–Wallis test as presented in Fig. 1.

Supplementary Fig. 2. Results of Kruskal–Wallis test as presented in Fig. 2.

Supplementary Fig. 3. Results of Kruskal–Wallis test as presented in Fig. 3.

Supplementary Fig. 4. Results of Kruskal–Wallis test as presented in Fig. 5.

Supplementary Fig. 5. Results of Kruskal–Wallis test as presented in Fig. 6.

Supplementary Fig. 6. Results of Kruskal–Wallis test as presented in Fig. 7.

Supplementary Fig. 7. Results of Kruskal–Wallis test as presented in Fig. 8.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Laboratory synthesis and preparation of thermo-responsive polymeric micelle and hydrogel for resveratrol delivery and release

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Resveratrol (RSV) exhibits anti-inflammatory, antioxidative, antiaging, and cardioprotective properties. However, due to its hydrophobic nature, it is prone to instability and oxidation, which significantly limit its biomedical applications.

Objectives. The aims of this study were: 1) To prepare and characterize hydrogels and micelles by mixing the synthesized PNIPAM-*b*-PEO-*b*-PNIPAM copolymer and RSV in an aqueous environment; 2) To investigate the molecular interactions between the polymer and RSV; 3) To evaluate various properties of the polymeric micelles and hydrogels; 4) To determine the efficiency of RSV release from the polymeric micelles.

Materials and methods. A well-defined PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer was synthesized and purified. Gel permeation chromatography and ^{1}H NMR were used to characterize the chemical composition and molecular weight of each copolymer. The encapsulation of RSV and its interaction with PNIPAM-*b*-PEO-*b*-PNIPAM were confirmed using 2D nuclear Overhauser effect spectroscopy (NOESY). The lower critical solution temperature (LCST), critical micelle concentration (CMC) and structure of the polymeric micelle were characterized using surface tension measurements, a viscometer, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The rheological behavior of the RSV-loaded hydrogels was also investigated.

Results. The results showed that the RSV-loaded micelles were successfully prepared. The LCST and CMC of PNIPAM-*b*-PEO-*b*-PNIPAM polymeric micelles were determined to be 35°C and 0.005 g/L, respectively. The micelles have a spherical profile with a particle size of 100 nm and a narrow size distribution.

Conclusions. Resveratrol can be encapsulated within polymeric micelles formed by PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer below the LCST. Its molecules are incorporated into the hydrophobic domains of poly(N-isopropyl acrylamide) (PNIPAM), forming a molecular complex. The point of molecular interaction is primarily at the phenolic region of RSV. Below LCST, PNIPAM-*b*-PEO-*b*-PNIPAM behaves as a polymeric surfactant at low concentrations and as an associating polymer at high concentrations. At high polymer concentrations, PNIPAM-*b*-PEO-*b*-PNIPAM formed a hydrogel. Above LCST, it was released from the polymeric micelles.

Key words: resveratrol, drug release, polymeric micelle

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Background

Cartilage regeneration is an important and innovative procedure for replacing damaged knee cartilage.[1–5] Due to the increasing aging population, there is a growing need for cartilage regeneration. Current methods include autologous chondrocyte implantation (ACI).[1] In this method, healthy cartilage cells are taken from the damaged knee and cultured in a lab for several weeks. They are then injected into the damaged joint to regenerate the cartilage along with the surrounding tissue. Although various therapeutic methods have been established, there remains a challenge in achieving cartilage regeneration with normal anatomy, morphology and function. Moreover, repaired cartilage often lacks sufficient mechanical properties, leading to further degeneration. These problems could potentially be solved by the application of hydrogels, which are commonly used as scaffolds for cartilage repair.[6,7]

Resveratrol (RSV, 3,5,4'-trihydroxy-*trans*-stilbene) is a natural phenol found in grape skins, blueberries, raspberries, peanuts, and other plants. It is anti-inflammatory, antioxidative, antiaging, and cardioprotective.[8–11] Importantly, RSV has been reported to suppress the interleukin (IL)-1b-induced inflammatory signaling pathway, making it a promising drug for potential arthritis therapies.[12–15] However, due to its instability under light and heat, as well as its susceptibility to oxidation, the biomedical applications of RSV are limited. Because RSV is a hydrophobic drug, one approach to overcome these challenges is designing an RSV-controlled release system via encapsulation to improve drug administration and therapy efficiency. Encapsulation is considered the most promising way to deliver hydrophobic drugs such as RSV. The primary strategy of encapsulation aims at incorporating a hydrophobic core material into a hydrophilic shell to facilitate the delivery of active agents or drugs into living cells.[16,17] Therefore, an encapsulated system with RSV as the core and biocompatible materials as the shell will not only enhance water solubility of RSV, but also increase its stability by improving photo-stability and resistance against oxidation, thereby enhancing its bioavailability during drug administration.

Stimuli-responsive hydrogel systems are unique due to their high swelling capacity and ability to undergo gelation. These hydrogels can change their appearance or physical properties in response to environmental stimuli such as pH, temperature and light.[18–22] Several thermoresponsive polymers form a gel-like material at temperatures higher than the lower critical solution temperature (LCST).[23–27] Some thermosensitive hydrogels shrink below and dissolve above the upper critical solution temperature (UCST). Typically, thermoresponsive hydrogels consist of a hydrophilic polymer network. Many studies have shown that the gel formation process involves cross-linking of molecular chains through intermolecular interactions (van der Waals forces, hydrophobic interactions, hydrogen bonding, etc.) rather than chemical reactions. A change in ambient temperature can affect these interactions, causing the hydrogel to form in aqueous solution through a simple reversible phase transition (sol–gel). Therefore, the preparation of thermoresponsive hydrogels is straightforward and does not require organic solvents, which is advantageous for delivering hydrophobic drugs. Some studies have shown that thermosensitive poly(N-isopropylacrylamide)-block-poly(ethylene oxide)-block-poly(N-isopropylacrylamide) (PNIPAM-*b*-PEO-*b*-PNIPAM) hydrogels exhibit ideal gel properties, capable of loading protein drugs below 30°C and undergoing a sol–gel phase transition at human body temperature.[23] The PNIPAM-*b*-PEO-*b*-PNIPAM hydrogel has been noted for its good biological degradation and safety.[24–27] However, it still has some unresolved issues such as the need for drug loading at lower temperatures and a short sustained-release cycle (only 7 days) for protein drugs, which limits its clinical application. In addition, an increase in PNIPAM block length can lead to the aggregation of protein drugs.

In this study, a thermosensitive PNIPAM-*b*-PEO-*b*-PNIPAM polymer was synthesized and used as a drug carrier for RSV. Above the LCST, PNIPAM segments become water-insoluble, resulting in the formation of hydrogel with a 3D polymeric network. Hydrophobic junctions within this network, formed by PNIPAM molecules, allow for the loading of RSV molecules. Below the LCST, PNIPAM becomes soluble in aqueous solutions, causing the hydrogel network to collapse and release the RSV molecules. The polymeric structure and incorporation of RSV into PNIPAM junctions were characterized using 2 NMR techniques. Additionally, the critical micelle concentration (CMC) was determined with surface tension. The rheology of PNIPAM-*b*-PEO-*b*-PNIPAM above the overlap concentration (C^*) and the micellar shape, particle size, distribution of the drug within micelles below C^*, and in vitro release properties were investigated.

Objectives

The aim of this study was to design a thermo-induced polymeric hydrogel as a potential carrier for RSV-loaded micelles and injectable hydrogels through: 1) characterizing the chemical structure of the synthesized block copolymer; 2) preparing and characterizing hydrogels and micelles by mixing the synthesized copolymer and RSV in an aqueous environment; 3) investigating the molecular interaction between the polymer and RSV; 4) evaluating various properties of the polymeric micelle and hydrogel; and 5) determining the efficiency of RSV release from the polymeric micelle.

Materials and methods

Materials

Resveratrol (98%) was purchased from Innochem Co Ltd (Beijing, China). The N-isopropyl acrylamide (NIPAM) monomer (M_n~113 g/mol, 98%) and poly(ethylene oxide) (PEO) (M_n~200,000 g/mol) were purchased from Sigma-Aldrich (St. Louis, USA). Ce(IV) ammonium nitrate ($Ce(NO_3)_6(NH_4)_2$) was provided by Wako Pure Chemical Co (Osaka, Japan). All experiments used deionized water obtained from the Millipore-Q water purification system (Merck Millipore, St. Louis, USA). The NIPAM monomer was purified by recrystallization from *n*-hexanes 3 times, while the PEO was purified by dissolving it in dichloromethane and precipitating it in diethyl ether. This purification process was repeated 3 times. Ce(IV) ammonium nitrate was dried at 105°C in a vacuum oven and dissolved in a 1 N aqueous solution of HNO_3 to give a 0.1 M aqueous solution of Ce^{4+} ions. Other chemicals were used as received without further purification.

Synthesis of PNIPAM-*b*-PEO-*b*-PNIPAM

The synthesis procedure was conducted as follows: PEO was dissolved in 20 mL of Milli-Q water in a 200 mL flask and degassed for 2 h under a gentle flow of pure N_2. Ce^{4+} solution was then added to give a Ce^{4+}/PEO molar ratio of 5:1. After stirring this solution for 10 min at 70°C, NIPAM dissolved in 15 mL of Milli-Q water was added under a nitrogen atmosphere. The polymerization was carried out under N_2 at 70°C for 1 h. Upon completion of the polymerization, the solution temperature was reduced to room temperature and dialyzed against Milli-Q water for 3 days using a regenerated cellulose bag with a molecular weight (MW) cutoff of 3,000 g/mol.

The Ce^{4+} and NIPAM solutions were degassed with nitrogen and transferred into the PEO solution using a syringe. Polymerization was carefully conducted to minimize oxygen exposure. Ce^{4+}, NIPAM and PEO solutions were placed in separate round-bottom flasks and degassed for 2 h using nitrogen. The solutions were then transferred with a cannula into the reaction vessel, where polymerization took place. Using high-purity nitrogen from Praxair (Xi'an, China; Ultra High Purity 5.0) improved the polymerization of NIPAM. The synthesis scheme for PNIPAM-*b*-PEO-*b*-PNIPAM is shown in Fig. 1.

The resulting product was dissolved in a 2 M $NaNO_3$ aqueous solution to achieve a 20 wt% solution, which was transferred into an ice bath to ensure complete dissolution. Centrifugation was performed using a Beckman Coulter Avanti J-301 centrifuge (Beckman Coulter, Brea, USA) at 40°C and 8,000 rpm for 60 min. The precipitate was recovered and fully dried with a Labconco Freezone 6 freeze dryer (Labconco, Kansas City, USA) before conducting gel permeation chromatography (GPC) and NMR analyses. Centrifugation was repeated until GPC and NMR data confirmed the absence of unreacted PEO. Excess salts were removed by dialysis against Milli-Q water for 3 days using a regenerated cellulose bag with an MW cutoff of 3,000 g/mol.

High-resolution ^{1}H NMR spectra of the samples were obtained using a 400 MHz Bruker NMR spectrometer (Bruker, Billerica, USA), with deuterated water (D_2O) as the solvent. The GPC device (Agilent 1260 series; Agilent Technologies, Santa Clara, USA) consisted of a single pump and multi-detector systems, including a light scattering detector, a refractive index detector and a viscosity detector.

Determination of the LCST by turbidimetry, absorbance and viscosity

Turbidimetry measurements were conducted using a DataPhysics MS-20 optical dispersion stability analyzer (DataPhysics Instruments, Filderstadt, Germany) at 0.5°C intervals from 25°C to 40°C. The polymer solution was prepared by dissolving 0.02 g of homopolymer PNIPAM (Sigma-Aldrich; MW = 20,000 g/mol) in 10 mL of Milli-Q water. Absorbance measurements were conducted using the BK4200 UV-vis spectrophotometer (Shanghai Huicheng General Instrument Co., Ltd., Shanghai, China) for a PNIPAM-*b*-PEO-*b*-PNIPAM solution at a concentration of 10 g/L mixed with RSV. The temperature was increased in 0.5°C increments from 25°C to 40°C. Viscosity measurements were conducted using a stress-controlled rheometer (Paar Physica DSR 4000; Anton Paar GmbH, Graz, Austria), with zero-shear viscosity (η_0) used as the viscosity data. The temperature was increased in 0.5°C increments from 25°C to 40°C.

Preparation of RSV-loaded polymeric micelles

Briefly, 0.4 g of PNIPAM-*b*-PEO-*b*-PNIPAM polymer and 0.01 g of RSV were accurately weighed and dissolved in 4 mL and 1 mL of dimethyl formamide (DMF), respectively. After mixing and stirring for 1 h, the solution was loaded into a dialysis bag and dialyzed against 120 L of deionized water for 2 days. The turbid solution obtained from dialysis was centrifuged at 2,000 rpm for 20 min. The supernatant was filtered using a nylon membrane with a pore diameter of 0.15 μm to remove RSV not encapsulated by micelles. The RSV-loaded micelles were obtained by freeze-drying the filtrate.

Characterization of micelle size, profile and structure

The average particle size and distribution of the polymeric micelles were determined using light scattering spectroscopy at 25°C and a wavelength of 486 nm, with

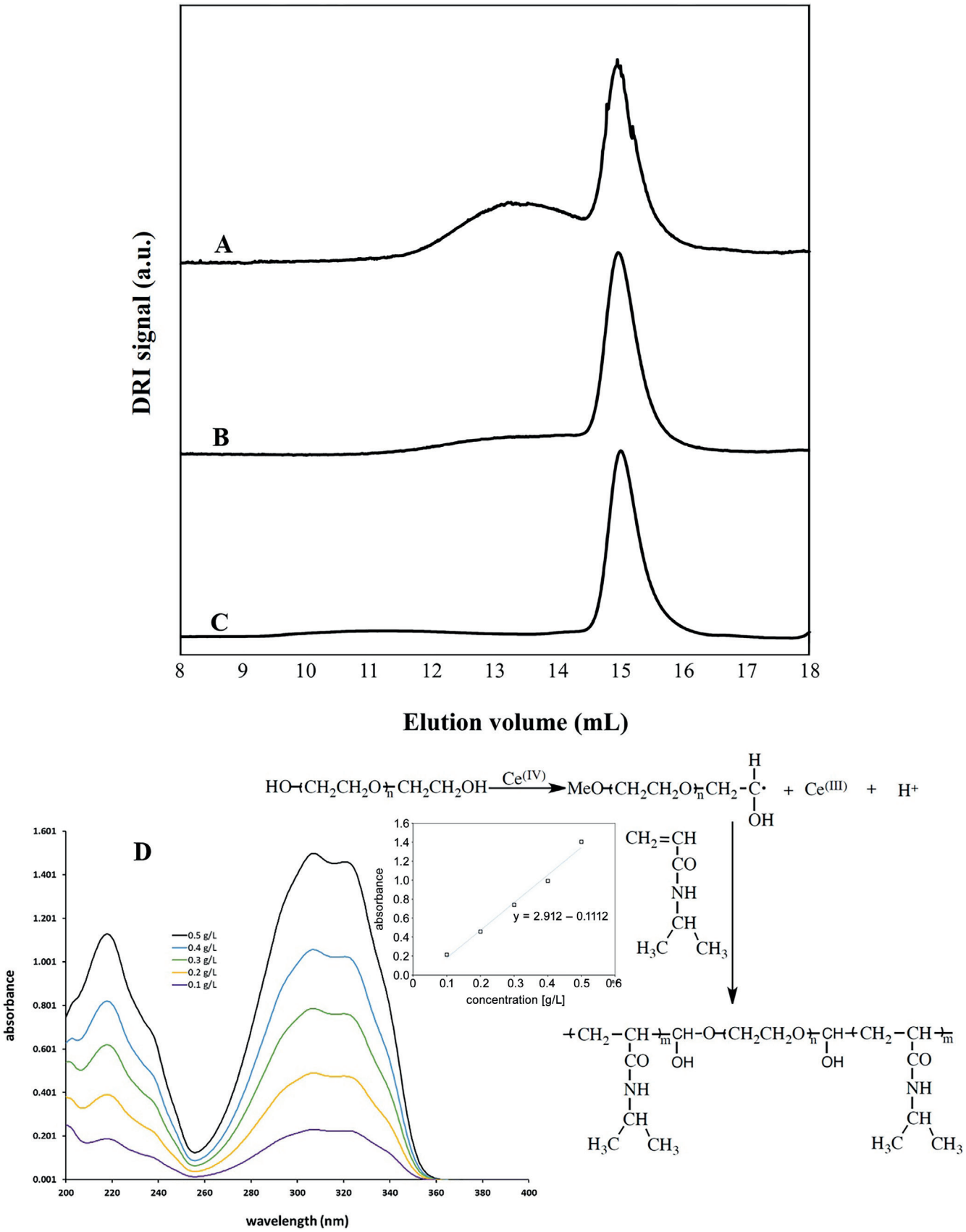

Fig. 1. Top: Synthesis scheme of PNIPAM-*b*-PEO-*b*-PNIPAM. Bottom: gel permeation chromatography (GPC) traces for the crude product synthesis with (A) reactants added through a cannula and using high-purity nitrogen, (B) reactants added through a cannula and degassed with an in-house nitrogen line, and (C) degassed in-house nitrogen; D. UV-vis spectra of samples with various resveratrol (RSV) concentrations and the calibration curve

a scattering angle set to 90°. Transmission electron microscopy (TEM) was used (Hitachi-HT7700; Hitachi Ltd., Tokyo, Japan) to observe the morphology and profile of the micelles. The freeze-dried micelle suspension was dropped onto a copper surface. Phosphotungstic acid (0.1% mass fraction) was used for staining, and the morphology was observed under a lens after drying.

Rheology

Rheological behaviors were investigated using a stress-controlled rheometer (Discovery HR-2; TA Instruments, New Castle, USA) Solutions with viscosities greater than 100 Pa·s were tested using a parallel plate geometry with a 25-mm diameter plate, whereas samples with viscosities below 200 Pa·s were analyzed using a double wall concentric cylinder setup. All experiments were performed at 25 ±2°C. Experimental data were recorded within the range of sensitivity for the rheometer based on the specifications provided by the instrument manufacturer. Angular frequency sweep experiments were conducted under conditions of linear viscoelasticity, where both G' and G'' values remained unchanged with applied stress. The angular frequency interval ranged from 0.01 rad/s to 1,000 rad/s. Various frequency intervals were applied to different sample solutions to ensure that viscoelastic responses were within the sensitivity range of the rheometer, as described in the instrument manual. For the acquisition of viscosity profiles, solutions were subjected to shear with a shear rate ranging from 0.0001 s^{-1} to 100 s^{-1}.

Standard curve of UV spectra of RSV

Resveratrol was dissolved in ethanol to prepare a standard stock solution at a concentration of 0.1 mg/mL. To construct the standard curve, a series of RSV solutions with different concentrations in ethanol were prepared. Ultraviolet–visible (UV-vis) spectra of samples at various RSV concentrations were obtained using a UV-vis spectrophotometer (Shanghai Huicheng General Instrument Co., Ltd.).

Morphology observation

Scanning electron microscopy (SEM) (Hitachi-SU8000; Hitachi Ltd.) and TEM were utilized to observe the morphological structure of polymeric micelles. The solutions were frozen with liquid nitrogen and coated with a thin layer of gold for 60 s at 40 W under vacuum to ensure the electrical conductivity of SEM samples. An excitation voltage of 10 kV was applied for all SEM images.

In vitro release of RSV from polymeric micelles

Two samples (26 mg each) of lyophilized polymeric micelles were accurately weighed and loaded into separate dialysis bags. The lyophilized micelles were dissolved with 5 mL of phosphate-buffered saline (PBS; pH 7.5). Each dialysis bag was sealed and immersed in 25 mL of sodium salicylate solution (1 mol/L) and phosphate buffer (pH 7.5) for an in vitro release experiment conducted in a water bath shaker at 35°C. The solution outside the dialysis bags was replaced with fresh solution of equal volume at set time intervals; the displacement was 10 mL. High-performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity; Agilent Technologies, Waldbronn, Germany) was used to detect the content of RSV in the released solution. The cumulative release amount was calculated using Equation 1:

$$E_r\% = \frac{V_e \sum_{i=1}^{n-1} m_i + V_0 m_n}{m_p} \times 100\% \quad (1)$$

where *Er*% represents the cumulative release amount of RSV in %; *Ve* is the displacement volume in mL (10 mL); *V0* is the volume of released solution in mL (30 mL); *mi* is the mass concentration of RSV at the i^{th} sampling in μg/mL; *mp* is the mass of RSV in the loaded polymeric micelles in μg; and *n* is the displacement number.

Results

Synthesis and characterization of PNIPAM-*b*-PEO-*b*-PNIPAM

Several attempts were made to synthesize PNIPAM-*b*-PEO-*b*-PNIPAM. Initially, the polymerization yielded PNIPAM blocks with a high MW of 12,000 g/mol, albeit with a low overall yield. Although the exact yield was not quantitatively determined, it was qualitatively estimated by examination of the GPC experiments. Depending on the polymerization conditions, the ratio of peak intensity to shoulder decreased, indicating an increase in polymerization yield. Using the high-purity nitrogen, the yield was further increased. After degassing the polymerization vessel with high-purity nitrogen, a significantly higher MW product was obtained, as evidenced by a substantial increase in the signal of crude product. The GPC curves for various attempts are shown in Fig. 1A–C. Analysis of the GPC trace using light scattering and differential refractometer (DRI) detectors revealed a MW of 242,000 g/mol and a polydispersity index (PDI) of 1.43.

The ^{1}H NMR spectrum in Fig. 2A shows assignments for all protons found in the chemical structure of the copolymer sample. Resveratrol was loaded into the polymeric micelle, and the ^{1}H NMR spectrum in Fig. 2B shows the chemical shifts of the protons from the RSV molecule. In contrast, Fig. 2C shows that RSV in D_2O without the polymer did not exhibit any peaks because RSV is highly hydrophobic.

To investigate the interaction and incorporation of RSV into PNIPAM hydrophobic junctions below the LCST, 2D nuclear Overhauser effect spectroscopy (NOESY)

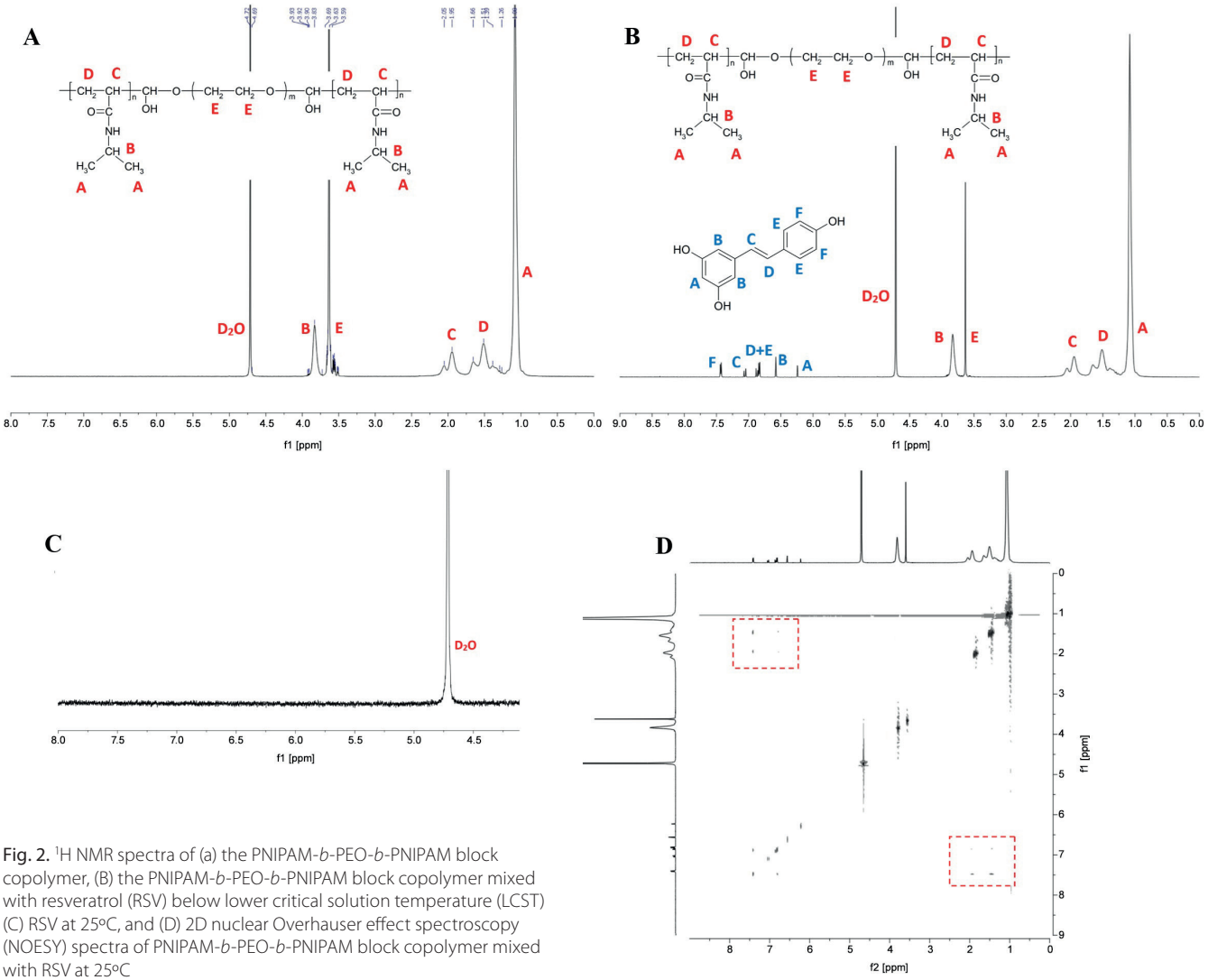

Fig. 2. ^{1}H NMR spectra of (a) the PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer, (B) the PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer mixed with resveratrol (RSV) below lower critical solution temperature (LCST) (C) RSV at 25°C, and (D) 2D nuclear Overhauser effect spectroscopy (NOESY) spectra of PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer mixed with RSV at 25°C

experiments were conducted, and the results are shown in Fig. 2D. In the spectra, there were some signals at the intersections of the peaks from RSV and the PNIPAM blocks, suggesting nuclear Overhauser effect (NOE) or proximity within 0.5 nm between protons of PNIPAM and the RSV molecule. Notably, Fig. 2D also indicates an interaction between the PNIPAM main chain and the RSV aromatic ring bearing 1 hydroxyl group, suggesting that the point of molecular interaction might be at the phenolic part of RSV.

Determination of CMC and *C**

Critical micelle concentration defines the concentration at which a surfactant or polymeric surfactant begins to form micelles. Above CMC, the concentration of free surfactant remains unchanged because additional surfactant molecules will only form more micelles. At CMC, the interface becomes saturated with surfactant molecules, resulting in constant surface tension with increasing surfactant concentration. Figure 3A shows the change in surface tension with increasing polymer concentration of PNIPAM-*b*-PEO-*b*-PNIPAM at 25°C, with CMC determined to be 45 mg/L from the cross point of 2 fits with different slopes.

At *C**, polymer chains begin to overlap and intermolecular interactions occur. This leads to a sharp increase in solution viscosity due to polymer chain entanglement for water-soluble polymers and hydrophobic association for associating polymers such as PNIPAM-*b*-PEO-*b*-PNIPAM below LCST. Figure 3B shows a plot of viscosity versus polymer concentration of PNIPAM-*b*-PEO-*b*-PNIPAM at 25°C, with *C** determined to be 10.1 g/L from the cross point of 2 fits with different slopes.

Determination of the LCST

The LCST of PNIPAM homopolymer and PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer were determined using a UV-vis spectrophotometer and rheometer. Below the LCST of PNIPAM, the solution remains clear and transmits light at 486 nm with 100% transmission. As the temperature of the solution increases past the LCST

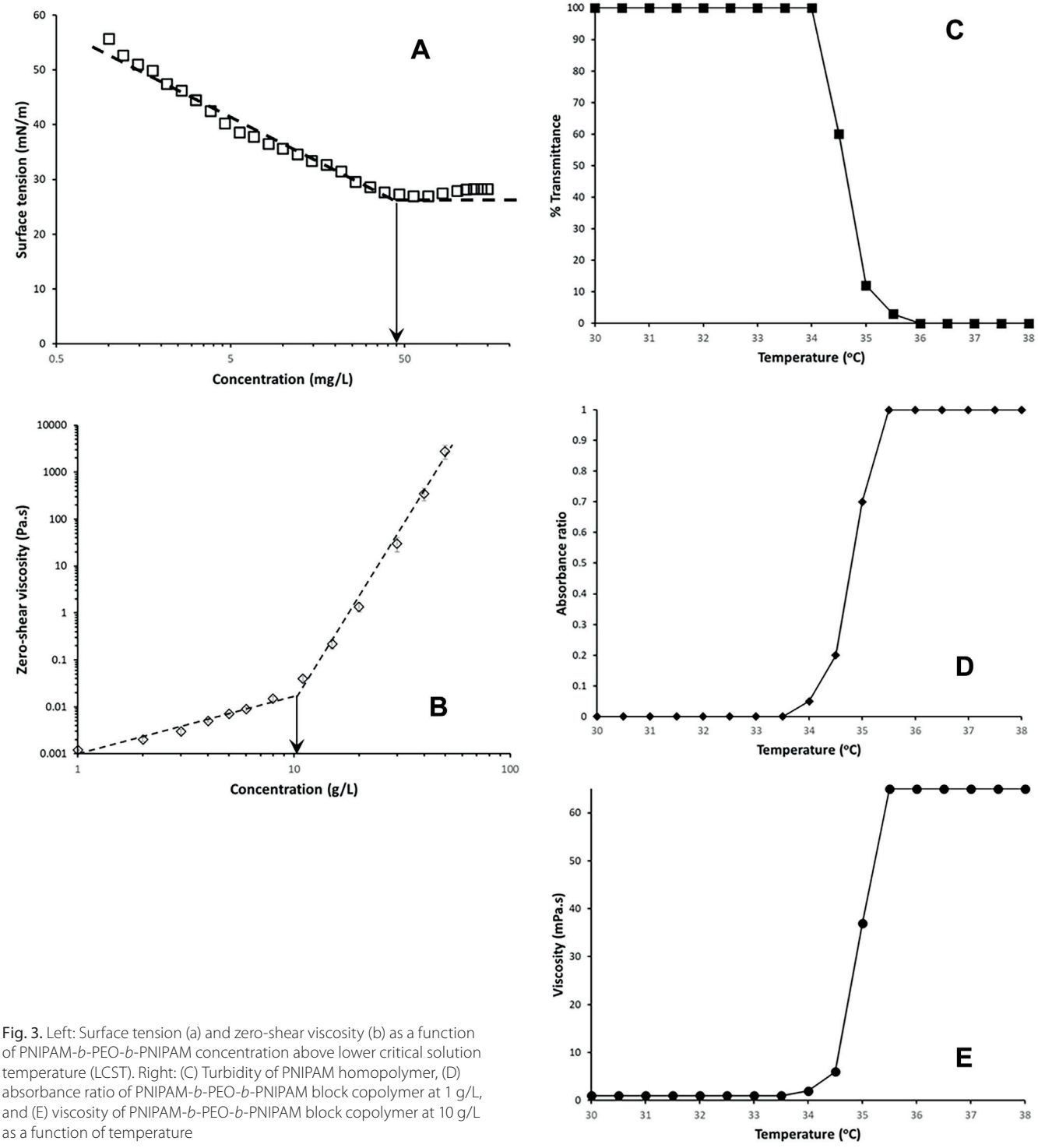

Fig. 3. Left: Surface tension (a) and zero-shear viscosity (b) as a function of PNIPAM-*b*-PEO-*b*-PNIPAM concentration above lower critical solution temperature (LCST). Right: (C) Turbidity of PNIPAM homopolymer, (D) absorbance ratio of PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer at 1 g/L, and (E) viscosity of PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer at 10 g/L as a function of temperature

of PNIPAM, phase separation occurs, the solution becomes turbid, and light transmission drops to 0%. This behavior is illustrated in Fig. 3C for the PNIPAM homopolymer.

For the LCST determination of PNIPAM-*b*-PEO-*b*-PNIPAM, RSV was mixed with the polymer solution at a concentration of 1 g/L. Below the LCST, PNIPAM segments are water-soluble, and hydrophobic RSV is released into the water, resulting in undetectable absorbance in the bulk solution and an absorbance ratio of zero. Above the LCST, PNIPAM becomes hydrophobic, and RSV dissolves in the hydrophobic core of the polymeric micelle. The absorbance at 307 nm matches that acquired with an equivalent amount of RSV in ethanol, resulting in an absorbance ratio of 1, as shown in Fig. 3D.

At a higher concentration (10 g/L) of PNIPAM-*b*-PEO-*b*-PNIPAM, the entire polymer chain is water-soluble below the LCST (Fig. 3E). Above the LCST, an associating polymer network forms to increase solution viscosity.

Using the above methods, results are presented in Fig. 3C–E. The LCST of PNIPAM-*b*-PEO-*b*-PNIPAM was determined to be 34.5°C, compared to 35°C for PNIPAM homopolymer.

Rheology and viscoelasticity

Figure 4A shows the steady-shear viscosity profiles as a function of shear rate obtained from aqueous solutions of the block copolymer at various concentrations above LCST. A Newtonian region was observed for all samples at lower shear rates, indicating constant viscosity regardless of shear rate. These viscosity values in the regimes are denoted as η_0 and represent the viscosity of the samples under no shear. Figure 4A demonstrates that increasing the block copolymer concentration from 1 g/L to 50 g/L significantly increased the solution viscosity by 10^6-fold, from 1 mPa·s to 2,000 Pa·s. These results suggest that above LCST, the PNIPAM segments become highly hydrophobic, facilitating efficient polymeric network formation through hydrophobic association. Figure 4A also shows that the viscosity significantly decreased, with an increase in shear rate when a shear force was applied to the sample solution. This phenomenon is defined as shear thinning, which is commonly observed in aqueous solutions of hydrophobically modified water-soluble polymers, and it has been widely studied in previous publications. Shear thinning typically results from a transition of inter-molecular hydrophobic associations to intra-molecular associations via the processes of "pull-out" and the rearrangement of hydrophobic units. Figure 4A shows significant shear thinning at lower onset shear rates for samples with higher viscosity, suggesting that this transition occurs more easily in polymer solutions with higher viscosity.

Figure 4B shows the G' and G" results as a function of ω for PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer solutions at various concentrations below LCST. Solutions above 40 g/L exhibited characteristic viscoelastic behavior resembling gels. At a concentration of 50 g/L, the G' and G" values changed only slightly with ω, and all G' values remained higher than G" values, suggesting the dominance of sample elasticity over the sample's viscous flow. Consequently, PNIPAM-*b*-PEO-*b*-PNIPAM at 50 g/L formed a physical gel in water via the hydrophobic association of PNIPAM segments.

Characterization of RSV-loaded polymeric micelles

Dynamic light scattering (DLS) experiments were conducted to measure the particle size distribution of resveratrol (RSV)-loaded polymeric micelles. Figure 5 shows the average diameter of micelles with 6.2% drug loading content (mass fraction). As seen in Fig. 5, the micelle diameter is approx. 100 nm, and the particle size distribution is relatively narrow.

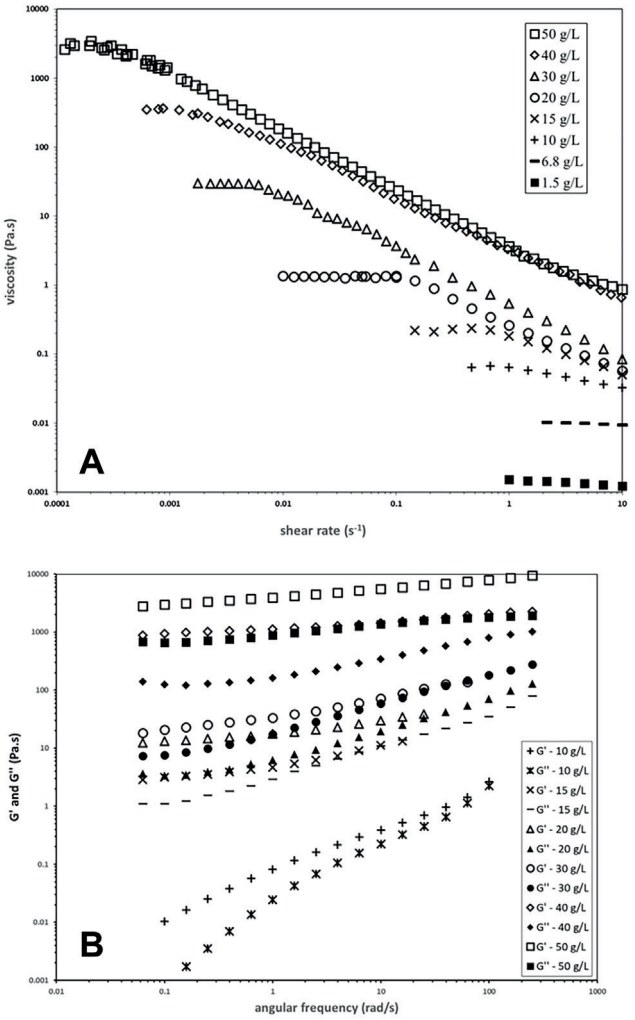

Fig. 4. A. Steady-shear viscosity as a function of shear rate for aqueous solutions of PNIPAM-*b*-PEO-*b*-PNIPAM at various concentrations above lower critical solution temperature (LCST); B. Storage and loss moduli (G' and G") as a function of angular frequency for aqueous solutions of PNIPAM-*b*-PEO-*b*-PNIPAM block copolymer at various concentrations above LCST

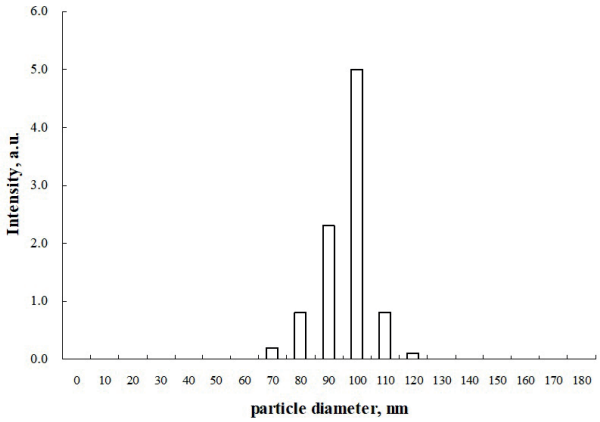

Fig. 5. Particle-size distribution of RSV-loaded polymeric micelles at 25°C

The same method was used to measure the particle size of RSV-loaded micelles with different drug dosages; the obtained data are listed in Table 1. With an increase in RSV

Table 1. The amounts of drug loading and releasing and particle diameter of polymeric micelle with various drug dosage

Drug dosage (by mass fraction, %)	Drug-loading amounts (by mass fraction, %)	Drug-releasing amounts (by mass fraction, %)	Average diameter of polymeric micelles [nm]
6.4	2.9	2.6	101.2
8.7	4.1	3.8	115.6
12.3	5.8	5.2	126.7
16.5	7.6	6.8	266.5

content, the size of polymeric micelles also slightly increased due to more drugs being loaded into the micelles, expanding the hydrophobic core of the polymeric micelle accordingly. At 16.5% RSV content, the micelle size changed significantly, likely due to exposure of a small amount of drugs to the aqueous environment, leading to secondary aggregation of micelles. The encapsulation efficiency of drug-loaded micelles prepared by the dialysis method was low, possibly due to the penetration of a small amount of RSV into the organic solvent from the dialysis bag during dialysis.

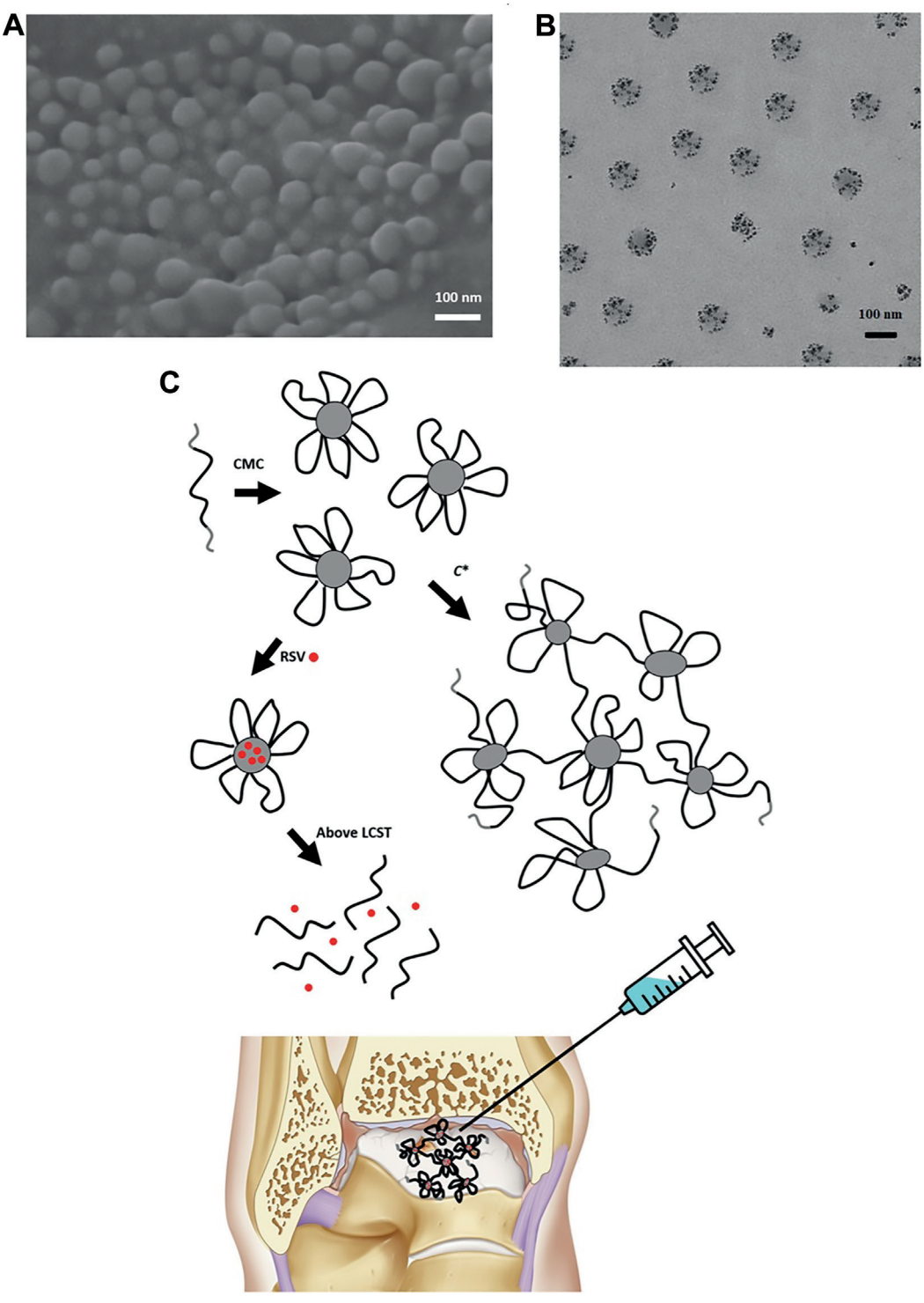

Fig. 6. A. Scanning electron microscopy (SEM) picture of resveratrol (RSV)-loaded polymeric micelles at room temperature; B. Transmission electron microscopy (TEM) picture of RSV-loaded polymeric micelles at room temperature; C. Proposed mechanism for injectable RSV-loaded polymeric micelles or hydrogels for cartilage regeneration

Figure 6A,B presents TEM images of the RSV-loaded polymeric micelles. Generally, the RSV-loaded micelles exhibit a spherical profile with an obvious core-shell structure, and the particle size is relatively uniform (around 100 nm), consistent with the results obtained using the DLS method.

Mechanisms of RSV-loaded polymeric micelles and hydrogel

As shown in Fig. 6C, an injectable RSV delivery system was developed by combining RSV-loaded PNIPAM-*b*-PEO-*b*-PNIPAM copolymer micelles with an in situ forming hydrogel at a high polymer concentration. This approach enhances the stability and retention time of the drug, thereby promoting the performance of cartilage regeneration. Below the CMC of 45 mg/L, the polymer exists as single chains. Above the CMC and the LCST, the PNIPAM blocks become hydrophobic, facilitating the formation of polymeric micelles with hydrophobic domains that accommodate the hydrophobic RSV molecules. As the concentration exceeds C^* = 10 g/L, a 3D polymeric network forms, increasing solution viscosity; a physical gel forms above 40 g/L. Below the LCST (34.5°C), the PNIPAM blocks become hydrophilic or water-soluble, causing the collapse of either the micellar structure or the polymeric network, thereby releasing RSV. Given that human body temperature exceeds the LCST of PNIPAM-*b*-PEO-*b*-PNIPAM block copolymers, RSV can be injected in vivo in the form of a hydrogel. Following injection, RSV will target damaged cartilage for repair and regeneration.

Discussion

Resveratrol is a hydrophobic drug. Theoretically, it can be encapsulated into a micellar system in an aqueous environment to establish a controlled release system for delivering RSV to a target site. However, such a system has never been studied or reported for RSV. Previous publications have successfully applied PNIPAM-based hydrogels for the delivery of various drugs, such as levofloxacin,[28] chemokine SDF-1 alpha,[29] melatonin,[30] ciprofloxacin,[31] doxorubicin,[32–34] ibuprofen,[35] and so on. These well-designed PNIPAM-based hydrogel systems have exhibited excellent behavior in drug delivery. Because all of the abovementioned drugs have properties similar to RSV, this study investigated the feasibility of using a PNIPAM and PEO-based hydrogel to deliver RSV. It demonstrated that RSV can be encapsulated into micelles formed by PNIPAM-*b*-PEO-*b*-PNIPAM below the LCST. Structural characterization of the complex indicated the specific position of RSV within the micelle, which is very important for molecular modeling and potential chemical modifications to improve the drug-loading performance. Resveratrol molecules insert themselves into the interior parts of PNIPAM hydrophobic domains to form a molecular complex. The methyl groups of PNIPAM and the resorcinol part of RSV are not involved in this complex because the aromatic structure is more compact and hydrophobic than linear alkyl chains. The π–π stacking interactions are easily formed between the conjugated structures of the aromatic ring. Polymeric surfactants containing aromatic structures may have a stronger interaction with RSV and could be used to encapsulate more RSV molecules compared to PNIPAM-*b*-PEO-*b*-PNIPAM.

The synthesized copolymer demonstrated a clear LCST. Below this temperature, PNIPAM-*b*-PEO-*b*-PNIPAM behaves like a polymeric surfactant at low concentrations and like an associating polymer at high concentrations. Above 50 g/L, the polymer formed a hydrogel through the hydrophobic association of PNIPAM segments. Resveratrol molecules can be loaded into the hydrogel for in vivo injection. After injection into an environment above the LCST, PNIPAM switches to a hydrophilic state to release the drugs. According to the results presented in Table 1, a large fraction of RSV was released from the polymeric micelle above the LCST, indicating significant potential for RSV delivery and release from this injectable hydrogel formulation.

Limitations

The effectiveness and performance of the RSV-loaded injectable formulation in the current study may be limited by in vitro experiments. Therefore, future studies should include in vivo experiments using experimental animals.

Conclusions

In this study, a thermo-responsive block copolymer consisting of a PEO main chain end-capped with PNIPAM blocks was synthesized for the preparation of RSV-loaded polymeric micelles and injectable hydrogels. The chemical structure of PNIPAM-*b*-PEO-*b*-PNIPAM was confirmed using ^{1}H NMR. The incorporation of RSV into the hydrophobic cores of polymeric micelles was confirmed with 2D NOESY, suggesting that RSV molecules associate with the interior of PNIPAM hydrophobic domains to form the molecular complex. However, the methyl groups of PNIPAM and the resorcinol part of RSV are not involved in this complex. The GPC results yield a polymer with MW of 242,000 g/mol and a PDI of 1.43. The CMC, C^* and LCST of PNIPAM-*b*-PEO-*b*-PNIPAM are 45 mg/L, 10.1 g/L and 34.5°C, respectively. We observed that the PNIPAM-*b*-PEO-*b*-PNIPAM solution exhibits a non-Newtonian plateau at very low shear rates, but at higher shear rates, the viscosity drops dramatically. The polymer at 50 g/L forms a physical gel in water due to the hydrophobic association of PNIPAM segments. The RSV-loaded micelles were successfully prepared; they exhibit a spherical profile and a particle size of 100 nm with a narrow size distribution.

In summary, this RSV-loaded hydrogel system prepared using thermal-responsive copolymers shows potential as an injectable formulation for delivering and releasing RSV for cartilage tissue regeneration.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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PPARγ alleviates damage to chorionic trophoblast cells induced by high glucose and high lipids through regulation of IGF-1

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Abstract

Background. Overweight and obesity are the most common high-risk conditions that increase the risk of adverse outcomes during pregnancy, childbirth, and the postpartum period. Dysfunctions in trophoblastic peroxisome proliferator-activated receptor gamma (PPARγ) contribute to a variety of related pregnancy disorders.

Objectives. This study investigated whether PPARγ influences chorionic trophoblast cell damage induced by high glucose (HG) and high lipid (HL) by regulating insulin-like growth factor-1 (IGF-1).

Materials and methods. Human trophoblast HTR-8/SVneo cells were exposed to HG and HL conditions to simulate damaged trophoblasts during pregnancy in vitro. Cell Counting Kit-8 (CCK-8) was used to assess cell proliferation. The Scratch test was used to test cell migration. Cell invasion ability was assessed by Transwell assay. ELISA was used to assess the inflammatory factor levels. Glucose, lactic acid, and adenosine triphosphate (ATP) levels were measured using biochemical kits.

Results. High glucose/HL inhibited the proliferation, migration, and invasion of HTR-8/SVneo cells. High glucose and HL increased tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 expression while decreasing IL-10 expression. High glucose and HL decreased glucose uptake and ATP levels. High glucose and HL reduced the expressiofns of PPARγ, IGF-1, insulin receptor substrate (IRS) 1, IRS2, GLUT1, and GLUT4. High PPARγ expression promoted cell proliferation, migration, and invasion induced by HG and HL, increased glucose uptake and ATP levels and inhibited inflammation. Low IGF-1 expression inhibited cell proliferation, migration, and invasion under HG and HL conditions, reduced glucose uptake and ATP levels, and increased inflammation. Low IGF-1 expression reversed the effects of PPARγ on HTR-8/SVneo cells under HG and HL conditions.

Conclusions. Peroxisome proliferator-activated receptor gamma alleviated HTR-8/SVneo cell damage induced by HG and HL by regulating IGF-1, suggesting a potentially effective approach for treating gestational obesity.

Key words: IGF-1, PPARγ, high glucose, high lipid, trophoblast cell injury

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Background

Epidemiological studies have shown that gestational obesity affects more than half of pregnancies in developed countries and is associated with obstetric complications and poor outcomes.[1] Gestational obesity is associated with various pregnancy complications, including gestational diabetes, gestational hypertension, preeclampsia, stroke, and venous thromboembolism.[2] Currently, dietary and lifestyle interventions for managing maternal obesity during pregnancy have shown limited effectiveness.[3] Therefore, further research into the molecular mechanisms underlying gestational obesity is crucial for developing new prevention and treatment strategies.

Obese pregnant women experience a lipotoxic placental environment characterized by increased inflammation and oxidative stress.[4] They also exhibit heightened insulin resistance, elevated plasma insulin, leptin, lipids, and potentially proinflammatory cytokines, along with lower plasma adiponectin, thereby increasing the risk of disease later in life.[5] Insulin signaling plays a crucial role in regulating cellular glucose uptake and maintaining blood glucose levels. Dysregulation of the insulin signaling pathway in placental trophoblastic cells, including insulin receptor, insulin receptor substrate (IRS) 1, IRS2, phosphoinositide 3-kinase, and glucose transporter (GLUT) 1, can lead to adverse outcomes in the context of obesity during pregnancy.[6]

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear transcription factor mainly expressed in adipose tissue. It promotes fatty acid storage in adipose tissue, regulates the expression of adipocyte-secreted hormones, and influences glucose homeostasis.[7] Peroxisome proliferator-activated receptor gamma is overexpressed in various types of trophoblasts during pregnancy.[8] However, unusually low PPARγ activity has been observed in placental pathology.[9] Studies have shown that PPARγ promotes trophoblastic differentiation and supports healthy placental function, potentially improving preeclampsia outcomes.[10] Peroxisome proliferator-activated receptor gamma agonists improve insulin resistance in human trophoblasts HTR-8/SVneo cell models exposed to high glucose (HG).[11] Additionally, PPARγ inhibits reactive oxygen species (ROS) production and inflammation levels in gestational intrahepatic cholestasis mice and taurocholic acid-treated HTR-8/SVneo cells.[12] Despite the known importance of PPARγ in trophoblast function, its precise role and mechanisms in protecting against or mitigating damage caused by HG and high lipid (HL) in HTR-8/SVneo cells are not yet fully understood. Further exploration and investigation are needed to elucidate the specific effects and underlying mechanisms of PPARγ in this context.

Insulin-like growth factor-1 (IGF-1), a key regulator of mammalian growth and metabolism, plays a crucial role in numerous cellular processes, including growth, differentiation, and transformation. It has become a focal point of research in various vascular diseases such as atherosclerosis, hypertension, angiogenesis, and diabetic vasculitis.[13] Previous studies have shown that activation of PPARγ by troglitazone leads to the modulation of IGF-1 gene expression in a dose-dependent manner.[14] Peroxisome proliferator-activated receptor gamma coactivator-1 alpha alternative splice variant 4 upregulates IGF-1 in cyclic AMP-regulated transcriptional coactivator 1/mastermind-like 2-positive mucoepidermoid carcinoma cells in a PPARγ-dependent manner.[15] MicroRNA (miR)-30a-3p is overexpressed in the placentas of preeclampsia patients, promoting HTR-8/SVneo cell invasion and inhibiting apoptosis by targeting IGF-1.[16] However, the roles and mechanisms of PPARγ and IGF-1 in cell damage in HTR-8/SVneo cells exposed to HG and HL remain unclear.

Objectives

This study aimed to investigate whether PPARγ influences HG- and HL-induced HTR-8/SVneo cell damage by regulating IGF-1.

Materials and methods

Cell culture and treatment

Human trophoblasts HTR-8/SVneo cells (AW-CNC496, Abiowell, China) were isolated from PMI-1640 supplemented with 5% fetal bovine serum (Hyclone, USA). The cells were grown in a CO_2 incubator at 37°C. As previously described,[17] low glucose (5.5 mmol/L glucose) is a typical condition for cell growth, mimicking normal physiological conditions, and an HG/HL cell model was constructed. Before treatment with HG/HL, the cells were treated with low glucose (5.5 mmol/L glucose) for 24 h. Subsequently, the cells were washed, and the medium was changed to HG (25 mmol/L glucose) and/or HL (0.4 mmol/L palmitic acid). The control group cells were cultured with low glucose for 24 h.

Cell transfection

HTR-8/SVneo cells were seeded in 6-well culture plates for 24 h. As previously described,[18] the cells were transfected with either the pcDNA3.1-PPARγ overexpression vector (2 μg) or pcDNA3.1-negative control (NC) (2 μg), and small interfering RNA (si)-NC (20 nM) or si-IGF-1 (HG-Si000875, Honorgene, China) (20 nM), using Lipofectamine 3000 reagent (ThermoFisher Scientific, USA). The sequence of si-IGF-1 used was 5'-ATTTCTTGAAGGTGAAGATG-3'. Cells were harvested 48 h after transfection.

Cell Count Kit-8 detection

As previously described,[19] the CCK-8 assay was used to measure cell viability. After 48 h of cell incubation, the CCK-8 reagent (AWC0114a, Abiowell, China) was added for 3 h. Cell viability was analyzed at 450 nm using a microplate reader (MB-530, HEALES).

Scratch test

A 20 µL pipette tip was used to form a wound through a single layer of cells. After removing the scratched cells, serum-free RPMI-1640 medium was added. Continuous images were captured under an optical microscope at 0, 24, and 48 h. Wound healing progress was assessed by measuring the width of the scratch.

Transwell assay

The Transwell membrane was pre-coated with 50 µL of 100% Matrigel (Corning) and then dried at 37°C. Cells were added to the top chamber with serum-free medium, whereas a complete medium containing 10% FBS was added to the bottom chamber. Following a 24-h incubation at 37°C with 5% CO_2, cells in the top chamber were removed using a cotton swab. Invaded trophoblast cells on the Transwell membranes were fixed in cold ethanol for 20 min, stained with 0.1% crystal violet for 5 min, and counted using an inverted microscope (Zeiss, Germany).

Enzyme-linked immunosorbent assay

As previously described,[20] enzyme-linked immunosorbent assay (ELISA) kits (CSB-E04740h, CSB-E08053h, CSB-E04638h, CSB-E04593h, CUSABIO, Wuhan, China) were used to measure levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-10.

Glucose uptake assay

Glucose uptake by cells was determined using the cell glucose colorimetric assay kit (A154-1-1, Nanjing Jiancheng Bioengineering Institute, China). As previously described,[21] 1×10^7 pretreated cells were seeded into 96-well cell culture plates and incubated overnight at 37°C. The cell suspension was removed and centrifuged at 1000 rpm for 10 min, and the supernatant was discarded, leaving the cell pellet. The pellet was incubated with 250 µL of working solution at 37°C for 10 min. The results were read and analyzed at 505 nm using a microplate reader.

Adenosine triphosphate levels

Intracellular adenosine triphosphate (ATP) levels were determined using the ATP assay kit (A095-1-1, Nanjing Jiancheng Bioengineering Institute, China). As previously described,[22] 100 µL of cell lysate was mixed with 100 µL of the ATP reaction mixture and incubated for 30 min. Absorbance was measured at a wavelength of 636 nm using a microplate reader.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from cells using TRlzol reagent (15596026, ThermoFisher Scientific, USA) and reverse-transcribed into complementary DNA (cDNA) using reverse transcription kits (CW2569, CWBIO, China). PCR amplification was performed with UltraSYBR mixture (CW2601, CWBIO, China) using the PIKOREAL96 fluorescent quantitative PCR system by ThermoFisher. β-actin mRNA was used for normalization, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Table 1. The primers used in this study

Genes name	Primer sequences
PPARγ	F: 5′-TGCTCCAGAAAATGACAGACC-3′ R: 5′-ATTTTCCCTCAGAATAGTGCAAC-3′
IGF-1	F: 5′-AGAGCCTGCGCAATGGAAT-3′ R: 5′- TTGGGTTGGAAGACTGTGA-3′
β-actin	F: 5′-ACCCTGAAGTACCCCATCGAG-3′ R: 5′-AGCACAGCCTGGATAGCAAC-3′

PPARγ – peroxisome proliferator-activated receptor gamma; IGF-1 – Insulin-like growth factor-1.

Western blot

RIPA buffer (AWB0136, Abiowell, China) was added, and the cell supernatant was collected after centrifugation. Protein concentration was determined using the BCA protein quantification kit. Subsequently, proteins were isolated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was then blocked with 5% skimmed milk for 1.5 h. Next, the membrane was incubated with a primary antibody (Table 2). After washing with TBST, the membrane was incubated with a secondary antibody for 2 h. The ChemiScope6100 system (CLiNX) captured the chemiluminescent signal from the membrane and produced a digital image of the protein bands. Image band densities were then calculated using ImageJ software (ImageJ 1.5, USA). Beta-actin was used as the internal reference.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software Inc., USA). Data are presented as median (max and min) (n = 6). The Mann–Whitney U test was used for comparisons between the 2 groups, whereas the Kruskal–Wallis H test, followed by Dunn's

Table 2. The antibody used in this study

Names	Dilution rate	Article number	Manufacturers	Country
PPARγ	1:500	16643-1-Ap	Proteintech	USA
IGF-1	1:1000	ab182408	Abcam	UK
IRS-1	1:1000	ab131487	Abcam	UK
IRS2	1:500	20702-1-AP	Proteintech	USA
GLUT1	1:500	21829-1-AP	Proteintech	USA
GLUT4	1:2000	66846-1-Ig	Proteintech	USA
β-actin	1:500	66009-1-Ig	Proteintech	USA
HRP polyclonal Goat anti-Mouse IgG (H+L) secondary antibody	1:5000	AWS0001	Abiowell	China
HRP polyclonal Goat anti-Rabbit IgG (H+L) secondary antibody	1:5000	AWS0002	Abiowell	China

PPARγ – peroxisome proliferator-activated receptor gamma; IGF-1 – insulin-like growth factor-1; IRS2 – insulin receptor substrate; GLUT1 – glucose transporter 1; IRS-1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1; GLUT4 – glucose transporter 1.

post hoc test, was applied for multiple group comparisons. Statistical significance was defined as $p < 0.05$. Supplementary Tables 1–5 contain statistical analysis results corresponding to the figures.

Results

High glucose and HL inhibited HTR-8/SVneo cell proliferation, migration, and invasion II rzedu

To investigate the effects of HG and HL on cell proliferation, migration, and invasion, CCK-8, scratch assay, and Transwell assay were performed. The results showed that HG and HL inhibited cell proliferation (Fig. 1A), cell migration (Fig. 1B), and cell invasion (Fig. 1C). High glucose and HL had superimposed effects. Compared with the HG group, HL further reduced cell proliferation, migration, and invasion (Fig. 1A–C). Our results suggested that HG and HL inhibited HTR-8/SVneo cell proliferation, migration, and invasion. Gestational obesity is a complex state involving multiple environmental changes, and the environment of HG and HL better simulates the pathological state of gestational obesity.[23,24] Therefore, HG plus HL cell models were constructed for subsequent in vitro studies.

High glucose and HL promoted cellular inflammation

Next, the effects of HG and HL on inflammation levels were further investigated. Proinflammatory factors TNF-α, IL-1β, and IL-6 play important roles during pregnancy in obese women.[25] CircSESN2 exacerbates HG-induced HTR-8/SVneo cell injury by binding to IGF2BP2 and increasing TNF-α, IL-1β, and IL-6 protein expressions.[26] Our results showed that HG and HL upregulated TNF-α, IL-1β, and IL-6 expressions while downregulating IL-10 expression in HTR-8/SVneo cells (Fig. 2A). Additionally, HG and HL decreased cellular glucose uptake and ATP levels (Fig. 2B). PPARγ, encoded by PPARG, is involved in angiogenesis, metabolic processes, anti-inflammatory responses, and reproductive development.[27] PPARγ promotes HTR-8/SVneo cell proliferation and migration while inhibiting inflammation.[28] IGF-1 is an important fetal growth factor, and LncRNA-SNX17 decreases miR-517a expression, thereby increasing IGF-1 expression in HTR-8/SVneo cells and enhancing cell proliferation and invasion.[29] Maternal obesity during pregnancy creates a proinflammatory environment that disrupts the response of beta cells to pregnancy endocrine signals, induces insulin resistance, and inhibits glucose uptake.[30] Klotho inhibits IGF-1 and insulin signaling molecules (IRS1, IRS2, and GLUT4) expression and glucose uptake in HG-induced HTR-8/SVneo cells, thereby decreasing cell viability.[31] Our results showed that HG and HL inhibited the expression of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4 (Fig. 2C). Overall, our findings suggest that HG and HL promote the inflammatory response in HTR-8/SVneo cells.

Upregulation of PPARγ inhibited cellular inflammatory response induced by HG and HL

To further explore the effect of PPARγ on inflammation under HG and HL conditions, HTR-8/SVneo cells were overexpressed with PPARγ. QRT-PCR results showed that oe-PPARγ increased PPARγ expression in cells (Fig. 3A). Oe-PPARγ enhanced proliferation under HG and HL conditions (Fig. 3B). Oe-PPARγ decreased TNF-α, IL-1β, and IL-6 expressions under HG and HL conditions, while increasing IL-10 levels (Fig. 3C). Additionally, oe-PPARγ increased glucose uptake capacity and ATP levels in cells under HG and HL conditions (Fig. 3D). Oe-PPARγ upregulated levels of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4 in cells under HG and HL conditions (Fig. 3E). In conclusion, high PPARγ expression reduced cellular inflammation levels under HG and HL conditions.

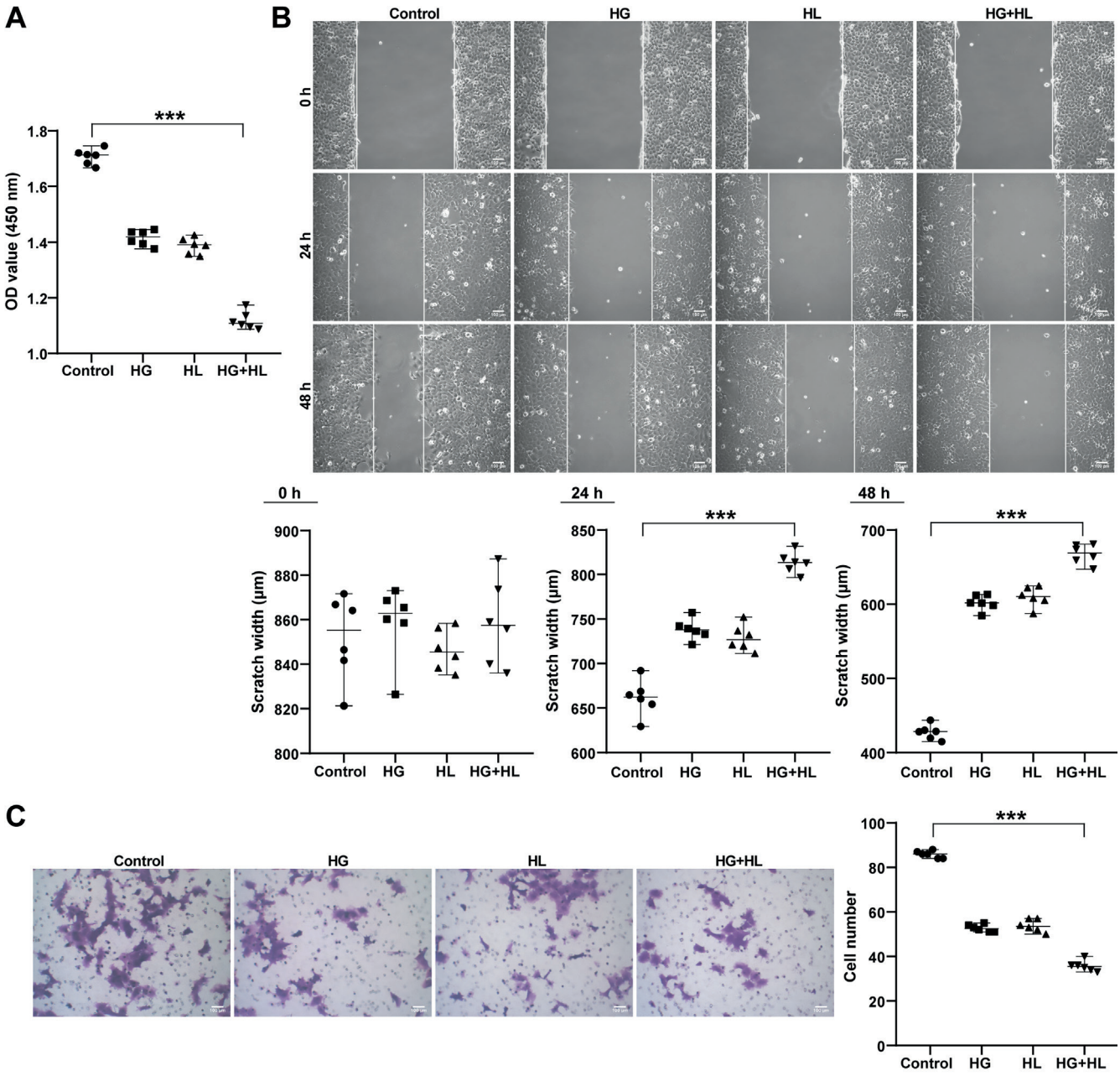

Fig. 1. High glucose and HL affected cell proliferation, migration, and invasion. A. Cell proliferation was measured by CCK-8; B. The scratch assay was used for cell migration; C. Transwell assay was used to measure cell invasion. Data are presented as median (max and min) (n = 6). Kruskal–Wallis H test and Dunn's post hoc test were performed among multiple groups; ***p < 0.001 compared to the control group

HL – high lipid; CCK-8 – Cell Counting Kit-8.

IGF-1 knockdown inhibited the cellular inflammatory response induced by HG and HL

Next, the effects of IGF-1 on HTR-8/SVneo cell inflammation under HG and HL conditions were investigated. QRT-PCR results showed that si-IGF-1 downregulated IGF-1 expression in cells (Fig. 4A). Si-IGF-1 inhibited cell proliferation under HG and HL conditions (Fig. 4B). Si-IGF-1 increased TNF-α, IL-1β, and IL-6 levels in cells under HG and HL conditions while decreasing IL-10 expression (Fig. 4C). Additionally, si-IGF-1 reduced glucose uptake capacity and ATP levels in cells induced by HG and HL (Fig. 4D). Si-IGF-1 downregulated the levels of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4 in HG and HL-induced cells (Fig. 4E). In conclusion, low IGF-1 expression promoted HG and HL-induced cellular inflammatory responses.

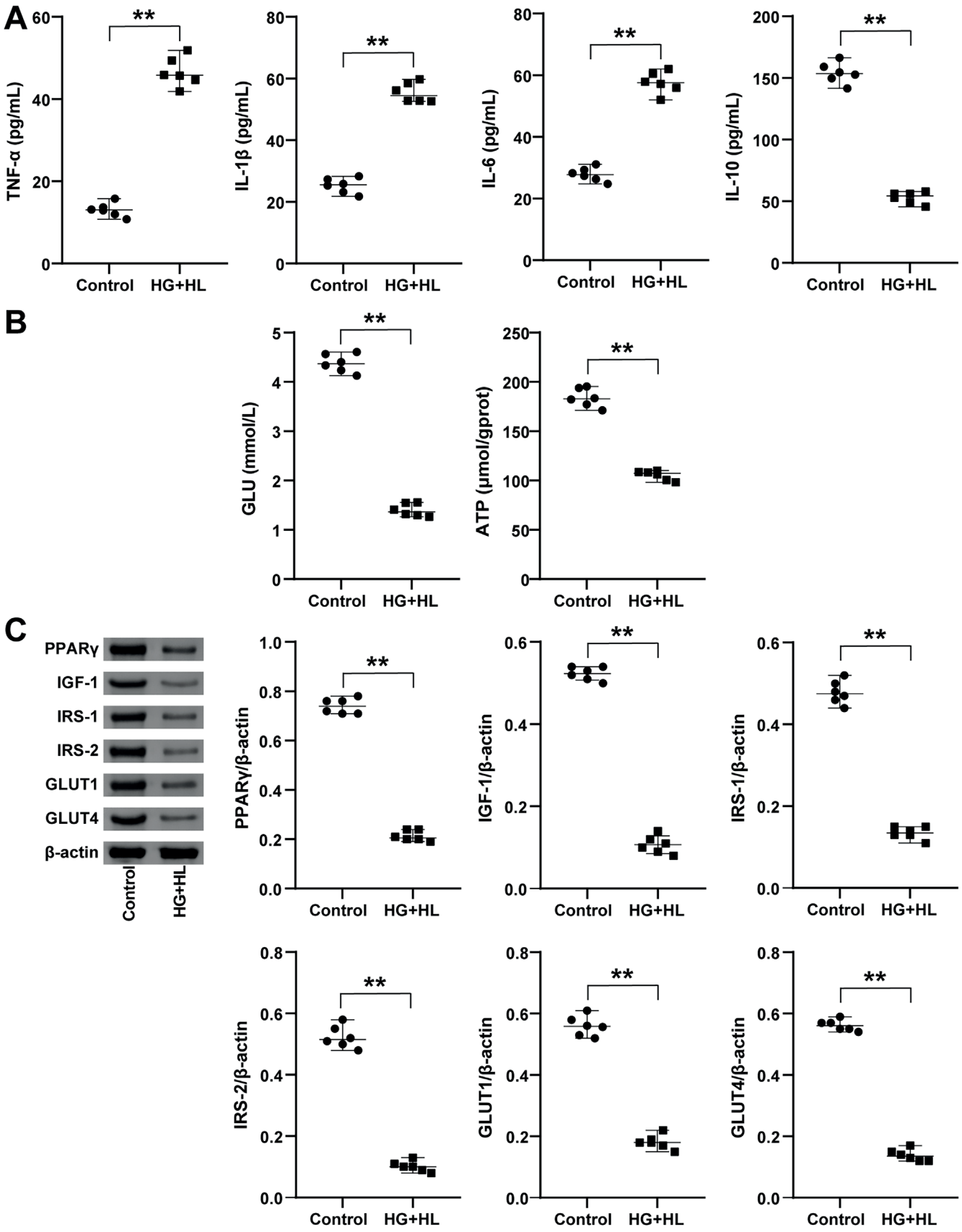

Fig. 2. High glucose and HL increased cellular inflammation. A. TNF-α, IL-1β, IL-6, and IL-10 levels; B. Glucose uptake and ATP levels were measured using biochemical kits; C. Western blot analysis of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4. Data are presented as median (max and min) (n = 6). The Mann–Whitney U test was performed between the 2 groups; *p < 0.05; **p < 0.01 compared to the control group

HL – high lipid; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; ATP – adenosine triphosphate; PPARγ – peroxisome proliferator-activated receptor gamma; IGF-1 – insulin-like growth factor-1; IRS1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1.

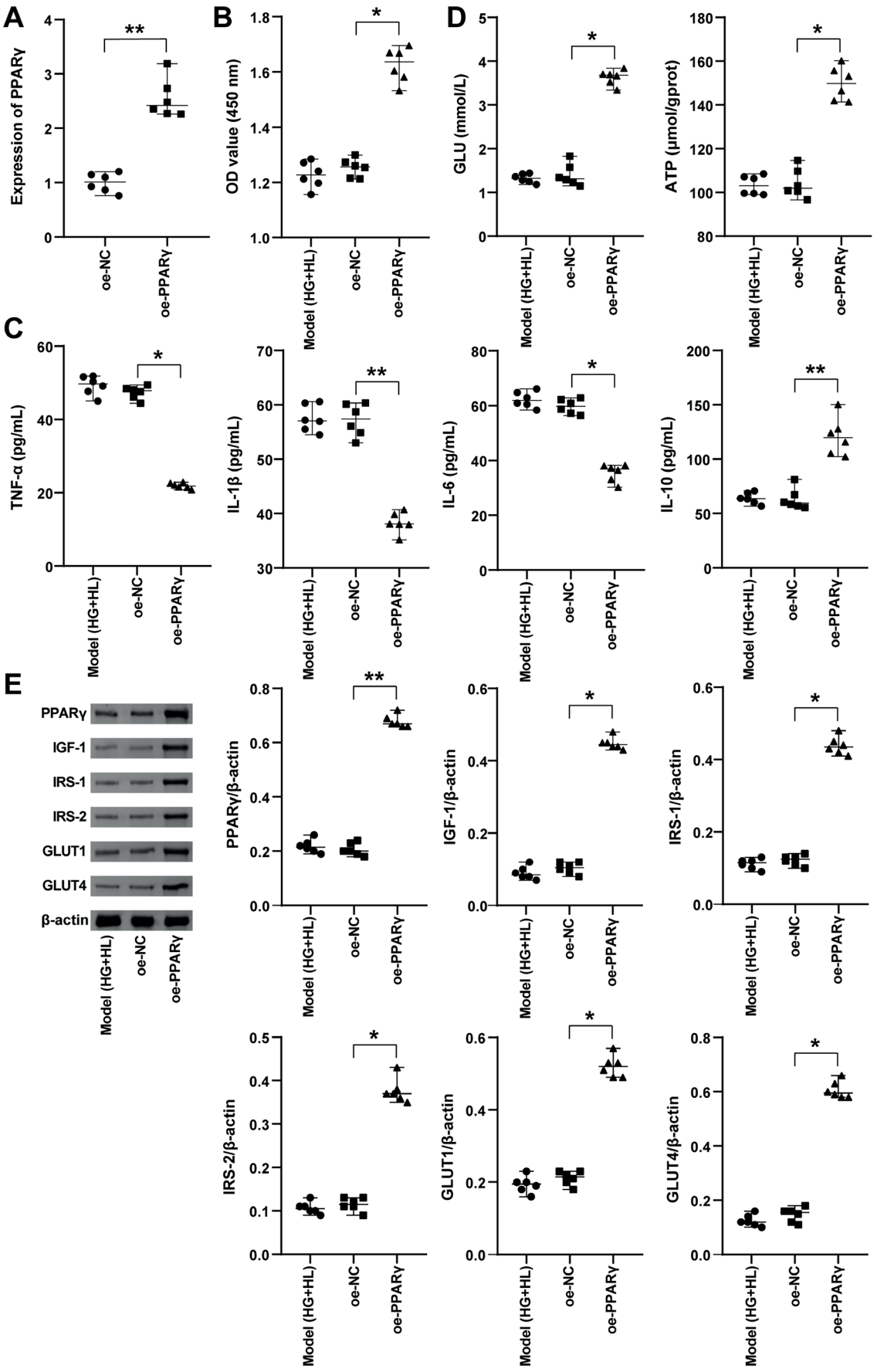

Fig. 3. PPARγ influenced cellular inflammatory response under HG and HL conditions. A. qRT-PCR analysis of PPARγ transfection efficiency; B. Cell proliferation; C. TNF-α, IL-1β, IL-6, and IL-10 levels; D. Glucose uptake and ATP production were measured using biochemical kits; E. Western blot analysis of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4. Data are presented as median (max and mini) (n = 6). The Kruskal–Wallis H test and Dunn's post hoc test were performed among multiple groups. The Mann–Whitney U test was performed between the 2 groups; *$p < 0.05$; **$p < 0.01$ compared to the oe-NC group

PPARγ – peroxisome proliferator-activated receptor gamma; Oe-NC – overexpression of negative control; Oe-PPARγ – overexpression of PPARγ; HG – high glucose; HL – high lipid; CCK-8 – Cell Counting Kit-8; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; ATP – adenosine triphosphate; IGF-1 – insulin-like growth factor-1; IRS1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1.

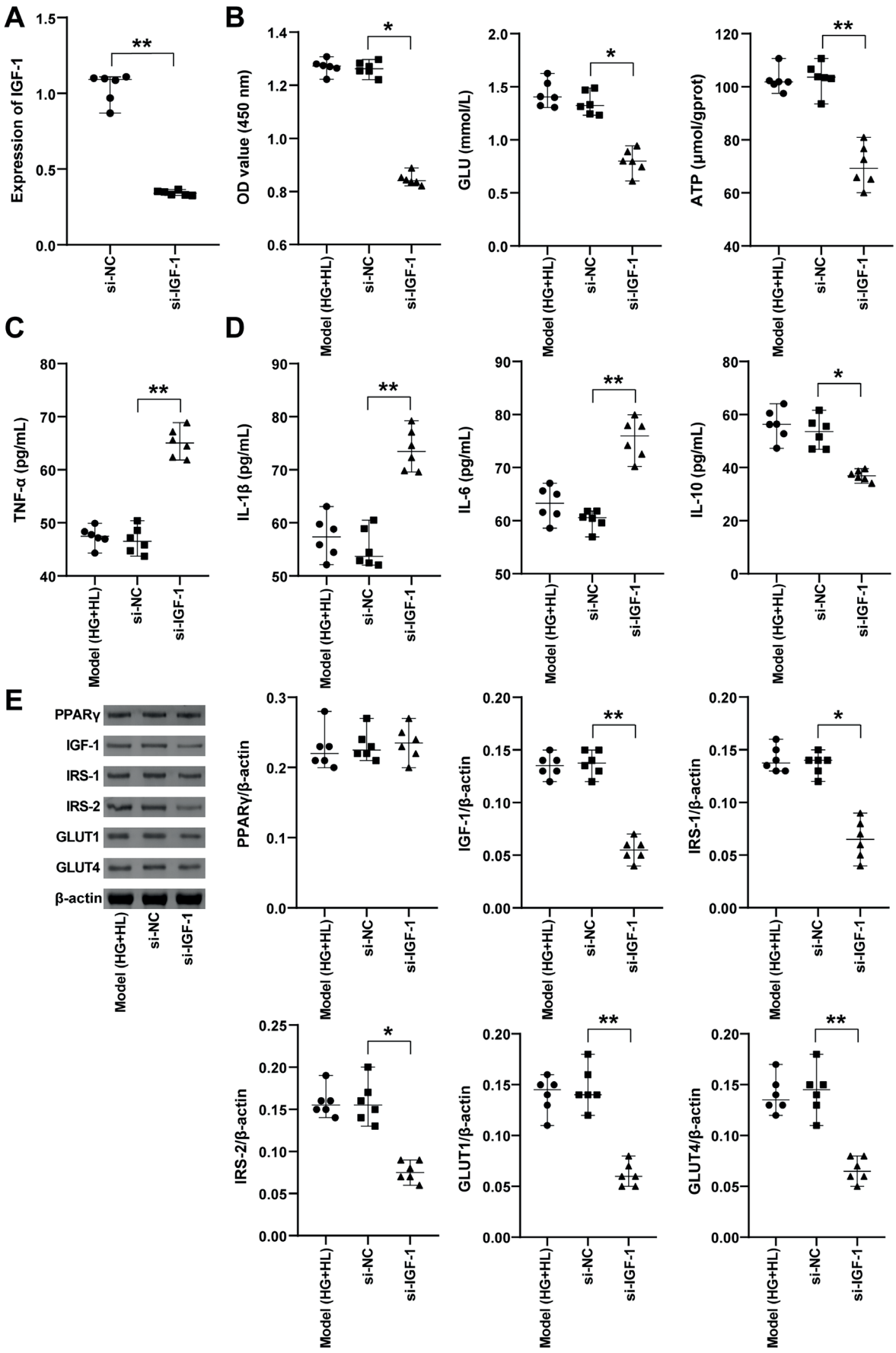

Fig. 4. IGF-1 affected cellular inflammatory responses under HG and HL conditions. A. Transfection efficiency of IGF-1; B. Cell proliferation was measured by CCK-8; C. TNF-α, IL-1β, IL-6, and IL-10 levels; D. Glucose uptake and ATP production were measured using biochemical kits; E. Western blot analysis of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4. Data are presented as median (max and min) (n = 6). The Kruskal–Wallis H test and Dunn's post hoc test were performed among multiple groups. The Mann–Whitney U test was performed between the 2 groups; *p < 0.05; **p < 0.01 compared to the si-NC group

IGF-1 – insulin-like growth factor-1; Si-NC – silencing of negative control; Si-IGF-1 – silencing of IGF-1; HG – high glucose; HL – high lipid; CCK-8 – Cell Counting Kit-8; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; ATP – adenosine triphosphate; IRS1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1.

PPARγ-mediated IGF-1 influenced the cellular inflammatory response and proliferation, migration, and invasion induced by HG and HL

The study further clarified whether PPARγ mediated IGF-1 to influence the inflammatory response and proliferation, migration, and invasion of HTR-8/SVneo cells under HG and HL conditions. PPARγ overexpression increased PPARγ and IGF-1 levels in cells, whereas si-IGF-1 downregulated IGF-1 levels (Fig. 5A). Oe-PPARγ enhanced the proliferation capacity of HG and HL-induced cells, whereas si-IGF-1 inhibited cell proliferation (Fig. 5B). Si-IGF-1 disrupted the promotion of HG- and HL-induced migration and invasion by oe-PPARγ, inhibiting these processes (Fig. 5C,D). Oe-PPARγ downregulated TNF-α, IL-1β, and IL-6 expression in HG and HL-induced cells, while upregulating IL-10 expression. Compared with the oe-PPARγ + si-NC group, TNF-α, IL-1β, and IL-6 expression was increased in the oe-PPARγ + si-IGF-1 group, whereas IL-10 levels were decreased (Fig. 6A). Glucose uptake capacity and ATP levels were increased in the oe-PPARγ + si-NC group compared with the oe-NC + si-NC group. Si-IGF-1 inhibited cellular glucose uptake and ATP levels (Fig. 6B). PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4 levels were upregulated in the oe-PPARγ + si-NC group compared with the oe-NC + si-NC group. In contrast, IGF-1, IRS1, IRS2, GLUT1, and GLUT4 levels were decreased in the oe-PPARγ + si-IGF-1 group compared with the oe-PPARγ + si-NC group (Fig. 6C). In conclusion, PPARγ promoted proliferation, migration, and invasion but reduced the cellular inflammatory response by upregulating IGF-1 under HG and HL conditions.

Discussion

We found that PPARγ overexpression reversed the effects of HG and HL on HTR-8/SVneo cells. PPARγ overexpression enhanced the cell proliferation, migration, and invasion induced by HG and HL, promoted glucose uptake and ATP levels, and reduced the inflammatory response. Knockdown of IGF-1 reduced the cell proliferation, migration, and invasion induced by HG and HL, inhibited glucose uptake and ATP levels, and promoted the inflammatory response. Knocking down IGF-1 reversed the effects of PPARγ overexpression on cells induced by HG and HL.

Diets containing HL and HG could lead to overweight/obesity.[32] Obesity in pregnant women is associated with impaired placental function, resulting in restricted development of placental blood vessels and fetal developmental disorders. Diallyl trisulfide reduces the expression of TNF-α and IL-1β in the placenta, promotes the expression of IL-10, and improves the reproductive performance of obese pregnant mice.[33] Palmitic acid increases TNF-α, IL-1β, and IL-6 levels in the placenta, inducing placental inflammation during pregnancy in mice.[34] The development of chorionic trophoblast cells, which comprise the outer layer of the placenta, is crucial for a successful pregnancy and plays a role in various pregnancy disorders, including gestational obesity.[35] Studies have shown that miR-134-5p inhibits FOXP2 expression, promotes TNF-α and IL-1β expression in HTR-8/SVneo cells under HG conditions, and inhibits IL-10 expression, thereby exacerbating gestational diabetes.[20] SESN2 upregulates TNF-α, IL-6, and IL-1β expression, exacerbating damage to HTR-8/SVneo cells under HG conditions.[26] We found that PPARγ decreased TNF-α, IL-1β, and IL-6 expression in HTR-8/SVneo cells under HG and HL conditions while increasing IGF-1 and IL-10 levels. IGF-1 knockdown played an opposite role in HTR-8/SVneo cells under HG and HL conditions, promoting TNF-α, IL-1β, and IL-6 expression while inhibiting IGF-1 and IL-10 levels. Furthermore, IGF-1 knockdown hindered the protective effect of PPARγ in HTR-8/SVneo cells under HG and HL conditions. Studies have shown that naringin upregulates IR-α and IGF1R expression in HTR-8/SVneo cells under HG conditions, increasing glucose uptake, cell proliferation, and migration.[6] Combined treatment with monounsaturated fatty acids can prevent palmitic acid-induced apoptosis of HTR-8/SVneo cells and reduce caspase 3/7 activity.[36] MiR-137 inhibits HTR-8/SVneo cell viability and migration under HG and HL conditions by downregulating FNDC5.[37] Blocking CYP1B1 inhibits HTR-8/SVneo cell proliferation, migration, and invasion under HG conditions.[38] Nesfatin 1 alleviates damage to HTR-8/SVneo cells under HG/HL conditions and improves cell viability.[17] Our results showed that PPARγ promoted proliferation, migration, and invasion of HTR-8/SVneo cells under HG and HL conditions, whereas IGF-1 knockdown reversed all of the above effects and inhibited proliferation, migration, and invasion. Our results were consistent with previous studies.

During normal pregnancy, the mother develops hyperinsulinemia, insulin resistance, hyperglycemia, and hyperlipidemia to provide nutrients to the fetus for growth and development.[39] Obesity during pregnancy may incorrectly induce or exaggerate metabolic and physiological changes and contribute to the risk of metabolic diseases for both the mother and the developing baby.[40] TDAG51 reduces blood glucose levels, enhances serum insulin, and reduces glucose and insulin resistance, improving gestational diabetes.[23] Studies have shown that β-carotene upregulates GLUT3, GLUT4, and IRS1 levels in insulin-resistant HTR-8/SVneo cells and increases glucose uptake to improve gestational diabetes mellitus.[41] ABHD5 promotes HG-induced expression of GLUT4, insulin receptor (INSR), IRS1, and IRS2 in HTR-8/SVneo cells to improve insulin resistance.[42] Klotho deletion reduces HG-induced cell viability, insulin signaling molecules (INSR-α, INSR-β, IRS1, IRS2, and GLUT4) expression, and glucose uptake while improving insulin resistance.[31] We found that the PPARγ promoted glucose uptake and

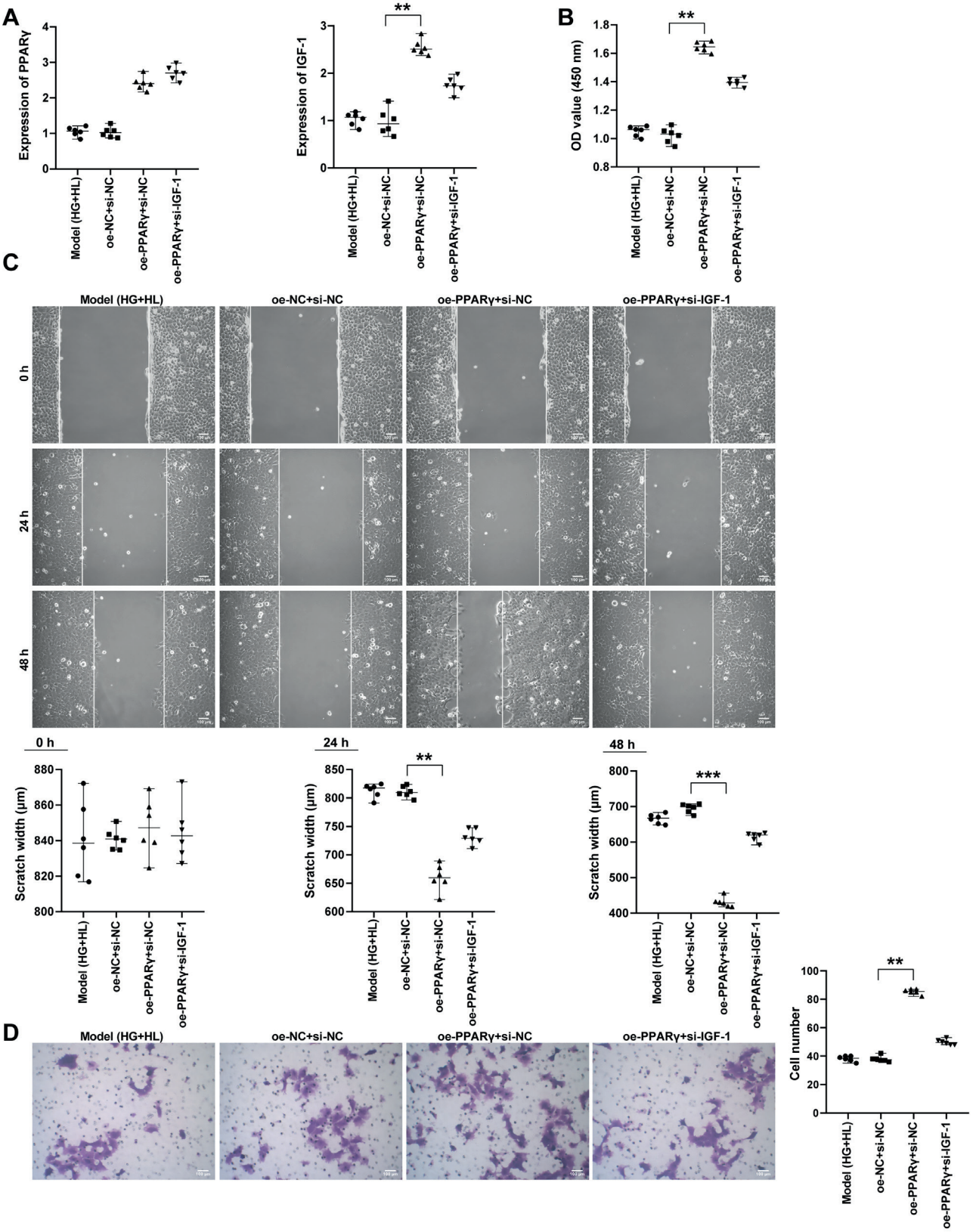

Fig. 5. PPARγ-mediated IGF-1 influenced cell proliferation, migration, and invasion interfered with HG and HL. A. PPARγ and IGF-1 mRNA expressions; B. Cell proliferation was measured by CCK-8; C. Scratch assay analysis of cell migration; D. Transwell was used to measure cell invasion. Data were presented as median (max and min) (n = 6). The Kruskal–Wallis H test and Dunn's post hoc test were performed among multiple group data; * $p < 0.05$, ** $p < 0.01$; ** $p < 0.001$ compared to the oe-NC+si-NC group

PPARγ – peroxisome proliferator-activated receptor gamma; Oe-NC – overexpression of negative control; Oe-PPARγ – overexpression of PPARγ; IGF-1 – insulin-like growth factor-1; Si-NC – silencing of negative control; Si-IGF-1 – silencing of IGF-1; HG – high glucose; HL – high lipid; CCK-8 – Cell Counting Kit-8; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; ATP – adenosine triphosphate; IRS1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1.

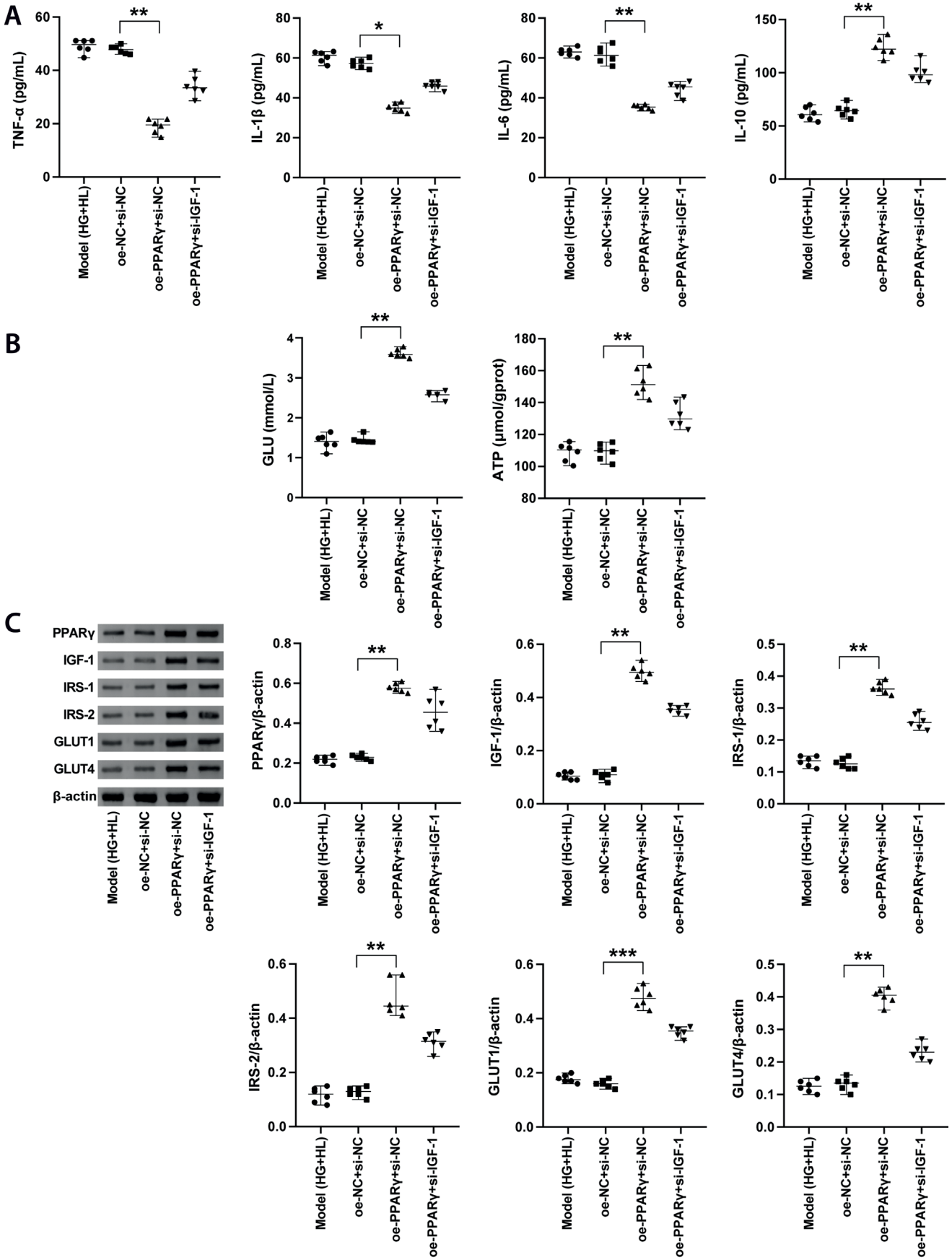

Fig. 6. PPARγ-mediated IGF-1 influenced cellular inflammatory response interfered with HG and HL. A. TNF-α, IL-1β, IL-6, and IL-10 levels; B. Biochemical kits were used to detect glucose uptake and ATP production; C. Western blot analysis of PPARγ, IGF-1, IRS1, IRS2, GLUT1, and GLUT4. Data were presented as median (max and min) (n = 6). The Kruskal–Wallis H test and Dunn's post hoc test were performed among multiple group data; * $p < 0.05$; ** $p < 0.01$; ** $p < 0.001$ compared to the oe-NC+si-NC group; PPARγ – peroxisome proliferator-activated receptor gamma

PPARγ – peroxisome proliferator-activated receptor gamma; Oe-NC – overexpression of negative control; Oe-PPARγ – overexpression of PPARγ; IGF-1 – insulin-like growth factor-1; Si-NC – silencing of negative control; Si-IGF-1 – silencing of IGF-1; HG – high glucose; HL – high lipid; CCK-8 – Cell Counting Kit-8; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; ATP – adenosine triphosphate; IRS1 – insulin receptor substrate 1; GLUT1 – glucose transporter 1.

increased ATP levels in HTR-8/SVneo cells under HG and HL conditions, increasing IGF-1, IRS1, IRS2, GLUT1, and GLUT4 expression. IGF-1 knockdown reversed the PPARγ effect on HTR-8/SVneo cells under HG and HL conditions, reducing glucose uptake and ATP levels and inhibiting the expression of IGF-1, IRS1, IRS2, GLUT1, and GLUT4. Therefore, PPARγ might emerge as a potential target for treating obesity in pregnancy.

Limitations

Certain limitations in this study should be taken into account when designing future studies. Because uterine smooth muscle cells,[43] endothelial cells,[44] and oocytes[45] influence the outcomes of obese pregnancy, further exploration into the potential molecular mechanisms of PPARγ in these cells is necessary. Successful pregnancy depends critically on well-regulated migration and invasion of placental villus trophoblasts.[46] The placenta is essential for nutrient and waste exchange between mother and fetus during pregnancy.[47] Obesity in pregnant women increases hypoxia, inflammation, oxidative stress, and mitochondrial dysfunction in the placenta.[48] Adipocyte-derived exosome NOX4 induces senescence of HTR8/SVneo cells, inhibiting cell proliferation and migration, and induces premature placental aging in obese pregnant mice.[49] Future studies should explore the potential molecular mechanisms of PPARγ in obese pregnant mice to strengthen the conclusions of this study based on these cell experiments. In this study, the sample size of the cell experiment was very limited. Future studies should aim to expand the sample size for exploration.

Conclusions

Our results suggest that PPARγ alleviates damage to HTR-8/SVneo cells induced by HG and HL by regulating IGF-1. These findings could offer new insights into potential strategies for the clinical treatment of gestational obesity.

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Venetoclax used alone, or in combination with cladribine, changes the expression of apoptosis-regulating genes in chronic lymphocytic leukemia cells in vitro

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Conflict of interest
None declared

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Abstract

Background. Venetoclax (VEN) and cladribine (2-CdA) are active agents in the treatment of chronic lymphocytic leukemia (CLL), although their precise pro-apoptotic mechanisms in CLL cells remain unclear. However, in vitro studies suggest that these drugs may alter the expression of several proteins involved in apoptosis.

Objectives. The aim of the study was to evaluate the effect of VEN and 2-CdA, used individually and in combination, on the expression of apoptosis-related genes in CLL cells in vitro.

Materials and methods. Mononuclear cells were collected from peripheral blood of 40 previously untreated CLL patients. The expression of 17 apoptosis-related genes was assessed using nCounter NanoString technology before and after 48-h in vitro incubation with VEN, 2-CdA or their combination (VEN + 2-CdA).

Results. Venetoclax + 2-CdA had a stronger effect on all tested genes except *BCL2* and *PMAIP* compared to VEN alone, and on *BID*, *BIK*, *FADD*, *P53*, and *SMAD3* compared to 2-CdA alone.

Conclusions. Venetoclax and 2-CdA may exert their pro-apoptotic effects on CLL cells in vitro, at least in part, by stimulating the expression of several apoptosis-related genes. The antileukemic activity of VEN is further enhanced when combined with 2-CdA.

Key words: apoptosis, cladribine (2-CdA), CLL, gene expression, venetoclax (VEN)

Highlights

- The influence of venetoclax (VEN), used alone or in combination with cladribine (2-CdA), on the expression of 17 apoptosis-involved genes was quantified in RNA using NanoString nCounter Analysis System (NanoString Technologies, Seattle, USA).
- Venetoclax not only inhibits the anti-apoptotic BCL-2 protein, which is its canonical function, but also alters the expression of several genes involved in apoptosis.
- The combination of VEN and 2-CdA more profoundly alters the expression of most apoptosis-related genes compared to either agent used alone.

Background

The most common leukemia in the Northern hemisphere is chronic lymphocytic leukemia (CLL). However, despite impressive progress in treatment, resulting in a considerable prolongation of survival, the condition remains incurable. Chronic lymphocytic leukemia peripheral blood lymphocytes have low proliferative potential, with the majority being blocked in the G0/G1 phase of the cell cycle in vivo.[1] Such inhibition of apoptosis prolongs their lifespan and is considered the primary reason for their accumulation in peripheral blood, lymph nodes and other organs.

For many decades, the cornerstone of CLL treatment has been the combination of alkylating agents and purine analogues with anti-CD20 antibodies. Recently, the role of immunochemotherapy has become increasingly important in many cases by the use of small molecules, i.e., oral treatments targeted at molecular phenomena involved in the inhibition of apoptosis, which are active also in cases with cytogenetic and/or molecular high-risk factors. Currently, routine treatment is based on the use of CLL inhibitors of Bruton's tyrosine kinase (BTKi) and the mitochondrial anti-apoptotic BCL2 protein, which is hyperexpressed in CLL.[2,3]

One of the most active drugs currently used in CLL is venetoclax (VEN), a highly selective BCL2 antagonist. Venetoclax is able to mimic the function of pro-apoptotic BH3-only proteins (BH3s). Its mechanism of action involves the intrinsic apoptotic pathway and it is able to directly induce the apoptosis independently of *TP53*, making it also active in patients with del17p/mut*TP53*.[3] When administrated in association with anti-CD20 antibodies, VEN is efficacious in the eradication of measurable residual disease (MRD), which is correlated with improvement of survival parameters. It is also active in patients after failure of BTKi.[4] However, a number of patients respond neither to BTKi nor to VEN or become resistant to both, and the management of these double-resistant patients is a challenge that has driven research into molecular-targeted drugs in CLL.[5,6] However, little is known about the possibility of combining VEN with classic chemotherapy in CLL. Such combinations were tested in acute leukemias, and the combination of VEN with azacytidine, decitabine or low-dose cytarabine is approved for the treatment of acute myeloid leukemias in frail patients.[7–9] Venetoclax has also been trialed with bendamustine, a drug combining the properties of alkylating agents and purine analogue, as a treatment for CLL.[10–12]

Our previous in vitro studies[13] examined the influence of VEN, cladribine (2-chlorodeoxyadenosine, 2-CdA) and their combination on peripheral blood lymphocyte viability and apoptosis in 103 previously-untreated patients with CLL; that study also determined the expression of some proteins involved in apoptosis. 2-CdA is a purine analogue, which has been found to be efficacious in CLL and hairy cell leukemia. It is believed to act by incorporating into newly synthesized DNA in place of deoxyadenosine, thereby disrupting the DNA helix, inhibiting its repair, and ultimately inducing apoptosis – primarily through the intrinsic pathway.[14] We found a strong synergy between VEN and 2-CdA regarding their proapoptotic activity and potential to modify the cellular content of several factors involved in apoptosis. In particular, treatment increased the levels of active caspase-3, caspase-9, p53, BIM (Bcl-2-interacting mediator of cell death), BAX (Bcl-2-associated X protein), NOXA (Phorbol-12-myristate-13-acetate-induced protein 1), PUMA (p53 upregulated modulator of apoptosis), and FADD (Fas-associated death domain protein), while reducing the proportion of BCL2-expressing cells. These findings suggest that the drugs exert their effects by modulating the expression of key regulators of apoptosis.[13] To further explore the effect of VEN and 2-CdA on CLL lymphocytes, the present study examines whether the 2 drugs, used alone or in combination, modify the expression of selected genes encoding apoptosis-regulating proteins.

Objectives

Building on previous research, the present study aims to determine the influence of VEN and 2-CdA, used individually or in combination, on the expression of apoptotic proteins in CLL cells in vitro. It examines the effect of the drugs on the expression of the genes encoding the previously tested proteins.

Table 1. General characteristic of patients

Feature		Number of patients	
Total number of patients		40 (15 F; 25 M)	
Age (x, range) [years]		66 (46–85)	
Number of patients with known *IGVH* mutational status	MUT-CLL	8 (3 F; 5 M)	21 (7 F; 14 M)
	UMUT-CLL	13 (4 F; 9 M)	
	ND	19 (8 F; 11 M)	
Stage	RAI 0–2	26	
	RAI 3–4	14	
Cytogenetic aberrations	del13q	11	
	del17p	2	
	del11q	3	
	tri12+	2	
	normal karyotype	7	
	ND	17	

F – female; M – male; x – average; CLL – chronic lymphoid leukemia; *IGVH* – variable region of the immunoglobulin heavy chain; MUT – mutated *IGVH(+)* cases; UMUT-CLL – unmutated IGVH (–) cases; ND – no information available; RAI – staging system for CLL; del13q – deletion of part of the long arm of chromosome 13; del17p – deletion of part of the short arm of chromosome 17; del11q – deletion of part of the long arm of chromosome 11; tri12+ – trisomy of chromosome 12.

Materials and methods

Materials

Peripheral blood mononuclear cells (PBMNCs) were collected from 40 untreated CLL patients (15 women and 25 men; mean age 66 years, range 46–85 years) during routine diagnostic procedures at the Department of Hematology, Medical University of Lodz, and the Regional Multispecialistic Center of Oncology and Traumatology in Łódź, Poland (Table 1). The diagnosis was established according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) 2018 criteria. All patients gave their written informed consent to participate in this study. The study was approved by the Ethics Committee of the Medical University of Lodz (approval No. KE/1287/18 issued October 16, 2018).

Drugs

Venetoclax was supplied by Abbvie USA (Warsaw, Poland) and was used at a concentration of 40 nM, alone or in combination with 2-CdA. 2-CdA was purchased from Institute of Biotechnology and Antibiotics (Warsaw, Poland) and used at concentration of 16 µM. These concentrations were chosen according to previous research.[13]

Isolation of RNA material for gene expression

The Syngen Blood/Cell RNA Maxi Kit (Syngen Biotech, Wrocław, Poland) was used to isolate total RNA from the cell pellet obtained before the culture and after 48-h incubation. RNA isolation was carried out manually, according to the instructions provided by the manufacturer's kit. RNA purity was assessed with spectrophotometry with an acceptable purity of the material range of 1.9–2.1 at A260/A280 absorbance. This assessment was performed using the Tapestation 4200 (Perlan Technologies, Warsaw, Poland), which enables fully automatic RNA gel electrophoresis, and thus allows for the assessment of the total RNA quality. In case of several samples, it was necessary to use a kit for cleaning and concentrating of total RNA (Syngen RNA clean-up Kit; Syngen Biotech).

Isolation of DNA material for *IGVH* mutational status assessment

Total DNA was isolated from the cell pellet material obtained before culture using the Syngen Blood/Cell DNA Maxi Kit (Syngen Biotech) according to the manufacturer's protocol.

Gene expression

Gene expression in the isolated mononuclear cells was tested before and after 48-h incubation. We selected 17 genes known to encode factors involved in apoptosis including those the products of which had been researched in our previous study[13]; *BCL2*, *BAX*, *BBC3*, *BIM*, *P53*, *APAF1*, *BAK*, *BID*, *BIK*, *CASP3*, *CASP8*, *CASP9*, *CFLAR*, *FADD*, *NOTCH1*, *PMAIP*, and *SMAD3*. In some cases, the gene products had been researched previously. Gene expression was quantified as RNA on the NanoString platform (NanoString Technologies, Seattle, USA) using the nCounter Digital Analyzer. Normalization for RNA loading was performed by application of 3 housekeeping genes (*B2M*, *BUSB* and *GAPDH*). The expression itself was determined using the NanoString method in the Laboratory of Molecular Diagnostics, First Department of Pathology and Experimental Cancer Research at Semmelweis University in Budapest, Hungary, in cooperation with Biomedica (Piaseczno, Poland).

Assessment of *IGVH* mutation status

The *IGVH* mutational status was evaluated with polymerase chain reaction (PCR) and Sanger sequencing according to manufacturer's protocol, with our modifications. The *IGVH-IGHD-IGHJ* FASTA sequences were submitted to the ARResT/AssignSubsets tool (https://station3.arrest.tools/subsets/), which reports the assignment to major CLL stereotyped subsets, and to the IMGT/V-QUEST tool (https://www.imgt.org/IMGT_vquest/input), for analyzing rearranged immunoglobulin (IG) sequences and comparing the submitted IG sequences with IMGT reference directory sets. Mutational status was determined using PCR amplification and sequence analysis

of *IGVH-IGHD-IGHJ* gene rearrangements; 98% identity cut-off to germline was used to define MUT-CLL (<98% identity) and UNMUT-CLL (≥98% identity).[15]

Statistical analyses

Statistical analysis of the expression levels of the 17 studied genes (n = 40) was performed using nSolver v. 4.0 software (NanoString, Seattle, USA), which is specifically designed for the NanoString platform. The program was also used to generate a graphical interpretation of the results in the form of heatmaps, based on the z-score values for individual genes. In heatmaps, the z-score presents the number of standard deviations (SDs) that the expression of a gene in a particular sample deviates from its mean expression across all samples, thus providing a standardized metric for relative comparison across different genes. The OmicSelector program was used to calculate the fold change (FC) value, estimating the fold changes in the test material relative to the reference material. A FC < 0.7 indicates reduced gene expression, FC > 1.3 indicates increased gene expression, and FC values between 0.7 and 1.3 are considered indicative of stable gene expression.

The difference between the tested genes and the housekeeping genes was evaluated using the Livak method (ΔΔCt method). This approach estimates FC in gene expression by first normalizing the expression of the target gene to a reference (housekeeping) gene within each sample (ΔCt), then comparing this normalized expression between 2 experimental conditions (ΔΔCt), and finally calculating the FC as $2^{-\Delta\Delta Ct}$. Statistical analysis of gene expression after normalization of the results was performed using the Student's t-test (Welch's correction for heteroscedasticity was applied) with Benjamini–Hochberg (BH) correction for multiple comparisons (n = 40, $p < 0.05$ was considered statistically significant).[16]

In the case of 1 gene (*BCL2*), the p-value was obtained using the Mann–Whitney U test due to the non-normal distribution of the data. It is essential to include normalization in gene expression analyses (e.g., RNA-seq and reverse transcription quantitative polymerase chain reaction (RT-qPCR)) to correct for variations in RNA input, reverse transcription efficiency and experimental conditions in general, thus ensuring that gene expression measurements reflect true biological differences.[17,18] Volcano plots were created using VolcaNoserR software (https://huygens.science.uva.nl/VolcaNoseR).

Results

Before the analysis of gene expression, the material was assessed for purity and degree of degradation. The results indicated that purity was over 90%, and the material was degraded in only 9.65%, which was sufficient for NanoString analysis.

The influence of the culture conditions on the expression of the tested genes was determined with a control group. The cells were cultured for 48 h without the addition of drugs, and the final expression levels were compared to the initial values measured prior to culture (i.e., the reference values). The culture resulted in a significant decrease in *PMAIP*, *BCL-2*, *BAX*, *BIM*, *BAK*, and *CFLAR* gene expression, and an increase in the expression of *FADD*. Increase in *BID* gene (FC > 1.3) was not significant (Table 2, Fig. 1A). Changes in the expression of the genes during the control culture in individual patients are shown on the heatmap with hierarchical clustering of samples (n = 59 including duplicates) and genes (n = 17) (Fig. 1B).

Culture with VEN (n = 65 including duplicates) resulted in a significant increase (FC > 1.3) in the expression of 7 genes: *APAF1*, *SMAD3* (p = 0.001), *NOTCH1* (p = 0.002), *BBC3*, *BID*, *BIK*, and *FADD* (p = 0.000), in relation to the corresponding values before culture. This was accompanied by a nonsignificant increase in 4 genes (*TP53*, *CAS3*, *CAS8*, *CAS9*) and a significant decrease in 2: *BCL2* (FC = 0.587; p = 0.009) and *PMAIP* (FC = 0.141; p = 0.000). The expression of 4 genes (*BAK*, *BAX*, *BIM*, *CFLAIR*) did not change. Compared to the control culture after 48 h,

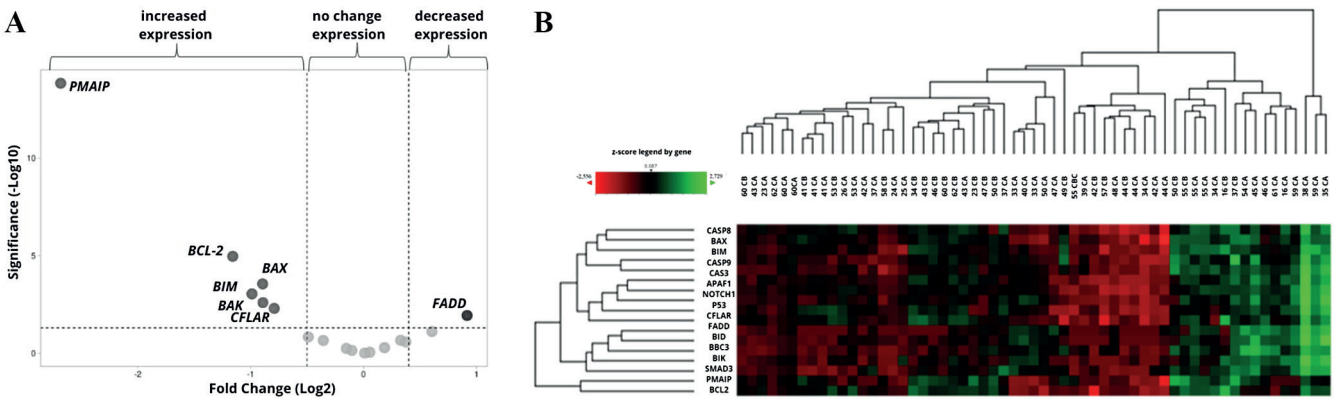

Fig. 1. A. Volcano plot showing differences in gene expression in control material before and after 48-h incubation; B. Heatmap with hierarchical clustering of samples (n = 59 including duplicates) and genes (n = 17) in the control material before and after 48-h incubation

Table 2. Changes in gene expression (FC) in control material before and after 48-h in vitro incubation

Gene	*APAF1*	*BAK*	*BAX*	*BBC3*	*BCL2*	*BID*	*BIK*	*BIM*	*CAS3*	*CASP8*	*CASP9*	*CFLAR*	*FADD*	*NOTCH1*	*P53*	*PMAIP*	*SMAD3*
FC (CA vs CB)	1.138	0.539	0.538	1.039	0.448	1.522	1.297	0.504	0.712	0.900	0.781	0.578	1.888	1.256	0.931	0.156	1.007
p-value	0.675	0.009	0.002	0.941	0.000	0.164	0.374	0.004	0.274	0.691	0.342	0.014	0.028	0.342	0.812	0.000	0.977

FC – fold change; CA – control after incubation; CB – control before incubation; p-value – Student's t-test with Benjamini–Hochberg (BH) correction; p-values <0.05 were considered statistically significant (bold); FC < 0.7 – reduced gene expression; FC > 1.3 – increased gene expression; FC (0.7–1.3) – no changes in gene expression.

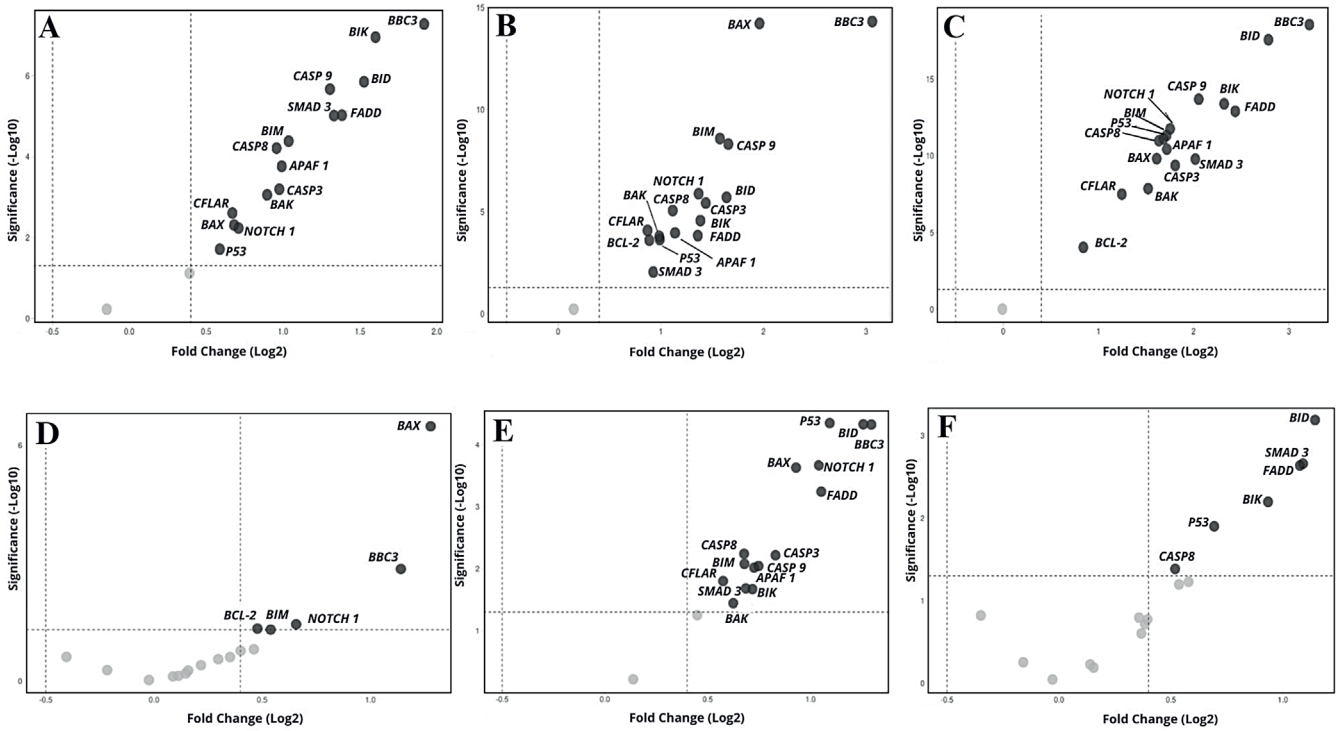

Fig. 2. Volcano plot showing differences in gene expression in samples treated: A. with 40 nM VEN; B. with 16 µM 2-CdA; C. with 40 nM VEN + 16 µM 2-CdA vs control material after 48-h incubation; D. with 16 µM 2-CdA vs 40 nM VEN; E. with 40 nM VEN + 16 µM 2-CdA vs 40 nM VEN; F. with 40 nM VEN + 16 µM 2-CdA vs 16 µM 2-CdA

VEN – venetoclax; 2-CdA – cladribin.

VEN significantly increased the expression of all genes except *BCL2* (FC = 1.312; p = 0.081), which showed a nonsignificant increase, and *PMAIP1* (FC = 0.904; p = 0.595), which remained unchanged (Fig. 2A, Fig. 3A).

The presence of 2-CdA in the culture (n = 58, including duplicates) resulted in a significant increase in the expression of *APAF1*, *BAX*, *BBC3*, *BID*, *BIK*, *CASP3*, *CASP8*, *CASP9*, *FADD*, *NOTCH1*, *TP53*, and *SMAD3* (FC > 1.3; p < 0.05), a nonsignificant increase in *BIM* (FC = 1.503; p = 0.076) and a significant decrease in *PMAIP1* expression (FC = 0.173; p = 0.000), compared to pre-culture values. No change in *BAK* (FC = 1.067; p = 0.768), *BCL2* (FC = 0.819; p = 0.324) or *CFLAR* (FC = 1.069; p = 0.768) was noted. Compared to the control cultures, 2-CdA significantly increased the expression of all genes apart from *SMAD3* (FC = 1.901; p = 0.095), which demonstrated an insignificant increase, and *PMAIP* (FC = 1.1103; p = 0.589), which did not change (Fig. 2B, Fig. 3B). Following culture with VEN and 2-CdA (n = 68, including duplicates), all genes showed increased expression compared to pre-incubation levels, except for *BCL2* (FC = 0.803; p = 0.233), which remained unchanged, and *PMAIP1* (FC = 0.155; p = 0.000), which showed a significant decrease.

The increase was not significant for *BAK* (FC = 1.548; p = 0.055) and *CFLAIR* (FC = 1.369; p = 0.129). The addition of both drugs resulted in a significant increase in all genes compared to controls, except *PMAIP* (FC = 3.209; p = 0.975), which was insignificantly higher, and *SMAD3* (FC = 0.994; p = 0.000) which did not differ (Fig. 2C, Fig. 3C).

To assess the impact of various drugs or drug combinations on gene expression, FC values were calculated relative to the expression levels observed under treatment with the comparator drug. Cultures treated with 2-CdA, *BAX* (FC = 2.427; p = 0.000) and *BBC3* (FC = 2.206; p = 0.012) showed significantly higher expression compared to cultures with VEN. Additionally, *BCL2*, *BIM*, *CASP3*, *NOTCH1*, and *TP53* exhibited nonsignificantly higher expression levels than those observed in the VEN-treated cultures (n = 75 including duplicates; FC > 1.3; p > 0.050) (Fig. 2D, Fig. 4A).

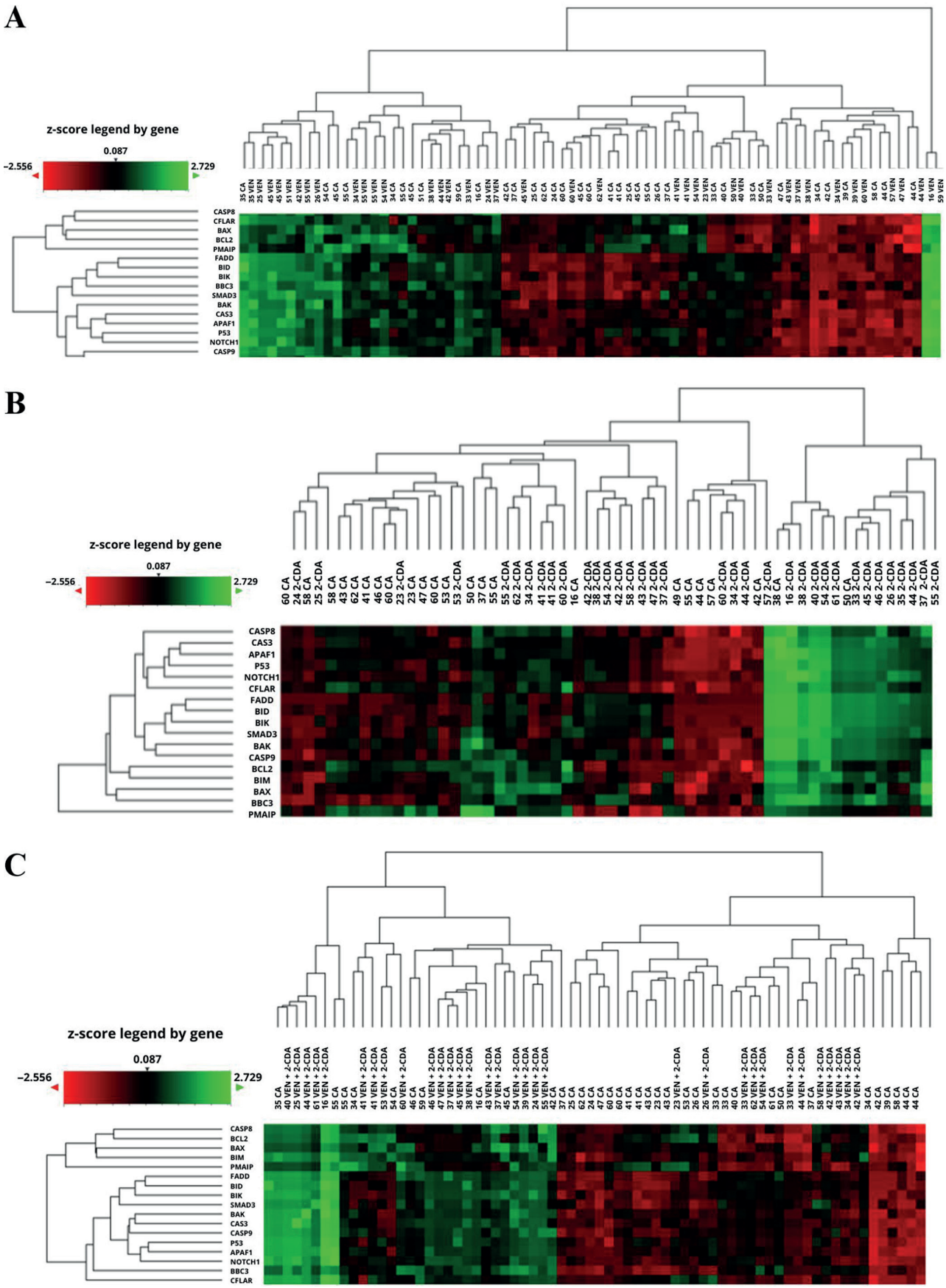

Fig. 3. Heatmap with hierarchical clustering of samples and genes (n = 17) in culture with VEN and control (n = 65 including duplicates (A) 2-CdA and control (n = 58 including duplicates) (B) and VEN + 2-CdAand control (n = 68 including duplicates) (C), after 48-h incubation

VEN – venetoclax; 2-CdA – cladrybin.

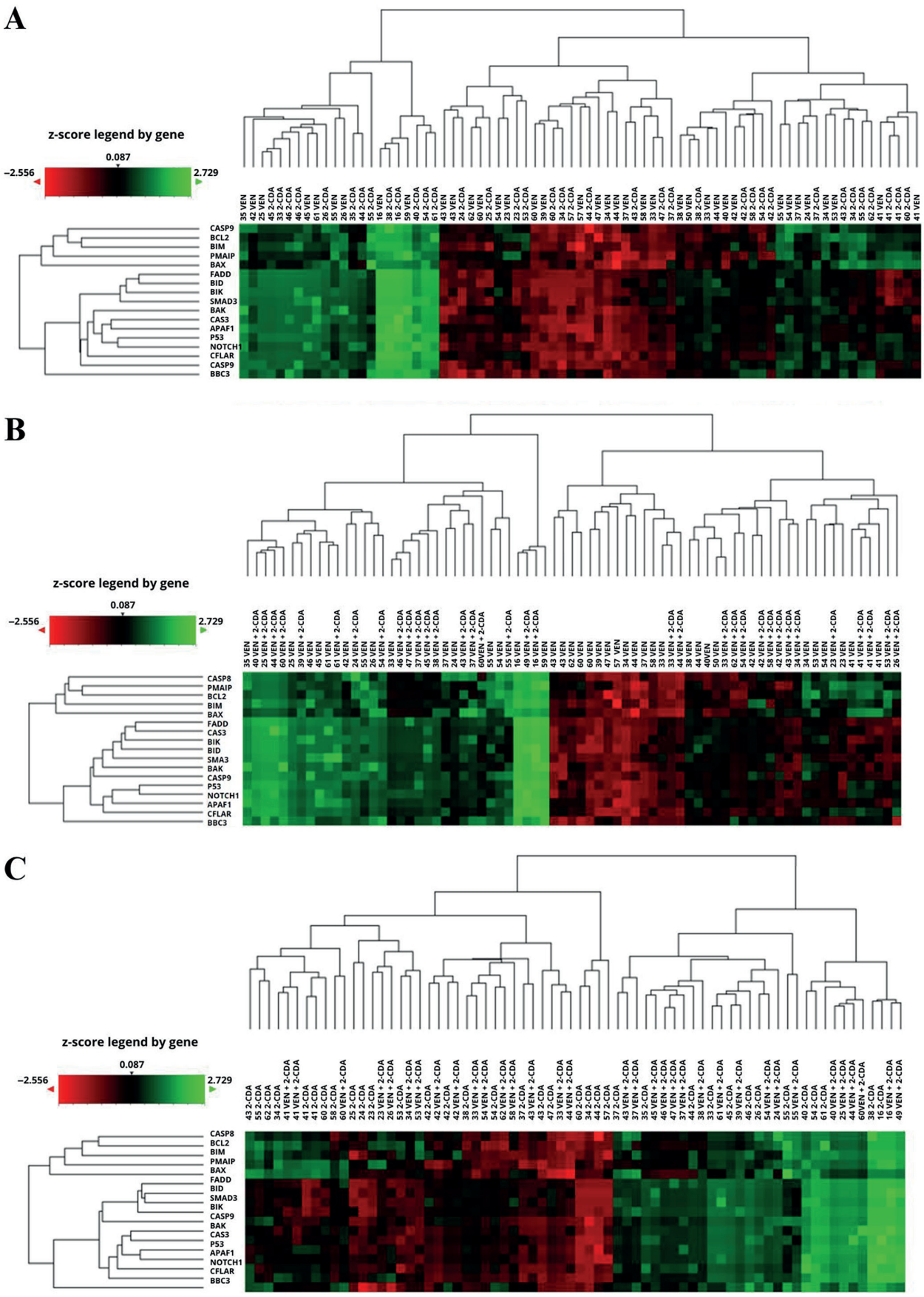

Fig. 4. Heatmap with hierarchical clustering of samples and genes (n = 17) in culture with VEN and 2-CdA alone (n = 75 including duplicates (A) VEN + 2-CdA and VEN alone (n = 73 including duplicates) (B) and VEN + 2-CdA and 2-CdA alone (n = 71 including duplicates) (C), after 48-h incubation

VEN – venetoclax; 2-CdA – cladrybin.

Similar changes in gene expression were observed under the influence of both drugs. All genes demonstrated significantly stronger stimulation under 2-CdA+VEN compared to VEN alone (n = 73 including duplicates), with the exceptions of *BCL2* (nonsignificant increase, FC = 1.3654, p > 0.050) and *PMAIP* (no changes, FC = 1.100) (Fig. 2E, Fig. 4B). In addition, *BID* (FC = 2.300, p = 0.011), *BIK* (FC = 1.911, p = 0.026), *FADD* (FC = 2.109; p = 0.013), *P53* (FC = 1.617; p = 0.043), and *SMAD3* (FC = 2.129; p = 0.013) demonstrated significantly higher expression under the influence of 2-CdA+VEN, compared to 2-CdA alone; only insignificant increases were noted for *APAF1*, *BAK*, *CAS 8*, *CAS 9*, and *NOTCH1* (p > 0.050).

To confirm whether *IGVH* mutational status influences the effect of the drugs on the expression of the genes tested, a graph was generated for each drug/drugs combination, and Spearman's correlation coefficient (r) was calculated for each experimental condition (n = 21). An r-value significantly different from 0 indicated that gene expression varied similarly under the experimental conditions in both mutated and unmutated cases – that is, IGHV mutational status did not appear to influence the genes' responses to the drugs (Table 3). As shown in Fig. 5 and Fig. 6, r was found to be statistically significant in all experimental conditions except for the relationship between VEN+2-CdA and VEN. Statistical analysis included Bonferroni correction for multiple comparisons (5 comparisons).

Discussion

The inhibition of apoptosis is a central feature of CLL pathogenesis, leading to the abnormal prolongation of leukemic lymphocyte survival. One of the causes of this phenomenon is believed to be the overexpression of the anti-apoptotic mitochondrial protein BCL2. The BCL2 family of proteins play a central role in the regulation of the mitochondrial pathway of apoptosis, thus exerting pro- or anti-apoptotic activity. The family encompasses anti-apoptotic agents (BCL-2, BCL-XL, MCL-1, BCL-W, BFL-1), as well as BH3-only sensitizers (BAD, BIK, NOXA, HRK, BMF) proposed to sensitize the mitochondrial outer membrane (MOM) for permeabilization by opposing anti-apoptotic family members, BH3-only activators (BID, PUMA, BIM), which induce the MOM via BAX/BAK, and pro-apoptotic effectors (BAX, BAK).[19,20]

Venetoclax has been found to be highly efficacious in CLL by inhibiting the anti-apoptotic protein BCL2. The mechanism of its action consists on direct binding to BCL-2 and displacing BIM from BCL2, which results in BAX/BAK homooligomerization. The BAX/BAK complex is responsible for the permeabilization of the mitochondrial outer membrane (MOM), the release of cytochrome c into the cytoplasm and the activation of caspase-mediated apoptosis.[4,13,21,22] Venetoclax is also effective in patients with a defective *TP53* gene, and when combined with an anti-CD20 antibody or BTKi, it enables the eradication of MRD in a significant proportion of patients, an outcome associated with prolonged progression-free survival and overall survival.[23]

However, a number of patients eventually develop resistance to drugs targeting pro-survival intracellular pathways, including VEN. Therefore, there is a need to determine the optimal use of molecular targeted drugs, which may include their combination with other antileukemic agents. 2-CdA belongs to a class of purine analogues, such as fludarabine and pentostatin, which until recently were widely used in both frontline therapy and in relapsed/refractory CLL patients. It acts by incorporating into newly synthesized DNA and inhibiting its repair, thereby triggering the intrinsic, p53-dependent apoptotic pathway. The induction of BAX protein expression leads to the translocation of cytochrome c from the mitochondria to the cytoplasm, formation of the apoptosome and initiation of the caspase cascade. 2-CdA may also act independently of P53 by interacting directly with mitochondrial proteins, thus leading to the release of cytochrome c and formation of apoptosome or apoptosis-inducing factor (AIF). In such cases, chromatin condensation and DNA fragmentation are triggered without caspase activation.[24–26] It has also been postulated to play a role in the extrinsic apoptosis pathway, but this remains controversial.[27,28]

In our previous study involving 103 treatment-naïve CLL patients,[13] we found that VEN, 2-CdA and their combination exhibited cytotoxicity and the ability to induce apoptosis. In addition, VEN and 2-CdA appeared to act synergistically, leading to an increased proportion of cells expressing proteins involved in both the intrinsic (BAX, PUMA, BIM, NOXA) and extrinsic (FADD) apoptotic pathways. We also observed a decrease of BCL-2 and increase of TP53 expression under the action of both drugs alone and in combination. To further investigate the mechanisms of action of VEN, 2-CdA and their combination on CLL cells, the present study examines their effects on a panel of genes known to be involved in the apoptosis of peripheral blood lymphocytes, using samples from 40 patients included in the previous study.[13] The influence of VEN and 2-CdA alone and in combination on the expression of these genes was tested. We analyzed the genes encoding the proteins analyzed in the previous study, as well as 7 others encoding other factors playing a part in apoptosis. Thus, the present analysis included genes encoding the proteins positively involved in intrinsic (BAX, BBC3, BIM, P53, APAF-1, BAK, BID, BIK, caspase-3 and -9, PMAIP, SMAD3) and extrinsic (caspase-8 and FADD) pathways of apoptosis, as well as having antiapoptotic activity (BCL2, CFLAIR, NOTCH-1).

First, we assessed the expression of these genes in a 48-h drug-free culture and found that the culture conditions alone modulated gene expression in various ways, depending on the gene: The expression of 2 genes (*BIDD* and *FADD*) increased, 6 (*BCL2*, *BAX*, *BIM*, *BAK*, *CFLAIR*,

Table 3. Fold change values for genes in individual comparative systems for the MUT and UMUT groups in terms of *IGVH*

Comparion groups	FC value																
	APAF1	*BAK*	*BAX*	*BBC3*	*BCL2*	*BID*	*BIK*	*BIM*	*CAS3*	*CASP8*	*CASP9*	*CFLAR*	*FADD*	*NOTCH1*	*P53*	*PMAIP*	*SMAD3*
MUT																	
CA vs CB	**1.481**	**3.974**	**4.508**	**1.488**	**4.384**	0.843	0.637	**4.491**	**2.117**	**2.179**	**1.778**	**2.946**	0.806	1.102	**1.864**	**16.075**	**2.112**
2-CdA vs CB	0.548	**1.528**	0.699	0.207	**1.805**	0.203	0.144	1.087	0.543	0.635	0.722	1.148	0.389	0.404	1.006	**10.526**	1.133
VEN vs CB	0.950	**2.451**	**4.538**	0.986	**4.793**	0.551	0.378	**3.388**	1.294	**1.430**	**1.576**	**1.920**	0.496	1.165	**1.868**	**26.981**	**1.306**
VEN + 2-CdA vs CB	0.380	1.263	0.990	0.211	**2.746**	0.107	0.108	**2.016**	0.483	0.685	0.681	1.260	0.210	0.380	0.716	**19.280**	0.475
2-CdA vs CA	0.370	0.384	0.155	0.139	0.412	0.241	0.227	0.242	0.257	0.292	0.406	0.390	0.482	0.366	0.540	0.655	0.536
VEN vs CA	0.642	0.617	1.007	0.662	1.093	0.654	0.594	0.754	0.611	0.656	0.886	0.652	0.615	1.058	1.002	1.678	0.618
VEN + 2-CdA vs CA	0.257	0.318	0.220	0.142	0.626	0.127	0.169	0.449	0.228	0.315	0.383	0.428	0.260	0.345	0.384	1.199	0.225
2CdA vs VEN	**6.498**	**4.759**	**3.116**	**2.626**	**2.710**	**2.617**	**2.381**	**2.251**	**2.887**	**1.858**	**2.563**	**2.182**	**1.735**	**1.604**	**1.672**	1.276	1.153
VEN + 2-CdA vs VEN	0.400	0.218	0.214	0.573	0.194	0.285	0.595	0.374	0.479	0.342	0.656	0.423	0.326	0.383	0.715	0.515	0.364
VEN + 2-CdA vs 2CDA	0.694	0.827	1.020	**1.522**	0.526	0.746	**1.854**	0.889	1.079	0.943	1.097	0.540	0.942	0.712	**1.832**	**1.417**	0.420
UMUT																	
CA vs CB	1.064	**2.040**	**2.395**	**2.270**	**2.835**	1.178	**1.857**	**2.358**	**2.761**	**1.520**	**1.898**	**1.902**	**1.637**	0.988	**1.678**	**5.889**	1.265
2-CdA vs CB	0.481	0.708	0.754	0.246	**1.825**	0.307	0.498	0.851	0.767	0.791	0.631	**1.308**	0.553	0.517	0.838	**3.701**	0.584
VEN vs CB	0.707	0.903	**1.363**	0.493	**2.763**	0.525	0.807	**1.486**	1.091	1.052	0.908	**1.684**	0.796	0.903	**1.305**	**5.438**	0.653
VEN + 2-CdA vs CB	0.430	**3.000**	1.103	0.354	**2.420**	0.237	0.490	0.780	0.665	0.717	0.648	1.099	0.403	0.436	0.576	**4.803**	0.579
2-Cda vs CA	0.452	0.347	0.315	0.108	0.644	0.261	0.268	0.361	0.278	0.520	0.333	0.688	0.388	0.523	0.499	0.628	0.461
VEN vs CA	0.664	0.443	0.569	0.217	0.974	0.446	0.435	0.630	0.395	0.692	0.479	0.885	0.486	0.914	0.778	0.923	0.516
VEN + 2-CdA vs CA	0.156	0.201	0.264	0.241	0.341	0.331	0.246	0.343	0.377	0.441	0.404	0.461	0.471	0.578	0.458	0.816	0.856
2-CdA vs VEN	**1.470**	**1.806**	**2.001**	**1.514**	**1.707**	**1.620**	**1.747**	**1.423**	**1.331**	**1.439**	1.287	**1.440**	**1.747**	**1.558**	**1.469**	1.276	1.119
VEN + 2-CdA vs VEN	0.609	0.851	0.717	0.876	0.451	0.607	0.525	0.610	0.681	0.713	0.653	0.507	0.483	0.441	0.883	0.810	0.887
VEN + 2-CdA vs 2CDA	0.896	1.086	**1.436**	**1.326**	0.770	0.984	0.917	0.867	0.907	1.026	0.840	0.729	0.844	0.688	1.298	**1.468**	0.992

VEN – venetoclax; 2-CdA – cladribine; CA – control after incubation; CB – control before incubation; FC – fold change; *IGVH* – variable region of the immunoglobulin heavy chain; MUT – mutated IGVH (+) cases; UMUT – unmutated IGVH (–) cases; FC < 0.7 – reduced gene expression; FC > 1.3 – increased gene expression; FC (0.7–1.3) – no changes in gene expression. Values in bold are statistically significant.

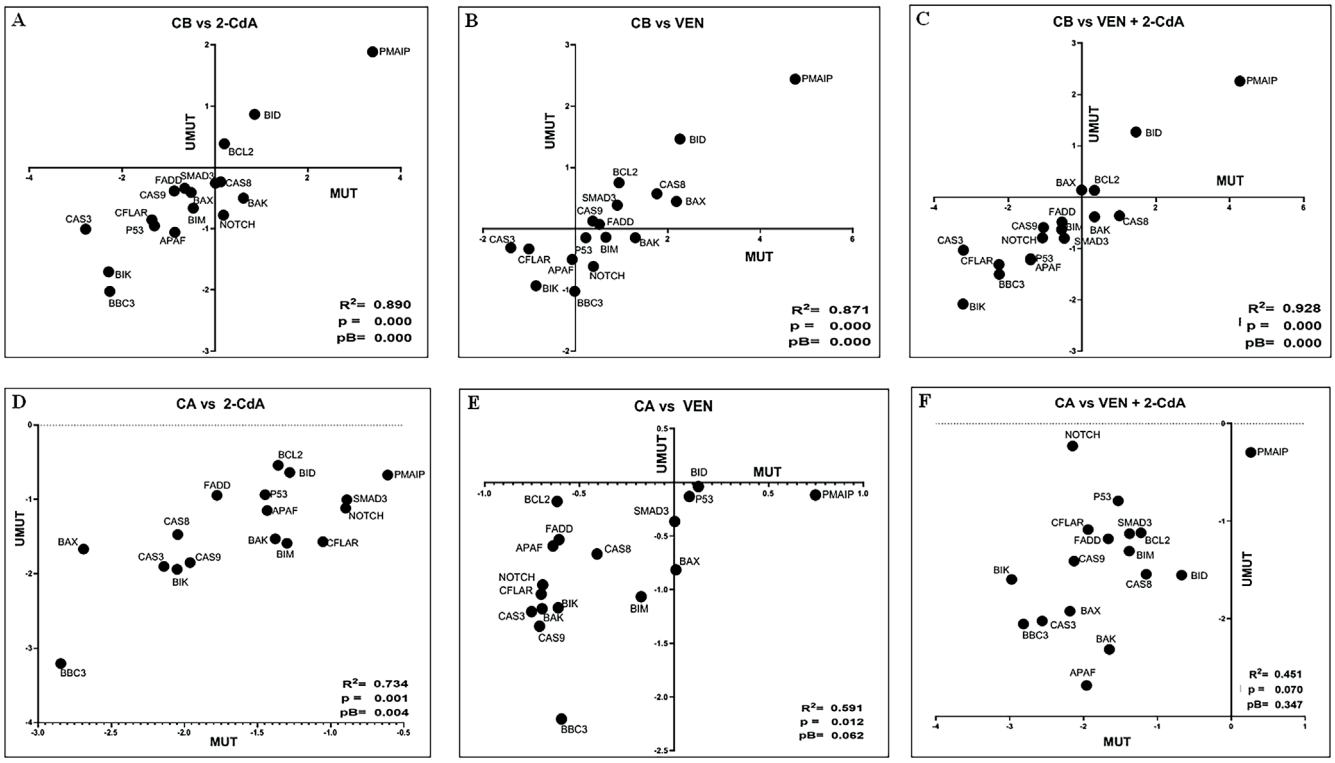

Fig. 5. Correlation graph of FC values for individual genes between the MUT and UMUT groups in comparison to the control. A. 2-CdA vs CB; B. VEN vs CB; C. VEN+ 2-CdA vs CB; D. 2-CdA vs CA; E. VEN vs CA; F. VEN+ 2-CdA vs CA

VEN – venetoclax; 2-CdA – cladrybin; MUT – mutated IGVH; UMUN – unmutated IGVH; CA – control after incubation; CB – control before incubation.

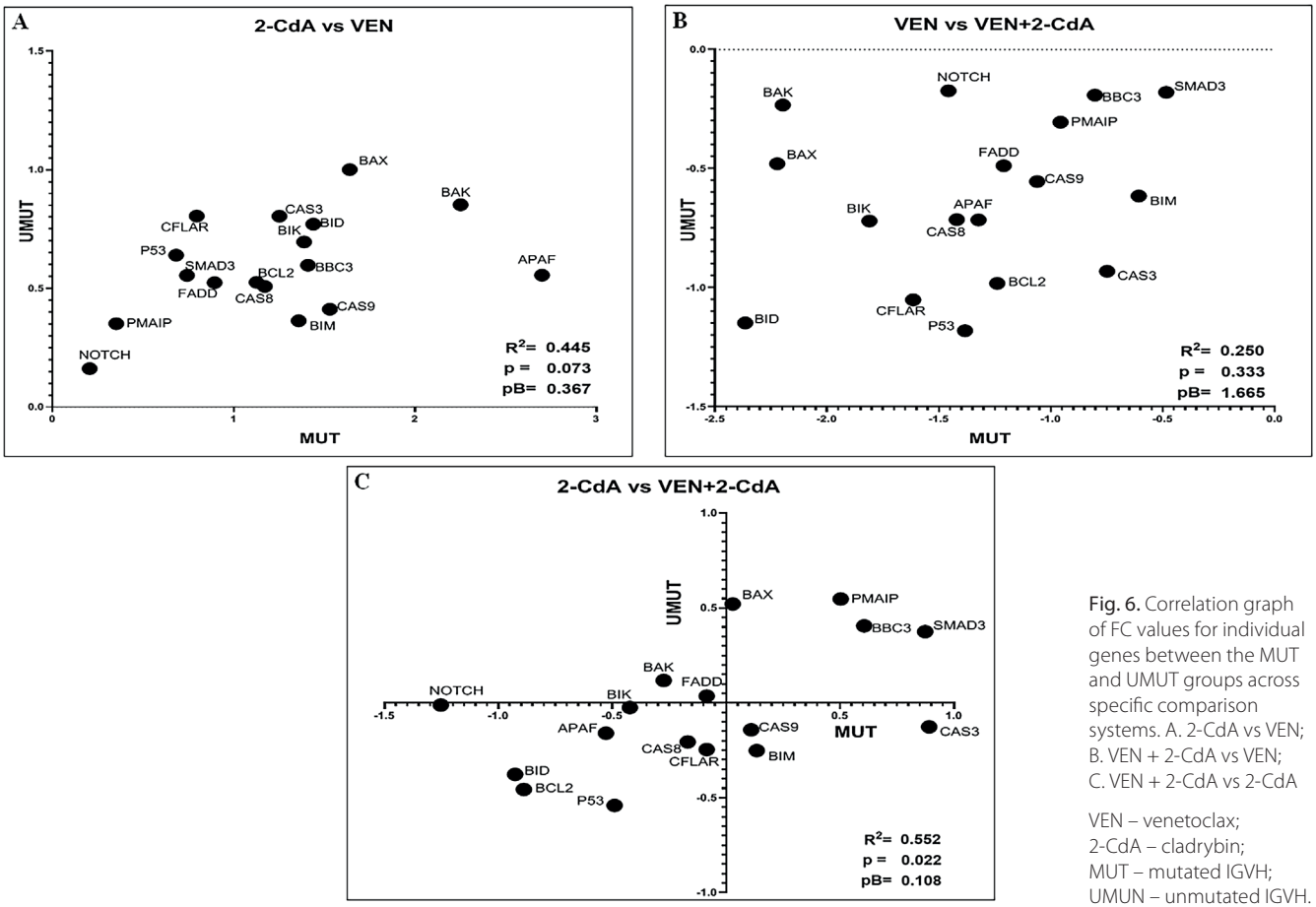

Fig. 6. Correlation graph of FC values for individual genes between the MUT and UMUT groups across specific comparison systems. A. 2-CdA vs VEN; B. VEN + 2-CdA vs VEN; C. VEN + 2-CdA vs 2-CdA

VEN – venetoclax; 2-CdA – cladrybin; MUT – mutated IGVH; UMUN – unmutated IGVH.

PMAIP) decreased, and the others remained stable. To account for the effect of the culture itself, the values obtained after the 48-hour control culture were compared to those obtained from the 48-hour cultures with the drug treatments.

Incubation of CLL lymphocytes with VEN or 2-CdA led to increased expression of all tested genes except *PMAIP1*, which encodes the protein NOXA. All changes were statistically significant, except for *BCL2* following VEN treatment and *SMAD3* following 2-CdA treatment. Similarly, the combination of both drugs brought about a significant rise in the expression of all genes tested except *SMAD3* (expression stable) and *PMAIP* (nonsignificant increase).

Therefore, it appears that both VEN and 2-CdA act on leukemic cells by stimulating the expression of genes encoding pro-apoptotic proteins. This may account for, at least partially, a rise in the percentage of leukemic cells expressing BAX, PUMA, BIM, P53, caspase-3, -8 and -9, and FADD, reported in our previous study.[13] Among the proteins reported in that study, only NOXA demonstrated increased expression after exposure to VEN and/or 2-CdA, without any simultaneous rise in the expression of its corresponding gene. NOXA is a BH3-only member of the BCL-2 family, containing only the BH3 domain (i.e., neither BH1 nor BH2). Ectopically expressed NOXA localizes to mitochondria and interacts with antiapoptotic factors of BCL-2 family, which leads to the activation of caspase-9. It has been found to demonstrate P53-dependent induction in primary mouse cells after exposure to X-ray irradiation[29]; however, NOXA expression can also be independent of TP53. In contrast to BIM and PUMA, which can form complexes non-selectively with all pro-survival BCL-2 family proteins, NOXA binds selectively to Mcl-1 and to Bfl1/A1.[30] The increase in the percentage of lymphocytes expressing NOXA observed following exposure to VEN and 2-CdA may be due to post-transcriptional regulation of its synthesis.

However, our previous observation that the percentage of BCL-2-expressing cells decreased, while *BCL2* gene expression increased following treatment with 2-CdA and the 2-CdA+VEN combination, remains unexplained. Venetoclax alone also led to an increase in BCL2 expression, though this change was not statistically significant. First, it is important to note that only a weak correlation may exist between the previously obtained protein data and the current gene expression findings. The expression of the studied proteins was given only as a percentage of positive cells, without any estimation of their intracellular content. It seems then possible that increased expression of *BCL-2* gene may be accompanied by a lower percentage of cells positive for that protein. It is all the more possible that BCL-2 is hyperexpressed in CLL, and that the median percentage of BCL-2-expressing cells in all samples, i.e., in both controls and treated cultures, was higher than 70%. In this setting, the estimation of intracellular BCL-2 content would have probably been a more accurate estimate of total BCL-2 protein expression than the percentage of BCL-2-positive cells.

It is also important to consider the complex nature of *BCL2* gene regulation in CLL, as the underlying causes of its overexpression remain unclear. Both transcriptional and post-transcriptional mechanisms have been proposed to explain BCL2 overexpression, including promoter hypomethylation, loss of miR-15a and miR-16-1 due to 13q14 deletion, and overexpression of nucleolin, which promotes BCL2 mRNA stabilization.[31–34] Additionally, BCL2 expression is negatively regulated by TP53.[35–37]

The observed stimulation of the antiapoptotic gene *BCL-2* expression by cytostatic drug 2-CdA, both alone and in combination with VEN, is probably a multifactorial phenomenon and one that is counter-balanced by the simultaneous stimulation of almost all the tested anti-survival genes. Notably, BCL2 expression was found to significantly decrease in the control culture (i.e., without drug treatment); therefore, its upregulation in response to all investigated drugs is unlikely to be a laboratory artifact.

Regarding the influence of different combinations of drugs on the expression of the genes tested, 2-CdA brought about a significantly higher rise in *BAX* and *BBC3* expression compared to VEN. Of note, the 2-CdA+VEN combination significantly enhanced the expression of all genes except *BCL2* and *PMAIP1* compared to VEN alone. However, only 5 genes (*TP53*, *BID*, *BIK*, *FADD*, and *SMAD3*) were more strongly upregulated by 2-CdA+VEN than by 2-CdA alone. The combined drugs hence seem to potentialize VEN to a greater degree than 2-CdA with regard to its effect on apoptosis-involved gene expression.

Unfortunately, it was not possible to perform any direct comparison between the changes in gene expression between patients with mutated compared to non-mutated *IGVH*. Therefore, a series of graphs was generated, with 1 for each experimental condition (drug/drug combinations) (Fig. 5,6). On each graph 1 gene was represented by a dot having the coordinates: X axis: log(FC) for mutated cases; and Y axis: log(FC) for unmutated cases. Spearman's correlation coefficient (r) was then calculated for each experimental condition.

Therefore, our findings do not support the hypothesis that the impact of VEN and 2-CdA on the expression of the genes involved in the regulation of apoptosis is dependent on *IGVH* mutational status. However, it should be noted that although clinical trials have shown the VEN + anti-CD20 antibody combination to be effective in both patients with mutated and unmutated IGHV genes, those with the mutated form tend to exhibit more favorable survival outcomes.[4,38,39] The impact of *IGHV* mutational status on the outcomes of CLL patients treated with 2-CdA-containing regimens has not been assessed.[40,41] The relationship between the effects of VEN and cytostatic drugs and *IGHV* mutational status should be further investigated in a larger patient cohort using more robust statistical methods.

Limitations

A key limitation of the study was the inability to perform statistical analysis comparing the expression levels of previously tested proteins with those of their corresponding genes.

Conclusions

To the best of our knowledge, this study is the first to evaluate the effects of 2-CdA and VEN on the expression of apoptosis-related genes in peripheral blood lymphocytes from patients with CLL. Our findings suggest that these drugs, whether used individually or in combination, exert their antileukemic effects not only by impairing DNA synthesis (2-CdA) or inhibiting BCL2 (VEN), but also by upregulating the expression of several genes encoding proteins essential for the execution of apoptosis. This effect of VEN is enhanced by its association with 2-CdA, which may justify further preclinical and clinical studies on its potential in the treatment of CLL.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.14535775. The package includes the following files:

Supplementary Fig. 1. Results of the normality tests (Shapiro–Wilk test) regarding the expression of the studied genes in the individual research groups: in the control group (A), in the VEN group (B), in the 2-CdA group (C), and in the VEN + 2-CdA group (D).

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Effectiveness of phentolamine mesylate, vibration and photobiomodulation in reducing pain and the reversal of local anesthesia: A systematic review

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Abstract

Background. Dental anesthesia administration often triggers unpleasant sensations, particularly needle injection-related pain, which can evoke fear among patients, especially in the pediatric population. Vibration and low-level laser therapy (LLLT) have been extensively studied as potential methods for alleviating pain. Additionally, phentolamine mesylate (PM) has shown promise in reducing the duration of anesthesia. From a clinical perspective, inadequate control over the persistence of the anesthetic effect may lead to complications associated with its prolonged duration, such as self-injuries or functional impairments.

Objectives. This review aimed to systematically summarize and compare methods of alleviating pain during local anesthesia and reducing its duration.

Materials and methods. In November 2023, an electronic search was systematically conducted across PubMed, Web of Science, and Scopus databases using keywords (pain) AND (anesthesia) AND ((phentolamine) or (vibration) or (LLLT) OR (PBM)). The initial pool consisted of 495 records, from which 241 duplicates were eliminated. After careful examination of the remaining articles, 40 were included. The study adhered to the PRISMA guidelines.

Results. Most studies reported beneficial effects of LLLT and vibration; however, some did not corroborate these findings. Four studies had inconclusive results. Regarding anesthesia duration involving PM and LLLT, the majority of studies exhibited notable reductions, although no significant differences were revealed in 1 study.

Conclusions. Vibration and LLLT appear to be advantageous methods in alleviating pain associated with local anesthesia administration. Phentolamine mesylate and LLLT are efficient in reversing local anesthesia.

Key words: LLLT, anesthesia, vibration, PBM, phentolamine

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Background

Local anesthesia (LA) is a routine and essential aspect of dental treatment, and it plays a crucial role in ensuring a patient's comfort during various procedures.[1,2] Patients may often experience fear and anxiety during dental appointments, primarily due to the discomfort or pain associated with the procedure or the needle administering LA before dental treatments.[3–5] There is also an aspect of temporary numbness, which some patients find unpleasant,[2,6–10] and because of its presence, dentists need to provide post-procedure guidelines and advise patients to avoid activities that could lead to oral injury due to the impaired sensation.[10]

In recent years, researchers have introduced several methods designed to alleviate the pain and discomfort commonly linked to the application of LA. Concurrently, the duration and management of numbness after oral injections are also an area of interest for the researchers. There are various methods to administer LA, but the most common techniques used in research of the aforementioned subjects are; topical anesthesia, which when applied to the mucous membranes helps numb the surface before an injection[1,3,11,12]; infiltration anesthesia, which is commonly used for procedures in the maxilla or treatments involving a single tooth or a small area of the mouth[1,13]; and nerve block anesthesia, which is deposited in proximity to a major nerve plexus and usually used in treatment of the mandibular region.[1,10]

Considering the pain that may be associated with the previously mentioned injection techniques, 2 notable methods that have gained attention for their potential to minimize pain and improve the overall dental anesthesia experience include vibratory stimuli and photobiomodulation (PBM). Applying vibration during the injection was investigated considering Gate Control Theory, which states that vibratory stimuli may activate large-diameter nerve fibers, which transmit signals faster than smaller pain fibers, and their activation may inhibit the transmission of pain signals, resulting in reducing the sensation of pain.[3,4,11,14] Additionally, vibration serves as a distraction technique with the idea that the vibration sensation may reduce the perception of pain by diverting the patient's attention away from the injection.[3,11] Photobiomodulation, also known as low-level laser therapy (LLLT) or laser therapy, involves the use of specific wavelengths of light to stimulate cellular processes.[15–19] In dentistry, it has been explored for its potential to reduce inflammation and promote tissue regeneration, and for its analgesic effects which can be useful in managing pain during and after dental treatment.[20–23] It may include lower pain sensations when PBM is combined with injection of a local anesthetic agent.[15,16,24,25] As PBM induces vasodilation, it increases the microcirculation in the anesthetic region and may accelerate the elimination of LA.[2,24]

In the context of solely regulating the duration of numbness after dental anesthesia, researchers are examining the use of phentolamine mesylate (PM). It acts as a non-selective alpha-adrenergic antagonist, promoting vasodilation, which enhances regional blood flow at the site of injection,[6,7,10,26] thereby accelerating the clearance of the local anesthetic agent from the tissues and leading to a potential reduction in the duration of postoperative soft tissue numbness.[6–10]

Objectives

There is no current published literature review that comprehensively synthesizes the existing research to evaluate the use of vibration or PBM for both pain reduction and acceleration of the elimination of anesthetic agents from the oral tissues and PM for reducing the duration of numbness after LA. This review aims to provide current insights into a multifaceted approach aimed at enhancing the patient experience during and after dental anesthesia. This involves optimizing the balance between effective pain management and minimizing the undesirable postoperative effects.

Materials and methods

Focused question

This systematic review followed the PICO framework as follows. PICO question: In dental patients undergoing LA (population), do interventions such as vibration, PBM, and PM (investigated condition) reduce pain and hasten the reversal of the LA effect (outcome) compared to conventional anesthesia administration (comparison condition)? (see Fig. 1).

Protocol

The selection process for articles in the systematic review was carefully outlined following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) flow diagram (Fig. 2). The systematic review was registered on the Open Science Framework (OSF) under the following link: https://osf.io/k9vub.

Eligibility criteria

For studies to be considered for inclusion in this review, they needed to fulfill specific criteria. These included utilizing vibrations or LLLT to alleviate pain during anesthesia administration, incorporating PM to reverse anesthesia effects, conducting in vitro studies, examining dental anesthesia applications, featuring a control group, maintaining a sample size of 10 or more

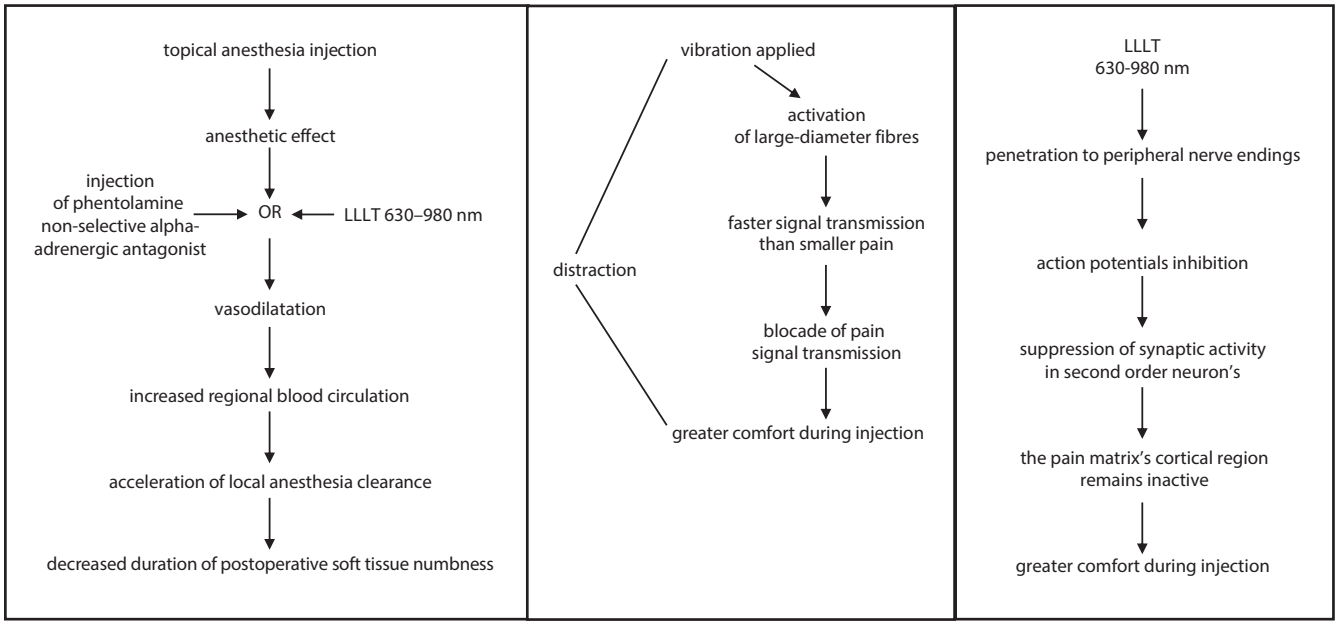

Fig. 1. The PICO framework

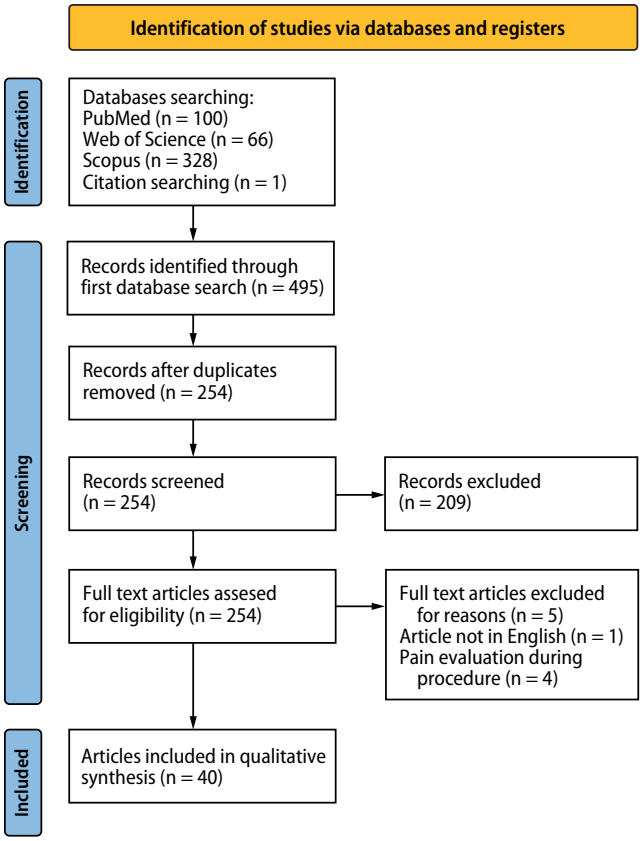

Fig. 2. The flow chart according to Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines

participants, being conducted in English, encompassing prospective case series, non-randomized controlled clinical trials (non-RCT), and randomized controlled clinical trials (RCT). On the other hand, the reviewers collectively established exclusion criteria. The included studies lacking a control group, those with a sample size of fewer than 10 participants, investigations carried out on animals, papers not in English, clinical reports, systematic reviews, opinions, editorial papers, or review articles, publications lacking full-text accessibility, and duplicates. No restrictions were applied with regard to the year of publication.

Information sources and search strategy

In November 2023, an electronic search using PubMed, Scopus, and Web of Science (WoS) medical databases was performed. Key words were used as follows: "pain"; "anesthesia"; "phentolamine"; "vibration", "LLLT"; and "PBM". In the Scopus database, the results were refined to titles, abstracts, and key words. In PubMed and WoS, the results were narrowed down to titles and abstracts. Only articles with full-text access were included.

Data collection and selection process and data items

Data, including authors, titles and abstracts of all results, were downloaded as a PDF file. The obtained information was subsequently entered into a standardized Microsoft Excel 2013 file (Microsoft Corp., Redmond, USA).

Risk of bias assessment

During the initial stages of study selection, the title and abstract of each paper were independently reviewed by 3 authors (D.F., A.S. and N.G.) to minimize the risk of reviewer bias. The level of agreement among the researchers

was assessed using Cohen's kappa test. If unanimity was not achieved, the decision on inclusion or exclusion was made by a 4th independent reviewer.

Quality assessment

Three independent reviewers (D.F., A.S. and N.G.), meticulously evaluated the procedural quality of each study encompassed in the article. Their assessment centered around crucial facets linked to the utilization of vibrations, LLLT and phentolamine in mitigating the discomfort and pain associated with LA administration, while also exploring their impact on the duration of anesthesia. The evaluation of study design, implementation and analysis hinged on several critical criteria: The adherence of all procedures to the prescribed manufacturer guidelines for the respective intervention was mandatory. Every intervention was conducted singularly by a designated operator, ensuring consistency and minimizing potential variability. The determination of the sample size was not only clearly elucidated but also justified comprehensively. Patients incorporated into the studies were exclusively those undergoing planned treatment without any emergent conditions, thereby ensuring a controlled and consistent participant profile. Moreover, the sample sizes surpassed the threshold of 10 patients/participants, thereby ensuring statistically significant power for the findings; a detailed and comprehensive depiction of the anesthesia employed was obligatory, encompassing its type, dosage and method of administration. Efforts were undertaken to blind the patients involved, mitigating potential biases in the reporting of outcomes. The scoring of studies adhered to a scale ranging from 0 to 9 points, with a higher score indicative of superior study quality. The assessment of bias risk scores was categorically classified into 3 groups: 0–3 points, signifying a high risk; 4–6 points, indicating a moderate risk; and 7–9 points, representing a low risk. Any discrepancies in scoring were meticulously resolved through extensive discussions until a consensus was collectively reached.

Results

Study selection

After conducting an initial search across three databases and eliminating duplicate entries, a total of 254 articles were initially identified as eligible for inclusion in the literature review. Following a preliminary assessment of the article titles and abstracts, 209 articles were excluded. Among the remaining 45 articles, 1 was eliminated because it was originally not in English, and 4 articles were excluded due to their incomplete relevance to the reviewed topic. Ultimately, 40 articles met the criteria for inclusion in the systematic review, all of which were clinical trials.

Effect of vibration

Investigations focused on assessing the efficacy of different vibrating devices (DentalVibe, Vibraject VAI, modified battery-powered shaver, sonic-powered toothbrush, HoMedics Atom massager, specialized Buzzy external tool) and dental instruments in alleviating patient discomfort associated with LA. In 13 of the included studies, a specialized wireless, rechargeable, handheld vibratory dental tool known as DentalVibe (DV) was employed.[3,12,13,27–36] In the research of Felemban et al.,[3] Erdogan et al.,[13] Raslan and Masri,[30] and Ramezani et al.,[35] a DV vibratory stimulus was used without the preceding desensitization of the mucosa with topical anesthesia. Hassanein et al.[29] similarly administered vibration, but the vibration-assisted injection was preceded by topical anesthesia with 20% benzocaine gel. Felemban,[3] Erdogan et al.,[13] and Raslan and Masri[30] found no statistically significant differences between the study and control groups. A significant difference in pain scores between the study and control groups, regardless of the injection method, was revealed in the study conducted by Ramezani et al.[35] With assumptions aligning with the aforementioned researchers, Joshi et al.,[31] Dak-Albab et al.,[32] Ching et al.,[33] and Salma et al.[34] evaluated the effectiveness of vibration in comparison with topical anesthetic gel and found significantly lower rates with vibration than anesthetic gel. In a comparative split-mouth clinical study by Shaefer et al.,[27] Nasehi et al.[28] and Tung et al.,[36] notable distinctions were presented, with the non-vibration group revealing higher scores for pain across all nerve blocks.

Albouni et al.[37] showed higher visual analogue scale (VAS) scores with the conventional injection (CI) method compared to the vibraject-assisted injection (VAI) method in all groups. Moreover, Hegde et al.[11] indicated significantly less pain in children using a special toy compared to conventional methods according to the Face, Leg, Activity, Cry, Consolability (FLACC) scale, Wong–Baker Pain Rating Scale and heart rate. In turn, Hutchins et al.[38] used a vibration stimulus produced by a modified battery-powered shaver compared to topical anesthetic in reducing pain during oral injections. The findings showed a notable difference in pain levels using 20% benzocaine across 2 categories: buccal anesthetic vs placebo and both buccal and palatal anesthetic vs placebo. In a study conducted by Bagherian and Sheikhfathollahi,[39] the authors investigated 48 children who received contralateral IANB or primary maxillary molar infiltration injections using cotton-roll vibration (topical anesthesia gel, cotton roll and vibration) and traditional methods as a control. The results showed significantly lower scores compared to the control method. A study by Gandhi et al.[40] found a statistically significant difference between the mucosal vibration group and the topical gel group in terms of Sound, Eye, Motor scale (SEM) and Wong–Baker FACES Pain Rating Scale (WBFPS) rates. The pain reaction assessed by Aminabadi et al.[41] in the topical anesthesia group was significantly

higher than in the other 2 groups (soft tissue vibration (C) and soft tissue vibration with a distraction exercise (C + SA), with pain being significantly less exhibited in the C + SA than in the C group.

Nanitsos et al.[42] proposed the use of a HoMedics Atom massager to apply vibration during LA. The assessment of pain using a VAS and McGill pain descriptors showed significantly lower mean rates on the vibration side during injections. The results of the study conducted by Meghana and Anjaneyulu[43] indicated that infiltration with topical anesthesia demonstrated the least pain perception, while infiltration without topical anesthesia and vibration resulted in higher pain scores, as supported by VAS assessments. Four studies[4,44–46] explored the synergistic effects of combining vibration and cold to alleviate pain during dental anesthesia using a specialized Buzzy external tool. Sahithi et al.[45] reported a significant decrease in pulse rate post-intervention and a reduction in Venham's Clinical Anxiety Rating Scale (VCARS) scores, indicating reduced anxiety, as well as a more pronounced reduction in discomfort during needle insertion, according to WBFPS and VAS scores. AlHareky et al.[4] demonstrated a significant decrease in pain post-injection compared to the control group, as indicated by VAS and FLACC scales, with no significant differences observed using the SEM scale. In the study by Marwah et al.,[44] only FLACC presented a statistically significant difference between groups, while in the study by Bilsin et al.,[46] the WBFPS demonstrated a notable contrast in favor of the vibration device.

Effect of photobiomodulation

Nowadays, researchers have been exploring PBM LLLT as a potential solution for pain reduction during anesthesia in the field of dentistry.[2,24,47–52] Part of these studies aim to not only illuminate its efficacy in pain management but also explore its potential to enhance microcirculation and accelerate the elimination of local anesthetics.[2,24]

In research by Jagtap et al.,[47] a significant statistical difference in VAS scores was found between the laser and placebo conditions in reducing pain caused by local anesthetic injections in 25 adult patients. Dehgan et al.[48] and Elbay et al.,[49] in a clinical trial involving 160 children, aimed to evaluate the impact of PBM, delivered by a 940 nm diode laser, in combination with 10% lidocaine topical anesthetic on pain experienced during LA injections. The results by Dehgan et al.[48] showed significantly lower pain scores in the groups receiving PBM compared to the placebo group. However, there was no significant difference observed among the 3 PBM groups. A study by Elbay et al.[49] showed no significant difference in injection pain among the groups.

Sharifi et al.[50] designed a triple-blind clinical trial involving 84 patients, which revealed a significant reduction in pain when LLLT was used compared to conventional injection. In a clinical trial by El Feghali et al.,[51] no significant differences in VAS pain scores between groups were found, but the results in the Verbal Rating Scale (VRS) showed significantly higher ratings of taste, undesirable numbness and overall satisfaction in the study group than in the control group. The study by Tuk et al.[52] involved 163 patients and showed significant differences in sweating rate in the extractions located in the mandibular region during maxillary or mandibular third molar anesthesia. Uçar et al.[24] revealed significantly lower PRS scores on the laser therapy side compared to the control side in a group of 60 children who required a bilateral pulpotomy in mandibular first primary molars. Annu et al.[2] demonstrated that the mean soft tissue LA reversal time duration was significantly shorter, with 660 nm wavelength therapy being more effective. Similar findings were obtained by Seraj et al.[53] in patients who received 810 nm laser irradiation 45 min after anesthesia injections.

Effect of phentolamine mesylate

Since the possibility of the use of PM in dentistry was noticed, researchers have made efforts to assess how the use of this non-selective alpha-adrenergic antagonist accelerates the disappearance of numbness and discomfort after dental anesthesia.

Tavares et al.[7] and Nourbakhsh et al.[9] researched the impact of PM on the duration of soft tissue anesthesia and the occurrence of soft tissue trauma following mandibular block injections in children aged 4–11. Tavares et al.[7] demonstrated a substantial reduction in recovery time (60 min in the PM group vs 135 min in the control group) and reported no differences in adverse events or vital signs. Nourbakhsh et al.[9] not only confirmed a significant decrease in recovery time but also introduced additional outcomes showing notable differences in the incidence of soft-tissue trauma in 43 patients divided into case and control groups.

Fowler et al.[8] and Shadmehr et al.[26] demonstrated the efficacy of PM in hastening soft-tissue recovery. Gago-Garcia[10] et al., based on data from 90 participants, claimed that the use of PM alongside 3 different substances (lidocaine, articaine and bupivacaine), compared to the average duration for each anesthetic, exhibited a strong potential to shorten the duration of anesthesia, with a particularly notable decrease observed when paired with bupivacaine. Similarly, the study by Michaud et al.,[6] which enrolled 40 adult participants, showed that PM injection significantly reduced the duration of soft tissue anesthesia in the lower lip and tongue, additionally hastening the recovery of function and reducing the time needed for smiling, drinking and speaking. General and detailed study characteristics are presented in Table 1 and Table 2, respectively.

Quality assessment

Out of the articles included in this review by 5 independent reviewers (D.F., A.S.,N.G., and A.O.),

Table 1. General characteristics of included studies

Study	Aim of the study	Materials and methods	Results	Conclusions
Annu et al., 2023[2]	Comparison of photobiomodulation (PBM) therapy at 660 and 810 nm wavelengths on the reversal of local anesthesia.	A group of 60 children (mean age: 73 months) was divided randomly into 3 equal groups. 45 min after IANB: The control group received no laser irradiation. The 2nd group underwent photobiomodulation therapy at 810 nm. The 3rd group underwent PBM at 660 nm. Reversal of local anesthesia tests: Palpation technique to check the numbness of lower lip, the pin prick test.	Reduction of the mean soft tissue local anesthesia reversal time duration by 55.5 min and 69 min with PBM at 810 nm and 610 nm wavelength, respectively. A statistically significant difference in the reversal time duration between the control group and the study group, between the 810 and the 660 nm LASER groups.	Both photobiomodulation (PBM) therapy at 660 and 810 nm wavelengths affected the mean soft tissue local anesthesia reversal time duration significantly. Photobiomodulation (PBM) therapy at 660 wavelengths was found to be more effective.
Felemban et al., 2021[3]	Assessment of vibration in reducing pain linked to LA compared to the conventional injection.	A group of 60 children was divided randomly into 2 groups. Before buccal infiltration anesthesia (BIA): The control group (31) received traditional BIA; the test group (29) received vibration with BIA. Pain assessment scales: FLACC scale by 2 external evaluators, the validated Arabic version of the Wong–Baker FACES scale, rating pain on a scale from 0 to 10 by subjects.	Regardless of age and treatment group, girls consistently maintained significantly higher average scores on both the FLACC and the Wong–Baker FACES scales than boys.	The utilization of DentalVibe did not significantly affect pain, discomfort, or time during buccal infiltration anesthesia (BIA) in pediatric patients when compared to the traditional method.
AlHareky et al., 2021[4]	Evaluation of the impact of device administering cold and vibrations during buccal infiltration injection.	A group of 51 children was divided randomly into 2 groups. Before anesthesia: Group 1: topical anesthesia of 20% benzocaine gel for 15 s. Group 2: cold + vibration, that remained active throughout the entire injection process. Pain assessment scales: visual analogue scale (VAS) by the child "behavioral/observational pain scale" by present parents, the Sounds, Eyes, and Motor (SEM) scale and FLACC scale by an external evaluator.	The VAS scale and the FLACC scale presented significant differences in post-injection pain in the study group than control. No significant difference observed using the SEM scale.	The use of external cold and vibrating devices is effective in diminishing the pain and anxiety among children undergoing infiltration dental anesthesia.
Michaud et al., 2018[6]	Evaluation of the effect of phentolamine mesylate on the duration of soft tissue anesthesia.	Forty participants were randomized into 2 groups, and in both groups, IANB was performed. Study group received phentolamine mesylate (PM) injection and the control group received an injection of sterile saline water.	Comparing to the control group, PM injection resulted in reduced duration of soft tissue anesthesia. In the lower lip: 66 min reduction In the tongue: 51 min reduction In terms of recovery of function, the reduced time was: for smiling: 55 min reduction for drinking: 66 min reduction for speaking: 68 min reduction.	Study showed that PM injection after performing IANB resulted in faster return to normal soft tissue sensation and function.
Tavares et al., 2008[7]	Assessing the safety and adverse effects (AEs) (primary objective) and effectiveness (secondary objective) of a phentolamine mesylate (PM) formulation as a local anesthesia reversal agent for pediatric patients.	A total of 152 pediatric patients randomized into 2 groups: 72 subjects receiving PM injection, 43 patients in control group with sham injection. The observation for safety and efficacy assessments was 4 h. Adverse events were categorized to severity (mild, moderate or severe). The duration of the LA measurement 6–11-year-olds group (4–5-year-olds were excluded) palpation technique.	A 60% and 55.6% reduction in the median time for the return of normal tongue and lip sensation, respectively. Thirty-seven AE reported, 36 mild or moderate, 1 severe.	Phentolamine mesylate injections exhibited no serious adverse effects. Phentolamine mesylate shortened the duration of soft tissue anesthesia in children in both the maxilla and mandible.

18 studies were considered of high quality (with a score of 7–9 points).[2,3,6,8–10,13,24,26,27,30,33,46–49,51,53] Three studies[35,36,40] were classified as low-quality (0–3 points). Additionally, 19 studies were considered to have a moderate risk of bias, scoring between 4 and 6 points[4,7,11,12,28,29,31,32,34,37–39,41–45,50,52] (Table 3).

Discussion

Pain management is a crucial element in building positive attitudes and cooperation between patients of different ages and dentists.[3,4,11,14] Although LA is commonly used

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Fowler et al., 2011[8]	Exploring the efficacy of phentolamine in reversing soft-tissue anesthesia.	Eighty-five adults with asymptomatic teeth in need of endodontic therapy were randomly assigned to receive either phentolamine or a sham treatment after the treatment with LA. Pain levels assessment at the injection site and in the tooth every half-hour during the initial 2 h, then every hour for the subsequent 3 h: VAS. Anesthesia reversal: Palpation technique at 15-min intervals for 5 h.	Phentolamine effect compared to the sham group: Maxillary, lip/cheek Disappearance of numbness: 35 min faster Return-to-normal sensation: 88 min faster Mandibular lip Numbness: 24 min faster Sensation: 47 min faster Tongue Numbness: 24 min faster Sensation: Not significantly reduced	Phentolamine presented quicker return of normal soft-tissue function and sensation following local anesthesia.
Babaei et al., 2011[9]	Evaluation of the LA duration after phentolamine mesylate (PM) distribution and the occurrence of soft-tissue trauma following this type of injections.	Split-mouth study including a group of 60 children was divided randomly into 2 equal groups. 30 min after LA: First group received PM. The 2nd group received sham injection. On the next visit the contralateral side was treated conversely. Palpation technique to check anesthesia duration. Monitoring of the safety, efficacy, and soft-tissue trauma every 15 min for 3–5 h. Vital signs were recorded 30 min after anesthesia and every 1 h.	Group 1: Sensation of soft tissue with PM injection was 29.47 min, and without – 135.52 min. Group 2: sensation of soft tissue with PM injection was 33.12 min, and without – 106.04 min. Statistically significant difference in time of return of a normal lip sensation between case and control groups. 19% of patients (8) without PM injection and 2% – 1 patient after PM injection (statistically significant) traumatized their lips a few hours after treatment. No trauma to tongue and cheek was found.	Use of PM can be beneficial to reduce the duration of LA in children needing dental procedures and can lower the incidence of soft-tissue trauma connected to dental anesthesia.
Gago-García et al., 2021[10]	Evaluation of the phentolamine mesylate (PM) distribution on anesthesia duration within 3 distinct local anesthetics, comparing it to the average duration for each individual anesthetic, and between all 3 types of anesthetics.	A group of 90 individuals was divided randomly into 3 equal groups: Group 1: lidocaine 2% 1/80000; Group 2: articaine 4% 1/200000 Group 3: bupivacaine 0.5% 1/200000. The untreated side served as the control. IANB was performed. After treatment PM was administered with a 1:1 ratio of anesthesia to reversal agent. Patients marked boxes for 15-min intervals after the reversal agent injection to note sensations: numbed, tingling or normal. Postoperative pain evaluation: The Heft–Parker visual analogue scale.	The average duration of anesthesia after injection of phentolamine mesylate: Group 1: Lip – 59.6 min, tongue – 52.5 min, normative value – 180 min. Group 2: lip – 88.5 min, tongue – 84.5 min, determined normative value – 258 min. Group 3: lip – 249 min, tongue – 214 min, normative value – 460 min.	Phentolamine mesylate has the potential to decrease the duration of anesthesia when used alongside various local anesthetics, especially bupivacaine.
Hegde et al., 2019[11]	Comparison of pain, anxiety and behavioral perception when administering local anesthesia with and without a vibrating and distracting toy.	A split-mouth study including a group of 30 children separated randomly into 2 equal groups 1: 6–8-year-olds, 2: 9–11-year-olds. Before injection of anesthesia the 1st group received vibration and at the 2nd appointment – conventional topical anesthesia. Group 2 inversely. Pain assessment scales: The Face, Legs, Activity, Cry, Consolability (FLACC) scale, pulse rate, the Wong–Baker faces pain rating scale (WBFPRS).	Statistically significant differences between conventional and device methods in pulse rate during treatment, FLACC and WBFPRS scores in both age groups.	These results suggest the device's effectiveness in decreasing pain across different age groups, leading to improved clinical outcomes.
Shilpapriya et al., 2015[12]	Comparison between the effectiveness of Dental Vibe® and topical local anesthetic in pain reduction	Split-mouth study including 30 children. Before anesthesia they were split into: Control group using topical anesthetic study group using DentalVibe during the 1st appointments. The other method was used at the 2nd appointment. Pain assessment scale: Universal pain rating scale.	Statistically lower mean pain scores were exhibited in the study group compared to the control group.	DentalVibe serves as a beneficial tool before dental injections, relieving pain and stress.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Erdogan et al., 2018[13]	Assessing the efficacy of a Vibratory Stimulation Device for intraoral local anesthesia administration.	Thirty-one participants received local anesthesia infiltration at the right maxillary incisors' apical region. They were randomly assigned to either conventional infiltration or conventional infiltration with DentalVibe. A 2-week interval between procedures. Pain assessment scales: VAS and WBFPRS.	No significant differences between groups.	The use of the vibration did not show any significant decrease in the perceived pain level linked to the administration of local anesthetic via maxillary anterior infiltration.
Uçar et al., 2022[24]	Evaluation of LLLT on pain during anesthesia administration, anesthesia efficacy and duration time of anesthesia.	Split-mouth study including a group of 60 children (mean age 7.11 ±1.12 years). Before injection: One side with topical anesthesia application and LLLT (810 nm diode laser). Opposite, control side with topical anesthesia application and placebo laser use. A 4–7 days interval between procedures. Pain assessment scales: WBFPRS – pain during injection of a needle and deposition of anesthetic solution; FLACC scale – anesthesia efficacy.	Injection pain significantly lower on the LLLT side than on the placebo side according to the WBFPRS scale but not the FLACC scale. Anesthesia efficacy and duration time not modified by LLLT. No pain response rate in relation to anesthesia efficacy higher on the LLLT side than on the control side.	Low-level laser therapy has an impact on alleviating injection pain; however, it did not affect anesthesia efficacy and duration time.
Shadmehr et al., 2019[26]	Evaluation of the LA duration after phentolamine mesylate (PM) distribution.	A group of 100 patients diagnosed with symptomatic irreversible pulpitis in their first or second mandibular molars were assigned to receive either OraVerse or a placebo following a treatment. Pain assessment scales: The Heft–Parker visual analogue scale – before and at 6, 12, 24, 36, 48, and 72 h after treatment. Soft-tissue anesthesia was monitored every 15 min for 5 h.	PM group: Lip sensation in about 120 min and tongue sensation in about 103 min. The control group: Lip sensation after around 152 min and tongue sensation after around 174 min. Patients administered phentolamine exhibited notably increased pain scores at 6- and 12-h intervals. The use of analgesics notably higher in the OraVerse group compared to the control group.	Despite phentolamine expediting the return of regular soft tissue sensation, it heightened postoperative pain in patients with symptomatic irreversible pulpitis, potentially restricting its administration within this patient group.
Shaefer et al., 2017[27]	Evaluate DV3 device mediation of injection discomfort during LB block and IANB without topical anesthetic, compared to the routine operator manipulation. Measure DV3's impact on the time required for complete anesthesia during an IANB.	Sixty volunteers. Bilateral intraoral LB block on one side using the DV3 device, while control injections involved routine operator manipulation. Subjects randomly divided into equal groups for IANB: One group with vibration and control without. Pain assessment scale: VAS, modified symptom severity index (SSI). Complete mandibular anesthesia duration post-IANB: A cold test on specific teeth.	Subjects receiving DV3 during the injection exhibited notably lower VAS scores. The mean numb time did not significantly differ between DV3 and non-DV3 groups.	The DV3 notably decreased discomfort during dental injections. However, its usage did not impact the duration for achieving complete mandibular anesthesia.
Nasehi et al., 2015[28]	To assess the pain experienced during LA using a vibrating intra-oral device (DentalVibe).	Ninety-nine subjects (mean age 39.18 ±17.45 years) underwent local anesthetic injections on each side of the oral cavity, randomly either with or without a vibration device. Anticipated and actual pain assessment: VAS scale.	The mean VAS scores for anticipated and actual pain significantly differed between study and control group with higher scores in the non-vibration group across all nerve blocks. Control group: No significant difference between anticipated and actual pain. Study group: Significantly lower actual pain scores than for anticipated pain in specific nerve blocks, no significant difference for palatal injections.	The vibration proved to be an effective method for relieving the clinical pain experienced during local anesthetic injections.
Hassanein et al., 2020[29]	Evaluation of vibration effectiveness in minimizing pain during local anesthesia comparing it to traditional injection methods.	Split-mouth study including 60 patients randomized into 2 equal groups. Group I: Injection with vibration. Group II: Traditional injection with topical anesthetic. A 2-week interval between procedures. Pain assessment scales: FACES Pain Rating scale, FLACC scale.	FACES Pain Rating Scale, FLACC Scale: There is a significant difference between study and control groups in self-reported pain.	Vibration during local anesthetic injections is more effective in reducing pain than traditional methods (with topical gel) in pediatric dental patients.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Raslan and Masri, 2018[30]	Comparison of perceived pain level during 3 types of anesthesia with and without vibration.	A group of 40 children (mean age 8.2 ±1.8 years) was enrolled into the study. Pain assessment during buccal and palatal infiltration on the maxilla and IANB on the mandible was performed. Each anesthesia on both sides of the arches with and without vibration. 6 injections during 4 visits. Pain assessment scales: WBFPRS, FLACC scale.	Pain scores did not differ significantly in all 3 methods according to both pain scales.	Pain perceived by children during 3 types of anesthesia did not differ regardless if vibration technique was used or not.
Joshi et al., 2021[31]	Evaluation of the effectiveness of vibration on decreasing the pain during anesthesia in comparison with topical gel.	Split-mouth study including a group of 50 adults (mean age 25.06 ±7.32 years). Before IANB: One side received a vibration. Opposite, the control side received topical anesthesia. Pain assessment scale: VAS.	Mean pain rate significantly lower on the vibration side than on the control side.	Application of vibration reduces the pain perceived by patients during local anesthesia more effectively than topical anesthesia.
Dak-Albab et al., 2016[32]	Evaluation of the effectiveness of vibration on decreasing the pain during anesthesia compared with topical gel.	Split-mouth study including a group of 30 children. Before injection: One side received a vibration. Opposite, the control side received topical anesthesia. A 1- or 2-week interval between procedures. Pain assessment scale: FLACC scale.	Mean pain rate significantly lower on the vibration side than on the control side. Significant differences in F, L and C components between vibration and topical gel sides.	Application of vibration reduces the pain perceived by patients during local anesthesia better than topical anesthesia.
Ching et al., 2014[33]	Comparison of perceived pain during anesthesia between vibration and traditional techniques.	Split-mouth study including a group of 36 children (mean age 14 years). Before injection: One side received a vibration. Opposite, the control side received just local anesthesia. Pain assessment scales: WBFPRS.	Mean pain rate significantly lower in the vibration group than in the control group.	Vibration reduces pain perceived during anesthesia more effectively than traditional method.
Salma et al., 2021[34]	Evaluation of vibration on reducing pain during anesthesia application.	Split-mouth study including a group of 166 adults (mean age 28.4 ±7.1 years). Before injection: One side received a vibration. Opposite, the control side received topical anesthesia. A 3-week interval between procedures. Pain assessment scale: VAS scale, during needle insertion (PP), midinjection pain (MIP). Heart rate: Baseline heart rate (BHR), penetration heart rate (PHR), and midinjection heart rate (IHR).	VAS scale: Median pain rates were lower in the study group than in the control: 43% less at penetration; 67% less during injection. Significantly lower pain during injection and anesthetic deposition in study group than control group. Heart rate: A. Per side Pain rates significantly higher during penetration than mid injection in both groups. Significantly higher increase in heart rate at penetration than during injection in both groups. B. Study group vs control group Significantly lower pain during injection in study group than control group. Significantly lower increase in heart rate in study group than control group. In control group pain rates at penetration and during injection were significantly different according to the: LA technique – values higher for IANB; gender – values higher in women.	Vibration has a pain-reducing effect during anesthesia administration compared to conventional methods.
Ramezani et al., 2017[35]	Effect of vibration on pain perceived during local anesthesia.	Split-mouth study including a group of 36 children (mean age 5.7 years). Before injection: One side received a vibration. Opposite, the control side received a placebo. Pain assessment scales: WBFPRS.	Significant difference in pain scores between study and control group. No difference in pain scores in terms of age, gender or injection type.	Vibration is an effective method of decreasing pain during anesthesia.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Tung et al., 2018[36]	Comparison between the effectiveness of vibration, manual stimulation and conventional anesthesia on decreasing the pain during administration of LA.	A group of 150 children (age 5–7 years). Control group: 11.1 ±2.4. Manual stimulation group 10.7 ±2.2 vibration group 11.1 ±2.3 years) was divided randomly into 3 equal groups. Before injection: The control group received just local anesthesia, the 1st study group – manual stimulation, the 2nd study group – vibration. Pain assessment scales: WBFPRS. Heart rate	No significant differences regardless of injection type. Mean pain rate significantly lower in the vibration group than control and manual stimulation group. No significant difference in pain scores between control and manual group. Significant difference in pain scores between vibration group and manual group. No significant differences in heart rate between groups.	Vibration is significantly more effective in decreasing pain during anesthesia than manual stimulation and conventional methods.
Albouni et al., 2022[37]	Comparing the pain levels between traditional syringe injections (CI) and those assisted by Vibraject technology (VAI – vibraject assisted injections).	A group of 75 children was divided randomly into 3 equal groups. I: Upper buccal infiltrations (UBI), II: Posterior palatal infiltrations (PPI) III: IANB All received both conventional and vibration-assisted injections in separate dental visits 2 weeks apart. Pain assessment scales: The VAS and FLACC scales.	Significant differences in VAS score between conventional CI and VAI syringe in Groups I, II, and III with higher VAS scores associated with the CI. In the FLACC scale, "mild" and "moderate" pain responses were significantly higher with VAI, while "severe pain" responses were significantly higher with CI.	VibraJect-assisted injection was more effective in minimizing pain during all 3 methods of local anesthetic administration in clinical dental procedures for children.
Hutchins et al., 1997[38]	Comparison of the effectiveness of vibration and topical anesthetic in reducing the pain felt during oral injections of local anesthesia.	A double-blind study consisted of 61 patients receiving a combination of topical anesthetic or placebo with or without 1-min vibration. The location of the injection was the palatal and buccal side of both maxillary 1st premolars. Pain assessment scale: A 5-point scale where 0 described no pain, 1 – mild pain, 2 – moderate pain, 3 – distressing pain, 4 – horrible pain, 5 – unbearable pain.	The use of topical anesthesia leads to a reduction in pain values across 2 categories: Buccal anesthetic vs placebo and both buccal and palatal anesthetic vs placebo. The presence of vibration alone or in the combination with anesthetic, the placebo alone and placebo plus vibration did not exhibit a statistically significant correlation with the pain level.	The topical anesthetic demonstrates a reduction in pain values, although the clinical relevance remains uncertain. The application of vibration appears to have limited effectiveness; however, exploring its other variations, using vibration during, not before the injection process or the use of a more effective vibration transfer could enhance its efficacy.
Bagherian and Sheikhfathollahi, 2016[39]	Children's behavioral responses to local anesthetic injections with the cotton-roll vibration method in comparison to the conventional topical anesthesia.	Forty-eight children (mean age 5.94 ±1.88 years) received contralateral IANB or primary maxillary molar infiltration injections randomly using cotton-roll vibration (topical anesthesia gel, cotton roll, and vibration) and control (routine topical anesthesia) methods. Each child received the alternate method on the other side in the next session. Pain assessment scales: FHFHTC scale, producing total scores from 0 to 18.	Regardless of gender, age and area of local anesthesia, the cotton-roll method showed significantly lower mean FHFHTC pain reaction scores compared to the control method.	The cotton-roll technique proves to be more effective than standard topical anesthesia in reducing children's behavioral pain responses. Topical anesthesia may provide greater psychological impact than pharmacological effects in reducing children's behavioral pain reactions.
Gandhi et al., 2008[40]	Effectiveness of vibration in minimizing pain during local anesthetic injections comparing to lignocaine jelly.	A group of 30 children.1-visit procedure: Application of topical anesthesia, traditional injection. 2-appointment procedure after 4–5 days on the contralateral side of the arch: Application of the mucosal vibrator before, during local anesthetic administration to the injection site and continued after needle removal. Pain assessment scales: The SEM scale, WBFPRS.	WBFPRS and SME scales. There is a statistically significant difference between the mucosal vibration and the topical gel group.	Using a vibration method during local anesthetic injections is more effective in reducing pain than traditional methods (with topical lignocaine gel) in pediatric dental patients.
Aminabadi et al., 2008[41]	Evaluate the efficacy of counterstimulation and distraction on pain during intraoral injection in pediatric patients.	A group of 78 children (mean age: 4.72 years) was divided randomly into 3 equal groups. Before IANB: Soft tissue vibration (C + SA), soft tissue vibration with distraction exercise (CD + SA), topical anesthesia (SA). Pain assessment scales: SEM scale.	Pain reaction in SA group significantly higher than in C + SA and CD + SA groups. Pain significantly less exhibited in CD + SA group than in C + SA group.	Both counter stimulation and distraction may effectively reduce pain.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Nanitsos et al., 2009[42]	Evaluation of the effectiveness of vibration on decreasing the pain during anesthesia.	Split-mouth study including a group of 62 adults (mean age 45 years). Before IANB, mandibular or buccal infiltration: One side received vibration. Opposite, the control side received no vibration. Pain assessment scales: VAS, McGill pain descriptors.	Statistically significant difference between anticipated and actual pain for block and infiltration injections. VAS: Mean pain rate significantly lower on the vibration side than on the control side during infiltration and IANB injections, as well as for each of these injections separately. McGill pain descriptors: Mean pain rate during injections significantly lower on the vibration side than on control side.	Application of vibration reduces the pain perceived by patients during local anesthesia.
Meghana Reddy, 2020[43]	Comparison between vibration and topical gel on decreasing the pain during anesthesia.	A group of 10 patients who required dental treatment in the 3 quadrants was enrolled into the study. Before infiltration: One quadrant received vibration; one quadrant received topical anesthesia with benzocaine 20% gel; the control side received just local anesthesia. Pain assessment scale: VAS.	Mild pain for topical gel application + anesthesia. Moderate pain for vibration + anesthesia. Severe pain for the anesthesia alone.	Topical anesthesia is better than vibration in decreasing pain during anesthesia.
Marwah et al., 2020[44]	Assessing patients' pain perception and comfort during the administration of local anesthesia by comparing the Buzzy system to the conventional syringe.	Fifty children were randomly separated into 2 groups: 1st group had LA administered using conventional syringe; 2nd group had Buzzy (vibration + cold) followed by administration of LA. Pain assessment scale: WBFPRS, pulse oximeter, FLACC scale.	The pulse rate, oxygen saturation levels and WBFPRS exhibited statistically insignificant results. The FLACC scale: Significantly higher score in the conventional than Buzzy group.	The combination of external cold and vibration can alleviate pain and anxiety experienced during the administration of local anesthesia.
Sahithi et al., 2021[45]	Evaluation of the external vibrating tools and counterstimulation effectiveness in reducing a child's dental anxiety and pain perception when receiving local anesthetic.	A group of 100 children was divided randomly into 2 equal groups: Group BD received vibration; Group CS received counterstimulation. Anxiety levels evaluation: Venham's Clinical Anxiety Rating Scale (VCARS), Venham Picture Test (VPT), and a Pulse oximeter. Pain assessment scales: WBFPS, VAS before, during, and after the administration of local anesthesia.	Post-intervention pulse rate measurements significantly decreased, indicating reduced anxiety, particularly notable in the BD group. The BD group exhibited a more pronounced reduction in pulse rate and subjective discomfort (WBFPS, VAS) during LA needle insertion compared to the CS group. VCARS scores showed a reduction only in the BD group.	Vibrating external stimulation demonstrated superior efficacy compared to counterstimulation in reducing needle-related anxiety among pediatric patients.
Bilsin et al., 2020[46]	Evaluation of the effectiveness of external cooling and vibration on decreasing the pain during anesthesia.	A group of 40 children (mean age 9.36 ±1.12 and 9.20 ±0.92 years for the control and study group, respectively) was divided randomly into 2 equal groups. In the study group external cold and vibrating device were used 2 min prior and during injection. In the control group anesthesia was performed without any additional application. Pain assessment scales: WBFPRS.	Mean pain score presented statistically significant difference between both groups. The mean age of the subjects negatively correlated with the mean scores of pain in both groups. A significant difference in pain rates between positive and definitely positive behaviors in control group.	Application of external cooling and vibration reduces the pain perceived by patients during anesthesia.
Jagtap et al., 2019[47]	Assessment of LLLT impact on the reduction of pain caused by local anesthetic injections.	Twenty-five patients (18–60-year-olds). Bilateral anesthesia was administered. The sites were divided into Condition A – LLLT 660-nm side and Condition B – Placebo side (without LLLT). Pain assessment scale: VAS scale.	Statistically significant difference in pain perception between the laser and placebo groups.	This study indicated a reduced perception of pain in the laser condition compared to the placebo condition.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Dehgan et al., 2022[48]	Evaluation of photobiomodulation used with different laser doses on reducing pain during anesthesia.	A group of 160 children (mean age 7 ±1.12 years) was divided randomly into 4 equal groups. Before anesthesia: 1st, 2nd and 3rd group received photobiomodulation at wavelength of 940 nm for 20 s with a power of 0.3 W, 0.4 W and 0.5 W, respectively. The control group with placebo application of laser. Pain assessment scales: WBFPRS, FLACC scale.	WBFPRS, FLACC scale scores. Significantly lower pain scores in the all-study groups than in the placebo group. No significant difference in the pain scores according to WBFPRS scale between the study groups.	Pain perceived during injection is reduced with the application of the photobiomodulation therapy prior to the injection regardless of the used dose in comparison to traditional method.
Elbay et al., 2022[49]	Evaluation of photobiomodulation used with different laser doses on reducing pain during supraperiosteal anesthesia and comparison of pain reduction during injection with and without photobiomodulation.	A group of 160 children (mean age 8.56 ±1.68 years) was divided randomly into 4 equal groups. Before anesthesia: 1st, 2nd and 3rd group received photobiomodulation at wavelength of 940 nm and a power of 0.3 W for 20, 30 and 40 s, respectively. The 4th group (control group) received a placebo application of laser. Pain assessment scales: WBFPRS, FLACC scale.	No significant differences between groups.	Pain perceived during injection did not differ between control group and photobiomodulation groups.
Sharifi et al., 2022[50]	Evaluation of LLLT with 810–980 nm wavelengths on pain during injection.	Split-mouth study including a group of 84 adults (mean age 24.76 ±2.63 years). Prior to the injection: One side received LLLT. Opposite, the control side received a placebo. A 14-day interval between procedures. Pain assessment scale: VAS.	Mean injection pain significantly lower on the LLLT side than on the placebo side. Injection pain significantly lower on the LLLT side than on the placebo side in women. No significant differences in men in terms of laser or placebo therapy and injection pain. No significant differences between overall pain scores in women and men with or without LLLT. No significant differences between women and men in terms of pain scores with LLLT therapy. Injection pain significantly lower in men without LLLT therapy. Significant differences between pain scores in women and men without LLLT.	Low-level laser therapy successfully reduced perceived pain during infiltration in the anterior region of the maxilla.
El Feghali et al., 2022[51]	Evaluation of the effectiveness of PBM on decreasing the pain during anesthesia in comparison with topical gel.	A group of 60 adults (mean age: Topical anesthesia group 42.27 ± 14.83 years, Laser group: 45.4 ± 15.84 years) was divided randomly into 2 equal groups. Before buccal infiltration: T group received topical anesthesia; L group received PBM. Pain assessment scales: VAS, Verbal Rating Scale (VRS).	VAS scale: No significant differences in pain scores between groups. VRS scale: Significantly higher ratings of taste, undesirable numbness, and overall satisfaction in L group than T group.	Photobiomodulation has a similar effect on decreasing pain as topical anesthesia; however, it exhibits better effects in terms of undesirable effects such as unpleasant taste and numbness.
Tuk et al., 2017[52]	Evaluation of LLLT therapy on pain perceived during local anesthesia.	A group of 163 adults (mean age 25.06 ±7.32 years) was divided randomly into 2 groups. Before IANB/local infiltration: Study group received LLLT therapy, the control group received placebo irradiation. Pain assessment scale: 11-point numerical rating scale (pain and anxiety), a blood volume pulse, a sweat conductance or galvanic skin response sensor.	Mandibular region: Heart rate a bit higher in control group than in LLLT group. Sweating slightly higher in LLLT group than in control group. Pain scores presented slight differences. Statistically significant difference between LLLT and control groups only in sweating rate. Maxillary region: Heart rate a little bit higher in control than LLLT group. Pain scores were lower for the LLLT group compared with control group. No significant differences between groups.	Low-level laser therapy is not effective in decreasing pain during LA.

Table 1. General characteristics of included studies – cont.

Study	Aim of the study	Materials and methods	Results	Conclusions
Seraj et al., 2020[53]	Assessing the effect of PBM at 810 nm wavelength on the reversal of local anesthesia.	Split-mouth study including a group of 34 children. Subjects were divided into 2 groups: In the laser side patients received 810-nm laser irradiation, 45 min after anesthesia injection, and the other side received placebo. A 7–10 days interval between procedures. Reversal of local anesthesia tests: Palpation technique every 15 min after the procedure.	Significant difference in the duration of anesthesia for both laser and sham laser groups. The time of anesthesia in the study – laser group was reduced by 43 ±24.03 min.	810-nm diode laser significantly reduced the duration of anesthesia time.

in dentistry, ongoing efforts are being made to improve techniques, methods and devices to alleviate injection anxiety.[11,14] The analysis of 40 studies investigating vibration-based and PBM methods alongside LA reveals promising outcomes in reducing pain during injection procedures across pediatric and adult populations. As pain is subjective in nature, the core indicators of the clinical effect of the presented methods were based on patient-reported sensations measured by different scales. The most frequently used were the VAS, FLACC scale, WBFPS, Wong–Baker Pain Rating Scale, and others, alongside more objective methods such as pulse and heart rate at baseline and after injection, pulse oximeter, blood volume pulse, sweat conductance, or galvanic skin response sensor. The analysis of the studies indicated a high degree of certainty for evidence and quality.

The impact on the level of pain perception during LA was analyzed in 32 scientific studies, 7 included PBM,[24,47–52] and 25 focused on the use of vibration.[2,4,11–13,27–46] Overall, 21 (65.5%) studies revealed significant pain reduction during injection, 7 (22%) found no significant differences, and 4 (12.5%) presented inconsistent results. In the area of vibration-based methodology, 17 research studies focused on the pediatric population,[3,4,11,12,29,30,32,33,35–37,39–41,44–46] 7 studies on adults,[13,27,28,31,34,38,42] and in 1 case, detailed characterization of the participants was not provided.[36] In the analysis of children and adolescents, a significant reduction in the incidence of pain was observed in 12 cases (70.5%).[11,12,29,32,33,35,37,39–41,45,46] No significant differences were observed in 2 (12%) publications[3,30] and inconsistent results were reported in 3 (17.5%) other studies.[4,36,44] In the analysis of the adult groups, a significant difference in decreasing pain during anesthesia was observed in 5 (72%) studies[27,28,31,34,42] and no significant difference in 2 (28%) studies.[13,38]

As a part of the research on PBM, 4 experiments were conducted on adults[47,50–52] and 3 on children and adolescents.[24,48,49] Children and adolescents were assessed with the WBFPS and FLACC scales in 3 studies.[24,48,49] Results indicated that in both scales, significant differences between groups were found in 1 study[48] and no significant differences in 1 paper.[49] Additionally, in 1 study,[24] significant differences between groups were presented in relation to WBFPS; however, regarding the FLACC scale, no significant differences were noted. In 4 studies conducted on adults, the VAS, numerical rating scale of pain, heart rate, and sweating were used to assess pain linked with injection. Among them, PBM measured with the VAS presented a statistically significant decrease in pain during injections in 2 studies,[47,50] while no significant differences were found in the remaining paper.[51] A significant difference in sweating was reported during mandible injections in 1 study.[52] However, in the same study, the numerical rating scale of pain, heart rate and sweating during maxillary anesthesia showed no significant differences.[52]

Reversal of the LA duration was evaluated in 9 studies.[2,6–10,24,26,53] For this purpose, 6 studies used PM[6–10,26] and 3 PBM.[2,24,53] Classifying papers according to the age of the respondents, 5 studies concerned children and adolescents[2,7,9,24,53] and 4 evaluated adults.[6,8,10,26] Significant differences in the duration of anesthesia were revealed in 8 studies. Only 1 study,[24] evaluating the use of PBM, in children did not observe any significant changes in anesthesia duration. In terms of adverse effects, no significant differences between the study and control groups were noted in 5 studies.[6–10] In 1 study,[9] which concerned children as an investigated group, nausea and elevated body temperature were reported. Postoperative pain was assessed in 2 studies,[8,26] both using a VAS. In the study by Shadmehr et al.,[26] pain 6 and 12 h after the procedure was significantly higher in the study group, whereas no significant differences were found in the other group.[8]

Limitations

Although all the selected studies were clinical studies, the samples were relatively small, and study participants were of different ages, which influenced the assessment methods/scales used. More studies are needed to verify the effects of PM, PBM and vibration. Since PM use is not permitted in all countries, the effect of the medication on patients of other nationalities could not be assessed. Moreover, significant heterogeneity among the included studies does not allow us to perform a meta-analysis. However, further research should be conducted to enable proceeding with a meta-analysis.

Table 2. Detailed characteristics of included studies

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Annu et al., 2023[2]	PBM	N/A	660 and 810 nm wavelengths 100 mW	45 min after injection, irradiation for 12 s, 6 places, continuous mode	lignocaine with adrenaline LIGNOX 2% A 1.5 mL	IANB	pulp therapy of mandibular molars	children: 4–8 years	palpation technique (anesthesia duration) the pin prick test	yes	mean soft tissue local anesthesia reversal time duration study groups: 660 nm – 130.5 min 810 nm – 144 min control group: 199.5 min
Felemban et al., 2021[3]	V	N/A	DentalVibe Injection Comfort System	5 s prior, during injection and 5 s after	mepivacaine 2% with 1:100,000 epinephrine	buccal infiltration anesthesia (BIA)	dental treatment	children: 6–12 years	FLACC scale, the validated Arabic version of the Wong–Baker FACES scale	no	N/A
AlHareky et al., 2021[4]	V	cold	The Buzzy device	just before and during injection	2% lidocaine with 1:100,000 adrenaline, 1.8 mL	BIA	dental treatment	children: 5–12 years	FLACC scale SEM scale VAS	yes no yes	N/A
Michaud et al., 2018[6]	PM	N/A	PM: 1.7 mL; 0.4 mg	after treatment	2% lidocaine with 1:100,000 adrenaline 1.8 mL	IANB	study	adults: over 18 years	anesthesia duration adverse effects	yes no	Study group lip – 120 min tongue – 105 min smiling – 96 min drinking – 104 min speaking – 72 min Control group lip – 170 min tongue – 134 min smiling – 151 min drinking – 170 min speaking – 140 min
Tavares et al., 2008[7]	PM	N/A	PM: if ½ cartridge of anesthetic = 0.2 mg; 1 cartridge = 0.4 mg	after treatment (20–49 min after injection)	2% lidocaine with 1:100,000 adrenaline 15–30 kg: ½ cartridge over 30 kg: ½ or whole cartridge	supraperiosteal injection; IANB; Gow–Gates nerve blocks	dental treatment (restorative or periodontal, crowns)	children: 4–11 years	anesthesia duration adverse reactions	yes no	75 min (55.6%) decrease in the median time for the return of normal lip sensation 67.5 min (60%) reduction in the median time for the return of normal tongue sensation.

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Fowler et al., 2011[8]	PM	N/A	PM: the same amount (1.8 mL/3.6 mL) as the cartridge of anesthetics	after treatment PM group: 71 min in maxilla 84 min in mandible Control group: 67 min in maxilla, 85 min in mandible	2% lidocaine with 1:100,000 adrenaline additionally 4% articaine with 1:100,000 or 2% lidocaine with 1:100,000 adrenaline 1.8 mL/3.6 mL	IANB, long buccal nerve block additionally: buccal infiltration, an intraosseous injection or infiltration	endodontic treatment	adults: 18–81 years	anesthesia duration VAS postop pain adverse reactions	yes no no	Study vs control Maxillary Lip/Cheek: Disappearance of numbness: 99 min vs 134 min Return-to-normal sensation: 136 min vs 224 min Mandibular Lip: Numbness: 121 min vs 145 min Sensation: 170 min vs 217 min Tongue: Numbness: 106 min vs 121 min Sensation: 142 min vs 169 min
Gago-García et al., 2021[10]	PM	N/A	PM the same amount (1.8 mL,1.7 mL) as the cartridge of anesthetics	after treatment	Group I lidocaine 2% 1/80,000 Group II articaine 4% 1/200,000, Group III bupivacaine 0.5% 1/200,000	IANB	tooth filling, tooth extraction, full mouth disinfection, implant placement, and root canal treatment	adults: over 18 years	anesthesia duration adverse reaction	yes no	On average Group I lip – 59.6 min tongue – 52.5 min normative value – 180 min Group II lip – 88.5 min tongue – 84.5 min normative value – 258 min Group III lip – 249 min tongue – 214 min normative value – 460 min

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Hegde et al., 2019[11]	V	N/A	Vibration device	2 min prior injection	N/A	inferior alveolar nerve block (IANB)	bilateral dental treatment	children: 6–11 years	FLACC scale	yes	N/A
									Wong–Bakers Pain Rating Scale	yes	
									pulses rate at the baseline and after injection	yes, after injection no, at the baseline	
Shilpapriya et al., 2015[12]	V	N/A	DentalVibe	1 minute before, during and 10 second after injeciton	N/A 2 mL	N/A	bilateral dental treatment	children: 6–12 years	universal pain assessment tool	yes	N/A
Erdogan et al., 2018[13]	V	N/A	DentalVibe	5 s prior, during and 5 s after injection	2% mepivacaine with 1:100,000 adrenaline 1 mL	infiltration technique at the apical region of the right maxillary incisors	study	adults: 18–26 years	Wong–Baker FACES Pain Rating Scale	no	N/A
									VAS	no	
Babaei et al., 2011[19]	PM	N/A	PM: 15–30 kg: 0.2 mg; above 30 kg: 0.4 mg	30 min after injection	lidocaine 2% 1/80,000 adrenaline max. 1 cartridge	IANB	bilateral dental treatment (except for extraction)	children: 4–11 years	anesthesia duration	yes	Group I Study vs control sensation of soft tissue 29.47 min vs 135.52 min. Group II Study vs control sensation of soft tissue 33.12 min vs 106.04 min.
Uçar et al., 2022[24]	PBM	N/A	810 nm wavelength 0.3 W 400-µm fiber	20 s continuous mode	4% articaine hydrochloride with 1/100 000 adrenaline 1 mL	local infiltration	bilateral pulpotomy in first primary molars in the mandible	children: 6–9 years	Wong–Baker FACES Pain Rating Scale	yes	Mean soft tissue anesthesia duration study group: 160.91 min control group: 161.83 min
									FLACC scale	no	
									anesthesia duration	no	
									adverse effects	no	
Shadmehr et al., 2019[26]	PM	N/A	PM: 1.7 mL; 0.4 mg	after treatment	2% lidocaine with 1:100,000 adrenaline 1.8 ml	IANB	single-session endodontic treatment	adults: over 18 years	anesthesia duration	yes	Study group lip – 120 min tongue – 105 min Control group lip – 152 min tongue – 174 min
									Heft–Parker visual analogue scale pre and postoperative	preoperative no postoperative adverse effect	

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Shaefer et al., 2017[27]	V	N/A counter-stimulation in control group	DentalVibe Injection Comfort System	10 s before, during and 5 s after injection	3% mepivicaine 0.5 mL for LB; 1.8 mL for IANB	intraoral long buccal (LB), IANB	study	adults: 21–32 years	VAS symptom severity index (SSI)	yes	N/A
Nasehi et al., 2015[28]	V	N/A	DentalVibe Injection Comfort System	DentalVibe used as per manufacturer's recommendations	Lignocaine hydrochloride with 1:200,000 adrenaline	IANB Long Buccal Infraorbital Palatal	bilateral dental treatment	adults: over 18 years	VAS	yes	N/A
Hassanein et al., 2020[29]	V	N/A	DentalVibe	5 s prior and during injection	mepivacaine HCl 2%, 1:20,000 levonordefrin	IANB	bilateral mandibular pulpotomy	children: 5–7 years	Wong–Baker FACES Pain Rating Scale FLACC scale	yes yes	N/A
Raslan and Masri, 2018[30]	V	N/A	Dentalvibe	buccal infiltration + IANB: 5 s prior, 60 s of anesthesia deposition and 5 s after injection palatal infiltration: 5 s prior and during anesthesia deposition	4% articaine hydrochloride with 1/100,000 adrenaline	buccal and palatal infiltration IANB	dental treatments in maxilla and mandible on both sides of the arches	children: 6–9 years	Wong–Baker Faces Pain Rating Scale FLACC scale	no no	N/A
Joshi et al., 2021[31]	V	N/A	DentalVibe	1 min prior, during and 10 s after injection	2% lidocaine with 1:200,000 adrenaline	IANB	bilateral dental extractions in the mandible	adults: 18–50 years	VAS	yes	N/A
Dak-Albab et al., 2016[32]	V	N/A	DentalVibe	30 s before injection	N/A	IANB	bilateral dental treatment	children: 8–12 years	FLACC scale	yes	N/A
Ching et al., 2014[33]	V	N/A	DentalVibe	5 s before, during anesthesia application, immediately stopped after needle	2% lidocaine with 1:100,000 adrenaline 0.85 mL	infiltration anesthesia	bilateral dental treatment	adolescents: 10–17 years	Wong–Baker FACES Pain Rating Scale	yes	N/A

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Salma et al., 2021[34]	V	N/A	DentalVibe	10 s before, during and 5 s after anesthesia	2% lidocaine with 1:100,000 adrenaline 1.8 mL	IANB, buccal, palatal infiltration	bilateral maxillary or mandibular extractions	adults: 18–55 years	VAS during needle insertion mid-injection pain	Yes	N/A
									heart rate baseline heart rate (BHR), penetration heart rate (PHR), and midinjection heart rate (IHR)	Yes	
Ramezani et al., 2017[35]	V	N/A	DentalVibe	5 s before, during and 5 s after injection	2% lidocaine with 1:80,000 adrenaline	IANB, infiltration block	bilateral maxillary or mandibular treatment	children: 5–7 years	Wong–Baker FACES Pain Rating Scale	yes	N/A
Tung et al., 2018[36]	V	manual stimulation with thumb (another group)	DentalVibe	10 s prior, during and 2 s after injection	2% lidocaine with 1:100,000 adrenaline	IANB/long buccal injections, maxillary infiltration injection	operative dental treatment	children: 7–14 years	Wong–Baker FACES Pain Rating Scale	yes	N/A
									pulse rate	no	
Albouni et al., 2022[37]	V	N/A	VibraJect	No specific data	2% lidocaine with 1:100,000 adrenaline	upper buccal infiltrations (UBI), posterior palatal infiltrations, IANB	bilateral dental treatment	children: 6–9 years	VAS	yes	N/A
									FLACC scale	yes	
Hutchins et al., 1997[38]	V	20% benzocaine	A modified battery-powered shaver	1 min before treatment	2% lidocaine with 1:100,000 adrenaline 0.2 mL	palatal and BIA of both maxillary first premolars	N/A	adults: over 18 years	5-point VAS	no, for vibration; yes, for topical anesthesia	N/A
Bagherian and Sheikhfathollahi, 2016[39]	V	verbal distraction	cotton roll	before, during and few seconds after injection	lidocaine 2% 1/80,000	IANB, maxillary infiltration	bilateral dental treatment	children	the author developed face, head, foot, hand, trunk, and cry (FHFHTC) scale	yes	N/A
Gandhi et al., 2018[40]	V	N/A	Rajasthan University of Health; Science (RUHS) mucosal vibrator	before, 1min during and 15 s after injection	N/A 1 mL	IANB	bilateral mandibular restoration, pulpotomy, pulpectomy or extraction	children: 6–11 years	SEM scale	yes	N/A
									Wong–Baker FPR scale	yes	

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Aminabadi et al., 2008[41]	V	verbal distraction	thumb and forefinger	N/A	2% lidocaine with 1/100000 epinephrine, 1 mL	IANB	mandibular restoration	children: 4–5 years	SEM scale	yes	N/A
Nanitsos et al., 2009[42]	V	N/A	HoMedics Atom massager	N/A	N/A	buccal or mandibular infiltration IANB	bilateral dental treatment	adults: 18–72 years	VAS	yes	N/A
									McGill pain descriptors	yes	
Meghana et al., 2020[43]	V	N/A	Waterpik Vibrator	N/A	N/A	infiltration injection	dental treatment in 3 quadrants	N/A	VAS scale	yes, but in favor of the topical anesthesia	N/A
Marwah et al., 2020[44]	V	cold	The Buzzy device	N/A	lidocaine 2% with 0.005 mg adrenaline	N/A	dental treatment	children: 5–10 years	Wong–Baker FACES Pain Rating Scale (WBFPRS)	no	N/A
									FLACC scale	yes	
Sahithi et al., 2021[45]	V	cold; counter stimulation in control group	BuzzyR device	N/A	lidocaine 2% 1/80,000	N/A	extraction and pulp therapy treatment in mandible	children: 4–11 years	Venham's Clinical Anxiety Rating Scale (VCARS)	pre and postop Yes	N/A
									Venham Picture Test (VPT)	pre no post yes	
									pulse oximeter	yes	
									Wong–Baker FACES Pain Rating Scale (WBFPS)	yes	
									VAS	yes	
Bilsin et al., 2018[46]	V	cold	The Buzzy device	2 min prior and during injection	2% lidocaine 2 mL	local mandibular anesthesia	extraction of mandibular primary molars	children: 7–12 years	Wong–Baker Faces Pain Rating Scale	yes	N/A
Jagtap et al.[47]	PBM	N/A	660-nm wavelength; 60 wM power	3 minutes before injection	N/A	N/A	bilateral extraction	adults: 18–60 years	VAS	yes	N/A

Table 2. Detailed characteristics of included studies – cont.

Study	Method	Additional pain reducing method	Parameters of PBM/PM/vibration appliance	Exposure time	Type of anesthetic + concentration + dose	Method of LA	Procedure type	Age category	Assessment method	Significant differences between study and control group	Duration of anesthesia study group vs control group
Dehgan et al., 2022[48]	PBM	N/A	980 nm wavelength 0.3 W, 0.4 W and 0.5 W a 400-µm fiber	20 s continuous mode	4% articaine hydrochloride with 1/100,000 adrenaline 1ml	buccal LA in maxilla or mandible	primary first molar treatment	children: 6–12 years	Wong–Baker FACES Pain Rating Scale	Yes	N/A
									FLACC scale	Yes	
Elbay et al., 2022[49]	PBM	N/A	940 nm wavelength 0.3 W a 400 µm fiber	20 s, 30 s, 40 s continuous mode	4% articaine hydrochloride with 1/100,000 epinephrine 1 mL	supra-periosteal anesthesia	dental treatment	children: 6–12 years	FLACC scale	No	N/A
									Wong–Baker FACES Pain Rating Scale	No	
Sharifi et al., 2022[50]	PBM	N/A	810–980 nm wavelengths; 50 mW, 810 nm, 100 mW, 810–980 nm or +50 mW, 980 nm	90 s prior the injection, dual continuous mode	2% lidocaine hydrochloride with 1:100,000 adrenaline 1.8 mL	buccal infiltration in anterior maxilla	bilateral restoration of central incisors in the maxilla	adults: over 18 years	VAS	Yes	N/A
El Feghali et al., 2022[51]	PBM	N/A	1,064 nm wavelength; 0.5 W; 10 Hz, 100 µs pulse width	60 s before injection	4% articaine with 1:100,000 adrenaline 2.2 mL	buccal infiltration injection	dental treatment in anterior maxillary region from canine to canine	adults	VAS	No significant differences	N/A
									VRS scale (taste, undesirable numbness, overall satisfaction)	Yes	
Tuk et al., 2017[52]	PBM	N/A	810 nm wavelength; 200 mW	Two times protocol: 30 s irradiation, 30 s interval, 30 s irradiation; 2 min of irradiation in total before injection continuous mode	articaine/hydrochloride 40 mg with epinephrine 0.01 mg 1.7 mL	IANB+ buccal infiltration/palatal and buccal infiltration	maxillary or mandibular third molar extractions	adults: 18–75 years	11-point numerical rating scale (pain and anxiety)	No	N/A
									blood volume pulse	No	N/A
									sweat conductance or galvanic skin response sensor	Yes, in mandible	N/A
Seraj et al., 2020[53]	PBM	N. d.	810 nm diode laser	45 min after injection, irradiation for 12 s in 6 spots	lidocaine 2% and 1: 100,000 epinephrine	infiltration	treatment of mandibular first molars	children: 4–8 years	palpation technique	Yes	Study laser group: 145.15 ±23.27 min. Sham group: 188.82 ±12.31 min.

N/A – no data; PBM – photobiomodulation; V – vibration.

Table 3. Quality assessment of included studies

Analyzed study	All procedures followed manufacturer's guidelines	Information about the anesthetic	Single operator	Clearly explained and justified sample size	No acute situations	Minimum 10 participants	Control group (split mouth included)	Use of single method	Single- or double-blinded	Sum	Risk of bias
Annu et al., 2023[2]	1	1	0	1	1	1	1	1	0	7	low
Felemban et al., 2021[3]	1	1	0	1	1	1	1	1	0	7	low
AlHareky et al., 2021[4]	1	1	0	1	1	1	1	0	0	6	moderate
Michaud et al., 2018[6]	1	1	1	1	1	1	1	1	1	9	low
Tavares et al., 2008[7]	1	0	0	0	1	1	1	1	1	6	moderate
Fowler et al., 2011[8]	1	1	1	0	1	1	1	1	1	8	low
Babaei et al., 2011[9]	1	1	1	0	1	1	1	1	1	8	low
Gago-García et al., 2021[10]	1	1	0	1	1	1	1	1	1	8	low
Hegde et al., 2019[11]	0	0	1	1	1	1	0	1	0	5	moderate
Erdogan et al., 2018[13]	1	1	1	0	1	1	1	1	0	7	low
Shaefer et al., 2017[27]	1	1	0	1	1	1	1	1	0	7	low
Uçar et al., 2022[24]	1	1	0	1	1	1	1	0	1	7	low
Shadmehr et al., 2019[26]	1	1	1	0	1	1	1	1	1	8	low
Shilpapriya et al., 2017[27]	1	0	0	0	1	1	1	0	0	4	moderate
Nasehi et al., 2015[28]	1	1	0	0	1	1	1	1	0	6	moderate
Hassanein et al., 2020[29]	1	1	1	0	1	1	1	0	0	6	moderate
Raslan and Masri, 2018[30]	1	0	1	1	1	1	1	1	0	7	low
Joshi et al., 2021[31]	1	0	0	1	1	1	0	1	0	5	moderate
Dak-Albab et al., 2016[32]	1	0	0	1	1	1	0	1	0	5	moderate
Ching et al., 2014[33]	1	1	0	1	1	1	1	1	0	7	low
Salma et al., 2021[34]	1	1	1	0	1	1	0	1	0	6	moderate
Ramezani et al., 2017[35]	0	1	0	0	1	1	0	0	0	3	high
Tung et al., 2018[36]	0	1	0	0	1	1	0	0	0	3	high high
Albouni et al., 2022[37]	1	0	1	0	1	1	1	0	0	5	moderate
Hutchins et al., 1997[38]	1	1	0	0	1	1	1	0	0	5	moderate
Bagherian and Sheikhfathollahi, 2016[39]	0	1	0	1	1	1	1	0	0	5	moderate

Table 3. Quality assessment of included studies – cont.

Analyzed study	All procedures followed manufacturer's guidelines	Information about the anesthetic	Single operator	Clearly explained and justified sample size	No acute situations	Minimum 10 participants	Control group (split mouth included)	Use of single method	Single- or double-blinded	Sum	Risk of bias
Gandhi et al., 2018[40]	0	0	0	0	1	1	1	0	0	3	high
Aminabadi et al., 2008[41]	0	1	1	0	1	1	1	0	0	5	moderate
Nanitsos et al., 2009[42]	1	0	0	0	1	1	1	1	0	5	moderate
Meghana Reddy, 2020[43]	1	0	0	1	1	1	0	1	0	5	moderate
Marwah et al., 2020[44]	1	1	0	1	1	1	1	0	0	6	moderate
Sahithi et al., 2021[45]	1	1	0	1	1	1	0	0	0	5	moderate
Bilsin et al., 2020[46]	1	1	1	1	1	1	1	0	0	7	low
Jagtap et al., 2019[47]	1	0	0	1	1	1	1	1	1	7	low
Dehgan et al., 2022[48]	1	1	1	1	1	1	1	0	1	8	low
Elbay et al., 2022[49]	1	1	1	0	1	1	1	1	1	8	low
Sharifi et al., 2022[50]	1	0	0	1	1	1	1	0	0	5	moderate
El Feghali et al., 2022[51]	1	1	0	0	1	1	1	1	1	7	low
Tuk et al., 2017[52]	1	1	0	0	1	1	1	1	1	7	low
Seraj et al., 2020[53]	1	1	0	0	1	1	1	1	1	7	low low

Conclusions

Significant reductions in pain perception, assessed using diverse pain scales, were observed in most cases evaluating the vibration-based and PBM methods. Furthermore, notable differences in anesthesia reversal using PM or PBM were documented with minimal adverse effects, underscoring the safety of these techniques.

Further research is warranted to explore the long-term efficacy, adverse event profiles and broader applications, particularly in the case of PBM, which has the least number of clinical trials regarding the subject evaluated in this review.

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Antigravity treadmill training after knee surgery: A scoping review

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Abstract

Antigravity treadmill training provides a viable option for physiotherapeutic care after knee surgery, especially for conditions that do not allow full weight bearing during the early phase post-intervention. This overview of the current state of knowledge identifies gaps and highlights areas where more research on antigravity treadmill training after knee surgery is needed. This review aimed to analyze and summarize the available evidence concerning the effects of antigravity treadmill training on patients after knee joint surgical procedures, including anterior cruciate ligament reconstruction (ACLR) and total (TKA) and unicompartmental knee arthroplasty (UKA). Several databases were searched for relevant material, including PubMed, Epistemonikos, the Cochrane Library, the Web of Science, and Google Scholar. Seven studies investigating antigravity treadmill training after various procedures were included, including ACLR and TKA. The studies were summarized, and the quality of evidence was evaluated using the appropriate tools. The evidence yielded by these studies suggests that antigravity treadmill training might be useful after knee surgery. However, the superiority over traditional physiotherapeutic measures has yet to be established. Therefore, future high-quality randomized controlled trials (RCTs) are needed to investigate the effect of antigravity treadmill training due to the low quality of available evidence. Also, a cost-effectiveness analysis is required to determine whether the investigated intervention fits the purpose.

Key words: knee arthroplasty, knee, total knee replacement, knee injuries, anterior cruciate ligament reconstruction

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Background

Rehabilitation interventions targeting the improvement in outcomes after surgical treatment on the knee primarily depend on the reason and type of procedure performed.[1–4] While early and intensive postoperative rehabilitation is allowed and even required for some conditions, weight-bearing restrictions are recommended for others.[5,6] However, partial weight-bearing primarily decreases muscular stimulation and, in the long run, a loss of muscle strength.[7]

It is commonly agreed upon that quadriceps and hamstring muscle strengthening should be a central target of therapy following knee surgery.[8,9] Mainly because of the restricted postoperative activity, the strength of the knee extensor muscles decreases significantly, impairing knee joint stability.[10–12] Also, knee flexor weakness is observed, which in anterior cruciate ligament reconstructed knees is linked to tendon harvesting for graft preparation purposes.[13–15] Subsequently, patients experience increased difficulty in performing daily activities, especially those requiring a more significant level of exertion with regard to the lower extremities.[16–18] This may result in a spiral where pain leads to inactivity, further exacerbating pain.[19] Moreover, a disturbed gait pattern has been shown to occur in patients undergoing knee surgery, such as total knee arthroplasty (TKA) and anterior cruciate ligament reconstruction (ACLR).[20,21]

Gait or run training using an antigravity treadmill is one method used during early rehabilitation following surgery. It aims to improve the functional outcome by early mobilization of patients despite weight-bearing restrictions. An antigravity treadmill, by either supporting the patient with ropes above a treadmill or using differential air pressures, enables patients to walk or run at a reduced body weight (BW) while maintaining a normal gait pattern.[22] Run training using an antigravity treadmill can also enhance sports performance.[23]

Antigravity treadmill training has been demonstrated to positively affect knee muscle strength in healthy individuals and those with different disorders.[23–25] In the field of orthopedics and sports medicine, antigravity treadmill training has been used in patients with hip replacements, ankle fractures, Achilles tendon rupture repairs, osteoarthritis, muscular dystrophy, and diabetic polyneuropathy.[26–33] However, there exists a need for evidence-based practices to consolidate, analyze and interpret the available literature and provide a foundation for future research and clinical decision-making in the context of antigravity treadmill training usage after knee surgery. The field of antigravity treadmill training after knee surgery is relatively new and rapidly evolving; therefore, a scoping review would be beneficial for providing an overview of the current state of knowledge, identifying gaps and highlighting areas where more research is needed.

Objectives

This systematic review aimed to analyze and summarize the available evidence concerning the usage of antigravity treadmill training in patients after knee joint surgical procedures, including ACLR and both TKA and unicompartmental knee arthroplasty (UKA). The evidence found was then ranked according to its power.

Materials and methods

Two reviewers independently searched multiple databases using the search strategy detailed in Table 1. The search was conducted using the Boolean operators of each column indexed with an "AND" in between. Between elements of the same column, an "OR" was introduced. The search strategy thus implicated the use of combinations of 1 search element per column with a search element for each of the other columns.

Table 1. The search strategy used for the present review purposes

Population	Intervention		Outcome
Knee* Ligament* TKR TKA ACL* LCL* MCL*	Anti*grav* Antigrav* Levitation* Zero-g Positive pressure Supported Suspended	Treadmill Running machine Walking machine	Pain Function Quality of life Adverse events Death Synovi* Cartilag* Osteo* *arthritis *nerv* Muscl* Blood Vascul* Imaging Radiography MRI CT Ultrasound

The asterisk (*) is used as a wildcard character in the search strategy. It represents any group of characters, allowing for the inclusion of all possible endings or variations of the term.

The searched databases included PubMed, Epistemonikos, the Cochrane Library, and the Web of Science. Additionally, Google Scholar was searched for relevant material. The search strategy included all articles published between 1980 and 2023 in English, German, Polish, French, and Arabic. The protocol for this review was not pre-registered, mainly because of its scoping character.

The obtained articles were then screened for eligibility. Articles eligible for inclusion included any original publication reporting clinically measured data. Title and abstract screening was performed independently by 2 researchers (H.T.H. and M.K.). Any conflicting views were resolved by a third party (R.P. and A.K.). Inclusion and exclusion criteria are detailed in Table 2. Additionally,

Table 2. Inclusion and exclusion criteria of the articles for the present scoping review purposes

Inclusion criteria	Exclusion criteria
• Original articles • Articles reporting findings of a clinical study, including: - Systematic reviews - Randomized controlled trials (RCTs) - Non-randomized controlled trials - Cross-sectional studies - Longitudinal studies - Cohort studies - Case-control studies - Case series - Case reports • Articles having a relevant PICO statement, including: - Population: Patients in the postoperative phase of any surgical procedures on the knee, including anterior cruciate ligament (ACL) repair, total knee arthroplasty, and unicompartmental knee arthroplasty - Intervention: Antigravity treadmill training in its various forms (rope suspension or positive pressure chambers) - Control: If present, any control, including conventional rehabilitative approaches or no treatment - Outcome: Any outcome, including patient-related outcome measures (PROMS), performance-based measures (PBMs), biomechanical or trigonometric as well as histopathologic or any other reported outcome.	• Non-original works: Studies reporting the work of a third research party. • Articles dealing with non-clinical data: - Expert and other types of opinions - Cost-effectiveness analyses - Literature reviews and any other type of reviews, excluding systematic reviews of randomized controlled trials - Editorials and any form of letters • Posters and conference papers, except for those reporting findings of clinical studies where no published article can be found • Any studies reporting data on non-human subjects, including animal and in vitro studies

the references of the included articles were screened for relevant material to ensure the comprehensiveness of the review. If the full text of the relevant article was not found, the authors attempted to contact the corresponding author to access it.

Relevant information extracted from the articles included the study design and level of evidence as well as the target population, the administered intervention, the comparators (control), the reported outcomes, the clinical and scientific recommendations, and the limitations of the study at hand. Full-text screening and subsequent data extraction were performed independently by 2 authors (H.T.H. and M.K.). Conflicts and discrepancies regarding the relevance of information were resolved by a third, more experienced party (R.P. and A.K.). Also, if necessary, additional notes were made during the data extraction. An appraisal using relevant Joanna Briggs Institute (JBI) instruments was conducted independently by the 2 previously mentioned reviewers. R.P. managed conflicts to assess the methodological quality of the studies.[34,35]

Results

The search yielded 8 articles that were deemed relevant to this review. However, the full text of one of them, a systematic review, was not found. Therefore, a request to provide the missing information was sent to the corresponding author indicated in the article. However, because no response was obtained, the systematic review was excluded from further analysis.

Finally, 7 articles were included in this scoping review: 1 randomized controlled trial (RCT),[36] 2 cohort studies,[37,38] 2 case series,[39,40] and 2 case reports.[41,42] A representation of the design and level of evidence of the included studies is presented in Table 3. Comparative analysis concerning the studied population, intervention and controls in the studies included in the present scoping review are shown in Table 4. Table 5 presents a comparative analysis of the main findings regarding the outcome, recommendations, limitations, and critical notes.

Table 3. A representation of the design and level of evidence of the included studies

Included study	Study design	Level of evidence
DeJong et al.[36]	randomized clinical trial	2
Bugbee et al.[37]*	cohort study	3
Sueyoshi and Emoto[38]	cohort study	3
Eastlack et al.[39]	case series	5
Huang et al.[40]	case series	5
Greig et al.[41]	case report	5
Hambly et al.[42]**	case report	5

*The study was a pilot and feasibility study; therefore, it was not assigned as a randomized clinical trial; **The report on the case was presented in 2 formats: a conference poster and a published article.

The results of the critical appraisal of the included studies using JBI critical appraisal checklists are presented in Fig. 1.

Discussion

Reviews are crucial in guiding and supporting the rationale for new clinical studies. They achieve this by identifying and addressing research gaps, thus minimizing the risk of redundant or wasteful research. The significance of different reviews in the context of improving evaluation standards for clinical studies in physiotherapy, orthopedics and sports medicine cannot be overstated.[43] This present review aims to analyze, summarize and critically appraise the available evidence on antigravity treadmill training in patients who have undergone knee surgery. The objective was achieved by searching multiple databases for

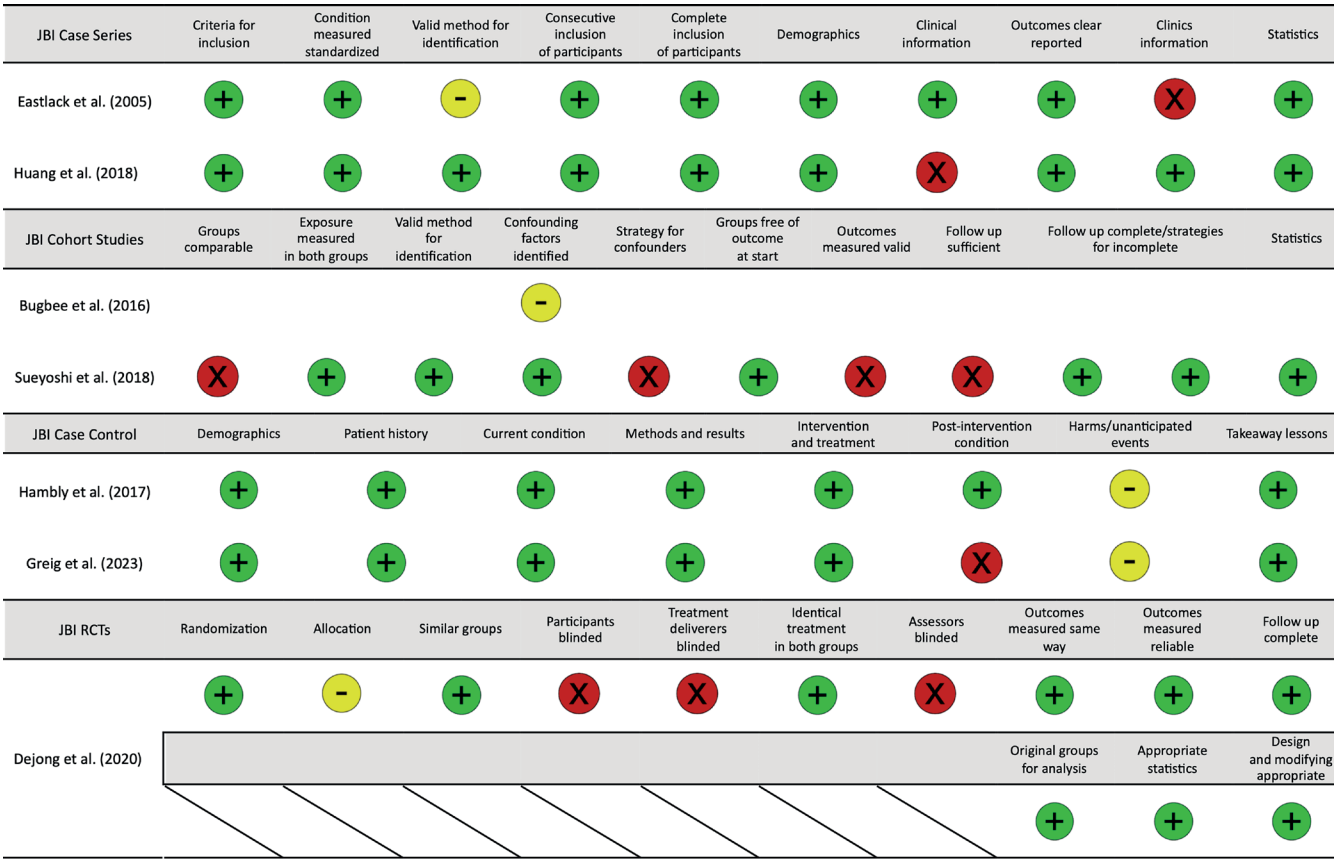

Fig. 1. The results of the critical appraisal of the included studies using the Joanna Briggs Institute (JBI) critical appraisal checklists

green – yes; yellow – unsure; red – no.

relevant materials. In short, the antigravity treadmill is a valuable device, and whether it is used in terms of gait or run training or for other purposes like balance exercises, it can improve outcomes of patients after knee surgery.[36–42] However, compared with procedures not involving an antigravity treadmill, its beneficial effects were not shown. The main findings of the particular analyzed studies will be discussed following the hierarchy of evidence.

The included RCT was deemed high-quality. However, no blinding was possible, and adverse events were not reported, even though they were a core outcome.[36] Although blinding decreases the risk of bias and improves a study's quality, it is rarely possible to blind patients to a physiotherapeutic intervention.[44–46] The main finding of the study of DeJong et al. was that no beneficial effects of using gait training with an antigravity treadmill were observed and that practitioners should, therefore, focus on the cost-effectiveness of the delivered interventions.[36,47,48]

Two cohort studies by Bugbee et al. and Sueyoshi et al. were included.[37,38] The Bugbee et al. study was analyzed as a cohort study, not as an RCT, because of its pilot and feasibility character, and it did not fulfill all the criteria of an RCT.[37] The study found no differences in the studied outcomes, including patient self-reported measures and mobility assessed using the Timed Up and Go test between the patients after TKA who received antigravity treadmill and land-based gait training.[37]Again, in light of cost-effectiveness, land-based gait training might be favored, although the pilot design of the study should be emphasized. In the other cohort study by Sueyoshi et al., patients after TKA, ACLR and other knee surgeries were divided into those performing balance exercises on an antigravity treadmill and those conducting the same balance exercises on the floor. In both studied groups, an improvement in timed single-leg stance was noted in the 2nd week postoperatively compared to the 1st week between the interventions carried out. However, a difference between the studied groups was not observed. It must be emphasized that the assignment to particular groups was based on patients' comfort level, precisely pain level, during the single leg stance on the floor using the involved limb. Patients who experienced a significant increase in pain during this test were assigned to balance exercises on an antigravity treadmill, while those who felt comfortable standing on the involved limb (no pain or a minimal increase in pain) were assigned to floor exercises.[38] Therefore, the study shows limited evidence due to the specific way assignments were issued for the studied interventions.

The included studies for the present scoping review purposes consecutively involved case series by Eastlack et al. and Huang et al.[39,40]

Table 4. Details concerning the studied population, intervention and controls (if applicable) in the studies included in the present scoping review

Authors	Population	Intervention	Control
DeJong et al.[36]	Patients after unilateral primary TKA Assignment, n = 368 Group 1, n = 95; Group 2, n = 96; Group 3, n = 96; Group 4, n = 99 Data analysis, n = 363 Group 1, n = 92; Group 2, n = 91; Group 3, n = 90; Group 4, n = 90 Follow-up analysis, n = 298 Group 1, n = 74; Group 2, n = 76; Group 3, n = 78; Group 4, n = 70 Gender: female 53–58% Mean age: 62.7–64.9 years Mean BMI: 31.2–32.2 kg/m^2 Inclusion criteria: Patients after elective unilateral TKA who initiated their outpatient PT within 24 days post-TKA; 40 years or older; weight less than 300 pounds. Exclusion criteria: Patients after a lower extremity joint replacement procedure, including a revision, 2nd, or bilateral TKA or total hip arthroplasty less than 1 year prior to their current TKA; whose payer was workers' compensation; in litigation related to injury or disease associated with their current TKA; pregnant or may be pregnant; a medical history of neurologic disorders, rheumatoid arthritis, or gout (unless 6 months since last exacerbation or flare up and under control medically); under active cancer treatment with history of malignancy in either or both lower extremities, or with evidence of signs or symptoms of cancer, chemotherapy, or radiation less than 1 year prior to their current TKA; developed deep vein thrombosis post-TKA; unable to proceed or continue the planned outpatient program because of complications such as wound infection related to the TKA and severe orthostatic hypotension; who required manipulation under anesthesia post-TKA; received more than 2 weeks of other care in another post-acute setting prior to outpatient PT. Random assignment to the studied groups.	The study had a parallel design comparing 4 different interventions: Group 1, a usual-care group that used a stationary recumbent bike Group 2, a group that used a BW-adjustable treadmill for gait training Group 3, a group that combined using a stationary recumbent bike with patterned electrical neuromuscular stimulation (PENS) Group 4, a group that combined using a BW-adjustable treadmill for gait training with PENS. All patients received up to 12 weeks of outpatient PT. Each visit included an exercise, treatment, and finishing and prevention phases. The exercise phase when the studied intervention was applied, lasted for 15–20 min. Regarding the BW-adjustable treadmill, the physical therapists identified the speed and amount of BW needed to be unloaded to minimize pain and allow patients to properly ambulate. Over time, physical therapists decreased BW support and increased speed as tolerated by the patient while maintaining a proper gait pattern. Used device: AlterG® Anti-Gravity Treadmill™. The information and results of the other interventions will not be displayed as it is not in the context of the current investigation.	The study had a parallel design comparing four groups with different interventions, as described in the Intervention column.
Bugbee et al.[37]	Patients after TKA Data analysis, n = 29 AlterG group, n = 14; Control group, n = 15 Gender: female 50–60% Mean age: 66.5–69.9 years Mean BMI: 28.4–28.8 kg/m^2 Inclusion criteria: Patients after unilateral primary TKA; discharged from the hospital to home (not to a skilled nursing facility); had only 3–4 home PT sessions; agreed to further outpatient PT at a single site; agreed to complete patient questionnaires. Exclusion criteria: inability to meet inclusion criteria; gross musculoskeletal deformity; uncontrolled chronic or systemic disease; inability to follow instructions because of mental impairment, substance abuse, or addiction. Random assignment to the studied groups.	Patients attended outpatient PT two days per week for 4 weeks for a total of 8 sessions. Therapy sessions lasted 45–60 min and included manual therapy, gait training, therapeutic exercises/activities and treatment modalities. Depending on the studied group the gait training antigravity (AlterG group) or land-based (control group). Regarding antigravity gait training, on day 1, the antigravity treadmill pressure chamber was set to allow only 50% of the patient's BW to be transmitted to the treadmill floor, and speed was controlled by the patient according to his/her comfort level. The percentage of BW was adjusted to allow for a safe and normalized gait pattern with a pain level no greater than 5 (0–10 scale) throughout the PT session. For subsequent visits, the body-weight setting was started from the end point of the previous session. Used treadmill: AlterG® Anti-Gravity Treadmill™	In the control group, land-based gait training was performed with or without an appropriate assistive device (AD) and appropriate assistance, tactile cueing, and verbal cueing from a physical therapist. Duration [min] and gait-training progression were dependent on the participant's functional goals, pain level (assessed throughout treatment), and level of fatigue.

Eastlack et al. studied the usage of gait training under lower body positive pressure (LBPP) conditions in patients after a unilateral arthroscopic meniscectomy or ACLR. Various parameters were measured under LBPP conditions, including ground reaction forces, dynamic knee range of motion, and electromyographic activity of the vastus medialis obliquus and biceps femoris. Also, pain during the interventions was assessed. It must be highlighted that the study was not intended to evaluate the effectiveness of LBPP as a rehabilitation modality. It was established

Table 4. Details concerning the studied population, intervention and controls (if applicable) in the studies included in the present scoping review – cont.

Authors	Population	Intervention	Control
Sueyoshi and Emoto[38]	Patients after TKA, ACLR and other knee surgery Data analysis, n = 49 AlterG group, n = 25 Control group, n = 24 AlterG group patients: n = 17 after TKA, n = 3 after TKA, n = 5 after other knee surgery Control group patients: n = 15 after TKA, n = 2 after TKA, n = 7 after other knee surgery Gender: not mentioned Mean age: 66.1–63.0 years Mean body mass: 56.6–58.6 kg Mean body height: 154.0–157.1 cm Inclusion criteria: not mentioned Exclusion criteria: not mentioned The assignment of patients to the studied groups depended on their performance in the initial balance test. Following the first assessment, individuals were allocated to either the AlterG group or the Control group based on their comfort levels. Patients who reported a "significant increase" in pain during the initial balance test were assigned to the AlterG group, while those who felt "comfortable" with no or minimal pain, or experienced no or minimal increase in pain, were assigned to the Control group.	Patients from the AlterG group performed balance exercise on antigravity treadmill. Performing antigravity or land-based balance exercise started at 1 week postoperatively and lasted 1 week. It was performed daily for at least 5 days a week. In each balance exercise session, patients were asked to stand on involved leg with their knee slightly bent targeting to stay on their foot for 30 s. This was repeated 3 times with 30 s rest in between trials. A balance exercise was made more challenging by having a participant stand on a form pad when appropriate. This decision was made by a licensed physical therapist. Regarding antigravity balance exercise the pressure on AlterG adjusted to a pain-free or minimal pain level at the beginning of each balance exercise session. Used device: AlterG® Anti-Gravity Treadmill™	Patients from the Control group performed described in the Intervention column balance exercise on a floor.
Eastlack et al.[39]	Patients after unilateral arthroscopic meniscectomy (n = 9) and ACLR with the use of autograft or allograft patellar tendon (n = 6); total number n = 15 Gender: female 33% Mean age: 41 years Mean body mass: 74.7 kg Mean body height: 175 cm Inclusion criteria: not mentioned Exclusion criteria: Pulmonary or cardiac disease; taking β-blocker medications; pregnancy; younger than 18 years.	Patients after meniscectomy exercised under lower body positive pressure conditions 1 week after surgery. Patients after ACLR exercised under lower body positive pressure conditions before surgery and once a week for 6 weeks postoperatively. Exercise under lower body positive pressure conditions was considered ambulation in the chamber in lower body positive pressure conditions (60% and 20% of BW). Each patient walked for 2 min at a comfortable walking speed of 0.67 m/s (1.5 mph) under 3 BW conditions (100%, 60% and 20% of BW). Used device: A developed device (Whalen and Hargens, US patent 5133339; Hargens waived rights to this patent to NASA) for unloading the lower extremities during walking or running. The device consist of a treadmill in a waist-high chamber that uses an airtight seal to create a pressure differential. By increasing pressure around the lower body in the chamber (called lower body positive pressure), the gravitational forces are counteracted.	None

to gain new knowledge about the effects of LBPP on gait after surgery. In patients after meniscectomies or ACLR, a significant decrease in ground reaction forces in both involved and uninvolved limbs was observed during gait training under LBPP conditions. The peak magnitude of electromyographic activity of the vastus medialis obliquus decreased as BW conditions were reduced, although the changes reached significance only at 20% of BW. Electromyographic activity of the biceps femoris trends towards decreased activity when exercising at 60% BW and 20% BW conditions, but the differences were not significant. Significant reductions in pain during LBPP training were observed in patients after ACLR. During the first 2 weeks after ACLR, no patient could ambulate on the involved limb under normal BW conditions. However, when ambulating under LBPP conditions, the same patients could participate in 2 min of exercise. All patients could tolerate ambulation at 100% BW by the 3rd postoperative week. One week after arthroscopic meniscectomy, patients could tolerate exercise at any BW condition with limited discomfort. Therefore, no significant differences in pain assessment were observed in this group of patients. Heart rate decreased along with a decreasing percentage of BW during training. No adverse events related to placement or exercise in the LBPP conditions chamber occurred.[39]

In the 2nd analyzed case series, the outcomes of patients after UKA significantly improved in terms of self-reported measures and gait parameters after 12 weeks of antigravity treadmill training in conjunction with a standard physical therapy program initiated within the 1st week following surgery.[40] It is crucial to highlight that the case series

Table 4. Details concerning the studied population, intervention and controls (if applicable) in the studies included in the present scoping review – cont.

Authors	Population	Intervention	Control
Huang et al.[40]	Patients after UKA n = 4 Gender: female 100% Mean age: 68.3 years Mean body mass: 68.5 kg Mean body height: 161.6 cm Inclusion criteria: Apart from information that there were included patients scheduled for UKA as a result of medial compartment osteoarthritis (OA) no specific criteria were mentioned. Exclusion criteria: Concomitant severe injury to contralateral knee; history of deep vein thrombosis or a disorder of the coagulative system; claustrophobia; general systemic disease affecting physical function; any other condition or treatment interfering with treadmill walking or rehabilitation.	Participants completed supervised antigravity treadmill training thrice weekly for 12 weeks in conjunction with their standard physical therapy program. Antigravity treadmill training and physical therapy were initiated the 1st week following surgery and progressed as follows: Weeks 1–2; weighting 50–55% of BW*; speed 1.0–1.4 mph; time 5–8 min; frequency 3 times per week; Weeks 3–4; weighting 55–60% of BW*; speed 1.4–2.0 mph; time 10 min; frequency 3 times per week; Weeks 5–8; weighting 60–75% of BW*; speed 2.0–2.5 mph; time 15 min; frequency 3 times per week; Weeks 9–10; weighting 75–85% of BW*; speed 2.5 mph; time 20 min; frequency 3 times per week; Weeks 11–12; weighting 85–90% of BW*; speed 2.5 mph; time 25–30 min; frequency 3 times per week. Physical therapy was performed 2 times a week and initially included icing, elevation and edema control. Treatment continued with passive and active range of motion exercise and progressed to strengthening. Soft tissue and joint mobilization techniques were added to improve joint range of motion. In addition, all patients could walk independently without an assistive device prior to initiating the treatment protocol described later. Used treadmill: AlterG® Anti-Gravity Treadmill™	None
Greig et al.[41]	One patient after ACLR with the use of autologous semitendinosus and gracilis tendons graft from the contralateral limb Gender: male Age: 26 years Body mass: not mentioned Body height: not mentioned Professional soccer player	At 4 weeks post-surgery, the patient completed 2-min running intervals at 10.2 km/h with linear progression from 70% to 95% of BW at 5% increments. Linear progression rather than a randomized allocation of speed was used to reflect the rehabilitation context of the player. This running speed was equivalent to 30% of the patient's maximum running speed determined from match-play and had been achieved during grass-based rehabilitation sessions in the preceding week. Before that, for the 4 weeks postoperatively, the patient was taking part in some kind of rehabilitation program; however, the details were not presented. Used treadmill: AlterG® Anti-Gravity Treadmill™	None

discussed did not include a control group, so care should be taken when attributing the improved outcomes solely to antigravity treadmill training. Also, it's crucial to note that the primary goal of the study of Eastlack et al. was not to assess the efficacy of LBPP as a rehabilitation method. Instead, the objective was to acquire new insights into the impact of LBPP on one's gait following surgery.[39]

The 2 case reports included in the present scoping review, representing the lowest level of evidence, were the studies by Greig et al. and Hambly et al.[41,42] Greig et al. assessed changes in parameters like uni-axial acceleration, vertical and mediolateral acceleration, and anteroposterior loading depending on the BW percentage during antigravity training in 1 patient after ACLR.[41] Hambly et al. assessed the effectiveness of a program comprised of 12 antigravity treadmill running sessions over an 8-week period in 1 patient after single-step arthroscopic osteochondral repair surgery comprised of microfracture and bone marrow aspirate concentrate (BMAC).[42] An improvement in the Self-Efficacy for Rehabilitation Outcomes and Knee Self-Efficacy scales and functional outcomes was noted in case report.[42]

Water-based rehabilitation is a popular treatment option that reduces BW due to buoyancy, so this alternative to antigravity treadmills might be considered. The advantage of antigravity treadmill training over water-based training is that the sterility of the wound is preserved, which makes antigravity treadmills an option that can

Table 4. Details concerning the studied population, intervention and controls (if applicable) in the studies included in the present scoping review – cont.

Authors	Population	Intervention	Control
Hambly et al.[42]	One patient after single step arthroscopic osteochondral repair surgery comprising microfracture and Bone Marrow Aspirate Concentrate (BMAC). Gender: female Age: 39 years Body mass: 60.3 kg Body height: 167 cm Endurance runner	The program comprised of 12 antigravity treadmill running sessions over an 8-week period taking the patient from 30% to 80% bodyweight as follows: Week 1; weighting 30% of BW; speed 6.7 km/h; time 5 min; RPE = 7 Week 2; weighting 30% of BW; speed 7.2 km/h; time 10 min; RPE = 7 Week 3 Session 1; weighting 40% of BW; speed 7.6 km/h; time 10 min; RPE = 8 Week 3 Session 2; weighting 40% of BW; speed 7.7 km/h; time 15 min; RPE = 9 Week 4 Session 1; weighting 50% of BW; speed 7.5 km/h; time 15 min; RPE = 9.5 Week 4 Session 2; weighting 50% of BW; speed 8.0 km/h; time 20 min; RPE = 11 Week 5 Session 1; weighting 60% of BW; speed 8.3 km/h; time 20 min; RPE = 11.5 Week 5 Session 2; weighting 60% of BW; speed 8.0 km/h; time 25 min; RPE = 11.5 Week 6 Session 1; weighting 70% of BW; speed 7.5 km/h; time 25 min; RPE = 11 Week 6 Session 2; weighting 70% of BW; speed 7.1 km/h; time 30 min; RPE = 11.5 Week 7; weighting 80% of BW; speed 8.0 km/h; time 30 min**; RPE = 11 Week 8; weighting 0% of BW; speed 7.5 km/h; time 30 min**; RPE = 10 The patient wore the Ossur Rebound® cartilage brace and the same running shoes during every session. The patient maintained their home exercises (including swimming, cycling and leg strengthening) as previously prescribed. Each treadmill session started with a 5 min. 100% BW self-paced walking warm up and ended with a 5 min 100% BW self-paced walking cool down. Used treadmill: AlterG® Anti-Gravity Treadmill™	None

*Progressed as tolerated; **Alternating 5 min running and 5 min walking; ACLR – anterior cruciate ligament reconstruction; BMI – body mass index; BW – body weight; n – number of participants; PT – physiotherapy; RPE – Rating of Perceived Exertion; TKA – total knee arthroplasty; UKA – unicompartmental knee arthroplasty.

be accessed earlier than water-based training regimens (wound infection and water-based therapy). One of the included cohort studies assessed the effectiveness of antigravity treadmills in reducing knee forces.[49] This study discussed that even though water provides buoyancy and thus reduces BW forces on the knee joint, the resistance due to hydrodynamic drag presents an anteroposterior component when walking in water. The 2nd advantage of LBPP is that it does not affect hydrodynamic or aerodynamic drags.

When conducting a study with comparators or control arms, the intervention and the control groups should be comparable.[50] It is safe to say that patients with different conditions cannot be taken into the same group as weight-bearing capabilities greatly affect the capacity of patients to exercise (weight-bearing and exercise). This is obvious considering the study that analyzed meniscectomy and ACLR patients.[39] While meniscectomy patients can ambulate with little to no pain at any percentage of their BW,[51] ACLR patients could not ambulate at all BWs.[52] Also, demographic variations such as age, gender and BW should be considered, as these are predictive factors for outcomes after knee surgery.[53–55]

Concerning outcome measurements, recommendations for future studies include the adherence to reporting core outcome measures. These outcomes include pain, function, quality of life and adverse events, and should be added to the measurements of the research agenda.[56,57] Surprisingly, most analyzed studies in the present scoping review did not include adverse events, although the importance of this measurement has long been established.[58,59] Only 2 studies assessed pain intensity, and interestingly, it was only assessed during the intervention, so no effectiveness of antigravity treadmill training on everyday pain intensity levels was evaluated.[37,39] Other recommendations would be to remember published details on the frequency of the intervention, the walking speed, the inclination, the duration of the intervention, and the percentage of BW applied.

Table 5. Details concerning the main findings concerning the outcome, recommendations, limitations, and critical notes of the studies included in the present scoping review

Authors	Outcome	Recommendations	Limitations	Notes
DeJong et al.[36]	Assessment at discharge from outpatient therapy: 1) Patient self-reported measure KOOS: Improvement when compared to baseline; no between-group differences both at the subscale level and for the combined KOOS 2) Patient performance-based measure Walking speed over 10-m curse: Improvement when compared to baseline; no between-group differences	Clinical practice: Neither BW-adjustable treadmill nor in combination with PENS provide benefits to TKA patients when compared to usual care. The choice of intervention defaults to the issue of costs. Research: Cost-effectiveness or a cost-savings analysis should be made to help providers make informed choices about which of the 4 equally effective interventions they should select for their patients. If the study is replicated, it is recommended to reduce the post-TKA enrollment window to a week or less. This is because the benefits of newer interventions may be more apparent in the early stages of post-TKA rehabilitation.	The main limitation is that it was confined to a single regional health system, albeit across 15 geographically dispersed outpatient centers. This should be seen in the light of variations in standard PT regimens nationwide.	None
Bugbee et al.[37]	Assessment at final therapy session: 1) Patient self-reported measure KOOS: Improvement when compared to baseline; no between-group differences 2) Mobility TUG: Improvement when compared to baseline; no between-group differences Assessment throughout intervention: 1) Pain NRS: Improvement when compared to baseline; no between-group differences Assessment at 3 months postoperatively: 1) Patient self-reported measure KOOS: Improvement when compared to baseline; no between-group differences. 2) Mobility TUG: Improvement when compared to baseline; no between-group differences.	None given	None given	The study was a pilot and feasibility study. It showed that use of the antigravity treadmill was safe and feasible during postoperative TKA rehabilitation. It was well tolerated by patients and rated highly satisfactory by physical therapists. Further studies are needed to determine the efficacy of antigravity compared to traditional land-based gait training.
Sueyoshi and Emoto[38]	Assessment at the end of protocol (2 weeks postoperatively): Timed single leg stance on a floor, improvement when compared to baseline; no between-group differences.	None given	The use of oral pain medication given by a surgeon was not controlled. No assessment of pain perception was taken. There was no group of patients with an increased pain level during the baseline balance test due to a safety concern.	The assignment to particular groups was based on patients' comfort level, precisely pain level during the single leg stance on the involved limb on a floor. Further investigation is required to examine the effects of using an antigravity treadmill during the acute recovery phase following knee surgery.

Adherence to the Template for Intervention Description and Replication (TIDieR) checklist is recommended when administering an intervention and its subsequent description in a publication.[60] Additionally, we advocate for participation in future high-quality RCTs to address existing gaps in knowledge and clarify the role of antigravity treadmill training in optimizing patient outcomes post-knee surgery.

Table 5. Details concerning the main findings concerning the outcome, recommendations, limitations, and critical notes of the studies included in the present scoping review – cont.

Authors	Outcome	Recommendations	Limitations	Notes
Eastlack et al.[39]	Outcomes measured under LBPP conditions: Peak Ground Reaction Force: Significant decrease in both involved and uninvolved limbs; values obtained in patients after ACLR showed similar reductions when compared with patients after meniscectomies Dynamic knee ROM: no significant reduction at 1 week postoperatively at 60% of BW but significant decrease at 20% of BW. In patients after ACLR the knees had a greater decrease in dynamic ROM than after meniscectomy. Longitudinal knee ROM gradually increased during the 6 weeks. Electromyographic activity of the vastus medialis obliques: decrease in peak magnitude as BW conditions were reduced, although the change reached significance only at 20% BW. Electromyographic activity of the biceps femoris: Trends toward decreased activity when exercising at 60% of BW and 20% of BW conditions, but the differences were not significant. Pain: Reduction as much as 80% in patients after ACLR. During the first 2 weeks after ACLR, no patient could ambulate on the involved limb under normal BW conditions. However, when ambulating under LBPP conditions, all of the same patients could participate in 2 min of exercise. All patients could tolerate ambulation at 100% of BW by the 3rd postoperative week. One week after arthroscopic meniscectomy, patients could tolerate exercise at any body weight condition with limited discomfort, therefore, no significant differences in pain assessment were observed. Heart rate: A significant decrease at 60% and 20% of BW, when compared with exercise at 100% of BW. No adverse events related to placement or exercise in the LBPP conditions chamber occurred.	Research: It is important to conduct further evaluation on patients who have undergone significant injuries or surgeries. In the future, studies should investigate how effective LBPP is as a rehabilitation measure following severe injuries and orthopedic surgeries that involve limiting lower-extremity loads.	None given	It is important to mention that the study was not intended to evaluate the effectiveness of LBPP as a rehabilitation modality. It was established to gain new knowledge about the effects of LBPP on gait after surgery. The authors mention a total of 15 patients (9 after meniscectomy and 6 ACLR), but they report 5 women and 9 men. The patient's pain was primarily reported based on their ability to ambulate. Although the study mentions the use of the VAS and an 80% decrease in pain in ACLR patients, it is not clear when the pain was measured (during rest, ambulation, under which BW, point in time before or after surgery). No data on VAS results were reported in the results. No reports on function or quality of life were assessed for in this study.
Huang et al.[40]	Assessment at post-intervention (12 weeks of intervention): 1) Patient self-reported measures KOOS: Improvement for each subscale when compared to baseline 2) Gait parameters Gait speed: Improvement when compared to baseline. Peak sagittal plane knee flexion angle and peak sagittal plane knee extensor moment during the weight acceptance phase of gait (0–15% of the gait cycle): improvement when compared to baseline, the average peak knee flexion angle and knee extensor moment reached respectively 99.3% and 90.2% of normal values	Research: Comparative studies are needed to establish the effectiveness of antigravity treadmill training usage in restoring joint function in UKA patients.	Care should be taken when attributing the improved knee kinematics and kinetics solely to antigravity treadmill training since this case series did not include a control group. Also, the presurgical gait status was not assessed.	None

Table 5. Details concerning the main findings concerning the outcome, recommendations, limitations, and critical notes of the studies included in the present scoping review – cont.

Authors	Outcome	Recommendations	Limitations	Notes
Greig et al.[41]	Outcomes measured under LBPP conditions: Uni-axial acceleration: No linear increase with step progression in % of BW. Vertical and mediolateral acceleration: Infliction point at 85% of BW. Antero-posterior loading: Infliction point at 80% of BW.	Clinical: Weighting amounting 70% of BW and 85% of BW represent discrete rehabilitative progressions. Being able to run at 85% of BW on antigravity treadmill could be considered sufficient to safely recommend land-based running with full bodyweight. Research: Possible future research could investigate changes in loading strategy during the rehabilitation process, compare running on grass with a speed-matched control group, and establish criteria for returning to training and playing. Additionally, statistical parametric mapping techniques could be used to examine temporal variations in loading during the stance phase.	None given	Eight months after ACLR the patient reported pain in the medial aspect of the involved knee and underwent a medial meniscectomy.
Hambly et al.[42]	Self-efficacy for rehabilitation outcomes scale and Knee Self-Efficacy Scale: The present and future self-efficacy scores showed an improvement from baseline to 8 week of the program. KOOS and IKDC: Improvement from baseline to 8 week of the program.	Research: Future studies should evaluate the role of antigravity treadmill intervention, supervised rehabilitation sessions, and/or the addition of a further 2 months of time post-surgery by comparing standard care with standard care plus an antigravity treadmill program in patients with knee cartilage lesions. Additionally, the psychometric properties of the Self-Efficacy for Rehabilitation Outcomes and Knee Self-Efficacy scales have not been evaluated for a knee osteochondral surgery population, and this also provides an opportunity for further studies.	Design of the study (case report).	A VAS pain score was collected before, during and after every session. No pain was reported throughout the program.

BW – body weight; IKDC – 2000 IKDC Subjective Knee Evaluation Form; KOOS – Knee injury and Osteoarthritis Outcome Score; LBPP – lower body positive pressure; NRS – Numerical Rating Scale; PENS – patterned electrical neuromuscular stimulation; ROM – range of motion; TKA – total knee arthroplasty; TUG – Timed Up and Go test; UKA – unicompartmental knee arthroplasty; VAS – visual analogue scale.

For control groups, future studies to determine the effectiveness of using antigravity treadmills should include appropriate comparators. The only difference between groups should be the intervention being investigated.[61] Both groups should also be comparable at baseline. It has already been mentioned that in one of the analyzed studies for the present scoping review, patients were assigned to studied groups based on their so-called comfort level during single leg stance on the floor using the involved limb, which, of course, may, in some way, undermine the evidence regarding the effectiveness of the tested methods.[38]

Potential practical implications of the scoping review may include rehabilitation protocol development and optimizing treatment strategies. Incorporating antigravity treadmill training into post-knee surgery rehabilitation protocols could offer valuable benefits, particularly for patients unable to bear full weight during the early recovery phase. Our scoping review highlights the potential utility of this intervention, especially in cases such as ACLR, TKA and UKA, where traditional physiotherapeutic measures may be insufficient. However, it is crucial to acknowledge the limitations of the current evidence, as our review underscores the need for further research to establish its superiority over conventional approaches.

As clinicians, it is essential to carefully consider patient selection criteria when contemplating the integration of antigravity treadmill training into rehabilitation plans. Engaging patients in shared decision-making processes, informed by discussions of the available evidence and potential benefits, can empower them to actively participate in their recovery journey. Moreover, while antigravity treadmill training shows promise, it should complement rather than replace traditional physiotherapy methods, emphasizing a comprehensive and multidisciplinary approach to postoperative care.

By identifying common trends or best practices in antigravity treadmill training protocols following knee surgery, the present scoping review can inform the development of evidence-based rehabilitation protocols for clinicians, potentially leading to improved patient outcomes and faster recovery times.

Based on the gathered evidence, clinical recommendations favoring antigravity treadmill training cannot be made at this stage as evidence from different studies failed to prove its superiority over other, more cost-effective treatment modalities. Consideration of cost-effectiveness is paramount. A thorough cost-benefit analysis will help elucidate the economic implications of incorporating antigravity treadmill training into rehabilitation protocols, ensuring that interventions are not only clinically effective but also financially sustainable in the long term. By adhering to these discussions and guidelines, clinicians can navigate the complexities surrounding antigravity treadmill training post-knee surgery, offering personalized and evidence-based care to their patients.

Limitations

The main limitations of this study consisted of the number of databases that were searched. Also, the search was limited to articles in English, French, German, Polish, and Arabic. Some studies might have been missed due to this limitation. Another limitation concerns the JBI appraisal, as the authors failed to identify some aspects not explicitly mentioned in the included studies. One systematic review on the effect of antigravity treadmill training was excluded as the study could not be found in full text, and the authors did not reply to the request.

Conclusions

The antigravity treadmill is a valuable device that allows the rehabilitation of patients who have restricted weight-bearing capabilities. Whether it is used in terms of gait or run training or for other purposes like balance exercises, it improves patients' outcomes after knee surgery. Compared with procedures not involving an antigravity treadmill, its beneficial effect was not shown; however, taking into account the low evidence of the analyzed studies, definitive conclusions cannot be made at this point.

Therefore, future high-quality RCTs should investigate the effect of antigravity treadmill training due to the low quality of provided evidence. Also, a cost-effectiveness analysis is required to determine whether the investigated intervention fits the purpose.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

No ethical approval was deemed necessary as this paper only provides a review of already conducted research. The individual studies were all compliant with relevant local ethical guidelines, as approval was provided by the relevant institutions.

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Prospective use of miRNAs as biomarkers in the diagnosis of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is the leading cause of dementia in the aging population. Pathogenic processes related to the accumulation of amyloid plaques (Aβ) and intracellular neurofibrillary tangles (NFTs) begin during the asymptomatic stage long before the onset of deterioration in cognitive functions and neurodegeneration, which makes rapid diagnosis and treatment difficult. Although biochemical diagnostic markers isolated from the body fluids of AD patients are currently used, scientists are engaged in research into molecular biomarkers that will significantly accelerate the diagnosis long before the first clinical symptoms appear. The research presented here focused on microRNAs (miRNAs), small, non-coding RNA molecules that are involved in the regulation of the post-transcriptional expression of many genes. A review of the literature revealed that miRNAs play an important role in regulating the expression of genes involved in the pathophysiological mechanisms of AD. Changes in the levels of miRNAs in a patient's body fluids can be used for rapid diagnosis. Original scientific articles published between 2014 and 2023 describing clinical and experimental studies on the role and expression levels of various miRNAs were selected from scientific databases such as PubMed, NCBI, Science Direct, and Google Scholar. The selected miRNAs were divided into 2 groups based on their expression level in AD: those with increased expression and those with decreased expression. A review of the latest scientific reports confirms that miRNAs may be a promising source of non-invasive and widely available biomarkers. Additionally, their modulation may prove to be an effective therapeutic strategy in AD.

Key words: Alzheimer's disease, microRNA, expression, downregulated, upregulated

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Introduction

Alzheimer's disease (AD) is a progressive and incurable neurodegenerative disease that accounts for approx. 70% of dementia cases among the aging population worldwide.[1,2] Individuals with AD may experience behavioral changes, visual disturbances, as well as visuospatial and perceptual problems. They also experience a progressive loss of recent memory, while older memories remain intact.[3]

The incidence of dementia increases with age, with up to a 15-fold increase observed between the ages of 60 and 85.[4] Additionally, gender plays a role, with 2/3 of AD patients being women due to their longer life expectancy and greater genetic risk. Even 1 copy of the *APOE-ε4* allele is enough to increase the risk of AD in women.[5]

Alzheimer's disease can be classified as familial early-onset AD (EOAD, approx. 5%), which has a genetic basis associated with autosomal mutations in the *APP*, *PSEN1* and *PSEN2* genes.[6,7] Around 95% of cases of AD are late-onset AD (LOAD), which is sporadic, idiopathic and of unknown origin. It is associated with a polymorphism in the *APOE* gene, which encodes apolipoprotein E (APOE).[8] The presence of a single *APOE ε4* allele increases the likelihood of AD in women by 4 times, while the *APOE ε4* homozygote increases the risk by several times in both women and men.[5] The clinical pathogenesis of AD is caused by the accumulation of extracellular neuritic plaques (amyloid) in the brain due to the accumulation of amyloid β (Aβ) peptides and intracellular neurofibrillary tangles (NFTs), mainly containing highly phosphorylated tau proteins.[9]

The Aβ peptides are produced by the proteolysis of the amyloid precursor protein (APP), which plays an important role in brain homeostasis. However, in individuals with AD, the Aβ peptide is produced through the amyloidogenic pathway, which is mediated by β-secretase (BACE1) and γ-secretase (presenilin 1 and presenilin 2). This pathway produces 2 peptides: one consisting of 40 amino acids (Aβ 1–40) and another consisting of 42 amino acids (Aβ 1–42).[10,11]

Hyperphosphorylation of tau proteins leads to the development of Alzheimer's-type neuronal fibrous degeneration (NFT). This reduces the number of synaptic connections between nerve cells, leading to neurodegeneration. There is a correlation between synaptic density and cognitive abilities at the different stages of AD.[12]

Identifying patients with mild cognitive impairment (MCI) who may progress to AD is crucial. Between 12% and 20% of MCI patients develop dementia annually, while others remain stable for many years or even recover from MCI.[13] Therefore, it is important to implement therapy in the initial phases of the disease to increase the likelihood of successful treatment and preservation of cognitive functions. However, detecting individuals in the prodromal stage of AD is challenging as current diagnostic techniques cannot accurately differentiate between the early stages of AD and age-related cognitive deficits and are impractical for routine use.[14]

Currently, positron emission tomography (PET) and cerebrospinal fluid (CSF) biochemical markers are used to diagnose AD. However, due to their invasive nature and cost, these tests are not routine. Furthermore, they are unable to detect the disease before clinical symptoms appear. Therefore, scientists are researching molecular biomarkers that could diagnose AD at an early stage.[15]

The expression of most genes is regulated at the post-transcriptional level by small, non-coding RNA microRNAs (miRNAs). Approximately 70% of known miRNAs are expressed in the brain. Therefore, their alteration and abnormal regulation may be involved in the etiopathology of AD. Numerous studies have shown that miRNAs not only affect the expression of genes with key importance in AD, such as *APP*, *BACE 1*, *PSEN 1*, *PSEN 2*, *MAPT*, and *APOE*, but also proteins associated with synapses, whose functional changes lead to synaptic dysfunction.[4,16] This article describes a selection of miRNAs with altered expression levels in AD (Table 1). [7,15,17–50] The research presented aimed to elucidate the role of miRNAs in the mechanisms involved in neurodegenerative processes and their potential use as biomarkers in AD. The scientific databases PubMed, NCBI, Science Direct, and Google Scholar were used to search the literature. Articles were identified through a keyword search using terms such as "Alzheimer's disease AND miRNAs'', "microRNAs AND biomarkers", "β-amyloid AND miRNAs", "tau protein AND miRNAs", "synaptic connections AND miRNAs", and "AD diagnostics AND miRNAs". The selection process involved the removal of duplicates, the elimination of articles based on their titles, and a review of the abstracts. The next limiting factor was the publication date and type of article. The authors selected only original articles published between 2014 and 2023. The selected miRNAs were tested on transgenic mice, cell lines, and human clinical material, including brain tissue, CSF, and the peripheral blood of patients with AD. The literature was also analyzed in terms of the regulation of genes related to Aβ metabolism, tau proteins, neuronal plasticity, synaptic deficits, and anti-inflammatory signaling by miRNA. A total of 84 articles met the adopted criteria. As a result of a comprehensive analysis, 20 miRNAs were selected and described, the research results of which were presented in at least 2 original articles. The selected miRNAs were classified into 2 groups according to their level of expression, namely those that downregulated and upregulated expression.

Objectives

The objective of this review was to demonstrate the significant role of miRNAs in the diagnosis of AD based on changes in their expression levels. The article presents potential biomarkers based on selected miRNAs involved in the pathogenesis of AD.

Table 1. MiRNAs associated with pathological features in AD

Direction of changes	miRNAs	Pathological process	Models	Reference
Upregulated	miRNA-128	increased level of inflammatory factors IL-1β and TNF-α	monocytes from AD patients	17
	miRNA-146a	inhibition of *ROCK1* expression increased level of tau protein	SH-SY5Y cells and 5xFAD mice; peripheral blood AD patients	18,19
	miRNA-455-3p	Aβ accumulation	fibroblasts and B cells from AD patients; AD brains cell line Neuro2a APPSwe/Δ9	20,21
	miRNA-125b	increased NF-Kb leading to a deficit of 15-LOX, NPD1 suppressed the expression of sphingosine kinase 1 (SphK1) protein		22,23
	miRNA-206	decreased in BDNF, HDAC4, Jun D; disorders of homeostasis, synaptic plasticity and neurotransmission in the CNS	serum from AD patients, transgenic mouse Tg2576; AD brains	15,24
	miRNA-331-3p miRNA-9-5p	inhibition the expression of Sqstm1 and Optn autophagy receptors; decrease in autophagic activity, accumulation of Aβ	SH-SY5Y cells, serum from AD	25,26
	miRNA-124	decreased PTPN1 level, increased tau protein hyperphosphorylation, loss of REST, suppression of *BACE1* expression	SH-SY5Y cells, AD brains	27–30
Downregulated	miRNA-222	increased in p27kip1 protein expression, cell cycle dysfunction	APP/PSEN1 mice, SH-SY5Y and HEK-293T serum AD patients	31,32
	miRNA-22	increased in the level of inflammatory factors IL-8, IL-1β and TNF-α, GSDMD, increased inflammation of microglia, promotion of pyroptosis	serum AD patients; APP/PS1 double transgenic mouse	33,34
	miRNA-188-5p	increased level of $A\beta_{1-42}$ oligomers, decreased density of dendritic processes, dysfunction of synaptic transmission	hippocampal neuron cultures; 5XFad mouse	35
	miRNA-132 miRNA-212	Aβ aggregation, tau protein phosphorylation, reduced intercellular signaling increase in *MAPK1* expression, neuronal apoptosis	3Xtg-AD mice and mouse Neuro2a cells; rats with AD	36,37
	miRNA-101	elevated expression of *APP*, accumulation of Aβ	rat hippocampal neurons	38,39
	miRNA-200a-3p	$A\beta_{1-42}$ accumulation, tau hyperphosphorylation	APP/PS1 mice; blood samples of AD patients	40,41
	miRNA-338-5p	*BACE1* overexpression, $A\beta_{1-42}$ accumulation, synaptic dysfunction, reducing brain amyloid plaque deposition	hippocampal samples from AD patients; ×5 FAD transgenic mice APP/PS1 transgenic mice	42,43
	miRNA-193b	*APP* overexpression, Aβ accumulation	APP/PS1 transgenic mouse, blood AD patients	44,45
	miRNA-137	*ERK1/2* overexpression, activation of caspase-3, promotion of apoptosis; inactivation of NF-κB pathway by targeting *TNFAIP1*	DIV14 rat primary cortical neurons; N2a cells and mouse cortical neurons	46,47
	miRNA-103a-3p miRNA-107	increase in the number of NFTs, increased expression of *BACE1*, *CDK5R1*, *DLG4*	microarray expression profiling in GEO	7,48–50

AD – Alzheimer's disease; GEO – Gene Expression Omnibus. IL-1β – interleukin 1β; TNF-α – tumor necrosis factor alpha; ROCK1 – Rho-associated coiled-coil containing protein kinase 1; Aβ – amyloid β peptides; NF-Kb – nuclear factor kappa B; 15-LOX – 15-lipoxygenase enzyme; NPD1 – D1 neuroprotectin; SphK1 – sphingosine kinase 1; Sqstm1 – sequestosome 1 receptor; Optn – optineurin receptor; PTPN1 – non-receptor-type protein phosphatase 1; BDNF – brain-derived neurotrophic factor; HDAC4 – histone deacetylase 4; Jun D – transcription factor Jun D; REST – silencing transcription factor R1; BACE1 – β-secretase; p27kip1 – cyclin-dependent kinase inhibitor 1B; GSDMD – gasdermin D; IL-8 –interleukin 8; SH-SY5Y – human neuroblastoma cell line; 3xTg – AD triple transgenic mice; MAPK1 – serine-threonine kinase pathway; APP – amyloid precursor protein; 5xFAD – mice model of familiar Alzheimer disease; SH-SY5Y – human neuroblastoma cells; CNS – central nervous system; ERK1/2 – protein-serine/threonine kinasis; TNFAIP1 – tumor necrosis factor alpha-induced protein 1; DLG4 – discs large MAGUK scaffold protein 4; CDK5R1 – cyclin-dependent kinase 5 activator 1.

Diagnostic markers in AD

The diagnosis of AD is primarily based on neuropsychological evaluation, CSF analysis or PET to determine pathological biomarkers.[25] Biomarkers of body fluids, such as Aβ, total tau (t-tau) and phosphorylated tau (p-tau), as well as PET imaging, may indicate synaptic dysfunction in the brain. Biochemical markers are frequently used in research. However, CSF and PET examinations are not commonly used due to their invasive nature and high cost.[51] Therefore, researchers have been studying the use of blood biomarkers for many years.

Biomarkers can be classified into 3 categories: those related to amyloid pathology in the brain ($A\beta_{1-40}$ and $A\beta_{1-42}$), those determining the degree of neurodegeneration (total tau protein (t-tau) and neurofilament light chain (NfL) and

Table 2. Selected biomarkers of AD in blood

Biomarker		Relevance in AD	Change in blood of AD
Amyloid pathology	$A\beta_{1-42}$	Distinguishing between AD, MCI, preclinical AD, and normal controls. Distinguishing AD from other neurodegenerative diseases.	Consistently decreased in both blood and cerebrospinal fluid. A decrease in $A\beta_{1-42}$ levels and the $A\beta_{1-42/40}$ ratio suggests a high degree of senile plaque burden, as confirmed with PET scans, and significant cognitive decline.[52,53]
	$A\beta_{1-40}$	Inconsistent results for $A\beta_{1-40}$ alone, $A\beta_{1-42/40}$ ratio may be a better biomarker than $A\beta_{1-42}$ alone.	
Degree of neurodegeneration	Total tau (t-tau)	Distinguishing between AD, MCI, and normal controls.	Elevated plasma t-tau levels in AD patients compared to control patients or MCI patients, but no difference between MCI patients who developed AD and patients with stable MCI.[53]
	NfL	Distinguishing between AD and normal controls but not from other neurodegenerative diseases. Valuable in assessing damaged neurons.	NfL proteins track the level of neuronal damage, but can only be detected in the blood after the onset of AD symptoms, which occurs 10 years after detection of abnormalities in amyloid deposition by PET or reduced levels of $A\beta_{42/40}$ in CSF.[54]
Tau protein pathology	p-tau 181 p-tau 217 p-tau 231	Distinguishing between AD and normal controls. p-tau 181 and p-tau 231 distinguish AD from other neurodegenerative diseases.	Changes in serum biomarker levels in AD patients. A significant increase in the level of p-tau 217, a slightly slower increase in p-tau 181 with a simultaneous decrease in $A\beta_{1-42}$ and the $A\beta_{1-42/40}$ ratio.[52] Individuals with β-amyloid pathology who were carriers of the *APOE4* allele had higher levels of p-tau 181 and p-tau 217 in plasma, but similar concentrations of $A\beta_{42/40}$, t-tau and NfL.[55] The correlation between the level of p-tau 181 in plasma and the presence of extensive amyloid-β pathology, as well as the aggregation of the t-tau protein typical of AD in PET, is evident. The association between plasma p-tau 181 levels and brain β-amyloid deposition is stronger in MCI than in AD.[56] Tau-231 levels increase more specifically in AD than in other neurodegenerative diseases.[53]

AD – Alzheimer's disease; MCI – mild cognitive impairment; PET – positron emission tomography; NfL – neurofilament light chain.

those indicating tau protein pathology (p-tau181, p-tau 231 and p-tau 217).[52] The above blood biomarkers for AD are summarized in Table 2.[52–56]

Blood marker tests show promise for diagnosing AD; however, the problem of early diagnosis persists. It is important to note that elevated levels of p-tau181, p-tau217 or p-tau231 are correlated with amyloid pathologies and NFT formation in the brain. The most optimal biomarkers are those involved in many regulatory brain processes and neuropathological signaling. These biomarkers can diagnose AD even before any neuropathology develops.

MiRNAs are potential biomarkers that can reflect changes in cellular homeostasis and indicate the presence of multiple pathologies.[57] Research has shown that levels of individual miRNAs vary during the development and differentiation of neurons in the human brain and in the aging of the central nervous system (CNS), with these changes being more prominent in AD than in MCI.[3,58]

In their meta-analysis, Moradifard et al. examined and analyzed miRNAs involved in AD, their target genes, mRNA-miRNA interactions between them, and signaling pathways. They compared a microarray dataset with expression profiles of miRNAs, from different areas of the brain. Moradifard et al. found that in AD, numerous miRNAs show differential expression, either decreased or increased. Furthermore, they identified associations between individual genes and AD-related pathways, including interactions with the ECM receptor pathway and CAM cell adhesion molecules. These pathways are crucial for neuronal development, synaptic activity, synaptic connection formation, and blood–brain barrier integrity.[9]

A family of miRNAs implicated in the pathology of dementia outlines the molecular genetics and epigenetics of AD. The mirRNAs have great potential due to their stability in extracellular environments, reactivity and ease of quantification using techniques such as real-time quantitative polymerase chain reaction (RT-qPCR). They are also resistant to the effects of thawing and freezing cycles, making them ideal for use in biomarker studies.[15] The significant changes in miRNA expression during diseases offer great potential for clinical diagnosis using miRNA signatures. Efforts are being made to create miRNA signatures that can diagnose different diseases, identify cancers, and predict therapeutic benefits and drug sensitivity.[59]

Levels of miRNAs may differ in patients at different stages of AD. Therefore, miRNAs have great potential as diagnostic and prognostic biomarkers. They have an advantage over the currently used biochemical markers $A\beta_{1-40}$, $A\beta_{1-42}$, t-tau, p-tau181, p-tau231, p-tau217, and NfL because they allow the detection of the disease at a very early stage, even before the appearance of pathological changes in the brain and clinical symptoms in various body fluids such as blood and serum. They can be obtained in a non-invasive way, making the procedure safer and cheaper than collecting CSF.

miRNAs and their role in gene expression

The miRNAs are small non-coding RNA molecules (snRNA) of 21–25 nucleotides that are involved in the post-transcriptional regulation of gene expression in eukaryotes.[13] These single-stranded nucleic acids are classified as either RNA polymerase II or III (RNA Pol II, RNA Pol III). The mechanism by which miRNAs regulate gene expression is base pairing with the 3' untranslated region (3'-UTR). The degree of complementarity within the RNA-induced silencing complex (RISC) determines whether it will lead to inhibition of the target mRNA translation or its degradation. Therefore, overexpression of mammalian miRNAs mainly works to reduce target mRNA levels and lower the miRNA expression to increase mRNA expression levels.[22,60]

The miRNAs originate from long transcripts with a double-stranded structure known as primary miRNAs (pri-miRNAs). Pri-miRNAs are transcribed by RNA polymerase II, to which they have a special affinity. In the cell's nucleus, pri-miRNA is recognized by the Pasha protein, a partner of the Drosha type III RNAase.[11] Pri-miRNA undergoes 2 stages of endonuclease processing before it becomes a mature, active miRNA. The first stage involves the Drosha enzyme, whose activity depends on the presence of the RNA-binding protein DGCR8.[59]

Next, the pre-miRNA is exported from the cell nucleus to the cytoplasm by Exportin 5, which interacts with the RAN protein. In the cytoplasm, RNase III (Dicer) cleaves the pre-miRNA, which is associated with the RNA-binding protein TRBP, to produce mature miRNA. Following cleavage, 1 strand of the miRNA molecule is incorporated into the RISC, while the other strand is degraded.[10,61]

Mature miRNAs that are embedded in RISC complexes bind to the 3'-UTR of the target gene RNA. The composition of this protein complex is not yet fully understood. However, it contains the key protein Argonaute, which binds mature miRNAs and searches for target mRNAs, causing translational repression. This results in a decrease in the expression of the gene encoded by the mRNA.[10,59,61]

Since the identification of the first miRNA in 1993, it has been estimated that at least 1% of the human genome is encoded by miRNAs. Each miRNA is capable of regulating up to 200 mRNAs.[62] One miRNA can target many genes, and 1 gene can be regulated by many different miRNAs. The miRNAs are a potential tool to study multifactorial diseases, e.g., neurodegenerative diseases, including AD.[11]

These molecules are involved in various biological processes, including growth, differentiation, regulation of the cell cycle, and metabolic cycles at the cellular level. In the nervous system, miRNAs regulate the proliferation, differentiation and apoptosis of nerve cells at various stages of development and also play a crucial role in memory formation.[31] MiRNAs found in neurons are associated with polyribosomes that affect protein expression, suggesting that they play a role in neural tissues and brain growth and development. These findings indicate that the dysregulation of miRNA expression may be associated with several neurodegenerative processes.[63]

MiRNAs involved in AD pathogenesis

The miRNAs regulate the expression of genes associated with AD development. Research is underway on many miRNAs that regulate the expression of genes such as *APP*, *BACE1*, *PSEN 1*, *PSEN 2*, *ROCK1*, *PTPN1*, and others, which are responsible for neurodegenerative processes such as the accumulation of Aβ in the brain, excessive phosphorylation of the tau protein, and induction of inflammation or apoptosis. Research is also being carried out on miRNAs that regulate genes involved in synapse function (e.g., *CPEB1* and *BDNF).*

Several miRNAs participate in the amyloidogenic pathway that results in the creation of harmful forms of insoluble Aβ. Many miRNAs target the 3'-UTR of *APP* mRNA. Overexpression of APP stimulates the production and accumulation of Aβ, which causes impaired neuronal activity, synaptic disorders, and ultimately, dementia.[10] Patel et al. found that overexpression of the miR-106a/520c reduced APP levels.[14] In addition, low levels of miRNA-101a-3p were shown in AD, and transfection of HEK-293 T cells with miRNA-101a-3p mimics and regulates autophagy in AD pathogenesis by targeting the *MAPK1* pathway. These studies suggest that miRNA-101a-3p may play an important role in future therapeutic activities.[64] Hébert et al. confirmed that overexpression of miRNA-106, miRNA-20a and miRNA-17-5a inhibits APP expression, which is consistent with similar research findings.[65] Liu et al. showed overexpression of miRNA-200b, miRNA-135a and miRNA-429 in their studies on APP/PSEN122 transgenic mice. An additional experiment was performed in primary hippocampal neurons and SH-SY5Y cells to confirm the effect of miRNAs on *APP* and *BACE1* expression. The study confirmed that *APP* expression was inhibited in cells transfected with miRNA-200b and miRNA-429, while *BACE1* expression was suppressed by the overexpression of miRNA-135a.[66]

BACE1 is involved in Aβ formation, and its expression is regulated by several miRNAs. Among them, overexpression of miRNA-29 leads to reduced BACE1 protein levels.[67] Similarly, miRNA-135b and miRNA-195a have shown a negative correlation with BACE1 protein levels, which results in lower Aβ levels. Zhang et al. conducted a study in which they transfected mouse hippocampal cells with miRNA-135b inhibitors and miNA-135b mimickers. The study found that overexpression of miRNA-135b improved learning in the maze test, confirming its protective role.[68] Rat neurons were transfected with a miRNA-195 mimetic to study its effect on the expression of APP and BACE1. The results showed that miRNA-195 effectively inhibited the expression of both *APP* and *BACE1* by almost 60% compared to the control group.[69]

MiRNA-34a is involved in the clearance of Aβ, which accumulates in the brain and leads to the formation of amyloid plaques. Studies have shown that miRNA-34a is overexpressed in microglial cells of people with sporadic AD. This microRNA targets the amyloid receptor *TREM2* on the surface of microglial cells, which is responsible for Aβ phagocytosis in the CNS. Significant *TREM2* deficiencies have been reported in the inflammatory neurodegeneration of AD.[70]

The process of tau protein phosphorylation and dephosphorylation is regulated by miRNAs. In a study by Wang et al., miRNA-138 was found to be overexpressed in AD and to promote tau phosphorylation in a mouse cell line model. The *RARA* gene is a direct target of miRNA-138. Downregulation of miRNA-138 increases *RARA* expression and attenuates the glycogen synthase-3β kinase activity of GSK-3b, thereby inhibiting tau protein phosphorylation.[71]

MiRNA-146a affects tau phosphorylation by post-transcriptional repression of *ROCK1*. Inhibition of miRNA-146a expression using antagomir reverses phosphorylation of key signaling pathway components (ROCK1-PTEN-Tau) in the brain.[18]

Several studies suggest that miRNAs are present in both neuronal axons and dendrites, indicating their potential role in synaptic functions. Synaptic miRNAs play important roles in various aspects of synaptic activity, including synaptic development. These include miRNA-134, miRNA-214, miRNA-188-5p, miRNA-138, miRNA-153, miRNA-124, miRNA-9-3p, miRNA-34, miRNA-125b, and miRNA-132, which are involved in synaptic plasticity, synaptogenesis, synaptic morphology, and synaptic excitability.[10,11,72]

miRNAs upregulated in AD

The changes in the levels of miRNAs in body fluids of individuals with AD and the greater stability of miRNAs compared to mRNA have increased the interest of scientists in these molecules as potential diagnostic biomarkers. Many miRNAs target several genes directly associated with neurodegenerative processes in AD, including *PSEN1*, *PSEN2*, *BACE-1*, *APP*, *TOMM40*, and *BDNF*. Some miRNAs are overexpressed, while others are suppressed due to their protective or inducing roles in AD.[62] It is currently believed that during the progression from MCI to AD, there is a change in gene expression direction, from predominantly overexpression in MCI to predominantly underexpression in AD, and this change in gene expression is an important factor in AD development.[73]

The following are the results of scientific research on 8 miRNAs, whose expression levels increased during AD.

miRNA-128

It was observed that some miRNAs show an increased expression in patients compared to control groups. Although earlier studies on miR-128 focused mainly on its role in tumors of the CNS, Zhang et al. directed their interest to miRNA-128 and the role of this molecule in the AD. The results of his research indicate a significantly elevated level of miRNA-128 in the serum of patients with AD when contrasted with the control group. As part of the study, they analyzed the expression of miRNA-128 and determined its diagnostic utility. A positive correlation was demonstrated between serum miRNA-128 levels and inflammatory factors interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α).[17]

miRNA-146a

Increased expression of miRNA-146a was demonstrated in areas of the brain most impacted by tau protein pathology, specifically the hippocampus and temporal cortex. In AD patients, miRNA-146a is involved in tau hyperphosphorylation and the pathogenesis of AD. MiRNA146a regulates ROCK1 mRNA in neural cells and directly targets the protein kinase ROCK1. Inhibition of ROCK1 may contribute to lower levels of PTEN phosphatase phosphorylation and induce aberrant tau phosphorylation. Moreover, in a transgenic mouse model of AD called 5xFAD, 3 mutations (2 in the *PSEN1* gene and 1 in the *APP* gene) inhibit miRNA-146a and led to increased ROCK1 protein levels and repression of tau protein hyperphosphorylation, partially restoring memory functions. The overexpression of miRNA-146a in the SH-SY5Y human neuroblastoma cell line was found to significantly increase tau phosphorylation and inhibit ROCK1 protein translation, leading to the disruption of neuronal microtubules and the cytoskeleton. PTEN phosphatase is known to dephosphorylate tau, and the loss of PTEN function has been associated with neurodegeneration mediated by tau hyperphosphorylation and neurofibrillary tangle formation.[18]

Huang et al.'s research indicates significantly higher levels of miRNA-146a in the peripheral blood of AD patients compared to a control group of healthy individuals. However, a negative correlation was found between the level of miRNA-146a and the concentration of $A\beta_{1-42}$, as well as the results of the MMSE test in AD patients. Additionally, tau protein levels were positively correlated with miRNA-146a.[19] Maffioletti et al. obtained different results in their comparative studies. No significant differences were found when comparing the level of miRNA-146a in the plasma of both AD patients and the control group. However, studies have shown that the level of miRNA-146a increases with age. Additionally, a gender-based relationship was detected, with lower levels of miRNA-146a observed in women compared to men.[74]

miRNA-455-3p

Although numerous studies on miRNA-455-3p indicate that it is involved in various human diseases, especially considering colon, prostate, liver, gastric, and small cell lung cancers, miRNA-455-3p may also play a role

of a potential biomarker in neurodegenerative diseases. Kumar et al. showed a higher expression of miRNA-455-3p in the serum of patients with familial and sporadic AD compared to healthy control. They conducted their study on post-mortem brains of individuals with AD and healthy individuals. They also extended their research by analyzing the fibroblasts and B cells of patients with familial and sporadic AD, as well as an age-matched control group. The levels of miRNA-455-3p were found to be increased in fibroblasts from patients with familial AD and in fibroblasts and B cells from patients with sporadic AD when compared to healthy controls. MiRNA-455-3p expression has been shown to increase in serum samples, cell lines, post-mortem brain models, and mouse models of AD.[20]

In a separate analysis, Kumar et al. investigated the molecular targets of miRNA-455-3p that affect AD development. They identified a correlation between miRNA-455-3p and multiple signaling pathways and their corresponding genes. The pathogenesis of AD is directly related to several signaling pathways and their respective genes, including extracellular matrix (ECM)-receptor interactions, adherens junctions, transforming growth factor beta (TGF-β) signaling pathways, and actin cytoskeleton regulation. These pathways involve genes such as *THBS1*, *COL3A1*, *HSPG2*, *COL6A1*, *RUXN1*, *MYC*, *Smad2*, *PLK1*, and *TNC*.[75]

Xiao et al. demonstrated that miRNA-455-5p targets the 3'UTR of the *CPEB1* gene, which plays a crucial role in protein synthesis in neurons. Higher levels of miRNA-455-5p inhibit the translation of *CPEB1*, leading to a decline in synaptic plasticity and memory.[21]

miRNA-125b

Upon analysis of the CSF of AD patients, it was demonstrated that miRNA-125b was overexpressed compared to the control group of healthy individuals. In addition, in vitro studies were conducted on the Neuro2a APPSwe/Δ9 cell line, where miRNA-125b mimetics were transfected, resulting in the overexpression of inflammatory factors TNF-α, IL-1β, IL-6, and IL-10, induction of apoptosis, and inhibition of cell proliferation. Transfected cells demonstrated increased levels of APP proteins, Aβ peptides and p-ERK proteins. The overexpression of miR-125b significantly suppressed the expression of sphingosine kinase 1 (SphK1) proteins, which affects cell death and survival by maintaining the sphingosine-1-phosphate (S1)/ceramide balance.[23]

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is overexpressed in AD tissues and strongly activates pro-inflammatory genes. Pro-inflammatory cytokines and peptides, including IL-1β, TNFα, Aβ42, as well as HSV-1 and aluminum, activate both NF-kB and possibly miRNAs. NF-kB has been shown to activate the transcription of several miRNAs, including miRNA-125b. Overexpression of miRNA-125b silences brain genes related to phagocytosis and neurotropism. Therefore, any biophysical or physiological stressor that activates NF-kB in a cell can also activate miRNA-125b, which is strongly transcriptionally controlled by NF-kB. MiRNA-125 regulates the levels of the enzymes 15-lipoxygenase-15-LOX and neuroprotectin D1 (neuroprotectin-D1-NPD1) derived from docosahexaenoic acid (DHA). A reduction in their levels impairs homeostasis as well as anti-apoptotic and neuroprotective effects of genes that are typically responsible for anti-inflammatory and neuroprotective signaling in brain cells.[22]

While studies have found elevated miRNA-125b levels in AD cell culture models and patient tissue samples; however, research on miRNA-125b levels in patient plasma is limited. In their study on changes in the expression of several miRNAs in the plasma of AD patients, including miRNA-125b, Vergallo et al. did not find any significant differences in the level of miRNA-125b compared to the control group of healthy individuals.[76]

miRNA-206

Another miRNA whose increased expression has been observed in the course of AD is miRNA-206. It was tested in the serum using RT-qPCR. In a group of MCI patients at higher risk of developing dementia and with worsening outcomes over 4 years displayed significantly higher levels of miRNA-206 expression in comparison to AD patients. Increased miRNA-206 expression was significantly associated with memory impairment and cognitive decline based on the Mini-Mental State Examination (MMSE). The mechanisms behind increased miRNA-206 expression, and its function in the CNS are not yet understood. However, the miRNA-206 targeting of brain-derived neurotrophic factor (BDNF), histone deacetylase 4 (HDAC4) and Jun D transcription factor has profound effects on the CNS. *BDNF* is a particularly important target of miRNA-206 due to its functions in maintaining CNS homeostasis, regulating neurotransmission, synaptic plasticity, dendritic branching, and neuronal survival. Individuals with MCI and AD show a decrease in *BDNF* mRNA and protein levels in their brains. Studies in mouse models of AD, with overexpression of the mutant human APP protein, have confirmed the silencing of *BDNF* by miRNA-206.[15]

Moon et al. confirmed the role of miRNA-206 in AD. The study analyzed a group of patients with early dementia and examined the level of miRNA-206 in the olfactory membrane of MCI patients. The results showed that the expression level of miRNA-206 was higher in MCI patients compared to the control group.[24]

miRNA-331-3p and miRNA-9-5p

Chen et al. conducted an experiment on a mouse model to demonstrate changes in the expression of miRNA-331-3p and miRNA-9-5p during the AD process. The study found that miRNA-331-3p and miRNA-9-5p targeted

sequestosome 1 (Sqstm1) and optineurin (Optn) receptors, which influence the selective autophagy pathway. Autophagic activity increased in the early stages of AD during initial Aβ deposition but decreased in the later stages of the disease. Following treatment of SH-S5Y5 cells with inhibitors of miRNA-331-3p or miRNA-9-5p, the levels of Sqstm1 and Optn increased. Therefore, the use of miRNA-331-3p and miRNA-9-5p antagonists may prevent memory loss and mobility impairment in AD. It is possible to distinguish between early and late stages of AD by assessing the expression of miRNA-331-3p and miRNA-9-5p, autophagic activity, and Aβ accumulation.[25]

To confirm the diagnostic value of miRNA-331-3p, studies were conducted in both AD patients and SH-SY5Y cells. The expression of miRNA-331-3p was significantly reduced in both the patients and the cell line. Correlations were found between miRNA-331-3p expression levels in the patient's serum and their MMSE scores, as well as pro-inflammatory cytokines. The studies on SH-SY5Y cells confirmed the results, showing that overexpression of miR-331-3p increased cell viability and inhibited inflammatory responses.[26]

miRNA-124

In their experiment on transgenic mice, Hou et al. demonstrated that the overexpression of miRNA-124 in the hippocampus resulted in reduced levels of non-receptor protein phosphatase 1 (PTPN1), hyperphosphorylation of tau and the formation of insoluble tau protein. To understand the regulatory mechanisms of the miRNA-124/PTPN1 pathway, the researchers examined levels of the R1 transcription factor (REST) under the influence of the stress factor $A\beta_{1-42}$ oligomer. R1 transcription factor mediates the expression of various classes of ncRNA, including miRNA-124. High levels of $A\beta_{1-42}$ oligomer in AD patients cause REST silencing in both cortical and hippocampal neurons in the CA1, CA3 and CA4 fields, as well as overexpression of miRNA-124. Therefore, the overexpression of REST suppresses miRNA-124 expression and reverses the decrease in PTPN1.[27]

Another group of researchers obtained contrasting results regarding miRNA-124 in the brains of patients with sporadic AD. Their research indicates that the level of miRNA-124 is lower compared to individuals from the control group. An et al. demonstrated in their studies on the human neuroblastoma cell line SH-SY5Y that high levels of miRNA-124 inhibit the activity of BACE1, which plays a crucial role in Aβ production. Therefore, miRNA-124 can be used as a BACE1 inhibitor.[30]

miRNAs downregulated in AD

Many studies on miRNAs as potential biomarkers in AD show differences in the expression of individual classes involved in neurodegenerative processes. Not only does miRNA overexpression indicate the development of the disease, but reduced expression levels also influence changes in the brain and the risk of progressive dementia.

This section describes 12 miRNAs that are downregulated in AD, based on animal studies, in vitro studies and in body fluids from AD patients.

miRNA-222

The group of miRNAs with reduced expression in AD includes miRNA-222. The expression of miRNA-222 was significantly decreased in mild and moderate AD groups compared to the healthy group. Moreover, miRNA-222 expression in the moderate AD group was considerably lower than in the mild AD group.[31]

Wang et al. demonstrated that miRNA-222 contributes to the development of AD by influencing cell cycle dysfunction and the expression of cyclin-dependent kinase inhibitor 1B (p27kip1). Normally, the expression of p27Kip1 proteins inhibits the phosphorylation of the retinoblastoma protein (pRb), which halts cell proliferation in the G1 phase of the cell cycle. Studies have shown that aberrant expression of cell cycle markers, such as p27Kip1, contributes to the pathogenesis of AD.[32]

miRNA-22

Han et al. confirmed the important role of miRNA-22 in inhibiting inflammation of microglia characteristic of AD. Compared to healthy controls, AD patients showed a significant decrease in circulating miRNA-22 levels, while the expression levels of pro-inflammatory factors, namely IL-18, IL-1β and TNF-α, were significantly increased in AD patients. The correlation analysis revealed a negative correlation between miRNA-22 and the inflammatory factors IL-18, IL-1β and TNF-α. This suggests that miRNA-22 plays a key role in the development of AD. It prevents pyroptosis and the release of inflammatory factors by regulating the mRNA expression of gasdermin D (GSDMD), a substrate of inflammatory caspases.[33]

Zhai et al. confirmed the protective role of miRNA-22 in APP/PS1 mice. The study induced inflammation and cell pyroptosis using Aβ, followed by transfection of cells with miRNA-22 mimetic using exosomes. The study demonstrated that miRNA-22 targets the 3'-UTR of the *GSDMD* gene mRNA, inhibiting GSDMD expression. This leads to a reduction in caspase-1 and inflammatory factors such as IL-1β, IL-6 and TNF-α. Research suggests that exosomes loaded with miRNA-22 can enhance cognitive performance in mice with AD more effectively by inhibiting inflammation.[34]

miRNA-188-5p

This study found that oligomeric $A\beta_{1-42}$ reduces the expression of miRNA-188-5p. The researchers examined the expression of miRNA-188-5p in brain tissue of patients

with AD and age-matched control subjects. The results showed significantly lower miRNA-188-5p expression in the cortex and hippocampus of AD patients. Furthermore, there was a significant increase in immunoreactivity against the protein neuropilin-2 (Nrp-2), which is the target of miRNA-188-5p. The elevated expression of miRNA-188-5p in 5xFAD transgenic mice restored synaptic transmission and dendritic spine density that was inhibited by oligomeric $A\beta_{1-42}$.[35]

miRNA-132 and miRNA-212

Hernandez-Rappi et al. demonstrated that a deficiency of miRNA-132 and miRNA-212 in mice promotes Aβ pathology. Both miRNA-132 and miRNA-212 play an important role in synaptic plasticity, neurite outgrowth and memory formation. The study found that levels of miRNA-132 and miRNA-212 are reduced in individuals with MCI and AD compared to healthy controls. This reduction is associated with memory impairment. The research group demonstrated the effects of miRNA-132/212 deletion on increased tau phosphorylation and accumulation, as well as the production and removal of Aβ, using miRNA-132/212 knockout mice. Additionally, using a transgenic mouse model (3xTg-AD), it was proven that miRNA-132 targets *SIRT1*, *MAPK1/ERK2* and *MAPT*, the levels of which were increased in 3xTg-AD mice, resulting in increased tau pathology and memory deficits. Reintroducing miRNA-132 and miRNA-212 into the body can partially halt this process.[36]

Furthermore, Deng et al. conducted studies in rats and found that miRNA132 inhibits the expression of the *MAPK1* gene, which is responsible for mitogen-activated protein kinase 1. The MAPK1 serine-threonine kinase pathway is involved in the p38 signaling pathway that enhances inflammation and apoptosis during oxidative stress. Therefore, low expression of miRNA-132 in AD leads to an increase in MAPK levels, resulting in inflammation and neuronal death.[37]

miRNA-101

To investigate the role of miRNAs in the regulation of *APP* gene expression, APP protein levels were measured in hippocampal cells from rats with reduced *Ago2* expression. Western blot analysis showed that APP levels were significantly higher in neurons with reduced Ago2 levels. Among the miRNAs that potentially target the *APP* 3'-UTR, miRNA-101 plays a key role. In this cell model, miRNA-101 showed a negative correlation with APP proteins. To determine the role of miRNA-101 in modulating the Ago2/miRNA pathway, hippocampal cells were transfected with specific microRNA inhibitors. The results showed that inhibition of miRNA-101 expression significantly increased APP protein levels, suggesting that APP expression in the hippocampus is regulated by miRNA-101.[77]

To confirm the regulatory role of miRNA-101 on endogenous *APP* in human cells, Long et al. performed a study in human HeLa cell line. In addition, the inhibitory effect of miRNA-101 on APP levels was compared not only in the human HeLa cell line but also in the human astrocyte cell lines and rodent neurons. For this purpose, miRNA-101 mimetics were used. These studies showed the strongest expression of miRNA-101 in model CNS neurons.[39]

miRNA-200a-3p

MiRNA-200a-3p, a member of the miRNA-200 family, is known to play a significant role in human cancers. Recent studies suggest that it may also have an impact on the pathology of AD. Studies in mice (APP/PS1) have shown that miRNA-200a-3p levels are decreased in AD. Similarly, significantly lower levels of miRNA-200a-3p were observed in the plasma of AD patients compared to the healthy control group. Experiments have shown that overexpression of miRNA-200a-3p inhibits the production of $A\beta_{1-42}$ in APPswe-transfected cells, while a reduction of miRNA-200a-3p leads to overproduction of the toxic form of Aβ. The target of miRNA-200a-3p has been identified as *BACE1* mRNA, and the expression level is negatively correlated with miRNA-200a-3p. Additionally, miRNA-200a-3p targets the *PRKACB* gene, which encodes one of the catalytic subunits of PKA that increases the level of phosphorylated tau. Studies have confirmed that miRNA-200a-3p mimetics significantly decreases the expression of both *PRKACB* mRNA and protein.[40]

Another research group suggests that miRNA-200a-3p is involved in Aβ-induced neuronal apoptosis. In studies conducted in the hippocampus of APPswe/PS mice, overexpression of miRNA-200a-3p and reduced levels of SIRT1 were observed. SIRT1 is an anti-apoptotic protein that inhibits neuronal apoptosis. Reporter gene assay confirmed the miRNA-200a-3p binding sites in the 3'-UTR of *SIRT1* mRNA. Suppression of miRNA-200a-3p inhibits apoptosis by targeting SIRT1. Therefore, miR-200a-3p may be a potential therapeutic target for the treatment of AD.[41]

miRNA-338-5p

MiRNA-338-5p is another miRNA that may serve as a biomarker for AD due to its reduced levels. It targets γ-secretase, a key enzyme involved in $A\beta_{1-42}$ production. To determine the impact of miR-338-5p on AD, its concentration was evaluated in individuals diagnosed with AD and a cohort of healthy participants. Studies have shown a significant decrease in miRNA-338-5p levels in people with AD. As expected, the overexpression of miRNA-338-5p suppresses *BACE1* gene expression. Neuroinflammation in the CNS is linked to an increased accumulation of Aβ in AD and plays a crucial role in the development of neuropathology. It results in a reduction of synaptic connections and cognitive abilities. Therefore, overexpression

of miRNA-338-5p may alleviate nervous system inflammation and restore synaptic function.[42]

Li et al. demonstrated the protective effect of miRNA-338-5p on cognitive dysfunction in APP/PS1 transgenic mice, achieved by reducing brain amyloid plaque deposition and delaying apoptotic neuronal loss caused by Aβ40 accumulation.[43]

miRNA-193b

In the case of miRNA-193b, decreased expression was found in hippocampal neurons of APP/PS1 transgenic mice, while the total level of miRNA-193b was overexpressed in exosomes. Research has indicated that a reduction in miRNA-193b levels in the hippocampus results in an increase in miRNA-193b found in exosomes labeled with a protein from the ATP-binding cassette transporter A1 (ATP-ABCA1). The difference in miRNA-193b expression observed in AD may be due to the body's effort to compensate for the loss of APP. The precise biological functions of miRNA-193b in AD have yet to be investigated. It is unclear whether ABCA1-tagged exosome signaling has any effects.[45] Inhibitory oligonucleotides capable of specifically binding and inhibiting miRNA-193b activity were used to demonstrate that miRNA-193b binding sites are located in the 3'UTR sequence of the APP gene transcript. The miRNA-193b inhibitor significantly increased APP expression compared to the control group.[44]

miRNA-137

Nong et al. investigated the protective role of miRNA-137 in neurotoxicity of DIV14 rat primary cortical neurons. They confirmed Aβ-induced neurotoxicity by testing for caspase-3 activity, which is closely related to cell apoptosis. Using miRNA-137 mimetics, it was found that the overexpression of miRNA-137 significantly alleviated the neurotoxicity caused by Aβ. Furthermore, they explained the molecular mechanisms responsible for the neuroprotective effect of miRNA-137. The study used a luciferase assay to detect a complementary binding site between miRNA-137 and extracellular signal-regulated kinase 1/2 (ERK1/2). Furthermore, the study found that miRNA-137 overexpression negatively regulates *ERK1/2* expression. These findings reveal the crucial role of miRNA-137/ERK1/2 signaling in AD.[46]

Experimental models of AD, including N2a cells and mouse cortical neurons, have been instrumental in elucidating the molecular mechanisms underlying the influence of miR-137 on Aβ-induced neurotoxicity. Reduced miRNA-137 levels have been observed to increase neurotoxicity, with this effect being attributed to the direct targeting of TNFAIP1 and the subsequent suppression of its mRNA and protein levels. Conversely, elevated miR-137 expression has been demonstrated to mitigate neurotoxicity by targeting TNFAIP1, thereby inactivating the NF-κB pathway.[47]

miRNA-103a-3p and miRNA-107

Using microarray expression profiling in Gene Expression Omnibus (GEO), Chang et al. confirmed that miRNA-103a-3p and miRNA-107 suppressed cofilin translation. The decreased levels of these miRNAs lead to high levels of cofilin protein in AD. Furthermore, a negative correlation was found between the number of senile plaques and NFTs and the level of miRNA-107 expression in the gray matter of the cerebral cortex during the early stages of AD. The study found that the *BACE1* gene is targeted by both miRNA-107 and miRNA-103a-3p. It also revealed that an increase in *BACE1* levels in AD patients was accompanied by a decrease in miRNA-107 expression. Profiling has confirmed that miRNA-107 and miRNA-103a-3p significantly contribute to the development of AD by regulating the expression of *BACE1*, *LRP1*, *CDK5R1*, and *DLG4*.[7]

Wang et al. confirmed that cases with low levels of miRNA-107 were associated with more severe AD pathology. This is usually characterized by increased *BACE1* levels compared to cases with high miRNA-107 levels.[48]

In their experiments on mouse brains, Shu et al. showed that as Aβ levels increase, miRNA-107 expression decreases. In their study, miRNA-107 mimetics were used to prevent the negative processes induced by increased levels of Aβ. MiRNA-107 protects cells against death in the CA1 region, impairment of synaptic transmission, memory loss, increases in $A\beta_{1-42}$ and p-tau levels, and depression of the BDNF-TrkB pathway.[49]

The role of miRNA-107 as an AD biomarker was confirmed through in vivo studies, which examined its level in the plasma of AD patients and a control group. The results showed reduced expression in people with AD. Furthermore, a positive correlation was found between miRNA-107 levels and MMSE test results, and a negative correlation was found with the level of dementia in AD patients.[50]

Limitations

The primary limitation of this review is the number of miRNAs described. Of the numerous miRNAs involved in the pathogenesis of AD, the authors selected only 20 (8 with increased expression and 12 with decreased expression in AD), a comprehensive listing of which would exceed the scope of this publication. The study is also limited by a lack of knowledge about the mechanism of action of all miRNAs regulating AD-related genes. It is important to remember that AD is a polygenic disease. Experimental models of AD, as well as human post-mortem and in vivo studies, indicate that dysregulation of several miRNAs may influence the pathophysiologic mechanisms of AD, including the Aβ pathway, tau pathology, the brain immune response, and inflammation, including regulation of oxidative stresses.

To ensure objectivity, patient selection must be based on several factors, including age, gender, comorbidities, medications, and stressors. It is also important to remember to select uniform biological materials, use consistent analytical techniques in research, adhere to standardized test methodology, and employ appropriate statistical analyses. Long-term studies are essential to accurately delineate the alterations in the expression levels of individual miRNAs at each stage of the disease.

Conclusions

Recent studies have confirmed that several miRNAs play a key role in the neurodegenerative processes associated with AD. These miRNAs can be identified in the CSF or peripheral blood of patients. The expression levels of miRNA-128, miRNA-455-3p, miRNA-206, miRNA-22, and miRNA-107 have been observed to vary in both experimental and clinical studies in AD patients. The use of different biological materials and analytical techniques may result in inconsistencies in miRNA expression, as evidenced by studies on miRNA-146a and miRNA-125b. Nevertheless, the majority of evidence indicates that miRNAs may be of significant value in the diagnosis and treatment of AD. Currently, there are no comprehensive miRNA profiles available for practical use. A major challenge is to standardize research methods and determine the expression ranges of all AD-related miRNAs. This will allow proper interpretation of blood test results in patients. We hope that miRNA-based research will become a viable method for diagnosing, preventing, delaying, and treating AD.

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Contemporary methods of treating venous lake lesions on the oral mucosa: A literature review

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Abstract

A venous lake (VL) is a vascular lesion arising from dilated venous vessels surrounded by thick fibrous tissue, located in the upper layers of the dermis. It can also appear in the oral cavity, especially on the lips, buccal mucosa and tongue. Recurrent bleeding or aesthetic complaints are the most common reasons for the treatment of these lesions. This review aims to present the current state of knowledge regarding the treatment of VL lesions in the oral cavity. PRISMA guidelines were followed. Articles were searched in the following databases: Pubmed, Medline and Scopus. The authors of this study analyzed scientific works concerning VL treatment. Keywords searched included "venous lake", "venous lake treatment", "sclerotherapy", "laser", "laser photocoagulation", "infrared coagulation", and "diathermocoagulation". Two articles described electrocoagulation, 10 articles focused on photocoagulation using laser devices, 2 articles studied photocoagulation with infrared, and 4 articles described sclerotherapy for the treatment of VL lesions. The most effective therapeutic options were electrocoagulation, 808 nm diode laser photocoagulation and 1064 nm Nd:YAG.

Key words: electrocoagulation, oral mucosa, sclerotherapy, laser photocoagulation, venous lake

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Introduction

A venous lake (VL) is a vascular lesion arising from dilated venous vessels surrounded by thick fibrous tissue, located in the superficial layers of the dermis.[1] It was first described in 1956 by Bean and Walsh.[2] It occurs most frequently in older people in areas of the body exposed to direct sunlight, such as the ears, face, hands, and lips.[3] Another location of these lesions is within the oral cavity with involvement of the labia, buccal mucosa and tongue. In the oral cavity, they manifest as well-defined, painless, navy blue or purple, usually single, convex lumps, ranging from a few to a dozen millimeters in diameter, and have a positive diascopic effect.[4] The characteristic clinical appearance is sufficient to make a diagnosis; therefore, collecting specimens for histopathological examination is usually not necessary. Research by Tobouti et al. reported that a VL is the 2nd most common vascular lesion in the oral cavity after pyogenic granuloma.[5] Recurrent bleeding or aesthetic complaints are the most common reasons for the treatment of these lesions.[6] An additional factor encouraging patients to undergo therapy is the fear of cancer transformation.[7]

Current methods of treating these lesions include surgical excision, cryosurgery using liquid nitrogen, electrocoagulation, infrared photocoagulation, laser photocoagulation, and sclerotherapy.[8–12] For the treatment of lesions located in the oral cavity, laser photocoagulation, infrared photocoagulation, electrocoagulation, and sclerotherapy are most often used. However, there is still no consensus which of these methods is the most effective. The gold standard is surgical excision, which has the lowest recurrence rate. However, it is associated with a longer recovery and more pain and scarring. These lesions are often located in aesthetically sensitive areas, which is a driving factor for the use of minimally invasive or selective treatment methods, free from the complications associated with surgical excision. Therefore, further research to standardize the treatment of VL lesions in the oral cavity using minimally invasive methods, considering the above facts, is justified.

There are few studies and articles in the scientific literature concerning the treatment of VLs in the oral cavity. Since this is a common clinical problem, especially in patients over 50 years of age, knowledge of treatment techniques is essential to improve this area of oral surgery.

Objectives

This review aims to present the current state of knowledge regarding the treatment of VLs in the oral cavity.

Materials and methods

This review aims to discuss the current treatment methods and the research conducted. The literature review was conducted in October 2023. It included publications from 1987–2023. Articles were searched in the following databases: PubMed, Medline and Scopus. The authors analyzed scientific works, especially in terms of innovative and minimally invasive approaches for the treatment of VL lesions. Key words searched included "venous lake", "venous lake treatment", "sclerotherapy", "laser", "laser photocoagulation", "infrared coagulation", and "diathermocoagulation". The inclusion criteria were full text articles in English published in peer-reviewed journals describing the treatment of VLs located exclusively within the oral cavity (case reports, case series and clinical trials). Letters to the editor, editorial comments and congress speeches were not analyzed in this study. Furthermore, articles that focused on the treatment of VLs located outside the oral cavity were not included in the analysis. Emphasis was placed on papers presenting unique information, expanding the review to include rarely published articles and innovative methods of treating VLs. Selected treatment methods were described, along with a discussion of the studies in which they were used.

Data selection

A total of 68 potentially relevant papers were found. Fifty publications were excluded due to a lack of a full text or duplications in the databases; those that were not relevant or included VL locations outside the oral cavity were also ommitted. Ultimately, 18 articles were included in the analysis (for most of the articles, PubMed and Medline gave similar results – we found 12 articles in the mentioned databases and 6 articles in Scopus). A workflow diagram of article selection is shown in Fig. 1.

Data collection

The collected data are presented in Table 1,[4,10,11,13–27] according to parameters such as authors, year of publication, number of lesions covered by the study, treatment method, healing period, results/conclusions, and post-treatment complications. We found 2 articles describing electrocoagulation, 10 articles focusing on photocoagulation using laser devices, 2 articles presenting photocoagulation with infrared lasers, and 4 articles describing sclerotherapy.

Results

Electrocoagulation

This method uses high-frequency electric current, causing a dramatic increase in the temperature of the tissues exposed to it.[28,29] It can be used to cut or burn soft tissue structures. The indisputable advantage of this method is the simultaneous coagulation of blood vessels that minimizes intraprocedural bleeding, which is highly desirable

in the case of VLs. The technical approach to VLs may be the excision of these lesions or their coagulation. However, only the 2nd option is found in the literature for the treatment of oral VLs.

Poonia et al. performed electrocoagulation of a VL located on the lower lip using monopolar coagulation in 1 patient.[11] A modification of this method consisted of introducing a 20 G needle into the lesion, to which a monopolar

Table 1. Summary of venous lake treatment methods

Author/year	Number of treated lesions	Number of treatment sessions	Treatment method		Healing period	Local anesthesia	Results	Adverse effects	Follow-up observation
Migliari et al. 2015[4]	16	1	laser type	Nd:Yag	2–4 weeks	absent	full recovery	swelling disappearing after 1–2 days	–
			λ	1,064 nm					
			mode	contactless					
			modulation	IMP					
			power	2 W					
			energy density	–					
			spot diameter	–					
Ah-Weng et al. 2004[10]	20	1 (85%) 2 (10%) 3 (5%)	photocoagulation with infrared light		–	applied	full recovery (80%), partial recovery with scar formation (20%)	post-procedural bleeding (15%)	1–6 months
			number of pulses	2–4 pulses					
			pulse length	1.0–1.5 s					
Poonia et al. 2019[11]	1	1	electrocoagulation inside the lesion		no data	applied	full recovery	absent	18 months
Weiss et al. 2014[13]	8	1	electrocoagulation inside the lesion		no data	applied	full recovery	absent	3 months
Azevedo et al. 2010[14]	17	1	laser type	laser diode	2–3 weeks	applied	full recovery	slight swelling resolved after 2 days, minimal post-procedure pain, 5.9% (1 patient required painkillers)	–
			λ	808 nm					
			mode	contactless					
			modulation	CW					
			power	power: 2–3 W					
			energy density	20 J/cm^2					
			fiber diameter	300 µm					
Voynov et al. 2016[15]	35	1	laser type	laser diode	2–4 weeks	applied	full recovery	moderate post-procedure pain/2.8% (1 patient) post-procedure bleeding	–
			λ	980 nm					
			mode	contactless mode					
			modulation	CW					
			power	power: 2–3 W					
			energy density	224 J/cm^2 334 J/cm^2					
			fiber diameter	optical fiber diameter: 300 µm					
Wang et al. 2021[16]	41	1 (90.24%) 2 (7.32%) 3 (2.44%)	laser type	alexandrite laser	–	–	80.49% full recovery, 19.51% partial recovery,	scar – 1 case	–
			λ	755 nm					
			mode	–					
			modulation	IMP: 3 ms					
			power	–					
			energy density	50–90 J/cm^2					
			spot diameter	8 mm					

Table 1. Summary of venous lake treatment methods – cont.

Author/year	Number of treated lesions	Number of treatment sessions	Treatment method		Healing period	Local anesthesia	Results	Adverse effects	Follow-up observation
Yang at al. 2017[17]	17	1–2 (82.4%) 1–3 (17.6%)	laser type	PDL – air cooling	4–16 weeks	optional	82.4% full recovery, 17.6% partial recovery, including (5.9%) no response to treatment – relapse after a year	swelling lasting 2–3 days	3 months –6 years
			λ	595 nm					
			mode	–					
			modulation	IMP: 2–10 ms					
			power	–					
			energy density	7–11 J/cm^2					
			spot diameter	7 mm					
			laser type	Nd:YAG – air cooling					
			λ	1,064 nm					
			mode	–					
			modulation	IMP: 15–40 ms					
			power	–					
			energy density	35–40 J/cm^2					
			spot diameter	–					
Roncero et al. 2009[18]	39	1 (89.25%) 2 (10.75%)	laser type	PDL	12–14 weeks	present in $^1/_3$ of patients	full recovery (95%)	5.12% scar	–
			λ	595 nm					
			mode	–					
			modulation	IMP: 20 ms					
			power	–					
			energy density	10 J/cm^2					
			spot diameter	–					
			laser type	Nd:Yag					
			λ	1,064 nm					
			mode	–					
			modulation	IMP: 20 ms					
			power	–					
			energy density	70 J/cm^2					
			spot diameter	–					
Armogida et al. 2023[19]	50	1	laser type	Nd:Yag	4 weeks	absent	full recovery	small scar (2%)	2 years
			λ	1,064 nm					
			mode	contactless					
			modulation	IMP					
			power	–					
			energy density	100 J/cm^2					
			spot diameter	2.5 mm					

Table 1. Summary of venous lake treatment methods – cont.

Author/year	Number of treated lesions	Number of treatment sessions	Treatment method		Healing period	Local anesthesia	Results	Adverse effects	Follow-up observation
Chenung and Lanigian 2007[20]	8	1 (12.5%) 2 (25%) 3 (50%) 5 (12.5%)	laser type	PDL	–	–	full recovery (25%), partial recovery (12.5%, no response (62.5%)	absent	–
			λ	595 nm					
			mode	–					
			modulation	–					
			power	–					
			energy density	8.5–13 J/cm^2					
			spot diameter	7 mm					
Neumann and Knobler 1990[21]	51	1 (76.4%) 2 (9.8%) 3 (7.8%) 4 (5.8%)	laser type	argon laser	10–20 days	optional (5.9% of patients received anesthesia)	full recovery (98.04%), recurrence (1.96%)	scars 10%	18 months
			λ	–					
			mode	–					
			modulation	IMP: 300 ms					
			power	1.8–3 W					
			energy density	–					
			spot diameter	1.5–2.0 mm					
Trafalski et al. 2021[22]	23	1 (83%) 2 (17%)	laser type	diode laser	4–12 weeks	applied	full recovery (83%), scarring (9%), partial recovery (4%), no response to treatment (4%)	scars (9%)	3–6 months
			λ	980 nm					
			mode	contactless					
			modulation	IMP: 100 ms, 50% duty cycle					
			power	6 W					
			energy density	–					
			spot diameter	–					
Colver and Hunter 1987[23]	10	1	photocoagulation with infrared light		2–3 weeks	applied	full recovery (100%)	slight recess (20%)	4 months
			number of pulses	1					
			pulse length	1.125 s					
Fernandez et al. 2020[24]	33	1 (85%) 2 (15%)	sclerotherapy		2–6 weeks	applied	full recovery	swelling, redness, burning sensation, lasting 1–3 days	3–6 months
			chemical compound	5% ethanolamine oleate					
			volume	0.3–0.9 mL					
Kuo and Yang 2003[25]	2	2	sclerotherapy		4 weeks	applied	full recovery (100%)	slight scar, hyperpigmentation (50%)	6 months
			chemical compound	1% polidocanol					
			volume	0.6–1.0 mL					
Cebeci et al. 2021[26]	25	1 (32%) 2 (28%) 3 (24%) 4 (8%) 5 (8%)	sclerotherapy		8–16 weeks	applied	full recovery (100%)	angioedema (8%), slight scarring and discoloration (8%), pain during the procedure	6 months
			chemical compound	1% polidocanol					
			volume	volume calculated based on the diameter of the lesion (0.3 mL/3 mm diameter of the lesion)					

Table 1. Summary of venous lake treatment methods – cont.

Author/year	Number of treated lesions	Number of treatment sessions	Treatment method		Healing period	Local anesthesia	Results	Adverse effects	Follow-up observation
Jung et al. 2008[27]	12	1 (38.46%) 2 (30.77%) 3 (15.38%) 4 (7.69%) 5 (7.69%)	sclerotherapy		2–12 weeks	applied	full recovery (100%)	pain and paresthesia during injection	10–49 months
			chemical compound	0.5% STS					
			volume	0.05–0.2 mL					

"–" – no data; CW – continuous wave; IMP – pulse mode; STS – sodium tetradecyl sulfate.

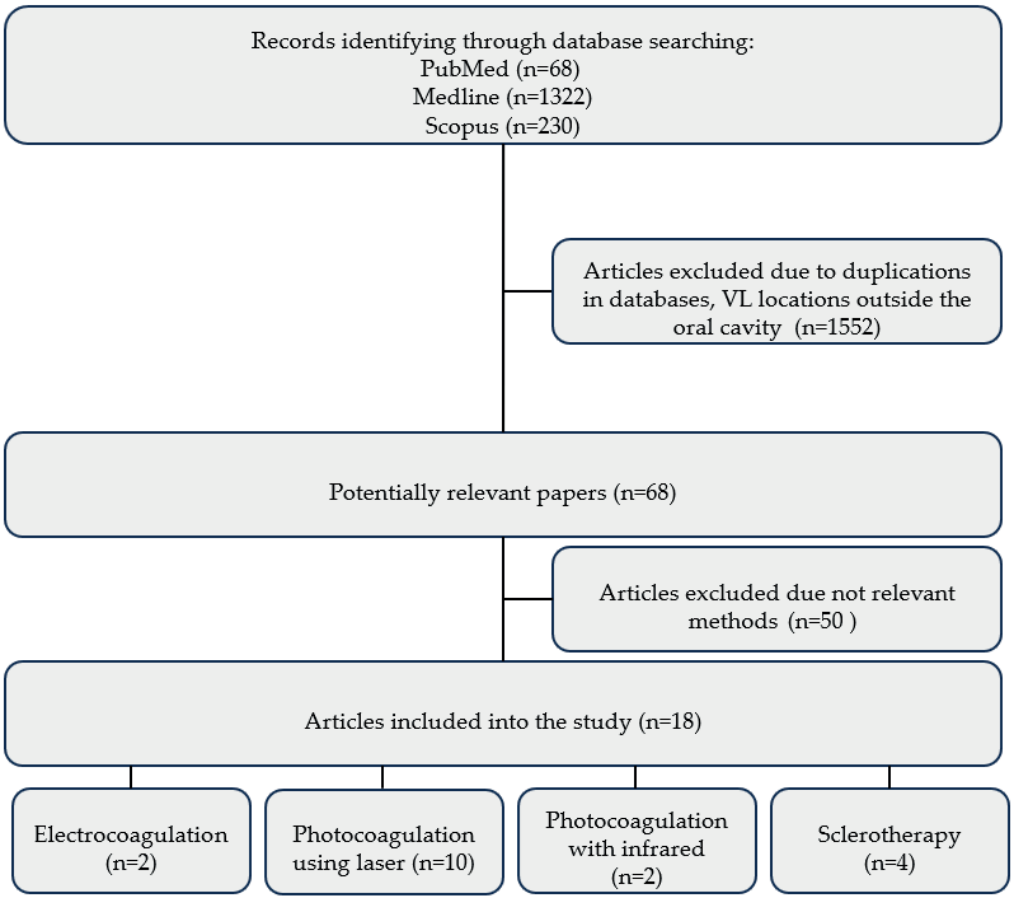

Fig.1. Workflow diagram of the articles selection

tip with an energy of 4 J was applied. Thanks to this solution, the energy was transferred directly to the inside of the lesion. The procedure was performed under local anesthesia. According to the researchers, the results were immediate, the healing process was uneventful, and full recovery was reached after 4 weeks. After 18 months, no recurrences were observed. This approach saves the mucous membrane overlying the lesion as well as the surrounding tissues, which proves the minimally invasive properties of this method.[11]

The same method of treating VLs was described by Weiss et al.[13] This study included 8 patients with lip lesions. The procedure was preceded by local anesthesia with 4% lidocaine. Through a 30 G injection needle inserted into the lesion, energy was delivered from a monopolar tip (McKesson 22-940™) with a power of 2 J. Immediate results were obtained with excellent cosmetic effects. After a 3-month observation period, full recovery of all lesions was observed and no side effects were detected. Researchers emphasize that this is a very effective and quick method for treating VLs with good aesthetic results. It also spares the mucosa overlying the lesion, proving the selectivity of this treatment option.[13]

Photocoagulation using laser devices

Lasers are a very attractive therapeutic option in the treatment of vascular lesions. Their operation is based on the theory of selective photothermolysis, described by Anderson and Parrish.[30] They proved that tissue chromophores can absorb specific wavelengths of light. Hemoglobin contained in the residual blood in the dilated vessels is 1 of the 4 tissue chromophores. Its largest absorption spectrum is in the range of 400–600 nm.

As the wavelength increases, its absorption decreases significantly, and it reaches another absorption peak, much weaker than the 1st one, which is in the range of 800–1,000 nm. The energy of the electromagnetic radiation is absorbed by the hemoglobin and is further transformed into thermal energy, causing blood coagulation and damage to the vessels, leading to their closure. The selection of laser devices in the treatment of VLs should consider the above properties of hemoglobin. Thanks to this, the impact of the laser beam is selective and limited mainly to the vascular lesion itself.[30]

Azevedo et al. researched a group of 17 patients using an 808 nm diode laser (Lasering 808; Revivre Italia SpA, Milan, Italy) to treat VLs of the oral cavity.[14] Exposure parameters were contactless mode, power of 2–3 W, optical fiber diameter of 300 μm, continuous wave operating mode, distance of the optical fiber from the lesion surface of 2–3 mm, average exposure time of 10 s, and an energy density of 20 J/cm^2. The procedure was preceded by local anesthesia and lasted until the lesion turned pale. If this result was not obtained, the procedure was repeated at 30-s intervals until successful to avoid overheating of the tissues. During the first 2 days after the procedure, participants observed slight swelling and minimal pain in the treated area. Only 1 patient required painkillers. After 2–3 weeks, all patients reached full recovery after only 1 session. Moreover, the recovery period was uneventful in all patients, without scarring or discoloration. The study authors emphasized that the contactless VL treatment technique using this laser is highly effective and simple.[14]

A different diode laser with a wavelength of 980 nm (LiteMedics, Milan, Italy) was used by Voynov et al., who included a group of 35 patients.[15] The procedure was preceded by superficial or infiltration anesthesia. The exposure was carried out in contactless mode with an optical fiber diameter of 300 μm, continuous mode and a power of 2–3 W. The exposure parameters and exposure time depended on the size of the VL and varied from 224 J/cm^2 at 2 W with a time of 20 s to 344 J/cm^2 at 3 W for 60 s. One session was sufficient for all patients to reach full recovery. Postoperative pain was minor, and 1 patient experienced transient bleeding. Recovery was reached within 2–4 weeks. The results of these studies also indicate that selective photocoagulation of VLs using a 980 nm diode laser is an effective and safe treatment method.[15]

Wang et al. used a 755 nm alexandrite laser (The Candela Gentle's Alexandrite Laser; Candela Medical, Marlborough, USA) to treat VL lesions on the lips in 41 patients.[16] The device was equipped with a Dynamic Cooling Device (DCD), and the area treated with the laser was covered with paper scarves with individually cut holes, exposing the lesion to avoid damaging adjacent tissues. The procedure was as follows: A spot width of 8 mm, pulse width of 3 ms and energy density of 50–90 J/cm^2. After the procedure, it was recommended to use erythromycin ointment for 3 days. Full recovery was reached in 80.49% of patients, and in 19.51% of patients, recovery was partial, with a reduction of the lesion in the range of 75–95%. Only 1 patient required 3 sessions, 3 patients required 2 sessions, and the remaining patients only required 1 session. One patient had a slight scar after treatment. Recovery was uneventful for all patients. The authors underscored the safety and efficacy of this method for the treatment of VLs. However, being aware of the use of many other types of lasers in the treatment of these lesions, they believe that the alexandrite laser requires comparison with other laser devices.[16]

A different approach was reported by Yang et al., who used 2 lasers emitting wavelengths of 595 and 1,064 nm.[17] The study included 15 Asians with skin phototype IV on the Fitzpatrick scale – a total of 17 VL lesions. Local anesthesia was used optionally before the procedure. First, a 595 nm pulse dye laser (PDL) was used, followed by a 1,064 nm Nd:YAG laser (Cynergy Multiplex; Cynosure Inc., Westford, USA). During exposure, continuous air cooling was used (Cryo 5a; Zimmer Medizinsysteme GmbH, Neu-Ulm, Germany). The exposure parameters were as follows for the PDL laser: A spot diameter of 7 mm, an energy density of 7–11.5 J/cm^2 and a pulse of 2–10 ms. For the Nd:YAG laser, an energy density of 35–40 J/cm^2 and a pulse of 15–40 ms was used. The authors reported that the selection of energy density was based on the color of the lesion, and the pulse width was based on the presumed diameter of the vessel. Applications of laser beams occurred consecutively without overlap. The treatments were repeated monthly until the changes disappeared completely. Post-treatment, it was recommended to use antibiotics (the study authors did not specify the type of antibiotic). After treatment, slight swelling was observed, which disappeared after 2–3 days. Full recovery was reached in 82.4% of patients after 1–2 sessions, while 17.6% reached partial recovery after 1–3 sessions. In 1 case, after 3 sessions, an improvement of 80% was achieved, with a recurrence of the lesion 1 year after the end of treatment. The authors explained this therapeutic approach by the fact that the absorption spectrum of hemoglobin coincided with the emission spectrum of both devices. However, a 596 nm PDL penetrates tissues to a depth of 1.5 mm, while the Nd:YAG 1,064 nm penetrates approx. 3.7–6.0 mm, which may result in the coagulation of vessels at various depths. The order in which the devices were used was because the PDL 595 nm transforms oxyhemoglobin into methemoglobin, which absorbs 1,064 nm Nd:YAG laser radiation 3 times more than oxyhemoglobin. Therefore, it is possible to use lower energy densities of the 1,064 nm Nd:YAG laser while maintaining its effectiveness and reducing side effects. The authors believe that this treatment method is effective and safe in Asians, although a lower energy density of the 1,064 nm Nd:YAG laser was used than in studies conducted on Caucasian patients.[17]

A similar therapeutic approach was characterized in the study by Roncero et al.[18] They used 2 PDL lasers: 595 nm and a Nd:YAG 1,064 nm laser (Cynergy Multiplex)

to treat 39 VLs in 30 patients. Infiltration anesthesia was used in $^1/_3$ of patients (mepivacaine 2%). For irradiation, a PDL laser of 595 nm, 20 ms, and 10 J/cm^2 was used first, followed by a Nd:YAG laser of 1,064 nm, 20 ms and 70 J/cm^2. The spot width in both cases was 7 mm. The procedures were performed under air cooling. After the procedure, a 2% mupirocin ointment was applied topically. Response to treatment was assessed after 3 months. One therapeutic session was required in 89.75% of patients and 2 sessions in the remaining patients. Full recovery was reached in 95% of patients. Among the side effects, the formation of a slight scar in 2 cases (5.12%) was observed. Researchers believe that the use of 595 nm PDL and 1,064 nm Nd:YAG lasers provides a safe, rapid and effective treatment option for oral VLs. By using these lasers in succession, better coagulation is achieved, especially in the case of deeper lesions.[18]

A 1,064 nm Nd:YAG laser (Synchro FT Deka, MELA s.r.l., Calenzano, Italy) was also used in the study by Armogida et al.[19] They examined a group of 47 patients with 50 VL lesions. They carried out exposure to lesions in a contactless mode with exposure parameters of 100 J/cm^2, spot diameter of 2.5 mm and total emitted energy of 4.9 J using continuous operation mode. The procedure was not preceded by anesthesia. The exposure continued until the lesion turned gray, the exposure time ranging from 30 to 120 s. Only 1 treatment session was required for all patients. Full recovery was reached in 47% of cases after 7 days and after 30 days in all lesions. Patients rated periprocedural pain at 1.86 on a 4-point scale. However, after 24 h, the pain level was 0. No complications were observed, except for 1 case of small scar formation. The observation period was 2 years. This procedure was shown to be effective and safe as it did not require anesthesia and resulted in the complete healing of all lesions.[19]

A 1,064 nm Nd:YAG laser (Power Laser TM ST6; Lares Research®, San Clemente, USA) was also used in the study by Migliari et al.[4] The study included 16 patients. The procedure was preceded by local anesthesia. The operating parameters of the laser device were as follows: a power of 2W, pulse frequency of 50 Hz and exposure time of 10 s. Irradiation was performed in a contactless mode with a 320 μm diameter optical fiber placed at 2–3 mm from the lesion, using fast circular movements. The procedure was performed until the VL turned pale and decreased. If necessary, another cycle was performed after 30 s to prevent heat damage to the tissues. In all patients, 1 therapeutic session was enough to reach full recovery. In all cases after treatment, investigators only observed swelling of the treated area, which lasted 1–2 days. No pain or bleeding was observed in any of them. The healing period was 2–4 weeks. After healing, none of the typical side effects, such as scars, discoloration or hyperpigmentation, were observed. Researchers demonstrate that this technique provides safe and effective treatment of VLs in the oral cavity with clear results.[4]

Chenung and Lanigan used a PDL 595 nm laser (Candela Vbeam; Candela Medical).[20] They included 8 patients in their study. The operating parameters of the laser device were as follows: an energy density of 8.5–13.0 J/cm^2, a spot diameter of 7 mm and a pulse length of 1.5 ms. Additionally, they used cryogenic spray cooling. The number of therapeutic sessions ranged from 1 to 5. In subsequent treatment sessions, the energy density was increased by 0.5–1.0 J/cm^2. Only 2 patients had full recovery (25%), and 1 had partial recovery (12.5%). The remaining patients did not respond to treatment, or the response was unsatisfactory. No complications were reported during healing. Due to the unsatisfactory results, the researchers suggested pressing the lesion with a transparent glass during exposure to increase the depth of beam penetration, as well as extending the pulse width, which could improve clinical results.[20]

An argon laser (Coherent Medical Group, Palo Alto, USA) with blue and green light with a peak output power of 488 nm and 514 nm was used by Neumann and Knobler.[21] The study included 51 patients with lip lesions. No anesthesia was used before the procedure except for 3 patients. The exposure parameters used a power of 1.8–3.0 W, a spot diameter of 1.5–2.0 mm and a pulse width of 300 ms. The healing period ranged 1.5–12 weeks. Patients were followed up for 18 months. Full recovery was reached in 98.03% of patients. Among the side effects, they observed scar formation in 10% of cases. One therapy session was required in 76.4%, 2 in 9.8%, 3 in 7.8%, and 4 sessions in 5.8% of participants. After 18 months of follow-up, 1 patient experienced a recurrence. According to researchers, the argon laser is a safe and effective therapeutic option for the treatment of VLs.[21]

Further research using a 980 nm diode laser (Smart M; Lasotronix, Piaseczno, Poland) was conducted by Trafalski et al. This group included 23 patients.[22] The procedure was preceded by local anesthesia with 10% lidocaine. Exposure parameters were a pulse mode, an output power of 6 W, a pulse width of 100 ms, and a 50% duty cycle. In 83% of patients, 1 therapy session was required, and in 17%, 2 sessions were required. The modification of the method consisted of contactless exposure through a microscope slide placed with light pressure on the lesion to reduce its vertical dimension and penetrate the beam deeper into the lesion. The healing assessment was based on an innovative method of fractal dimension analysis (FDA), texture analysis (TA) and graphic images of these changes taken before treatment and 1 and 12 weeks after treatment. Full recovery was reached in 83% of patients and scar formation was observed in 9%. In the remaining 4%, partial recovery occurred, and in 4%, there was no response to treatment. Researchers reported no side effects. They emphasized that the use of a 980 nm diode laser is effective and safe in the treatment of VLs. Moreover, they reported that FDA and TA is a useful and objective method for assessing the effects of the treatment for these lesions using a diode laser.[22]

Photocoagulation with infrared light

This method uses an infrared coagulator that emits incoherent radiation in the range of 400–2,500 nm. The maximum output power of the device is in the infrared spectrum. The radiation is delivered through optical fibers to a quartz tip, which is applied directly to the lesion being treated, causing tissue heating and coagulation. The dose of emitted energy can be adjusted in terms of power and pulse length. Coagulators can typically generate pulses in the range of 0.5–3 s. The coagulation depth is several millimeters and is approximately equal to the pulse duration. However, pulses longer than 3 s cause charring and burning of tissues. This device provides excellent hemostasis without the release of gases that are present when using laser surgery or electrocoagulation. Infrared light photocoagulation is used to treat various lesions, such as hemorrhoids, condylomas and benign cervical lesions.[31,32] It is also a therapeutic option in the treatment of VLs located in the oral cavity.

Colver and Hunter treated 9 patients with a total of 10 VL lesions using an infrared coagulator (Model IRK 151, MBBAT; Lumatec GmbH, Deisenhofen, Germany) with a quartz tip diameter of 6 mm.[23] The therapy was preceded by local anesthesia in the form of 1% lidocaine hydrochloride. Before activation, the sapphire tip was applied with light pressure to the lesion to empty it of residual blood. The treatment was performed with 1 pulse lasting 1.125 s. After 2–3 weeks, full recovery of all lesions was observed, but in 2 patients, a small depression occurred in the treated area. After 4 months, no recurrences were noted. In all cases, only 1 treatment session was required. The authors suggested that in subsequent studies, the pulse duration should be shortened to 1 s to minimize the complications they noted in 2 cases. Researchers consider this method effective, quick and safe in the treatment of VLs.[23]

Ah-Weng et al. treated 18 patients with 20 VL lesions on the lips using an infrared coagulator (IRK151; Lumatec).[10] The procedure was preceded by local infiltration anesthesia with adrenaline, and the tip was placed with slight pressure on the lesions to empty them of residual blood. The number of pulses ranged from 2 to 4. The initial infrared pulse had a length of 1 s with subsequent pulses being increased by 0.125 s, reaching a maximum value of 1.5 s. The procedure continued until the lesion faded, along with a 2-mm margin around the VL. Patients were observed for 3–6 months after the end of treatment, with an average of 3 months. Full recovery was reached in 16 cases, and in 4 cases, partial recovery was reached with the formation of a slight scar at the treatment site. In 17 cases, 1 therapeutic session was sufficient, in 2 cases, 2 sessions, and in 1 case, 3 sessions were required. No healing complications were observed, except for 3 cases of postoperative bleeding. The observations of the authors of this study show that this method is effective, resulting in good therapeutic and cosmetic effects.[10]

Sclerotherapy

This is a therapeutic method that involves closing a fragment or longer section of a vein or artery by administering an appropriate substance causing obliteration. The mechanism of action of sclerosing substances is to react with the vascular endothelium, bringing about its destruction.[3,33] The effect on the endothelium additionally leads to the formation of a plug made of dead endothelial cells, fibrin and blood elements. The plug is firmly attached to the wall of the obliterated vessel, which prevents it from traveling along with the blood flow. As a result, the vessel lumen is closed and filled with fibrous connective tissue.[34,35] The most commonly used sclerosing agents include hypertonic glucose solution, alcohol (ethanol), ethanolamine oleate (EO), bleomycin, polidocanol, sodium tetradecyl sulfate (STS), and OK-432.[36] However, it is still unknown which sclerosing agent is best in terms of effectiveness and safety. Sclerotherapy is used primarily to treat venous and lymphatic diseases, telangiectasias, esophageal varices, hemorrhoids, and varicose veins.[36–39] It may also be considered a therapeutic option for VLs located in the oral cavity. Due to their low costs and satisfactory results, this therapeutic approach is widely practiced.

Fernadez et al. conducted a study on a group of 33 patients using 5% ethanolamine oleate (EO) (Ethamolin; Zest Pharma Ltda., Rio de Janeiro, Brazil).[24] The volume of the solution was calculated based on the diameter of the lesion: 0.1 mL/1 mm of lesion diameter. The total volume ranged from 0.3 mL to 0.9 mL. Before the procedure, infiltration anesthesia with a vasoconstrictor was used. One therapy session was required in 85% of patients, and in the remaining cases, 2 sessions were needed. The procedure was repeated after 3 weeks in cases where 1 session was insufficient to obtain satisfactory results. The size of the treated lesions ranged from 3 to 10 mm. Complications in the form of scarring and discoloration were not reported, but most patients reported some discomfort after the application of the sclerosing agent, such as pain, swelling, redness, and burning, which lasted from 1 to 3 days. Healing of the lesions occurred within 2–6 weeks. In all cases, there was a complete regression of the changes, and observations were carried out for 3–6 months after the procedure. The authors' conclusions support that sclerotherapy with EO is an effective, inexpensive and predictable method for the treatment of VLs on the lips in elderly patients.[24]

Kuo and Yang used 1% polidocanol in 2 patients with VLs on the upper lip.[25] They injected the sclerosing agent into the lesion using a needle and insulin syringe. The volume of sclerotization was 0.6–1.0 mL. The preparation was administered until the residual blood was removed from the lesion; then, the researchers applied 10 min of pressure to the treated area. The changes disappeared after 2 sclerotherapy sessions. The healing period was 4 weeks. In 1 case, an inconspicuous scar and hyperpigmentation occurred. The observation period was 6 months.

The authors believed that sclerotherapy with this agent is efficacious and represents a viable alternative to other therapeutic methods for treating VLs.[25]

Another group of 25 patients with lip lesions treated with 1% polidocanol was described by Cebeci et al.[26] The volume of the sclerosing agent was calculated based on the diameter of the lesion – 0.1 mm^3/1 mm of lesion diameter. The agent was administered using a syringe and insulin needle until the blood was emptied from the lesion. Then, pressure was applied for 5 min. All changes regressed completely. The healing period was 2–4 months. The follow-up period was 6 months from the last treatment. Only 1 session was required in 32% of patients, 2 sessions in 28%, 3 sessions in 24%, 4 sessions in 8%, and 5 sessions in 8%. The number of sessions depended on the size of the VL. In the case of a diameter not exceeding 3 mm, the number of sessions ranged from 1 to 2. For lesions with a diameter of 4–6 mm, the number of sessions ranged from 1 to 4. However, in the case of lesions with a diameter of 7–8 mm, 3–5 therapeutic sessions were required. The interval between subsequent sessions was 3 weeks. In 2 cases, minor scarring and discoloration occurred. The adverse effects included local angioedema in 2 cases. Cebeci et al. emphasized that this treatment method is both effective and simple, with excellent therapeutic and cosmetic results.[26]

Jung et al. included 12 patients with 13 lesions on the lips in a study using 0.5% STS.[27] The size of the lesions ranged from 2 to 10 mm. To establish the correct diagnosis, a 2 mm punch biopsy was performed before treatment. The biopsy material was assessed by histopathological examination. The sclerosing agent was injected slowly into the lesion using a needle and insulin syringe until it was emptied of residual blood. Then, alternating pressure was applied for 10 min. The volume of this agent was 0.05–0.20 mL. Patients were followed up after 2 weeks, and the treatment was repeated until the lesion disappeared completely. All patients reached full recovery. In 32% of patients, only 1 session was required. In 28% of patients, 2 sessions were sufficient. In 24% of patients, 3 sessions were necessary. Eight percent of patients required 4 sessions and 8% of patients required 5 sessions. The recovery period ranged between 2–12 weeks. The mean follow-up period was 29.58 months (10–49 months). No complications, such as necrosis, hyperpigmentation, swelling, or inflammation, were observed. Moderate pain and paresthesia during injection were observed in some patients but disappeared quickly. The authors emphasized that the above method was effective and acceptable to patients.[27]

Discussion

Therapeutic options for VLs that occur in aesthetically sensitive areas should primarily include a minimally invasive approach and highly cosmetic treatment results. Based on the data collected in this review, it can be concluded that the most frequently chosen therapeutic option for these lesions is photocoagulation using various laser devices. Their usefulness is supported by ease of use, good treatment results and a small number of adverse effects. The lasers used in this area included a PDL 595 nm, diode laser 980 nm, diode 808 nm, Nd:YAG 1,064 nm, alexandrite laser 755 nm, and argon laser with peak output powers of 488 nm and 514 nm. According to the theory of selective photothermolysis, their highly effective results are attributed to the perfect absorption of the emitted electromagnetic radiation by a tissue chromophore, which is hemoglobin.[30] It absorbs the laser energy and turns it into heat, which is then transferred to the walls of the vessels, causing them to coagulate and close. Thanks to this, laser surgery is becoming the gold standard for the treatment of minor vascular lesions. Due to the greatest absorption of radiation in the range of 400–600 nm by hemoglobin molecules, devices such as PDL 595 nm and argon lasers of 458 nm or 514 nm should be promoted.[40] However, the depth with which the beam of these devices penetrates the tissues is only 1.5–2.0 mm, covering only superficial changes. It should also be noted that this wavelength of light is absorbed by melanin much more strongly than near-infrared radiation. This is important when the treated lesions are on the verge of the lips and in patients with a dark complexion. This may result in a greater risk of skin damage if a VL is on the lips. Especially in these patients, devices emitting radiation in the near-infrared range should be preferred to limit epidermis damage and complications such as skin discoloration. It should be remembered that as the wavelength increases, the laser beam penetrates deeper into the tissues, which significantly improves the treatment of larger or deeper lesions. Therefore, diode lasers emitting radiation at 808 nm and 980 nm and Nd:YAG 1,064 nm, whose emission spectrum is less absorbed by hemoglobin than the emission spectrum of lasers emitting shorter wavelengths, provide better results in the treatment of VLs. An interesting solution is the use of lasers with different emitted wavelengths – PDL 595 nm with a Nd:YAG 1,064 nm laser. The justification for this method is the previously mentioned fact of a different depth of tissue penetration and the electromagnetic radiation emitted by them. In addition, the PDL 595 nm laser used as the first one converts oxyhemoglobin into methemoglobin, which has 3 times the absorption of 1,064 nm ND:YAG laser radiation than normal blood. This effect allows the use of lower energy densities of the 1,064 nm Nd:YAG laser, maintaining its effectiveness and reducing the risk of side effects.[41] However, the test results do not confirm that this option is significantly more effective than using only the 1,064 nm Nd:YAG laser.

Analyzing the treatment results, the best results were obtained using an 808 nm diode laser and a 1,064 nm Nd:YAG laser, reaching full recovery in all treated lesions.[4,14,19] The use of a 980 nm diode laser was characterized by full recovery in 83–100% of lesions,[15,22] an argon laser with a peak output power of 488 nm and 514 nm

in 98.03%,[21] an alexandrite laser 755 nm in 80.49%,[16] and a PDL 595 nm in 25% of cases.[20] The use of a 595 nm PDL laser with a 1,064 nm Nd:YAG laser resulted in a full recovery rate of 82.4–95.0%.[17,18] The above data support a higher effectiveness of devices emitting radiation in the near-infrared range, even though hemoglobin is characterized by a lower absorption of radiation in this range. However, it is difficult to compare the results of studies conducted using different lasers. This is due to the different number of treated patients and the use of different radiation parameters. Moreover, the lesions treated varied in size, which may additionally influence the treatment results. Therefore, further research on the standardization of irradiation parameters of VLs is of key importance in this field.

The 2nd most common therapeutic option for the treatment of oral VLs is sclerotherapy. The most used agents are 1.0% polidocanol, 5.0% EO and 0.5% STS. The research results indicate that this is a simple, very effective and inexpensive treatment method. A VL is considered to be a low-flow lesion. Therefore, injecting a sclerosing agent inside the VL allows the achievement of a therapeutic concentration, resulting in an appropriate response to treatment. Sclerosing agents such as polidocanol, EO and STS are characterized by low toxicity. However, it should be remembered that when using them, side effects may occur, such as tissue necrosis, allergic reactions or discoloration. In studies using these agents, full recovery was reported in all cases, which is only possible when using a 1,064 nm Nd:YAG laser and an 808 nm diode laser and electrocoagulation. However, complications in the form of scaring and hyperpigmentation were more common than complications when using diode 808 nm lasers, Nd:YAG 1,064 nm lasers and electrocoagulation. Moreover, for sclerotherapy, the number of therapeutic sessions necessary to reach full recovery is much larger and ranges from 1 to 5. Therefore, this may encourage clinicians to use other, more effective methods that require fewer treatment sessions. This discrepancy is primarily due to the different sizes of the lesions treated and the agents used. Analyzing the results of sclerotherapy, it appears that as the size of the VL increases, the number of sessions needed to reach full recovery may increase. This is particularly visible in the cases using 1.0% polidocanol and 0.5% STS, where the number of sessions increased significantly with the increased size of the lesion and ranged from 1 to 5 sessions.[25–27] However, for 5% EO, the number of sessions ranged from 1 to 2, which indicates that the size of the treated lesions has a much smaller impact on this factor.[24] Therefore, the use of 5.0% EO is more beneficial than 1.0% polidocanol and 0.5% STS.

Another method, less frequently used, is photocoagulation with infrared light.[10,23] The emitted incoherent infrared radiation results in similar results to the use of laser devices. Full recovery in the analyzed studies was reached in 80–100% of cases. It is a contact technique, and an important element of it is compressing the lesion before irradiation to empty it of residual blood. This minimizes the energy needed to coagulate pathological vessels, thus increasing the precision of the procedure and preventing the formation of scars. Despite very good treatment results, it is difficult to consider it as the method of choice in the treatment of VLs in the oral cavity. This is because the number of therapy sessions needed to reach full recovery ranges from 1 to 3. Moreover, researchers using it have observed complications in the form of depressions at the treatment site, which is undesirable in the case of aesthetically sensitive areas. Clinicians using this method suggest shortening the pulse length to 1 s to improve its effectiveness and minimize side effects. Therefore, further research in this direction, especially on larger numbers of patients, should be conducted.

The least frequently used treatment method for oral VLs is electrocoagulation. In the analyzed studies, it was modified by introducing a needle into the lesion, to which an active monopolar diathermy electrode was applied.[11,13] Thanks to this, the energy of the device is transferred directly to the interior of the lesion, which provides the selective nature of this technique. This procedure allows you to save the surrounding mucosa, which is confirmed by the results of the conducted research. Moreover, using this method, it is possible to reach full recovery of lesions with only 1 therapeutic session. Such results were obtained only when using an 808 nm diode laser and a 1,064 nm Nd:YAG laser. Additionally, no side effects were observed using this technique. Therefore, this therapeutic option can be considered the method of choice in the treatment of VLs in the oral cavity. However, the small number of patients treated with this method does not allow us to clearly state that it is as effective as photocoagulation with an 808 nm diode laser and Nd:YAG.

It is also worth mentioning that the assessment of the results of the healing of VLs may be influenced by the bias of the researchers or the inaccuracy of the assessments. There are no qualitative and fully objective methods for assessing the healing of VLs. Subjective scar assessment scales are used, such as The Stony Brook Scar Evaluation Scale (SBSES), Manchester Scar Scale (MSS), Patient and Observer Scar Assessment Scale (POSAS), and Vancouver Scar Scale (VSS).[42,43] It is worth mentioning that Trafalski et al. took up such a challenge using TA and FDA on graphic images of the treated lesions.[22] They analyzed photographs of VLs before the procedure, 7 days, and 3 months after the procedure to monitor the healing process. Additionally, after 3 months, they compared the healed areas with the adjacent healthy mucosa, which served as a control group for the treated lesions. Their results were based on the mathematical analysis of digital images, which made them free from subjective assessment. The authors emphasize that FDA and TA are useful and objective methods for assessing the effects of diode laser treatment of VLs, which should encourage other clinicians to use it when evaluating other treatment options.

Analyzing the research results in this review, it was shown that the most effective therapeutic options for

the treatment of VLs in the oral cavity are electrocoagulation, photocoagulation with an 808 nm diode laser, and Nd:YAG.[4,11,13,14,19] This is supported by reaching full recovery in all treated cases using only 1 therapeutic session. Another equally effective method is sclerotherapy using 1.0% polidocanol, 0.5% STS and 5.0% EO.[24–27] Full recovery was reached in all cases; however, the number of treatment sessions ranged from 1 to 5 for 1.0% polidocanol and 0.5% STS.[25–27] In contrast, 5.0% EO required only 1–2 therapeutic sessions.[24] Additionally, side effects in the form of scarring and discoloration are much more common than with other methods. This may make sclerotherapy less attractive compared to the previously mentioned options.

Considering the healing period of VLs treated with lasers, it ranged from 2 to 16 weeks. The shortest time was 2–3 weeks in the case of an 808 nm diode laser[14] and an argon laser with a peak output power of 488 nm and 514 nm.[21] For other devices, the healing period was as follows: for the 980 nm diode laser it was 2–12 weeks,[15,22] for the Nd:YAG 1064 nm it was 4 weeks,[4,19] and for the PDL 595 nm with Nd:YAG it was 4–16 weeks.[15,16] This is another factor that favors the greatest effectiveness of the 808 nm diode laser, the argon laser with a peak output power of 488 nm and 514 nm, and the Nd:YAG 1064 nm laser. However, in the case of the argon laser, the number of treatment sessions ranged from 1 to 5,[21] and for the 808 nm diode and Nd:YAG lasers, only 1 session was required.[4,14,19] Considering the ratio of full recovery to the number of necessary treatment sessions, the 808 nm diode laser and Nd:YAG are characterized by the highest effectiveness. Referring to other methods, the healing time is 2–16 weeks for sclerotherapy,[24–27] there is no data for electrocoagulation, and for photocoagulation with infrared light, it is 2–3 weeks, but no data were available in the analyzed studies.

The most common adverse effects of all laser devices included minor swelling that disappeared after 2–3 days, moderate pain after the procedure, bleeding, and the formation of minor scars at the treatment site. Lack of response to treatment was noted in the case of a diode laser 980 nm – 4%,[22] and a PDL 595 nm laser – 65%.[20] In 1 study, researchers employed photocoagulation with infrared light and reported a 20% complication rate in the form of depression at the surgical site.[23] In the case of sclerotherapy, the most common adverse effects are bleeding after the procedure, short-term pain and local swelling, but also angioedema. In the case of electrocoagulation, the authors did not identify any adverse effects.

Authors should discuss the results and how they can be interpreted from the perspective of previous studies and the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

There are few studies and articles in the scientific literature regarding the treatment of VLs in the oral cavity. Additionally, their treatment protocol has not been developed yet. Since this is not an uncommon clinical problem, knowledge of treatment techniques is essential to improve this area of oral surgery.

Limitations

The above publications are narrative in nature and may be characterized by the authors' bias. However, such reviews are of great value for the development of scientific and clinical concepts. Due to the small number of studies on the treatment of VLs located in the oral cavity, it is impossible to perform a reliable systematic review.

In the case of lesions treated with a laser, some of the publications do not describe all the parameters characterizing the laser beam, which makes it impossible to repeat the experiment and objectively compare the results.

The topic of the treatment of VLs located in the oral cavity is not widely described. There are only few studies on this topic written after 2020. In order to provide the most comprehensive account possible, we have drawn upon the existing literature on this subject.

Conclusions

Venous lakes in the oral cavity are mainly an aesthetic problem, negatively affecting the quality of life of patients. Modern methods of treating VLs of the oral cavity are characterized by low invasiveness and are safe and effective, which is why they are promoted among clinicians instead of surgical excision. Among modern methods of treating VL in the oral cavity, only sclerotherapy, electrocoagulation and photocoagulation with an 808 nm and Nd:YAG 1,064 nm diode laser are 100% effective. Regardless of the method chosen, patients should be informed about potential side effects associated with their use, such as slight postprocedural pain and swelling, bleeding and paresthesias. Additionally, the possibility of leaving scars should be considered.

In the opinion of the authors of this review, it cannot be clearly stated which method of treating VLs in the oral cavity is best due to the small number of publications on this topic. Research reports support the use of 808 nm and Nd:YAG 1,064 nm diode lasers and electrocoagulation. In these cases, the response to treatment reached 100% with only 1 treatment session. However, it should be mentioned that the cost of these devices may be a limitation and can encourage the selection of other cheaper methods. Therefore, sclerotherapy using 5% EO may be an attractive treatment option due to its low costs, 100% effectiveness and the small number of therapeutic sessions necessary to reach full recovery.

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Factors influencing patients' decisions regarding participation in clinical trials: Review of current literature

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Abstract

Obtaining timely data in clinical trials (CTs) is critical for drug registration. This depends directly on the speed at which patients are recruited. This paper provides an overview of selected scientific works and literature from different countries about patients' motivators and barriers to participating in CTs From 55 articles retrieved from PubMed, 5 were selected for the analysis. Additionally, 6 publications, including 2 by Polish authors, were reviewed. As a result, we identified 10 factors for further investigation: altruism, hope for personal benefit, access to better care, the role of a doctor, the opinions of close friends or relatives, financial compensation, side effects, the patient's role as a guinea pig, effort and time, and the use of placebo. Regardless of the therapeutic area, health status, study phase, country, geographic area, economic situation, or healthcare system, patients indicated very similar reasons when deciding to participate in a CT. Even if patients as a group had similar motives and concerns, there are individual elements or unusual factors that need to be better understood and evaluated to accelerate the recruitment process in order to avoid certain drugs or therapies being overlooked or underestimated. In this way, investigators can help patients make the best decisions and more effectively support the process of registering a new drug. Future research on factors influencing patients' decisions is still necessary: We do not know how the COVID-19 pandemic may have influenced patient motivation, how new regulations on CTs are changing patients' perceptions of CTs, and what may be important depending on the study, country, therapeutic area and other factors.

Key words: cancer, patient, clinical trial, motivation, altruism

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Background

The critical aspect of every clinical trial (CT) is the recruitment of a planned number of participants. The speed and efficiency of recruitment often determine the success of a study. Understanding the volunteers' motivations for participating in a study has been the subject of numerous investigations and analyses over the years. The diversity of CTs due to different phases, therapeutic areas, criteria, and types, such as platform or basket trials, is not the only complication. There are also cultural, social and ethical differences, as well as the changing awareness and knowledge of societies in different regions of the world over time.[1,2] Particularly notable is the change in the perception of the significance of CTs in the last 3–5 years, primarily due to the COVID-19 pandemic[3] and the rapid progress in developing modern drugs and therapies used in cancer and rare diseases treatment as well as other diseases. In many therapeutic areas like oncology or rare diseases, patient needs are still inadequately met. In 2019, according to data from the report of the National Cancer Registry, over 176,000 new cancer cases and more than 100,000 deaths related to cancer were recorded in Poland, making malignant neoplasms the 2nd leading cause of death in the country (25.7% of male deaths and 23.2% of female deaths in 2019).[4,5] The issue of potential participants' consent to participate in a study or to decline is of utmost importance from social, economic and healthcare perspectives and directly impacts the wellbeing of each individual.

According to the current state of knowledge, the factors positively influencing patients' decisions regarding participation in CTs include altruism, hope for improvement in health and better medical care related to participation in the study. In turn, the elements that evoke fears and negative reactions include the risks related to the safety of treatment with a new investigational product, requirements regarding the patient's time and effort, and the possibility of being treated as a guinea pig. The studies also paid attention to other factors affecting patients in different ways, such as trust in the doctor, the opinion of relatives and financial compensation. The review of selected works shows that the process of providing information to patients does not raise serious objections and that doctors and patients are appropriately informed, regardless of the country or therapeutic area. In some papers, there are less typical observations depending on the specificity of the examination or the disease. This article provides an overview of selected scientific works and literature from different countries about consent to participate in CTs and related barriers.

Objectives

The aim was to select universal factors influencing patients' decisions and identify variables that may modify them. Finally, it was assumed that a possible way to achieve this goal would be a selection of publications from different geographic areas, concerning very different therapeutic areas and different types of CTs, including CT phases from 1 to 3. This publication may contribute to the design of further scientific research aimed at better understanding the issues of participation in CTs and lead to the development of tools and methods to enhance recruitment effectiveness in CTs.

Materials and methods

Between May and July 2023, the PubMed database was queried using the following search terms: "patient*" [ti] AND "clinical trial*" [ti] AND ("motivation" OR "determinant*" OR "element*") AND "decision*". We excluded articles of the "Review" or "Systematic review" type, and as a result, 550 publications from 1990–2023 were obtained. After the initial elimination of papers whose titles indicated analyses in areas other than those of interest to the authors, the abstracts of 16 publications were reviewed, of which 5 publications were included in the analysis.[6–10] The main reasons for excluding articles from this review were: analysis of matter not aligned with the primary objective of our publication; specifically focused, tailored or bespoke studies; very specific or narrow patient population represented; and mismatched aspects regarding consent for study participation. In addition, based on reviews of other publications and reports, including references in the previously mentioned articles, another 4 publications were selected.[11–14] Due to the special interest in the observations resulting from the Polish works, 2 publications in Polish have been added.[15,16] Works published before 2006 were excluded from the analysis. Finally, 11 papers were selected for analysis and are presented and characterized in Table 1.

Results

The authors of the selected publications used various methods and techniques for collecting and analyzing information from respondents, e.g., multiple choice questionnaires, closed questions or free text, semi-structured interviews done by trained investigators, self-administered questionnaires, semi-structured telephone interviews, etc. These methods have been classified and compiled in Table 2. Following an exhaustive review of 11 research studies, repeatable factors influencing patients' decisions were identified. These factors are outlined below.

Altruism was the most common factor affecting the patient's consent to participate in a CT; it was mentioned in 10 out of 11 analyzed studies. In some works, altruism appears directly[3,9,15]; in others, it is presented descriptively. For instance, there is an understanding that the trials will contribute to scientific knowledge,[6] and the knowledge

Table 1. Publications included in the review of factors influencing patients' decisions regarding participation in CTs

Authors, year of publication, reference	Country	Number of respondents	Age [years], median (range)	Instrument characteristic	Areas addressed in instrument (questionnaire/interview)	Respondent and method	Patient characteristic	Therapeutic area or disease	CT/study characteristic (RCT)	Period of collection responses	Site/s	Phase of CTs
Bleidorn et al., 2015[6]	Germany	20 (100% F)	37 (20–65)	Semi-structured telephone interview. Interviews were recorded and transcribed. Data were discussed by team of physician, senior researcher and psychologist	– motivation and influencing factors, – trial decliners' reasons, – barriers for trial participation	an independent interviewer (physician) made interview with patient	CT participants, interview after study follow-up visit	urinary tract infection	urinary tract infection, RCT, 3 phase	November 2012–May 2013 and September–October 2013	Departments of Family Medicine of Hannover Medical School and University Medical Center Göttingen in cooperation with 42 family practices in Lower Saxony and Bremen, Germany	3
Burgess et al., 2009[7]	South Africa	250 (52.6% F)	56.3 (±10.9)	18 statements, self-administered questionnaire	– access to services, – emotions and social motivations, – contribution to scientific and learning more about condition, – influence of others	patient	95.6% – currently participating in CT	cardiovascular	cardiovascular: Atherosclerosis, other chronic disease	January 2005–May 2006	TREAD Research, Tygerberg hospital, Parow, South Africa	3
Catania et al., 2008[8]	Italy	102 (73.5% F)	58 (21–78)	17-item, multiple choice questions, self-administered questionnaire	– patients' fears, afraid, reticence, prejudices, – patient's expectations towards a clinical experiment, – communication	patient	patients affected with advanced breast or lung cancer, never before participated or discussed the possibility of taking part in CT	malignant tumors, advanced breast (n = 64) or lung cancer (n = 38)	no CT theoretical considerations about participation in CT	September 2006–April 2007	walk-in clinic or day hospital, European Institute of Oncology, Milan, Italy	not specified
Caroll et al., 2012[9]	USA	26 (85% F)	56 (41 for 25th percentile, 61 for 75th percentile)	semi-structured interviews done by trained investigators and standard prompt questions	Initial thoughts and reactions of patient in case of study proposal to participate. Factors important for patient's decision to participate or not. Factors motivating to participate/not participate. Concerns about participating. Medical benefits, medical risks or harms, nonmedical benefits, nonmedical burdens	patient semi-structured interviews done by trained investigators	patient group I (WHO) with PAH (NYHA I–III)	PAH	Hypothetical 2 RCTs in Pulmonary Arterial Hypertension. The 1st study with excluded all PAH medications and the 2nd with placebo or new drug (PAH is a rare disease (6.2%)).	July 2009–August 2009	University of Pennsylvania, Pulmonary Vascular Disease Program, USA	not specified

Table 1. Publications included in the review of factors influencing patients' decisions regarding participation in clinical trials – cont.

Authors, year of publication, reference	Country	Number of respondents	Age [years], median (range)	Instrument characteristic	Areas addressed in instrument (questionnaire/interview)	Respondent and method	Patient characteristic	Therapeutic area or disease	CT/study characteristic (RCT)	Period of collection responses	Site/s	Phase of CTs
Moorcraft et al., 2016[10]	UK	276 (32% F)	64 (19–85)	2 questionnaires A and B, both of multiple choice and free text. A – 25 questions for patients who consented, B – 21 questions for patients who declined participation in CT.	1. Reasons for trial participation or not, factors which motivated and influenced patients' decision to participate or not in CT and the main reason of the decision. 2. Patients' views on cancer research and biopsies 3. Patients' understanding of cancer diagnosis. 4. Patients' views on the written trial information, the verbal explanation, trial discussions, and the consent process.	patient	Patient just after decision re: Participation in CT. The questionnaires could be completed in clinic or taken home and returned at the patients' next clinic appointment.	oncology	36 recruiting studies: IMP, palliative and prescreening in lymphoma and gastrointestinal. Oncology trials: 1st phase (2), 2nd phase (10), 2nd/3rd phase (2), 3rd phase (13), not applicable (9), cancers: colorectal, esophagogastric, pancreatic, hepatobiliary, other GI, Hodgkin's lymphoma, non-Hodgkin's lymphoma	August 2013–July 2014	Gastrointestinal and Lymphoma Unit, Royal Marsden Hospital, Sutton, UK	1, 2, 2/3, 3 and N/A
Godskesen et al., 2015[11]	Sweden	88 (60% F)	61 (SD ±9.1), range 39–80)	60 items questionnaire	– decision-making process, – trial information, – understanding and experiences	patient	current CT participant	oncology	9 ongoing RCT, 3 phase (2 breast, 1 prostate, 1 melanoma, 1 gastric, 1 rectal, 1 colorectal, 1 pancreatic, 1 lymphoma)	January–April 2012	Department of Oncology, Uppsala University Hospital, Sweden	3
Kurt et al., 2017[12]	USA	1836 (71% F)	3 age categories per each patients' subgroups	prospective, cross-sectional, self-administered questionnaire-survey	1. Motivational factors: Doctor, research, money, closest, benefit someone in the future. 2. Barriers: Doctors, time, family, beliefs, research, side effects, transport 3. Helpful resources: Additional materials, other patients, language, medical interpreter	patient	active clinic patient, not participating in CT	emergency medicine (n = 523) family medicine (n = 493) infectious disease (n = 435) obstetrics/gynecology (n = 566)	emergency medicine, family medicine, infectious disease, obstetrics/gynecology no CT theoretical considerations about participation in CT	June 2014–March 2015	Outpatient practices at 3 hospital sites affiliated with a single health network: 2 OB/GYN clinics, 4 FM clinics, 2 ID clinics, 3 separate emergency departments in the USA	not specified
Agrawal et al., 2006[13]	USA	163 (44% F)	57.7 (48–68 IQR	questionnaire of 61 questions in 8 domains	(1) alternative treatment (2) pressure to participate, (3) prognosis, (4) understanding of the protocol, (5) motivations for participation in phase I studies, (6) risk/benefit preferences for cancer treatment, (7) information-gathering behavior about phase I oncology studies (8) sociodemographic characteristics	patient and trained interviewers administered the survey	patient just consented to participate in phase I oncology trial, before receiving any therapy	oncology, advanced cancer	phase 1 oncology trials, advanced cancer patients (had cancer for average 4.8 years)	not specified	5 sites: 1) National Cancer Institute (Bethesda), 2) Institute for Drug Development of the Cancer Therapy and Research Center (San Antonio), 3) Northwestern Cancer Center (Chicago), 4) The University of Texas MD Anderson Cancer Center (Houston), 5) Fox Chase Cancer Center (Philadelphia), USA	1

Table 1. Publications included in the review of factors influencing patients' decisions regarding participation in clinical trials – cont.

Authors, year of publication, reference	Country	Number of respondents	Age [years], median (range)	Instrument characteristic	Areas addressed in instrument (questionnaire/interview)	Respondent and method	Patient characteristic	Therapeutic area or disease	CT/study characteristic (RCT)	Period of collection responses	Site/s	Phase of CTs
Catt et al., 2011[14]	UK	40 (45% F)	58.8 (29–76, SD ±11.1)	Consultation of researcher with patient, semi-structured interview with patients and 3 questionnaires to be completed at home by patient: (1) CT accept/decline questionnaire, (2) Life Orientation Test-Revised (LOT-R), (3) General Health Questionnaire-12 item version (GHQ12).	Questionnaire regarding CT accept/decline consists of 19 items – reasons of decision, for example hope, expectations of benefit, altruism, concerns, general perceptions of the trial information.	patient, researcher consultation and semi-structured interview	Patients attending clinics for phase 1 trial discussion. 46% (n = 18) of patients have earlier experience in CTs.	oncology	colorectal/upper GI (n = 22), breast (n = 6), gynecological (n = 5), skin (n = 3) other (n = 4)	August 2007–December 2008	5 UK centers: Beatson Oncology Centre, Glasgow; Royal Marsden Hospital, Sutton; Royal Free Hospital, London; Southampton and Oxford CR-UK Medical Oncology Units, UK	1
Kotowski, 2021[15]	Poland	401 (63.8% F)	5 age categories per 2 patients' subgroups (cf. Moorcraft et al.[10])	paper questionnaire including 20 closed questions	1.Knowledge about CT. 2. Level of confidence and associations with CT as opportunities and threats. 3. Readiness to participate in a CT including factors influencing the decision. 4. Expectations re: Future communication and education about CT	patient	Hospitalized patients from oncological and non-oncological clinics, chronic diseases	chronic diseases (oncology and non-oncology)	oncology (n = 211): lung cancer (n = 82), breast cancer (n = 103), colorectal cancer (n = 12), other cancers (n = 18) non-oncology chronic diseases (n = 190): respiratory (n = 149), digestive diseases (n = 20), cardiovascular (n = 12), other chronic diseases (n = 31)	not specified	National Oncology Institute, Military Institute of Medicine – National Research Institute, Institute of Hematology and Transfusion Medicine, University Clinical Center of the Medical University of Warsaw, Institute of Tuberculosis and Lung Diseases, Warsaw, Poland	N/A
Preus and Preus, 2022[16]	Poland	2301 (62% F)	3 age categories per 2 patients' subgroups (cf. Godskesen et al.[11])	questionnaire for patient	knowledge about CT, access to information about CT, attitude towards taking the study drug, factors affecting participation in a CT opinions on healthcare in Poland evaluation of CT informed consent in a CT an opinion on the participants of CTs	patient	patients participated in CT (n = 418), patients not participated in CT (n = 1883)	various diseases and different therapeutic areas	various diseases, e.g., cardiology, oncology, pulmonology, neurology, orthopedics, ophthalmology	not specified	Public hospitals in Poland	N/A

N/A – not applicable. F – female; RCT – randomized clinical trial; PAH – pulmonary arterial hypertension; CT – clinical trial; GI – gastrointestinal; OB/GYN – obstetrics/gynecology; FM – family medicine; ID – infectious disease; SD – standard deviation; IQR – interquartile range.

Table 2. Summary of identified factors influencing patients' decisions regarding participation in CTs

Authors, year of publication, reference	Country	Therapeutic area or disease	Number of respondents	Respondent status[A]	Altruism	Hope of personal benefit	Access to better care	Role of a doctor	Closest	Financial compensation	Side effect/ trial safety	Guinea pig	Time and effort	Selected extra element identified or analyzed in publication	Unique or special observations related to patients' motivation in publication
Bleidorn et al., 2015[6]	Germany	urinary tract infection	20 (100% F)	+	yes	yes		yes	–	–	yes	–	yes	spontaneity	Individual treatment preferences of patients: An attractive option was treatment of UTI with no antibiotic.
Burgess et al., 2009[7]	South Africa	cardiovascular	250 (53% F)	+	yes	yes	yes	yes	yes	yes	–	–	yes	social outing	Social aspect of participation: Patients appreciated the opportunity to meet other patients with similar problems. Placebo is not a problem for patients.
Catania et al., 2008[8]	Italy	malignant tumors, advanced breast or lung cancer	102 (74% F)	–	yes	yes	yes	yes	–	–	yes	yes	yes	Distance to study site is no important.	28% of patients thought that they were being asked to participate in CT, because doctors are interested in advancing their own research.
Caroll et al., 2012[9]	USA	PAH	26 (85% F)	–	yes	yes	yes	yes	–	yes	yes	yes	yes	placebo	Patients expressed the heightened concerns regarding the risk in studies with placebo use, without any background therapy.
Moorcraft et al., 2016[10]	UK	oncology	276 (32% F)	±	yes	yes	yes	yes	yes	–	–	–	–	biopsy	The verbal explanation of the trial was rated as excellent, good, fair or poor by 96.4% of patients; also decliners rated it as excellent/ good.
Godskesen et al., 2015[11]	Sweden	oncology	88 (60% F)	+	yes	yes	yes	yes	yes	–	yes	–	–	duty to help	Altruism appeared spontaneously in the pilot test.
Kurt et al., 2017[12]	USA	emergency medicine, family medicine, infectious disease, obstetrics/gynecology	1836 (71% F)	+	yes	–	yes	yes	yes	yes	yes	–	yes	beliefs	Impact of the same factor may vary in treatment specialties, for example relationship with doctor is less important in case of emergency medicine patients in comparison to other specialties.

Table 2. Summary of identified factors influencing patients' decisions regarding participation in clinical trials – cont.

Authors, year of publication, reference	Country	Therapeutic area or disease	Number of respondents	Respondent status[A]	Altruism	Hope of personal benefit	Access to better care	Role of a doctor	Closest	Financial compensation	Side effect/ trial safety	Guinea pig	Time and effort	Selected extra element identified or analyzed in publication	Unique or special observations related to patients' motivation in publication
Agrawal et al., 2006[13]	USA	oncology, advanced cancer	163 (44% F)	±	–	yes	yes	yes	yes	yes	yes	–	–	The information patients found most useful is that "drug kills cancer cells".	Side effects like nausea, fatigue, a bone marrow biopsy, spending a night in the hospital; even a 10% chance of dying would not deter 90% of patients from enrolling in a phase 1 research study.
Catt et al., 2011[14]	UK	oncology	40 (45% F)	±	yes	yes	–	yes	yes	–	yes	yes	yes	Patients' personalities and their tendency to be optimistic: More optimistic patients may be more likely to participate in studies.	According to authors, patients of phase 1 studies comparing to phase 3 gave lower ranking to altruism as a primary motivation for study participating in comparison to patients of phase 3. Possible explanation is that participants of 3 phase studies have enough resources to think beyond their own situation and outcome and feel they have capacity to be selfless. Another explanation was using some components in communication with patients of phase 1.
Kotowski, 2021[15]	Poland	oncology and non-oncology chronic disease	401 (64% F)	–	yes	yes	yes	yes	yes	–	yes	yes	–	Possibility to withdraw from the study at any time	For patient's undecided to participate in the study, their decisions would ultimately be influenced by: The terminal phase of the disease or the lack of other options of treatment.
Preus and Preus, 2022[16]	Poland	various diseases and different therapeutic areas	2,301 (62% F)	±	yes	yes	yes	yes	–	yes	–	–	–	Access to information about CT	no comment

Respondent status: + participant of CT; – not participated in CT; ± mixed group of patients: Participants and non-participants. F – female; UTI – urinary tract infection; CT – clinical trial; PAH – pulmonary arterial hypertension; GI – gastrointestinal.

gained will help others in the future[12] or benefit others directly.[10] Of patients participating in palliative studies (lymphoma and gastrointestinal), 25–62% indicated altruism as the main reason for their consent to participate, and 84–96% noted that it was a factor that influenced their decision positively.[10] Godskesen et al. reported similar findings: although altruism is mentioned as the most important decision factor by 28% of the respondents, it is also highly expressed as the median for agreement, reaching the value of 9.7 on a 10-point scale. Altruism as a motivator appears in every study, independently of the therapeutic area, phase, population, or country. Sometimes, altruism appeared spontaneously as a motive for trial participation in the pilot test.[11]

Hope for personal benefit was another common motivation for respondents. This category includes hope itself, medical benefits, hope for a cure, and the desire for the best available treatment or access to medical care. Hope is usually ranked high in terms of frequency and it also stands out when the question concerns the most important motivator. For example, in a study by Catt et al., 21% of people indicated that their primary motivation for participating in the study was that "the trial offered me some medical benefit." Furthermore, 15% said that "joining the trial would give me hope." The frequency of agreement reached 77% for the 1st statement and 85% for the 2nd.[14] The same inseparable link between participation in the trial and hope is mentioned in the study from the USA, where the use of a new chemotherapeutic drug had a positive effect on patients' outlook.[13]

Personal benefit was also mentioned in the study among German patients with urinary tract infections (UTI); they perceived the benefit as being able to handle future UTI treatment themselves without doctor consultation and avoiding "harmful" antibiotic treatment.[6] In the Kotowski's study, hope was defined as "an opportunity for a new drug" and was indicated as the reason for consent to join a CT by 78.6% of non-oncological and 67.0% of oncological patients.[15] Another analysis from Poland showed that "access to innovative therapies" was a motivator for 50% of patients previously participating in CTs and 74.6% with no such experience.[16] This factor is, therefore, extremely important regardless of the therapeutic area, patient experience, health condition, or geographic location.

Access to better care covers access to better or extra examinations, investigations or medication. Compared to hope for personal benefit, this motivation is distinguished by a more measurable and real, material nature, e.g., more tests or access to a test that the patient would not have had if he had not participated in the study. This factor also includes access to certain drugs.

This motivation was expressed by patients from different countries and different care systems, including those from Sweden,[11] Italy,[8] Poland,[15,16] USA,[13] UK,[10] and South Africa.[7] Discussing this factor, the authors point to various grounds for justifying such a choice. In South Africa, "access to medication" was mentioned by 81% of respondents ("agree" or "strongly agree") and "access to investigations" by 80%. The study drugs used in trials in South Africa are often already registered in the USA or Western European countries yet are still not available for patients in South Africa.[7] The high rate (mean = 8.7 on a 10-point scale) of Swedish respondents (n = 86) agreed that "access to extra exams" was the reason they had decided to participate in randomized CTs[11] A Polish study showed that both oncological (76%) and non-oncological (63.3%) patients indicated easier access to additional tests.[15]

An additional motivation was the role of a doctor, which included trust in the doctor, the relationship, the doctor's advice, any pressure from the doctor, and the doctor's reputation. In various studies, this element always refers to the doctor who is the investigator; it can be a specialist like an oncologist working at a university hospital or a general practitioner, as in the study from Germany. In one study, 97% of patients from the UK confirmed that they trusted the doctor who treated them; for 1%, it was even the most important factor in the decision to join a CT.[14] In a separate study, authors from South Africa asked patients about being under pressure from the staff, and 94% of participants confirmed they did not feel any. In the same study, 67% of participants strongly agreed or agreed that they had received advice from the doctor to take part in the study.[7] In the Swedish study, 70% reported that they did not sense the doctors' expectation that they would agree to participate in the trial. The mean value for the opinion that patient participation was based on the doctor's expectation (expressed by the statement "my doctor thought so") was 4.1 on a 10-point scale. According to the authors, half of the respondents preferred to share the responsibility for making the decision by seeking consultations with a doctor or relatives.[11]

A similarly important and positive role of a doctor is the trust the doctor builds with patients. In the study from the USA related to an active drug, trust was the motivation for 60% (one of the top 3 most often indicated factors), while in the palliative study, it was the motivation for 49% (also in the top 3).[10] In another American study from 2006, the authors reported that only 7% felt moderate or significant pressure from the study's clinical researchers.[13]

A different scenario was observed within the subgroup of emergency medicine patients in the study by Kurt et al. They were less likely to be influenced by the doctor's reputation or their relationship with the investigator, but these patients, due to the emergency, may have lacked the opportunity to establish a relationship or learn about the investigator's reputation.[12] Overall, studies showed that the doctor's role is crucial, and investigators should appropriately balance positive communication about the study without exerting pressure in order to recruit patients. However, there are situations where the doctor's significance might not be as pronounced.

Several studies also analyzed the influence of close individuals on a patient's decisions.[7,10–15] In the case of close relatives, their influence on decisions was primarily examined in terms of what level of pressure they exerted on the patient; however, according to results, patients did not generally feel pressure from their loved ones. In a Swedish study, the opinion that "those close to me thought I should agree to participate" only reached a mean of 2.4 on a 10-point scale. These results were too low to conclude that the patient was under the pressure or significant influence of close relatives.[11]

Almost half of all patients from South Africa claimed that they were influenced by their family or friends, and the remaining 50% confirmed that the opinion of friends or relatives was significant for them.[7] In a paper from the UK, a questionnaire revealed that 51% of patients agreed with the statement "Others (e.g., family or friends) wanted me to join the trial"; however, there was nothing included about the strength of such an influence.[14] This suggests that not only in Scandinavia, where autonomy is a fundamental ethical principle, patients do not perceive pressure from their families or friends as a primary motivating factor. Additionally, in an American study from 2006, the authors explained that only 9% of patients in phase 1 oncological study felt a moderate or significant amount of pressure from their families.[13]

The study by Kurt et al. identified family concerns as a significant barrier to agreeing to participate in a CT for patients in the emergency medicine group (mean response = 2.4). The authors explain that this is a result of difficulties associated with involving loved ones in the patient's decision-making process during emergencies. Often, they are not present or near the patient, or they may also be under significant stress.[12] This example shows that the specificity of the trial should always be taken into consideration.

Financial compensation as a motivation was analyzed in 5 studies.[7,9,12,13,15] Investigators, bioethicists and ethics committees usually regard this as essential information. Based on the included studies, this factor does not appear to be particularly significant in patients' decision-making process. In a study from South Africa, 80% stated that money had little importance in their decision to participate in a CT. On the other hand data showed that almost 5.5% strongly agreed, 8% agreed and 7% partly agreed with the statement that trial was an "easy way to obtain money."[7]

In a Polish study, reimbursement of travel costs ranked 7th out of 8 factors determining respondents' potential participation in clinical study.[16] This element was mentioned by 22.2% of previous CT participants and 23% of those who had not participated yet. In a study from the USA, motivation for all patient subgroups (emergency medicine, family medicine, infectious diseases, obstetrics/gynecology) seemed to be minimally influenced by the money offered for participation.[12] The mean response for financial compensation (value: 1.77–2.17) was low, but not as low as for the category "the doctor conducting the research is the same race/ethnicity as me" (value: 1.02–1.56). In another study from the USA, the financial costs of participation were deemed unimportant in patients' opinions: information about participation costs was most useful for only 1% of patients.[13]

In yet another American study, the compensation or reimbursement was somewhat relevant to the patient's decision.[9] Based on quoted patient statements, the attitude towards reimbursement seemed pragmatic: "Compensation would help, but if I would not be compensated, that would not prevent me from doing it." Thus, while the financial aspect was slightly important, it did not play a decisive role in the decision-making process. The lack of cost reimbursement did not seem to significantly alter the percentage of patients agreeing to participate in the study. In most cases, a pragmatic approach was observed among patients.

In addition to the aforementioned factors that positively impacted study participation, some factors exerted negative influence, referred to as barriers.

First, several studies examined the potential side effects of the trials, including trial safety and drug toxicity. In 1 study, 53% of Italian patients expressed concerns about potential and lesser-known side effects.[8] Similarly, in the UK study, 59% of respondents claimed, "I was worried about the side-effects of the study drug/s."[14] In addition, 65% of those in an American study expressed concerns about side effects.[9] The risk of unknown side effects emerged as the primary barrier across all patient subgroups from the study (mean values: 2.59–3.25).[12] In the German study on UTI treatment, many interviewees held the opinion that a UTI is not a serious condition, and thus they were willing to participate in the study. Conversely, had the condition been more severe, they might have declined participation.[6] A Polish study reported that patients were afraid of higher toxicity of the experimental treatment, with rates of 64.7% for oncology and 48% in case of non-oncology patients.[15]

In summary, the potential for adverse effects significantly impacted patient decisions, acting as a notable barrier to participation. Concerns about potential and lesser-known side effects were widespread, influencing willingness to engage in CTs. The perceived risk associated with unknown side effects was a primary deterrent for various patient subgroups.

Patient concern about being guinea pigs appeared in 4 of the analyzed studies.[8,9,14,15] The fear of being a "guinea pig" emerged as a significant barrier. This concept has not been precisely defined, but it can refer to the perceived objectification of the patient in the study. In the study by Catania et al., this concern was expressed by 36% of patients,[8] and a similar result was observed in the study from the UK (33%).[14] In a study by Carroll et al., 15% of the participants communicated their fears about being a guinea pig.[9] The same factor was noted in the Polish study; of the patients who would not agree to participate in the study (n = 59, 14.9% of respondents), 59.3% (n = 35) stated that they did not agree to be an experimental "guinea pig."[15]

Effort and time were 2 factors considered together. They were considered in 5 studies.[6,8,9,12,14] In the German patients with UTI, the authors noted that trial-related time and effort may have kept patients from participating, which is particularly true for employed patients.[6] Patients expressed that visits and phone calls were always difficult to reconcile with work. Based on a study from the USA involving pulmonary arterial disease (PAH) patients, it is evident that the time demand of the trials could be challenging for some patients and may have hindered enrollment.[9] Patients were afraid of missing work or having to travel to sites. Of those who were asked to participate in 8 visits within 16 weeks, 65% of participants claimed that this was a burden or inconvenience related to the time demands. In the same study, 23% identified "duration of trial" as an issue. Another American study showed that time commitment was more visible as a barrier for those who graduated college or had a higher degree in comparison to those with less than a high school education.[12]

Contrary to the above examples, patients from South Africa expressed that time was not a huge issue. Only 7% of patients felt that they did not have the time required to participate in a CT; 81% of them "strongly agreed" or "agreed" with the statement "I have the time." However, this response could be related to the fact that 66.5% of the respondents from the South Africa trial were unemployed.[7]

In an Italian study, 55% of patients were afraid of wasting time, but the reason was completely different: They were afraid of using inefficient drugs.[8] In a study from the UK, 16% of patients agreed with the questionnaire statement "I thought the trial needed too much effort from me"; however, this percentage included only 6 patients and is therefore not a representative sample for conclusive findings. In summary, it should be noted that the time commitment necessary to participate in CTs can be a barrier for professionally active people.

In CTs, the issue of placebo usage can often create misunderstandings. In the case of 1 study, the potential issue of a placebo was analyzed.[9] The authors presented 2 hypothetical randomized CTs to the patients: One with the continuation of background therapy and another without any treatment in which only a placebo was allowed. Based on the responses, it is evident that patients were not interested in studies where they did not receive any treatment. Concern about placebo was expressed by 38% of the potential participants in a hypothetical study. This finding may indicate that more clarification is necessary for randomized placebo-controlled trials, emphasizing that patients are never left without treatment.

Discussion

A review of publications shows that regardless of the therapeutic area, health status, phase of the study, country, geographic area, economic situation, or healthcare system, patients point to the same or very similar factors when deciding to participate in a CT. This area is researched in different ways, using various methods and techniques of data collection and analysis, but regardless of the methods, similar results were obtained.

In most cases, motivations include altruism, hope for personal benefit and access to better care. In addition, barriers include concerns about safety, potential side effects, and the fear of being treated as a guinea pig. These patterns are independent of the size of the study group or the patient's previous experience with CTs.

In some studies, patients indicate factors that do not appear in other analyses, which are most often determined by the specifics of the study or possibly by local factors due to socioeconomic or cultural circumstances. An example is a study from South Africa, in which social motivation, i.e., the desire to interact and meet other patients, was highlighted.[7] In contrast, for studies conducted on diseases that are not directly life-threatening (e.g., UTIs), patients were willing to take more risks by their own choice and were guided by individual therapeutic preferences.[6] Overall, patients as a group or community were driven by similar motives and concerns.

Analyzing the statements of individual patients cited in the papers, there were clear differences on the individual level in the rationale for justifying the patient's decision. The individual patient's decision to participate in the study was multifaceted and motivated by very different individual factors and circumstances. Examples of these factors would be the severity of the disease, the level of anxiety or concern about side effects, and the risk of losing one's job due to the study length and participation required.

Repeated factors in the analyzed works, which, although they influenced patients' decisions to a lesser extent, were important for patients, include doctor's advice and opinions of relatives. At the same time, the data presented showed that patients were not subjected to pressure from the environment. A factor that turned out to be appreciated by patients but did not have a decisive influence on their attitude or decision was financial compensation.

Based on a review of the literature mentioned above, the determinants of consent can be divided according to several categories, regardless of the phase of the study, therapeutic area, patient population, etc. The following are 3 classifications of motivators developed based on selected literature.

1) All factors influencing patients' decisions to participate in the study can be assigned to 1 of the following 2 categories:

1. Factors motivated by personal benefit, satisfying the individual needs of the patient as an individual, such as hope for improvement in health, absence of the occurrence of adverse symptoms, financial compensation, and the sense of belonging to a group.

2. Factors based on benefit and good for the community such as altruism, acting for the world, science, and medical progress.

2) Motivators for participation in a CT can also be arranged using the criterion of frequency and universality:

1. Common, standard factors: e.g., hope for personal benefit, better medical care, altruism, willingness to support science, fear of side effects, burdensome procedures, medical risk, and safety

2. Atypical and unique factors that depend on the project and the specifics of the study. These factors could include project-specific requirements such as procedures required in the study (e.g., number of biopsies), treatment preferences (antibiotic compared to non-antibiotic treatment), patient's situation (e.g., employment or lack of work), time commitment and effort required (e.g., missing work as addressed in a study from the USA compared to the opportunity for unemployed patients in the South African study), and the investigator's gender in gynecology studies.

The factors mentioned in the first point appear in every study regardless of the therapeutic area, phase or research method (interview, questionnaire, others). They play a role in each patient's decision regardless of previous study experience or knowledge of the study, and they affect the patient's final decision. Atypical factors should be kept in mind when analyzing study specificity, cultural differences and unique study procedures. Often, these factors are less studied due to their rarer occurrence in studies, and it is easier to overlook them or underestimate their role.

3) Criterion of importance/significance:

1. Critical: Primary factors or direct motivations/barriers, e.g., personal benefit, lack of other treatment options, individual treatment preference, prior experience, and altruism.

2. Major: Secondary factors or supporting motivations/barriers, e.g., opinion of loved ones, doctor's advice, faith, and fear of side effects.

3. Minor: Other factors, e.g., sense of belonging to the community, reputation of the center or trust in the institution, financial compensation, and race of investigator.

In the case of these factors, their assignment to different groups may overlap; in other words, the same motivator may appear in different categories, transitioning among adjacent groups rather than transitioning from extreme minor to critical categories. For example, doctor's advice might appear in both the minor and critical categories, although it is generally assigned to the major category. In contrast, a sense of belonging to the community (minor) will not be observed in the critical category.

Limitations

Limitations of this review are due to methodological differences and different sizes of patient groups, as well as various therapeutic areas and phases of trials. In some publications, the respondents were participants in CTs, while in others, they were not. In addition, papers that addressed patient participation in hypothetical, non-existent CTs were included in the analysis.[8,9,15] The data presented in this review were collected by authors from 2005 to 2015 (note: There was a lack of information in Polish papers about collection time). Thus, all of these studies were performed before the outbreak of the COVID-19 pandemic. This period may have significantly affected the level of knowledge about and attitudes towards CTs, and thus, the factors motivating patients to participate may have altered. Additionally, in recent years, 2 extremely important regulations have appeared in European Union countries, affecting the way CTs are conducted.[17,18] Therefore, it is necessary to analyze and determine the motivations and barriers to patient agreement to participate in a CT.

Conclusions

Based on the conducted review, it is evident that there are various correlations between the examined factors and the patient's decision to participate in a CT. It is recommended to examine which dependencies exist between the type of clinical study, the characteristics of the study participant candidate, and the final decision of the patient regarding their participation in the CT. Future studies on factors influencing patients' decisions regarding participation in CTs should include more diverse groups of patients examined using similar methods. It would also be worthwhile to conduct such studies to see how the COVID-19 pandemic may have affected patient motivation.

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