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Editorial

Beyond the boundaries: Transitioning from categorical to dimensional paradigms in mental health diagnostics

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Abstract

Mental health diagnostics is undergoing a transformation, with a shift away from traditional categorical systems like the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), and the International Classification of Diseases, 11th Revision (ICD-11), and toward innovative frameworks like the Hierarchical Taxonomy of Psychopathology (HiTOP) and the Research Domain Criteria (RDoC). These emerging models prioritize dimensional and biobehavioral approaches in order to overcome limitations such as oversimplification, comorbidity and heterogeneity. This editorial explores the challenges of implementing these paradigms, such as the need for empirical validation, interdisciplinary collaboration and clinician training. It highlights the importance of advanced tools, biomarkers and technological integration to improve precision in diagnosis and treatment. Future research directions include creating reliable dimensional assessment methods, conducting longitudinal studies and fostering interdisciplinary networks. By bridging traditional and emerging frameworks, the field can progress toward personalized, biologically informed mental health treatment. This transition necessitates collaboration among researchers, clinicians and policymakers to improve diagnostic accuracy and treatment outcomes for those affected by mental health disorders.

Key words: precision medicine, Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-5), International Classification of Diseases 11th Revision (ICD-11), Hierarchical Taxonomy of Psychopathology (HiTOP), Research Domain Criteria (RDoC)

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Introduction: The established frameworks

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), and the International Classification of Diseases, 11th Revision (ICD-11), are foundational tools in the field of mental health. Developed through extensive research and international collaboration, these categorical classification systems have provided clinicians with standardized criteria for diagnosing mental disorders.[1,2] Understanding their historical context and clinical significance sheds light on their pivotal role in shaping contemporary psychiatric practice.[3,4] One of the key strengths of categorical diagnosis, as embodied by DSM-5 and ICD-11, is the facilitation of clear communication among healthcare professionals. By providing specific diagnostic labels, these manuals help ensure that practitioners across different settings and regions can accurately identify and treat mental health conditions.[5] This standardization also supports epidemiological studies and informs public health policies by offering consistent data on the prevalence and incidence of disorders[6](Table 1[7–17]).

However, despite their widespread use, DSM-5 and ICD-11 have faced criticism regarding their limitations.[18] The categorical approach can sometimes oversimplify the complexity of mental health by forcing symptoms into rigid boxes, potentially overlooking the nuanced spectrum of individual experiences.[19] Issues such as comorbidity and heterogeneity within diagnostic categories highlight the need for a more dimensional understanding of mental disorders.[20–23] The DSM-5 has increasingly integrated dimensional information into its traditionally categorical framework, recognizing that personality disorders can be more accurately described along a spectrum of trait dimensions.[24–26] This includes encouraging clinicians to rate the severity of key symptoms in the schizophrenia spectrum and other psychotic disorders, as well as specifying dimensional levels of severity for autism spectrum disorders and substance use disorders.[27–29] In addition, Section III of the DSM-5 features cross-cutting symptom measures and severity rating scales that can be applied across multiple diagnostic categories – enhancing precision and reflecting the manual's broader shift toward spectrum-based approaches.[30–32] Acknowledging these criticisms and recent trends is essential as the field considers transitioning to new frameworks like the Hierarchical Taxonomy of Psychopathology (HiTOP) and the Research Domain Criteria (RDoC), which aim to address these limitations[33] (Table 1[7–17]). This editorial seeks to advance beyond existing commentaries by synthesizing cutting-edge dimensional frameworks and established categorical approaches, thereby offering a uniquely comprehensive perspective that not only bridges critical gaps in the literature but also sets a new standard for clinical application and future research.

Emerging paradigms: the Hierarchical Taxonomy of Psychopathology (HiTOP) and the Research Domain Criteria (RDoC)

The HiTOP represents a significant shift from traditional categorical models by adopting a dimensional perspective on mental disorders.[14,34,35] Rather than viewing mental health conditions as distinct categories, HiTOP organizes psychopathology along a spectrum of symptom dimensions and hierarchical structures.[12,14,36] This approach acknowledges the overlap and comorbidity often seen in mental health diagnoses, aiming to provide a more nuanced and accurate representation of an individual's psychological functioning.[14,34,37,38] By focusing on symptom severity and patterns rather than strict diagnostic labels,

Table 1. Summary table of mental disorder classification systems

System	Descriptions	Strengths	Weaknesses	Ref.
DSM-5	Primarily used in the USA for clinical diagnosis and research	Provides detailed criteria for diagnosis, widely used in research settings, and has a long history of use	Criticized for lack of validity, influenced by commercial factors, and slow to incorporate new findings	7–9
ICD-11	Used globally for clinical diagnosis and public health purposes	Harmonized with DSM-5 to some extent, focuses on clinical utility, and is widely accepted internationally	Contains some disorder categories not present in DSM-5, and differences in priorities and uses can lead to inconsistencies	1,10,11
HiTOP	Classify mental disorders based on empirical data and dimensional traits	Integrates maladaptive personality traits into a single system, offers a dimensional approach that may better capture the complexity of mental disorders	Still under development and less widely adopted compared to DSM-5 and ICD-11	12–14
RDoC	Integrate basic behavioral and neuroscience research to understand mental disorders	Focuses on understanding the biological bases of mental disorders, offers a dimensional approach that can enhance research precision	Lacks extensive validation, and its practical application in clinical settings is still limited	15–17

DSM-5 – Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; ICD-11 – International Classification of Diseases, 11th Revision; HiTOP – Hierarchical Taxonomy of Psychopathology; RDoC – Research Domain Criteria.

HiTOP facilitates personalized assessments and interventions, potentially improving treatment outcomes.[13,38]

The RDoC initiative, developed by the National Institute of Mental Health (NIMH), seeks to redefine mental health diagnoses through the lens of biobehavioral systems and neurobiological mechanisms.[15,16,39] It emphasizes the importance of understanding mental disorders based on underlying genetic, neural and behavioral components across 5 domains: negative valence systems, positive valence systems, cognitive systems, social processes, and arousal/regulatory systems.[40–42] This framework encourages researchers to investigate the fundamental processes that contribute to mental health conditions, promoting a more integrated approach that spans from basic neuroscience to clinical practice.[16,40,41,43] By aligning diagnostic criteria with biological markers and behavioral indicators, RDoC aims to enhance the precision of mental health assessments and foster the development of targeted treatments.[39,44–46]

Challenges in transitioning to new frameworks

Transitioning to frameworks like HiTOP and RDoC introduces significant research barriers and methodological challenges. One primary hurdle is the need for extensive empirical validation of these new models across diverse populations.[47] The dimensional and biobehavioral nature of HiTOP and RDoC requires large-scale, longitudinal studies to establish reliability and validity, which demands considerable time and resources.[48] Additionally, researchers must develop new assessment tools and metrics that can accurately capture the continuous spectrum of mental health symptoms, moving away from traditional categorical measures.[49,50] There is also the challenge of integrating biological data with psychological assessments, necessitating interdisciplinary collaboration between neuroscientists, psychologists and psychiatrists.[51] Navigating these methodological complexities is crucial for the successful adoption of these emerging paradigms.[52]

Implementing HiTOP and RDoC in clinical settings presents challenges related to practitioner training and acceptance. Clinicians are accustomed to the DSM-5 and ICD-11 systems, and shifting to new frameworks requires substantial education and adjustment.[53] The dimensional approaches may initially seem abstract or less intuitive compared to categorical diagnoses, potentially leading to resistance among practitioners.[53] Training programs must be developed to familiarize clinicians with the new concepts, assessment methods and implications for treatment planning.[48] Moreover, there is a need to demonstrate the practical benefits of these frameworks in improving patient outcomes to encourage their adoption.[47,54] Ensuring that clinicians are adequately supported during this transition is essential for the frameworks to gain traction in everyday practice.[55]

Integrating HiTOP and RDoC with existing diagnostic systems poses significant logistical and conceptual challenges.[55] The current healthcare infrastructure, insurance policies and legal frameworks are deeply intertwined with the DSM and ICD classifications.[53] Transitioning to new models requires careful alignment to avoid discrepancies in diagnosis, billing and treatment authorization.[38] There is also the risk of fragmentation if some practitioners adopt the new frameworks while others continue with traditional systems.[56] Developing a coherent strategy that allows for compatibility between old and new models is imperative.[57] This might involve creating crosswalks between diagnostic criteria or establishing hybrid models that incorporate elements of both categorical and dimensional approaches.[58] Successfully navigating this integration is key to ensuring a smooth transition without disrupting patient care.[59]

Proposing future research directions

Advancing the implementation of HiTOP and RDoC frameworks hinges on the development of reliable and valid dimensional assessment tools.[47] Current diagnostic instruments are largely rooted in categorical models, which may not capture the nuanced spectra of mental health symptoms emphasized by HiTOP and RDoC.[49] Future research should focus on creating and validating tools that measure symptoms along continuous dimensions, allowing for more precise and individualized assessments.[48] This involves leveraging psychometric techniques to ensure these tools are sensitive to variations across different populations and settings.[60] Integrating technological advancements such as digital assessments and machine learning algorithms can enhance the accuracy and utility of these instruments in both research and clinical practice.[61–63] Moreover, harnessing advanced artificial intelligence (AI) tools for predictive modeling, integrating multi-omic datasets (e.g., genomic, proteomic and metabolomic profiles) to identify novel biomarkers,[64–66] and employing sophisticated human models (such as induced pluripotent stem cells or organ-on-a-chip platforms) can further refine and personalize diagnostic strategies.[67–69] These approaches not only improve the sensitivity and specificity of assessments but also open avenues for innovative, tailored interventions, ultimately bridging the gap between theoretical constructs and pragmatic clinical solutions.[70–72]

Longitudinal studies are essential for understanding the developmental trajectories and causal mechanisms underlying mental disorders within the HiTOP and RDoC frameworks.[47,73] Such studies can illuminate how genetic, environmental and neurobiological factors interact over time to influence psychopathology.[15,74,75] Future research should prioritize long-term, multi-wave studies that incorporate a variety of biobehavioral measures, including neuroimaging, genetic analyses and physiological

assessments.[16,76–78] To achieve these goals, researchers can employ advanced data-integration platforms and standardized protocols to streamline participant tracking across multiple time points.[79,80] Collaborative, multi-site consortiums can leverage pooled datasets to enhance statistical power and cross-validate findings,[81,82] while novel analytical approaches – such as machine learning, network analyses and Bayesian modeling – can discern subtle patterns of risk and resilience.[83,84] Additionally, incorporating ecological momentary assessments via mobile devices, collecting wearable sensor data and integrating electronic health records can provide rich, context-sensitive information that complements traditional laboratory-based measures.[85,86] Such comprehensive, technology-driven methodologies will ultimately enable more nuanced insights into the dynamic interplay of risk factors and resilience processes, paving the way toward more predictive, preventative and personalized mental healthcare.[87,88] These studies can help identify early biomarkers of mental health conditions, track changes in symptom dimensions and evaluate the effectiveness of interventions over time.[49,89,90] By embracing a longitudinal approach, researchers can contribute to more dynamic and predictive models of mental health.[35]

The complexity of mental health necessitates collaboration across multiple disciplines, particularly emphasizing preclinical research.[91] Future research should encourage partnerships between psychologists, psychiatrists, neuroscientists, geneticists, and other related professionals to integrate diverse perspectives and methodologies.[92] This integrated approach could involve leveraging advanced genomic and neuroimaging techniques, harnessing machine learning analytics, employing preclinical models (such as induced pluripotent stem cells or organoid systems) and fostering multi-institutional collaborations to drive the development of more predictive, preventive and personalized interventions.[67,88,93–95] Interdisciplinary teams can facilitate the exploration of mental disorders from biological, psychological and social angles, aligning with the comprehensive aims of HiTOP and RDoC.[96] Establishing collaborative research networks and consortia can enhance data sharing, standardize methodologies and accelerate scientific advancements.[97] Such cooperation is vital for developing holistic models of psychopathology and translating research findings into practical applications.[98–100] Preclinical models, including advanced technologies like optogenetics and chemogenetics, are crucial for this integration, as they allow for the exploration of genetic and environmental factors in mental health.[101–103]

For HiTOP and RDoC to be effectively integrated into clinical practice, concerted efforts are needed to address policy and standardization challenges.[38,47] Future research should inform policy development by providing evidence on the benefits and feasibility of these new frameworks.[104] Engaging with policymakers, professional organizations and regulatory bodies can facilitate the incorporation of dimensional and biobehavioral approaches into diagnostic guidelines and reimbursement structures.[105,106] Additionally, establishing standardized protocols and training programs will ensure consistent application among practitioners.[107] Research should also explore strategies for bridging the gap between existing categorical systems and the new models to ease the transition and minimize disruptions in clinical care.[48,54,60]

Conclusions

The integration of dimensional, biobehavioral and categorical perspectives heralds a transformative era in mental health diagnostics. By merging the established strengths of frameworks like the DSM-5 and ICD-11 with the transdiagnostic insights of HiTOP and RDoC, the field stands poised to achieve unprecedented diagnostic precision, more personalized treatments and improved clinical outcomes. Emerging empirical evidence – from large-scale, longitudinal studies to compelling case-based examples – further underscores the value of expanding beyond narrow diagnostic boundaries. Realizing the full potential of these approaches, however, will demand concerted efforts on multiple fronts. Researchers must refine and validate comprehensive assessment tools that capture the complexity of psychopathological phenomena, while clinicians require training and resources to confidently apply these methods in diverse settings. Policymakers, educators and professional organizations will play pivotal roles in promoting interdisciplinary collaborations, providing supportive infrastructures and encouraging data sharing across institutions. Such integrative efforts will be bolstered by advanced computational techniques, the establishment of shared data repositories and the embrace of interdisciplinary teams capable of synthesizing varied perspectives. Moreover, global engagement and cross-cultural studies will be critical to ensuring that emerging models are broadly applicable, equitable and culturally sensitive. Although many of these proposals remain conceptual at present, ongoing empirical endeavors promise to anchor them in robust, evidence-based practice. By harmonizing traditional diagnostic schemas with cutting-edge dimensional frameworks, the mental health community can forge a new path – one that better captures individual differences, guides more targeted interventions, reduces stigma, and ultimately improves the lives of individuals affected by mental health disorders worldwide.

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Anti-amyloid treatments in Alzheimer's disease: elegance, evidence and ethics

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Abstract

The so-called "amyloid cascade hypothesis" provides an elegant explanation of Alzheimer's disease (AD), has motivated the amyloid-lowering therapeutic strategy, and led to the elaboration of a rich experimental and conceptual toolkit for the field to progress. But it might be incorrect. The scientific evidence base supporting the efficacy and safety of current anti-amyloid antibody treatments in AD is weak. Nevertheless, we argue that there is a bias towards the amyloid-lowering therapeutic strategy amongst key opinion leaders in the research and advocacy communities. To demonstrate this, we first focus on the AD lexicon: while any accrual of amyloid on a brain PET scan can now permit diagnosis/definition of AD, lowering positron emission tomography (PET) amyloid is considered disease modification, and treatment-induced side-effects are hidden behind neutral-sounding acronyms: ARIA (amyloid-β (Aβ)-related imaging abnormalities: brain bleeding and swelling) and ARPA (amyloid-β (Aβ) removal-related pseudo-atrophy: brain shrinkage). Second, we underline that drugmakers did not test anti-amyloid antibodies against the best proven interventions and did not adequately inform trial participants of risks, thus violating research ethics of the Declaration of Helsinki on 2 counts. In conclusion, we are critical of over-reliance on the idea that PET amyloid-lowering treatments for AD are a therapeutic revolution as claimed, and consider that optimism does not excuse a lack of scientific, regulatory, and ethical integrity. We argue for rigorous, properly controlled (e.g. donepezil) anti-amyloid trials demonstrating cognitive and functional benefit before accepting amyloid-lowering drugs as the new standard of care for AD patients.

Key words: ethics, clinical trials, bias, Alzheimer's disease, amyloid hypothesis

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Introduction

After 20 years since the approval of memantine, some novel drugs have been introduced for the treatment of patients with Alzheimer's disease (AD) beginning in late 2021. In clinical trials, these drugs, Aduhelm (aducanumab), Leqembi (lecanemab) and Kisunla (donanemab), all of which are monoclonal antibodies against amyloid-β (Aβ) peptides made of 39–43 amino acids which make up the amyloid in the brain and elsewhere in the body, have been found to reduce Aβ on a positron emission tomography (PET) scan in the brain.[1]

Based on this scientific achievement and a statistical delay of cognitive decline, they have been applauded as wonder drugs amidst a backdrop of rhetoric suggesting that the AD treatment landscape is undergoing substantive transformation.[2] However, their effect on reducing cognitive decline remains beyond clinically-meaningful detection, whereas what are frequently visible, though often treated as a footnote, are their adverse side effects that include brain edema and microhemorrhages, which can, in rare instances, be fatal.[3] We have previously argued that survival time is an important factor in determining the therapeutic value of these and other drugs for long-term use in AD.[4] On the back of recent scandals of scientific image manipulation and controversial decisions made by drugmakers, we continue our critique of over-reliance on amyloid-lowering as a therapeutic strategy in AD.

In this editorial, we examine these issues in detail. In particular, we wish to argue that there is a community-wide bias towards these drugs which facilitates a lack of scientific, ethical and regulatory integrity that does disservice to the growing community affected by AD.

The amyloid hypothesis: an elegant engine to motivate clinical trials

According to the famous amyloid cascade hypothesis (ACH), AD is caused by the sequential deposition of proteins that define the disease, Aβ and tau. Amyloid is thought to act as an upstream "trigger", whereas tau is the "bullet" of AD pathogenesis.[5] We recognize the strength of the evidence in favor of the ACH from genetics and neuropathology of the early contemporary history of AD research.[6] Moreover, the ACH has contributed to significant experimental and conceptual progress in the field by motivating thousands of experiments and dozens of clinical trials testing its central claim, as well as providing a rich conceptual toolkit to protect it from empirical refutation.[7]

Here, our concern is what Nguyen calls "the seductions of clarity".[8] In other words, because the ACH provides an elegant explanation of AD, it must be right. We worry that the ACH's elegance has taken precedence over evidence in favor of the amyloid-lowering therapeutic strategy. Thus, the ACH may be "a conclusion in search of support",[6] instead of the other way round. For instance, if we take the example of the re-definition the disease itself as a biological entity, "AD = A+T+N+"[9] – where A+ stands for biomarkers of amyloid, T for tau, and N for neurodegeneration, this essentially summarizes the hypothetical amyloid cascade (A→T→N), suggestive of the role of this hypothetical explanatory schema in defining the disease itself. In 2024, it is now possible to diagnose AD in A+ people alone,[10] though this idea has provoked much controversy among neurologists.[11]

However, the ACH could be wrong. Prior to 2021, all clinical trials lowering amyloid had failed to improve or even succeeded in worsening AD patients' cognition.[12] All such trials were resting on the assumption that amyloid-β is an underlying cause of AD. This idea has certainly evolved since the early 2000s, but has not undergone anything like a paradigm shift.[7] In 2011, Castellani and Smith[13] noted:

> *With each failure of anti-amyloid-β therapy in clinical trials, new trials are initiated with no hint of slowing down [...]. With dozens of clinical trials targeting amyloid-β either under way or having failed, and with no signs of slowing down, a legitimate concern is that the hypothesis has become 'too big to fail'. With so much time, money and, indeed, faith invested in the construct, is a negative outcome simply intolerable for the scientific community and society who depends on it?*

More recent criticism – including our own – against over-reliance on monoclonal Aβ antibodies as a therapeutic strategy in AD, is thus not novel, but an iteration of earlier attempts to expose what should be regarded as over-reliance on a seductive idea.

Recent hope from amyloid-lowering trials in AD has been provided by results from clinical trials. Patients treated with infusions of lecanemab over 18 months had a worsening of the CDR-SB cognitive scoring system by 1.21 points, while placebo worsened 1.66 points, and the relative difference between these two is 27% (0.45 points), less than half the level of minimal clinical relevancy of at least 1–2.5 points, and similar to other drugs of the same category as lecanemab, which on average produce a 0.18 point difference according to the largest meta-analysis yet of amyloid-lowering clinical trials.[3] Yet as Kurkinen[14] states:

> *1.21 and 1.66 are not measured values of the study population with and without lecanemab but are calculated ... as a weight-adjusted change from the data for men and women. Isn't this like comparing apples and oranges? Clearly, a −0.73 difference for men and a −0.20 for women [...] are too different to originate (statistically) from the same population. Therefore, I suggest that 1.21, 1.66 and −0.45 do not represent*

any population, do not characterize anybody, have no meaning, and are useless value … commentaries and popular media have interpreted the −0.45 difference as a 27% (0.45/1.66) less cognitive decline in the lecanemab group compared to the placebo group. This is a very trivial miscalculation. The correct value is 9.3% (0.45/4.86), which pays attention to the 3.2 baseline.

However, listening to its advocates gives the impression that the ACH has finally been vindicated after a decades-long search that never truly questioned the target, but the trials testing them.

The pathologization of Aβ PET scans in AD

As of 2024, the Alzheimer's Association workgroup considers a positive amyloid PET scan to be sufficient for diagnosis, suggesting the separability of AD and dementia.[10] This rethink of the AD concept has ushered in "a fundamental shift from syndrome-based Alzheimer's dementia care to early, biomarker-guided treatment of Alzheimer's disease".[15] On the front line of this shift, a recent class of "high-clearance anti-Aβ antibodies" has been approved in different health systems, including the USA – aducanumab, lecanemab, and donanemab.[1]

These antibodies provide a set of powerful immunotherapies that significantly reduce Aβ on PET scans, while also modifying the brain's highly sensitive and coordinated immune response, inducing severe neuronal disturbances.[16] The precedent for the approval of these drugs was set on June 7, 2021, when the U.S. Food and Drug Administration (FDA) approved aducanumab (Aduhelm®; Biogen, Cambridge, USA), the first new drug in 18 years for the treatment of patients with AD, citing the "evidence that Aduhelm reduces amyloid beta plaques in the brain and that the reduction in these plaques is reasonably likely to predict important benefits to patients".[17] This drug would then be withdrawn in 2024 amidst a backdrop of rhetoric about its supposed importance to research as a "groundbreaking discovery".[4] Nevertheless, lecanemab and donanemab would fare better in different health systems on the way to almost universal approval.

However, recent literature has brought serious question marks as to whether the PET-signals captured in the clinical trials testing these monoclonal anti-Aβ antibodies are misinterpreted as Aβ clearance, rather than general brain shrinkage and tissue damage. Høilund-Carlsen et al.[18] observe that

…decreased amyloid PET signal in these trials is unlikely to be a one-to-one reflection of amyloid removal, but rather a reflection of increased therapy-related brain damage, as supported by the increased incidence of ARIAs and reported loss of brain volume […]. [The authors therefore] fear that reported decreases in cerebral amyloid deposits more likely reflect decreased uptake of unspecific amyloid PET tracers.

This leads the authors to question the use of amyloid PET as a single primary outcome measure for anti-amyloid treatments and argue that outcome data in these studies should therefore be supplemented with brain magnetic resonance imaging (MRI) scans, to show the effect of the drugs on brain size and atrophy.[19] Given the uncertainties about the meaningfulness of statistical delays in cognitive decline, PET Aβ reduction, as well the possibility of side effects, we argue that language used to describe the therapeutic relevance of reducing PET Aβ with anti-Aβ antibodies in AD should be based on a sober interpretation of clinical trial data.

Indeed, the concept of "ARIA," short for Aβ-related imaging abnormalities, emerged to describe treatment-related brain edema and hemorrhage seen in MRI of AD, particularly following Aβ-lowering. ARIA represents 2 phenomena: edema (ARIA-E) and hemorrhage (ARIA-H). ARIA-E or cerebral edema results from the accumulation of fluid due to the opening of the blood–brain barrier.[20] Symptoms common to ARIA-E and ARIA-H include headache, confusion, dizziness, nausea, tremor and gait disturbances. ARIA-H occurs frequently in the aging population and AD patients, whereas ARIA-E is more specifically related to amyloid-lowering.[21]

The phenomenon of ARIA has variable severity as detected by MRI depending on different background factors. Approximately half of AD patients have what is known as cerebral amyloid angiopathy (CAA), in which Aβ accumulates in the walls of cerebral arteries.[22] When antibodies remove Aβ from the walls of blood vessels, the weakened vessels increase a person's susceptibility to edema, hemorrhage and mortality in more severe cases.[23] Other risk factors include the use of anticoagulant drugs, and also apolipoprotein E (*APOE4*) genotype, a risk factor for dementia.[24]

Moreover, lecanemab did not appear to slow cognitive decline in *APOE4* carriers, and appeared to accelerate decline in participants with 2 copies of the *APOE4* gene.[14] Most people with symptomatic AD carry 1 or 2 copies of the *APOE4* gene, limiting the impact of amyloid PET-lowering drugs outside of clinical trials.[25]

A meta-analysis found that one related effect of anti-amyloid antibodies is accelerated brain atrophy.[26] Belder et al. propose a novel acronym within the emerging AD lexicon: Aβ removal-related pseudoatrophy or "ARPA", a loss of brain volume associated with treatment with Aβ-lowering therapies, which the authors consider not to be harmful.[27]

The acronym "ARIA" was intended to refer to an imaging phenomenon rather than a clinical syndrome.[28] We argue that this is a rhetorical strategy that means that ARIA

cannot be, by definition, a cause of death. Moreover, ARPA differs by 1 letter to ARIA. These are paronyms: words with similar forms, but different meanings. We believe that this rhetorical choice may have been made to make "ARPA" sound more palatable alongside its well-accepted amyloid-lowering neighbor, ARIA. Although acronyms are necessary to standardize language in the scientific literature, they should not trivialize important side-effects.

In summary, the use of anti-amyloid treatments can lead to swelling and bleeding in many cases, sometimes very serious. An important feature of these 2 Aβ-lowering acronyms is how neutral they sound. Yet some cases of ARIA can be very serious and result in death due to related causes, and further data will ultimately determine ARPA's significance. If we return to the contrast between the neutral-sounding lexicon of PET Aβ-lowering (ARIA and ARPA) with the lexicon of PET Aβ increase, the new biological diagnostic criteria of the Alzheimer's Association working group considers a positive Aβ-PET scan to be sufficient for diagnosis of biological AD, a "pathogenic condition".[10] Yet, most asymptomatic Aβ-positive individuals who have "biological AD" will not actually develop dementia in their lifetimes, shedding doubt on the usefulness of a potentially harmful label to patients in the absence of cognitive decline.[29] We consider that this asymmetry that pathologizes PET Aβ accumulation and banalizes side effects of PET Aβ lowering is suggestive of bias in the language used to talk about AD towards the amyloid-lowering strategy.

Doubts about scientific, ethical and regulatory integrity

Going back to our point about seductive clarity and amyloid, we wish to first draw attention to a hot topic in AD and neuroscience research: image manipulation, or the faking of research findings. In AD research, so far these relate to a fake amyloid oligomer Aβ*56[30] and dozens of further manipulated papers supporting the amyloid-lowering strategy.[31] We do not claim that these findings directly refute the ACH. But they do raise concern about the vulnerability of those seduced by the ACH's clarity and how it may have led them to fake images in favor of the ACH, "a conclusion in need of support".[6] Whatever the reasons and pressures that led leading scientists to manipulate their images, it means that the ACH evidence base is now partly lacking in scientific integrity.

However, integrity issues in the field extend beyond preclinical science to the ethics of clinical research. In trials of both lecanemab and donanemab, *APOE4* genetic tests showed that certain patients were predisposed to ARIA if they took the drugs, but these participants were not informed, creating a recent scandal in the lay press in the *New York Times*.[32] We consider this withholding of genetic risk for ARIA to be a violation of Paragraph 26 of the Declaration of Helsinki,[33] which states:

> *In medical research involving human participants capable of giving informed consent, each potential participant must be adequately informed in plain language of the aims, methods, anticipated benefits and* ***potential risks and burdens****.*

We draw on the "social value requirement for clinical research", where social value is understood as "collecting data which might be used to improve health"[34] to ask the question: why did drug developers, who aim to improve health, not share genotype data they knew to have a significant impact on participants' health, i.e., data with high social value to those people who could use them to make informed decision to continue or forego participation?

Beyond this hard violation of informed consent, there are also widespread soft violations of providing inaccurate information about the risk-benefit profile of these antibodies on the part of clinicians and advocates, based on seductive language used to describe the effects of anti-amyloid antibodies as giving people more time with their loved ones, despite the fact that this claim is not backed up by data from clinical trials.[35] For instance, on September 12, 2024, the leading AD scientist Henrik Zetterberg published an article in *Nature*.[36] We consider that this clinician-scientist and industry-backed key opinion leader has overstated clinical facts and made ungrounded claims about the drug. Zetterberg states that the drug can "buy a person invaluable months or years to spend with loved ones before dementia sets in". As another example, in the above *New York Times* piece,[32] a leading Alzheimer's Association spokesperson repeats this "precious time bought" narrative:

> *I think it's transformational. It is not a cure. We understand that. And it has side effects. So it may not be for everyone. But for those that could benefit, it offers more time during the most critical stage where you're still independent, you still have a lot of opportunity to enjoy time with family, baptisms, weddings, graduations.*

As alluded to above, we consider these to be misguided claims. As Professor Robert Howard, an old age psychiatrist specializing in dementia argues[37]:

> *The benefits of lecanemab are so modest as to be undetectable in an individual treated patient. Although 27% slowing of disease course sounds impressive, this is not strictly what the analysis of the trial data showed. It's important the results are discussed honestly, accurately and without spin.*

Without truthful reporting of the risks and benefits of these drugs for individuals, truly informed consent is not possible.[35] But beyond the problem of informed consent, let us turn to the use of placebo, since Paragraph 33

of the World Medical Association (WMA) Declaration of Helsinki[33] explicitly states:

> *The benefits, risks, burdens, and effectiveness of a new intervention must be tested against those of the best proven intervention(s) […]. Extreme care must be taken to avoid abuse of this option.*

Several cholinesterase inhibitors have received full approval for use in early AD, which is the disease stage at which the tests of lecanemab and donanemab were conducted.[38] However, in neither the lecanemab nor the donanemab trial were cholinesterase inhibitors administered systematically, but instead used as part of the randomization criteria. As measured by ADAS-Cog 14 or CDR-SB, the slowing of cognitive decline accomplished by lecanemab at 18 months was only half of that achieved by the vastly cheaper donepezil by only 6 months.[39] If anti-amyloid antibodies are indeed disease modifying, then they should have lasting effects on the disease course regardless of the supposedly symptomatic effects of previously-approved drugs. Finally, the use of inactive placebo as a control may have led to unblinding effects due to ARIA, since on more objective measures of cognition, the effect size of antibody treatment was lower.[40]

Drug sponsors have an ethical duty to inform research participants of genetic profiles, risks and benefits, and also to test these antibodies against the standard of care[4] via "head-to-head" comparisons with available treatments, none of which seem to be a priority for drug developers.[3]

Here, we make a final point about conflicts of interest. A US Congressional report found that the FDA's relationship with sponsor Biogen during approval process of Aduhelm was "atypical and failed to follow the agency's own documentation protocol", and a recent BMJ inquiry found that the FDA committee that approved donanemab contained conflicts of interest.[41] The European Medicines Agency (EMA), who initially rejected lecanemab in July 2024, has a no-tolerance policy on conflicts of interest in their advisory board. However, after re-considering the data with "excluded data from 274 patients who carried 2 copies of the *ApoE4* gene and were therefore at highest risk of ARIA", as well as "submissions from patients, carers, clinicians and professional organizations, who shared their perspectives on the unmet needs of patients with Alzheimer's disease and the data on cognitive decline and risks", the EMA also recommended approval for use in early AD with *ApoE ε4* non-carriers or heterozygotes in November 2024.[42] It is not clear whether any of the groups in the "re-examination procedure" had conflicts of interest. We believe that advocacy from industry-backed leaders like Zetterberg will have certainly played a role in the change of decision. Nevertheless, other researchers have reported difficulties when contacting the EMA for information regarding conflicts of interest.[43]

However, the financial interests of these companies (Biogen, Eisai and Eli Lilly & Company) extend into drug advocacy in patient organizations, such as the Alzheimer's Association, whose 2023 Annual Report states that Eli Lilly and Eisai (the primary sponsors of donanemab and lecanemab, respectively) donated between $500,000 and $999,999, whereas Biogen (the secondary sponsor of lecanemab) donated between $250,000 and $499,999. We are concerned that this industry-backed advocacy is based on a foregone conclusion, inspired by over-reliance on the ACH, that lowering amyloid PET is ultimately the best strategy available for finding a treatment for AD. We consider that amyloid-lowering should not distract us from the need to explore more treatment avenues in the AD pipeline, which is full of potential treatments that have a variety of non-amyloid and tau targets.[44]

Conclusion

The ACH has long been believed to be the code that would crack the enigma of age-related pathological cognitive decline, and the Alzheimer's Association 2024 workgroup's criteria for biological AD[10] essentially write the ' amyloid→tau→neurodegeneration' hypothetical cascade into history. Here, we remind readers that for cognitively-unimpaired older adults, being amyloid-positive on a PET scan means being in a state of risk, such that converting to dementia is the exception rather than the rule.[29]

In an interview,[45] the geriatrician Jason Karlawish discusses an advert by Biogen (sponsor of Aduhelm and Leqembi), "ID AD: Identify Alzheimer's earlier." The ad portrays a middle-aged man, and from above, white paint is being poured over half of his head, and has closed his covered eye. Below him, the ad reads: "Our understanding of Alzheimer's disease is evolving. So should the way we manage it." The paint represents amyloid build-up on the brain, and as Karlawish points out:

> *The ad by Biogen … depicts a person with an amyloid image that looks like the living dead, "The Phantom of the Opera", in which half the person's face looks like a skull and the other half looks alive. That's not the kind of imagery that's going to help us respect the person, recognize the mind of the person living with Alzheimer's disease.*

The "tragedy discourse"[46] of AD can be seen in the aforementioned New York Times piece in a quote from a researcher at the Alzheimer's Drug Discovery Foundation[32]: "People are robbed of everything that makes them human […]. They're like infants in a human body." We worry that the tragedy discourse of AD[46] has contributed to the above non-respect of the rights of people with dementia as research participants with a right to informed consent.

Absent longer, more rigorous tests of amyloid-lowering treatments that emphasize clinical endpoints, respect informed consent and test antibodies against approved drugs for AD, we therefore argue for a scientific and

ethical reassessment of PET amyloid-lowering treatments. We argue for rigorous, properly controlled (e.g., donepezil) anti-amyloid trials demonstrating long-term cognitive and functional benefit before accepting amyloid-lowering drugs as the new standard of care for AD patients. Given the limited resources available for health care and research, the "high-tech" approach to dementia prevention should not distract away from cost-effective "low-tech"[47] action to rethink ambitious public health action against behavioral and social determinants of brain health.[48]

We do not deny the impact of dementia on selfhood, relationships and well-being that urgently requires safe and effective treatments. But we urge researchers, advocates and regulators to put the rights of people living with dementia before the promotion and testing of ideas so as to "promote, protect, and ensure the full enjoyment of human rights by persons with disabilities" as articulated in the UN's Convention on the Rights of Persons with Disabilities,[49] in force in 186 countries. We close with a quote by philosopher Jiddu Krishnamurti,[50] who mentioned a trap humankind can fall into in both daily life and hypothesis-driven science:

> *We sacrifice the present for the future – and it does not matter what means we employ as long as our declared purpose is to produce a result which we say will be beneficial to man. Therefore, the implication is that a wrong means will produce a right end and you justify the wrong means through ideation.*

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The only constant in life is change: Summary of the last 4 years of *Advances in Clinical and Experimental Medicine*

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Abstract

In the last 4 years, the journal *Advances in Clinical and Experimental Medicine* has made significant strides in adapting to changes in scientific publishing. It has maintained high levels of citations and submitted manuscripts, publishing a considerable number of articles ahead of print releases to minimize wait times. With a solid reputation and a growing base of over 7,000 reviewers, the journal upholds rigorous ethical standards and thorough statistical verification for all manuscripts. Bibliometric indicators showcase the impact of *Adv Clin Exp Med*, including an increased impact factor, CiteScore, Index Copernicus Value (ICV) and recognition in indexes and databases like Scopus and PubMed Central (PMC). International cooperation support for early-career researchers are key focuses, with efforts to provide guidelines, online meetings, and social media promotion. Various materials for authors prepared by the editorial staff are discussed, including detailed instructions for authors, tips regarding graphical abstracts and choosing a checklist, as well as ethical guidelines, a brochure on the rules of statistical analysis and data presentation, and technical requirements for figures. The journal also emphasizes data sharing, detailed procedures for errata and retractions, and clear policies concerning the use of artificial intelligence (AI) tools. Calls for submissions show authors the optimal direction for creating original, innovative scientific papers. Financing from the Polish Ministry of Science and Higher Education ensures the financial stability of the journal. By adapting to the evolving landscape of scientific communication, *Adv Clin Exp Med* remains dedicated to facilitating open access publishing and disseminating high-quality medical research to its readers.

Key words: scientific journal, bibliometrics, research ethics, early-career researchers, ahead of print.

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Introduction

It has been 4 years since *Advances in Clinical and Experimental Medicine (Adv Clin Exp Med)* entered a new phase of its development. Since its foundation in 1992, successive editors-in-chief, section editors and the editorial staff have consistently contributed to building the journal's reputation (with an impact factor obtained in 2009 and inclusion in Scopus since 2001). Over the past 4 years, the journal has focused on:

1) keeping pace with standards set by international bodies and leading journals in the medical field;

2) making *Adv Clin Exp Med* more author-friendly by offering precise guidance concerning our requirements, submission process, research ethics, and other issues;

3) publishing as many papers as possible ahead of print releases in order to shorten the wait time for publication.

Constant changes in scientific publishing also mean that authors' reasons for choosing journals to submit their work to may vary, and editors should not assume that the popularity of their journals is guaranteed and constant, as Seeber[1] outlined. The increasing popularity of preprints[2–4] and the emergence of artificial intelligence (AI) tools[5] are other examples of the deep reshaping of the scientific communication landscape observed in recent years. Scientific journals lose their importance in some aspects while remaining paramount for appropriate scholarly communication in others.[6] Similar to the "publish or perish" rule, editors of scientific journals must understand that "change or perish" should be their motto in this era of acceleration in all fields, areas and dimensions. The present summary of *Adv Clin Exp Med's* performance in the last 4 years proves that we successfully adapted to change and are ready and prepared for further development.

Reputation of *Adv Clin Exp Med*

Both the number of citations of published articles (Web of Science: 2021 – 2836, 2022 – 3137, 2023 – 3303) and the number of submitted manuscripts (817 submissions in 2021, 1146 in 2022, 972 in 2023, and already 848 by October 25, 2024) remain high. The rejection rate for 2021 was 82.38%, for 2022 – 80.58%, for 2023 – 80.24%, and for 2024 (until October 25) – 81.35%. In 2023, we published 230 articles – 150 in the 12 issues of 2023 and 80 from the 2024 issues as ahead of print (in 2022 – 195 articles – 146 in the 12 issues, and 49 from the 2023 issues as ahead of print). This means that on December 31, 2023, issue 12/2023 was published (the issue for a given month is published on the last day of that month), but a large number of articles from issues 1–8/2024 were already available ahead of print releases at that time. From January 1 to October 25, 2024, we published 101 articles in issues 1–9/2024, and as of October 25, 2024, 91 articles from further issues (up to and including 11/2025) were available ahead of print.

Our reviewer base includes more than 7,000 researchers and continues to grow. We set high ethical requirements for reviewers (working in double-blind review mode), as formulated in the guidelines available on our website.

A survey conducted in 2020 by Hardwicke and Goodman[7] among editors of 107 across 57 scientific fields showed that 34% (36/107) rarely or never use specialized statistical review, 34% (36/107) used it for 10–50% of their articles and 23% used it for all articles. These numbers have changed little since 1998, despite the dramatically increased concern about research validity, underscoring the continuing importance of thorough statistical verification for all peer-reviewed manuscripts. The statisticians working in the Centre for Statistical Analysis of Wroclaw Medical University assess the use of statistical tools in submitted papers. At the same time, editors of English-language publications employed by the journal are responsible for editing the content of articles. Although there are voices, like the commentary by Zhou,[8] which emphasize the importance of papers in languages other than English, the latter will remain the language of global scientific communication for decades. Its widespread use does not mean the editors should relax their standards regarding language correctness. We have also developed formalized procedures for errata, retractions (https://advances.umw.edu.pl/en/complaints-corrections-retractions) and appeals against the section editors' decisions (https://advances.umw.edu.pl/en/appeals). Retraction rules are particularly important in light of the rising number of retracted papers in scientific journals – only in 2023, more than 10,000 scientific papers were retracted.[9] In 2009, the Committee on Publication Ethics (COPE) published its retraction guidelines[10,11]; clear retraction procedures are therefore necessary and obvious.

Bibliometric indicators

In 2010, the journal achieved an impact factor (IF), which stands at 2.1; the 5-year impact factor is 2.2, while the Journal Citation Indicator (JCI) value for *Adv Clin Exp Med* in 2023 has increased to 0.4. This indicator considers differences between scientific fields, document types, and years of publication and provides an authoritative assessment of a journal's impact on global science. In 2024, *Adv Clin Exp Med* moved from Q4 to Q3 in the Medicine, Research & Experimental category within the Journal Citation Reports provided by Clarivate Analytics (London, UK).

Additionally, the Scopus database shows a steady increase in the Scopus CiteScore from 3.0 for 2021 to 3.4 for 2022 and 3.7 for 2023 (CiteScore Tracker is 4.1 as of December 2024). According to Scopus CiteScore, the journal is in Q1 in the Reviews and References (medical) category, and in Q2 in Medicine (miscellaneous), Biochemistry, Genetics and Molecular Biology category.

This year's evaluation score by Index Copernicus International (ICI) database is 171.00 (the journal is on the ICI Journals Master List).

Indexes and databases

The journal is indexed in PubMed/MEDLINE, Directory of Open Access Journals (DOAJ), Science Citation Index Expanded (SCIE), Scopus, Embase/Excerpta Medica and CrossRef, and in the CLOCKSS dark archive, among others (https://advances.umw.edu.pl/en/indexation-and-statistics). In March 2024, we successfully completed the qualification process for the PubMed Central (PMC) database and are depositing all articles from the issues published in 2023 and 2024.

As of 2023, we have also been tracking the resonance of our publications in lay media (press, news outlets) and social media using the Altmetric provided by Digital Science (London, UK), and metrics reflecting mentions of our articles are available on our website – both for individual articles and for the journal as a whole (https://advances.umw.edu.pl/en/altmetrics). Williams[12] provided a substantive review and evaluation of altmetrics for academics to consider when adopting, utilizing and researching these tools.

International cooperation

We have patronized 13 conferences organized by global and European scientific societies, and published 9 books of abstracts from these events as special issues of the journal (https://advances.umw.edu.pl/en/special-issues).

The Scientific Committee and Editorial Board members are award-winning researchers of various specialties from all over the world – their reputation is highlighted by a high Hirsch index. The expansion of the Scientific Committee to include researchers from the Far East and Latin America is due to Clarivate's guidelines and the fact that many of the authors publishing in our journal come from these regions of the world.

Collaboration with authors and support for early-career researchers

We offer the authors (particularly early-career researchers) a wide range of materials prepared by our editors to facilitate publication. *Advances in Clinical and Experimental Medicine* is often the first journal in which they publish their work. To better suit their needs, we have introduced a research letter category of papers. As Kukafka et al.[13] have put it, this article type is optimal for presenting new, early, or sometimes preliminary research findings.

We regularly organize online meetings with authors, which occur monthly via GoogleMeet. Our social media – Facebook, LinkedIn, Instagram, BlueSky, and X (formerly Twitter) – are platforms for promoting publications and communicating with the scientific community. At least 1 post is published daily on each of these sites. The role of disseminating publication via social media by scientific journals themselves was analyzed, among others, by Özkent[14], Lopez et al.[15] and Pandey et al.[16]

We are also updating our instructions for authors to be as precise as possible, based on best practices from well-established journals known for their high standards (https://advances.umw.edu.pl/en/instructions-for-authors). The issue of clear guidelines on formatting the submitted manuscripts was discussed, i.a., by Iwaz,[17] who emphasized the importance of regular and accurate revisions of journals' instructions to ensure consistency, conciseness and specificity, as well as of matching essential instructions from journals with simple requirements from submission systems.

Since March 2023, attaching graphical abstracts to submitted papers has been mandatory to enhance the possibilities of promoting the publication – both through our website and on social media. We have developed detailed guidelines on how to prepare such an abstract (https://advances.umw.edu.pl/en/graphical-abstracts). The benefits of employing graphical abstracts in scientific publications were discussed by Klaassen et al.[18] (on the example of urology journals), Chapman et al.[19] (in surgical journals) and Stahl-Timmins et al.[20] (in *BMJ*).

As of August 15, 2024, we also have made it mandatory to share the data underlying the analyses presented in an article (https://advances.umw.edu.pl/en/data-sharing). It is worth mentioning that such a requirement can also benefit the authors in the long run, as presented by Popkin[21] and particularly by Christensen et al.,[22] who showed the impact of data sharing on article citations.

There is also a clear policy in *Adv Clin Exp Med* on using AI tools by authors: https://advances.umw.edu.pl/en/ai-usage. Such a policy is still rare among Polish and foreign journals. Since the potential of AI usage in research and scientific writing is dynamically rising, as recently outlined by Khalifa and Albadawy,[23] employing such tools should not be banned outright, but rationally regulated.

Based on our experience, we have also prepared other materials for authors:

– tips on the selection of a checklist – a tool to ensure the proper structure and layout of the article[24];

– updated guidelines for the analysis and presentation of statistical data (https://advances.umw.edu.pl/source/ACEM%20Statistical%20guidelines%202024.pdf);

– guidelines for early-career researchers on the principles of developing scientific papers (https://advances.umw.edu.pl/upload/files/ACEM_toolbox_www(1).pdf);

– detailed presentation of technical requirements regarding figures (https://advances.umw.edu.pl/source/ACEM%20Figures%20technical%20requirements%202024.pdf);

– an outline of preprints as means of scientific communication.[25]

The requirements and procedures for publishing ethics follow the guidelines of the COPE, the International Committee of Medical Journal Editors (ICMJE) and Clarivate Analytics (our editors learned them during online training – we make sure to provide regular training for the editorial team).

Section editors manage calls for submissions – initiatives designed to encourage specialists in specific fields to submit submissions on specific research areas. These submissions undergo standard peer review, but have priority in publication as ahead of print papers (https://advances.umw.edu.pl/en/calls-for-submissions/). Calls for submissions are designed to support authors by helping them focus on the latest trends in experimental and clinical research and to guide them in the optimal direction to write original, innovative scientific papers.

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Summary

Many authors offer varying predictions and ideas concerning the future of scientific publishing – some are rather radical, like in the opinion piece of Habibzadeh,[26] who envisions that in near future, a universal AI-based system with ready access to a collective database will be formed to analyze the gigantic amount of data being created; consequently, there will be no ready articles, no scientific journals, no indexing system, no peer review, no research or publication ethics concerns, and no editors. Others argue that the changes will be less dramatic, e.g., there will be no journals, but only a stream of articles disseminated by larger entities (which is nothing new – Brown et al.[27] already in 1967 prophesied the twilight of scientific journals). As the editors of *Adv Clin Exp Med*, we are dedicated to providing – as long as needed – a platform for open-access publication for the authors and top-quality scientific content covering all fields of medicine for the readers.

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AHEAD OF PRINT

Effect of proton pump inhibitors compared to histamine-2 receptor antagonists on bleeding management and wound healing after endoscopic mucosal resection or endoscopic submucosal dissection: A meta-analysis of randomized clinical trials

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Abstract

Introduction. Proton pump inhibitors (PPIs) and histamine type-2 receptor antagonists (H2RAs) are generally effective in preventing delayed bleeding and healing artificial wounds after endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD). This study aimed to review the therapeutic effects of PPIs and H2RAs on damage caused by EMR and ESD.

Materials and methods. Thirteen articles were collected between 2002 and 2022 by searching Medlib, ScienceDirect, PubMed, International Scientific Indexing (ISI), Embase, and Scopus databases using valid keywords. The main inclusion criteria were delayed wound healing, bleeding, epigastric pain, intraoperative bleeding, and perforation. The odds ratio (OR) and 95% confidence interval (95% CI) were evaluated using a random or fixed effects model. Data analysis was performed using Stata v. 14.2.

Results. A total of 13 articles including 1,483 patients were analyzed. The results showed that delayed bleeding was significantly less frequent in the PPI group than in the H2RA group (OR = 0.6; 95% CI: 0.39–0.92). Subgroup analysis showed that PPI was more effective in preventing delayed bleeding than H2RA for ESD wounds (OR = 0.65; 95% CI: 0.44–1.08). There was no statistically significant difference between both groups regarding the incidence of epigastric pain, intraoperative bleeding, wound healing, and perforation after endoscopic treatments.

Conclusions. The meta-analysis results reveal that PPI is more effective than H2RA in preventing delayed bleeding after endoscopic treatment, particularly in patients treated with ESD. However, there was no significant difference between PPI and H2RA in terms of intraoperative bleeding, epigastric pain, wound healing, and perforation from endoscopic therapy.

Key words: endoscopic mucosal resection, endoscopic submucosal dissection, proton pump inhibitor, histamine H2 receptor antagonist

Introduction

Endoscopic mucosal resection (EMR) is a common treatment for gastric and colonic neoplasms, such as early gastric cancer and adenomas.[1] The procedure involves injecting physiological saline into the submucosa to remove the lesion using a snare device with electrocautery.[2] However, EMR is less effective for block resection of lesions larger than 2 cm.[3]

In late 1990s, endoscopic submucosal dissection (ESD) emerged as a procedure enabling the resection of lesions larger than 2 cm. The procedure involves 3 main steps: 1) injecting fluid into the submucosa to elevate the lesion, 2) cutting the surrounding mucosa of the lesion, and 3) dissecting the submucosa beneath the lesion.[4] Endoscopic submucosal dissection facilitates histological evaluations and minimizes the risk of local recurrence.[5,6] However, it has a higher risk of complications and causes more profound and extensive artificial ulcerations than EMR.[7,8] Both methods carry significant difficulties, including bleeding, perforation and aspiration pneumonitis.[9,10] Postoperative bleeding is the most common complication of ESD, occurring in 5–10% of cases, although this percentage varies in different studies.[11] Ulcer bleeding is more likely to occur after ESD than EMR due to the larger resected area, and delayed bleeding is closely linked to lesion size.[12]

For the management and control of ulcer bleeding, mainly 2 groups of gastric acid secretion inhibitors are administered: proton pump inhibitors (PPIs) and histamine type-2 receptor antagonists (H2RAs). The healing rate of peptic ulcers treated with PPIs is faster than that of patients treated with H2RA because of their more robust antiacid effectiveness. Studies have shown that H2RA activity is substantially quicker and less expensive than PPIs despite having a lower potency.[13,14] Proton pump inhibitors and H2RAs have been compared therapeutically in randomized controlled trials[1,15,16] to treat artificial ulcers following endoscopic treatments. Proton pump inhibitors and H2RAs neutralize pH levels and allow to avoid bleeding following ESD. In several earlier investigations, PPIs were found to be favored over H2RA.[15] However, PPIs, or substituted benzoimidazoles, decrease the generation of acid by blocking the parietal cell hydrogen-potassium adenosine-triphosphatase enzyme system in the gastric mucosa.[16]

Objectives

We performed a systematic review and meta-analysis of randomized trials in this study to examine the therapeutic effects of PPIs and H2RAs for treating iatrogenic stomach ulcers following ESD or EMR.

Methods

Search strategy

The aim of this meta-analysis was to compare the effectiveness of PPIs and H2RAs in controlling bleeding and speeding up wound healing after EMR or ESD. The study was conducted by reviewing literature and electronic databases from 2000 until November 2022. Studies were selected from scientific journals and articles available in PubMed, Medlib, ScienceDirect, International Scientific Indexing (ISI), Scopus, and Embase. The search was conducted using valid keywords, such as "endoscopic mucosal resection," "endoscopic submucosal dissection," "PPIs," and "histamine h2 receptor antagonists". Keywords were standardized in MESH prior to searching. The search strategy, screening and data selection were performed according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Inclusion and exclusion criteria

The meta-analysis inclusion criteria consisted of :1) studies on patients undergoing EMR or ESD treatments, 2) patients who were treated with PPIs and H2RA for endoscopy-induced ulcers, and 3) surveys reporting at least 1 outcome, such as post-endoscopy bleeding, and epigastric pain, and wound healing. The exclusion criteria included: 1) non-randomized and uncontrolled reviews, 2) qualitative and descriptive studies, 3) articles presented at conferences, 4) review articles, systematic reviews and meta-analyses, as well as 5) articles published in language other than English.

Study selection

Using Endnote X8 (Clarivate Plc, London, UK), 2 researchers examined article titles and abstracts and then screened them according to the inclusion and exclusion criteria (Fig. 1). Papers meeting the criteria were further evaluated by reading their full text. In cases of disagreement between the 2 researchers, a 3rd expert made the final judgement. Quality assessment was conducted using the methods recommended in the Cochrane Handbook for Systematic Reviews of Interventions (Fig. 2,3).[17] The articles' bias risk was evaluated by 2 reviewers using 7 criteria: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), and selective reporting (reporting bias). Each criterion was classified as "low risk," "high risk" or "unclear risk."

Data extraction and analysis

All articles were evaluated for their homogeneity. In case of significant heterogeneity, subgroup analysis and

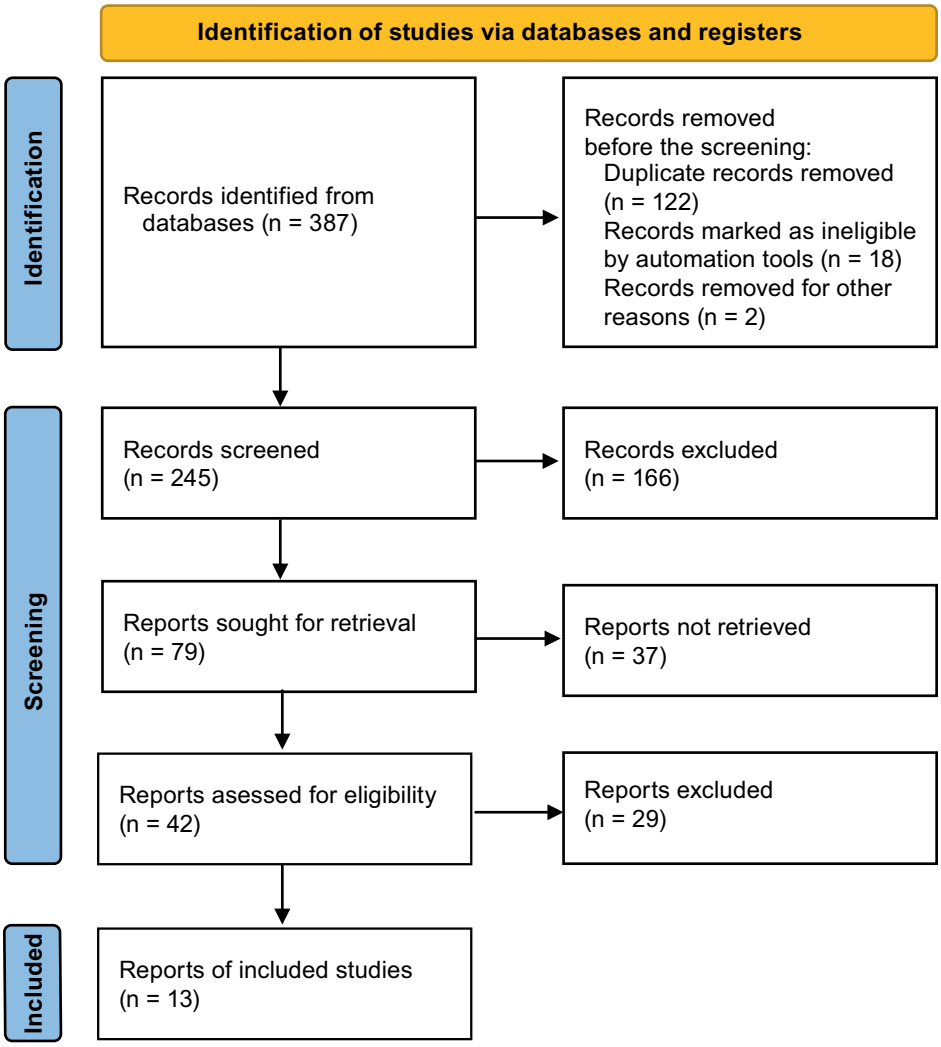

Fig. 1. Study flowchart

meta-regression were performed to examine heterogeneity. The selected papers were thoroughly reviewed, and their information was entered into a form designed and prepared for data extraction. The data was then transferred from Excel 2019 (Microsoft Corp., Redmond, USA), Review Manager v. 5.3 (RevMan 5.3; Cochrane Collaboration, London, UK) and Stata v. 14 software (StataCorp LLC, College Station, USA). The data collected included the names of the authors, year of publication, place of research, number of patients, average age and gender of participants, endoscopic treatment, type and dosage of medication, duration of the drug and follow-up, *Heliobacter pylori*-infection percentage (%), and size and location of the lesion. The main complications observed included delayed bleeding after endoscopy, epigastric pain, perforation, and change in wound size 28 days after endoscopy.

Statistical analyses

The studies were classified according to the number of samples, mean (M) and standard deviation (SD). Each study was evaluated based on its variance. To investigate heterogeneity, we tested the Q test and I^2 index for significance at the error level of less than 5% for α. If the results of the studies were heterogeneous, we analyzed them using meta-analysis (fixed- and random-effects model). Subgroup analyses were performed to consider the duration of drug use (4 and 8 weeks), the type of PPI (omeprazole and rabeprazole), the kind of endoscopy (EMR and ESD), and the use of PPI and H2RA alone or in combination with cytoprotective agents. Publication bias in the included studies of the meta-analysis was assessed using the Beggs and Egger plot. Data analysis was performed using Stata v. 14.

Results

After removing duplicate and unrelated articles, 79 studies were reviewed. The process of selecting the analyses is shown in Fig. 3. Overall, 13 articles were included in the study. Among these articles, 10 were published as full texts and 3 trials as abstracts (with sufficient information) between 2002 and 2021 (Table 1).[1,15,16,18–27] Seven studies were conducted in Japan and 6 studies in South Korea. The study included 1,483 participants from the southern region, with 793 subjects in the PPI group

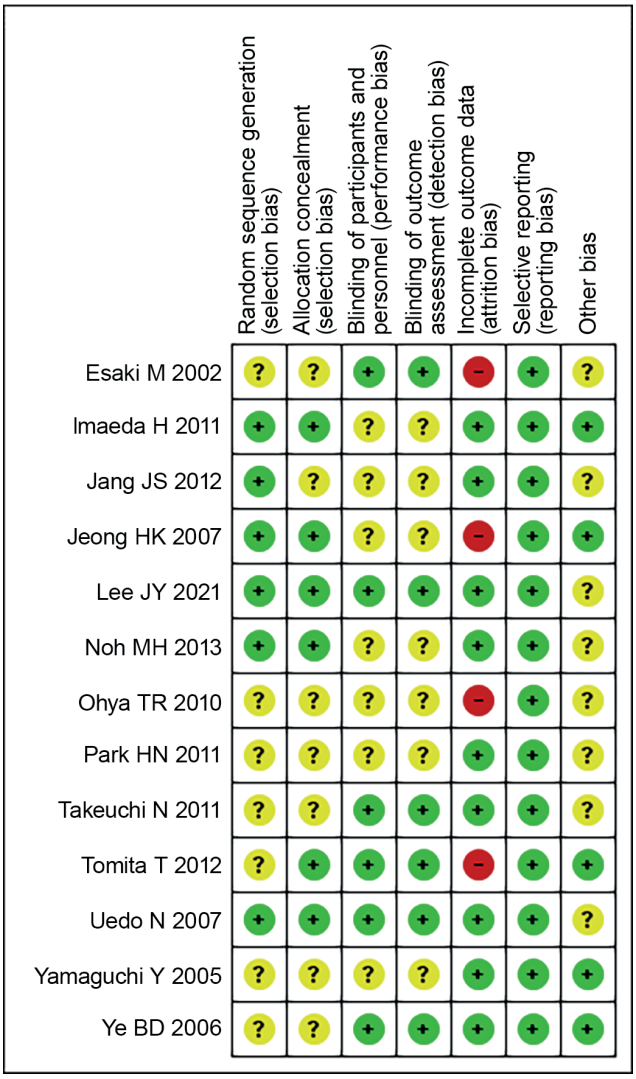

Fig. 2. Risk of bias summary across studies

and 790 in the H2RA group. The average age of the participants was 66.09 years in the PPI group and 66.6 years in the H2RA group. Additionally, the prevalence of *H. pylori* was 0.73% and 0.71% in the PPI and H2RA groups, respectively. The average pH was 6.2 in the PPI group and 5.3 in the H2RA group.

The duration of drug use was 28 days in 8 articles and 56 days in 5 papers. Proton pump inhibitors and H2RA were examined alone in 10 studies and in combination with cytoprotective agents in 3 articles. The funnel plots show the publication bias symmetrically, the p-value was 0.484, showing no possibility of publication bias across studies based on the delayed bleeding data (Fig. 4). Similarly, no possibility of publication bias across studies was shown based on the wound healing data, with a p-value of 0.348 (Fig. 5).

Delayed bleeding

In 11 trials involving 1,407 patients (755 receiving PPI and 752 receiving H2RA), the effect of PPI was compared to that of H2RA on delayed bleeding after endoscopic treatments. Based on the fixed-effects model, in comparison to H2RA, PPI treatment was significantly effective in preventing bleeding after gastric endoscopic treatment (odds ratio (OR) = 0.6; 95% confidence interval (95% CI): 0.4–0.90; p = 0.01; Fig. 6). For sensitivity analysis, excluding the 3 trials that used cytoprotective agents, did not change the results (OR = 0.66; 95% CI: 0.43–1.02; p = 0.04; Table 2). No significant difference was observed between PPI and H2RA in the delayed bleeding prevention subsequently endoscopic treatments with omeprazole (OR = 0.99; 95% CI: 0.41–2.41; p = 0.98) and rabeprazole (OR = 0.53; 95% CI: 0.21–0.23; p = 0.13). In the subgroup analysis with 4-week medication, PPI was pointedly more operative than H2RA in preventing delayed bleeding after endoscopic treatment (OR = 12.9; 95% CI: 5.56–30.26; p = 0.000). The same result was found in the subgroup that received 8-week drugs (OR = 0.53; 95% CI: 0.31–0.91; p = 0.02). Both PPI and H2RA were tested separately (OR = 0.66; 95% CI: 0.43–1.02; p = 0.04) and in combination with cytoprotective agents (OR = 0.31; 95% CI: 0.13–0.95; p = 0.03). The PPI was more efficient than H2RA in preventing bleeding. In the subgroup undergoing EMR, there was no significant difference between PPI and H2RA in preventing bleeding (OR = 0.94; 95% CI: 0.73–1.32; p = 0.56), while in the EDS subgroup, PPI was more effective than H2RA in preventing bleeding after endoscopy (OR = 0.65; 95% CI: 0.44–1.08; p = 0.03).

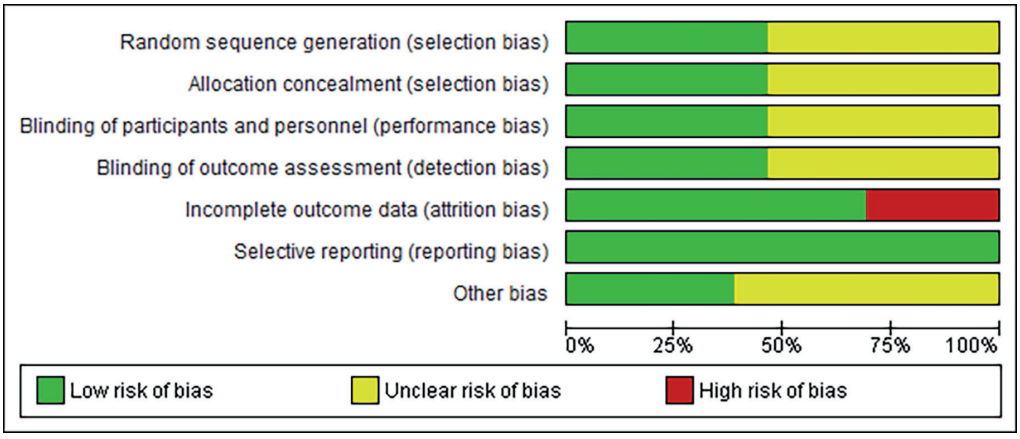

Fig. 3. Details of quality assessment for each included study are represented for each risk of bias item: low risk of bias (green), unclear risk of bias (yellow) and high risk of bias (red)

Table 1. Study characteristics

Authors and reference	Country	Year	Study design	Lesion size [mm]	Percentage of tumor location [upper/ middle/ lower]	Hp positive – city [%]	Percentage of gender [M/F]	Age [years]	Dosage [mg/day]	Treatment (n)	Follow-up time [days]	Medication duration [days]	Endoscopic therapy	Number of participants
Ye et al.[1]	South Korea	2006	prospective randomized controlled trial	11 11	2/32/66 2/27/71	61 56	68/32 59/41	61 59	20 40	omeprazole (41) famotidine (41)	28	28	EMR + ESD	82
Esaki et al.[15]	Japan	2002	prospective randomized controlled trial	18 20	0/12/88 0/37/63	88 63	88/12 63/37	62 70	20 40	omeprazole (8) famotidine (8)	28	28	EMR	16
Uedo et al.[16]	Japan	2007	prospective randomized controlled trial	41 1	0/47/53 0/38/62	81 86	78/22 79/21	68 66	20 800	rabeprazole (73) cimetidine (70)	56	56	ESD	143
Tomita et al.[18]	Japan	2012	prospective randomized controlled trial	43.8 40.3	12/32/33 16/33/30	–	76/24 74/26	70.4 70.6	20 40	omeprazole (77) famotidine (79)	56	56	ESD	156
Ohya et al.[19]	Japan	2010	prospective randomized trial	35 33	12/59/29 4/53/43	77 79	74/26 72/28	65 65	10 20	rabeprazole (31) lafutidine (29)	28	28	ESD	60
Imaeda et al.[20]	Japan	2011	prospective randomized trial	37.7 36.3	0/42/20 0/36/25	61 62	76/24 85/15	68.4 67.6	30 150	lansoprazole (62) roxatidine (61)	56	56	ESD	123
Jeong et al.[21]	South Korea	2007	prospective randomized trial	18 19	0/31/69 0/28/72	62 64	65/35 67/33	63 64	40 40	pantoprazole (85) famotidine (79)	90	56	ESD	164
Lee and Jang[22]	South Korea	2021	prospective randomized controlled trial	28 30	5/4/42 4/5/43	–	60/40 75/25	62.4 70.6	20 + 100 400 +100	rabeprozole + rebamipide (52) cimetidine + rebamipide (52)	28	28	ESD	104
Noh et al.[23]	South Korea	2013	prospective randomized controlled trial	–	–	–	–	–	–	PPI + cytoprotective (92) H2RA + cytoprotective (98)	28	28	ESD	190
Jang et al.[24]	South Korea	2012	prospective randomized controlled trial	–	–	–	–	–	–	PPI + cytoprotective (110) H2RA + cytoprotective (111)	28	28	ESD	121
Yamaguchi et al.[25]	Japan	2005	prospective randomized trial	28 22	3/62/35 0/71/29	83 81	69/31 82/18	72 73	20 40	omeprazole (29) famotidine (28)	60	56	EMR + ESD	57
Takeuchi et al.[26]	Japan	2011	prospective randomized trial	–	14/10/6 12/9/9	76 80	66/34 70/30	68.7 67.4	10 150	rabeprazole (30) roxatidine (30)	56	28	ESD	60
Park et al.[27]	South Korea	2011	prospective randomized controlled trial	–	–	–	–	–	–	PPI (103) H2RA (104)	28	28	ESD	207

PPI – proton pump inhibitors; H2RA – histamine-2-receptor antagonists; EMR – endoscopic mucosal resection; ESD – endoscopic submucosal dissection.

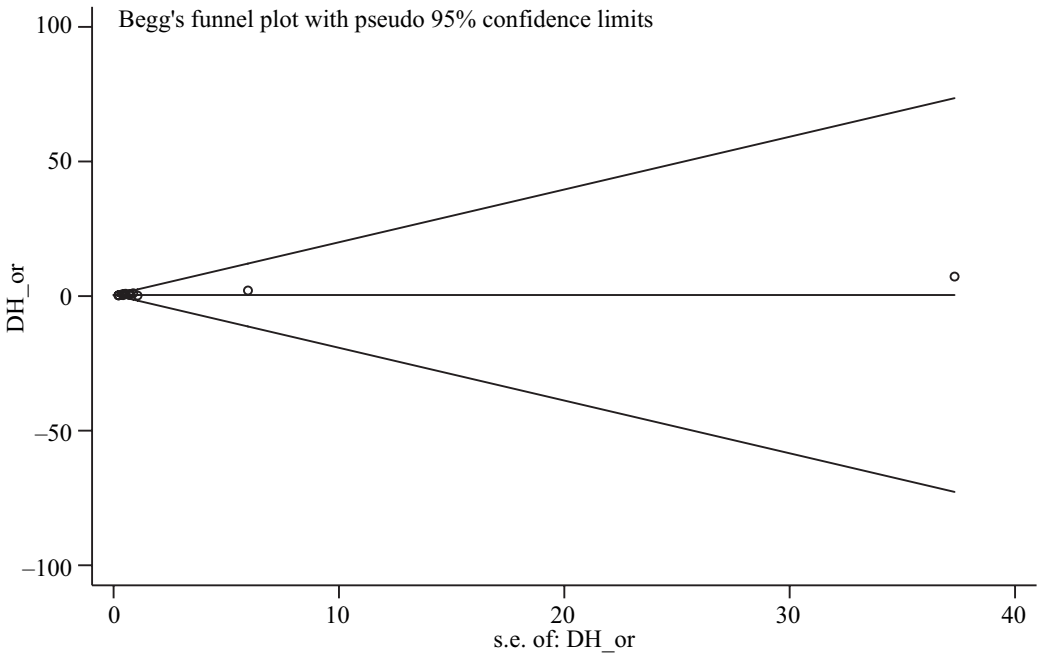

Fig. 4. Publication bias diagram. The circles show the weight of the studies based on the delayed bleeding data (p = 0.484)

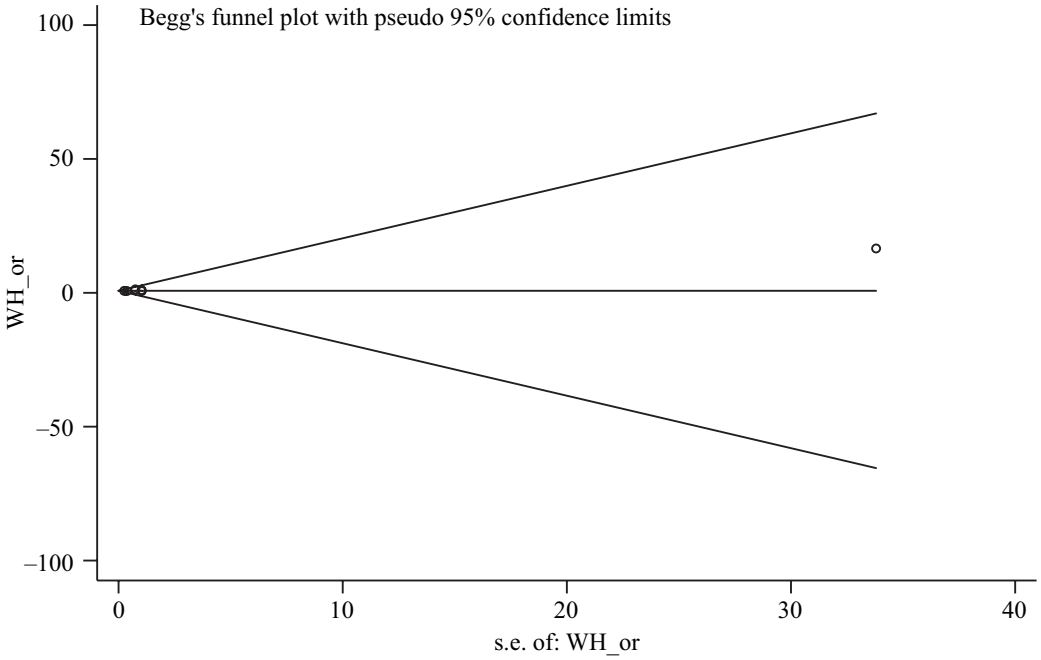

Fig. 5. Publication bias diagram. The circles show the weight of the studies based on the wound healing data (p = 0.348)

Intraoperative bleeding

In 3 trials, intraoperative bleeding was reported in 287 patients receiving PPI and in 281 patients receiving H2RA. No significant difference was detected in intraoperative bleeding in the 2 patient groups (OR = 1.55; 95% CI: 0.93–2.61; p = 0.094).

Wound healing

Wound healing caused by EMR or ESD was investigated in 6 trials. These studies included 668 patients – 335 in the PPI group and 333 in the H2RA group. Based on the random-effects model, there was no significant difference between PPI and H2RA in wound healing after endoscopy (OR = 0.92; 95% CI: 0.6–1.4; p = 0.7; Fig. 7). The sensitivity analysis of all 6 trials, excluding 1 trial that used cytoprotective agents, did not change the results. In addition, no significant difference between PPI and H2RA in wound healing was found between 4-week (OR = 2.1; 95% CI: 0.4–11.02; p = 0.37) and 8-week (OR = 0.74; 95% CI: 1.21–0.45; p = 0.23) medication.

Epigastric pain

Three trials reported epigastric pain after endoscopy in 122 patients receiving PPI and 119 patients receiving H2RA. The results of the trials were combined, and

Table 2. Odds ratio for therapeutic endoscopic outcomes

p-value	Pooled OR (95% CI)	Subgroup	Outcome
0.01	0.6 (0.39–0.92)		delayed bleeding
0.98	0.99 (0.41–2.41)	omeprazole	–
0.13	0.53 (0.23–1.21)	rabeprazole	–
0.00	12.9 (5.56–30.26)	4 weeks	–
0.02	0.53 (0.31–0.91)	8 weeks	–
0.04	0.66 (0.43–1.02)	alone	–
0.03	0.31 (0.13–0.95)	combined	–
0.56	0.94 (0.73–1.32)	EMR	–
0.03	0.65 (0.44–1.08)	ESD	–
0.09	1.55 (0.93–2.61)	–	surgery bleeding
0.2	0.64 (0.30–1.38)	–	epigastric pain
0.8	1.05 (0.71–1.57)	–	wound healing
0.37	2.1 (0.4–11.02)	4 weeks	–
0.23	0.74 (0.45–1.21)	8 weeks	–
0.5	1.5 (0.43–5.26)	–	perforation

OR – odds ratio; 95% CI – 95% confidence interval; PPI – proton pump inhibitors; H2RA – histamine-2-receptor antagonists; EMR – endoscopic mucosal resection; ESD – endoscopic submucosal dissection.

a consolidated OR = 0.64 was obtained. Also, there was no statistical difference between PPI compared to H2RA for treating epigastric pain after EMR or ESD (OR = 0.64; 95% CI: 0.30–1.38; p = 0.25).

Perforation

Four trials involving 119 patients receiving PPI and 290 patients receiving H2RA reported endoscopy-related perforation. The combined results of the trials indicated a consolidated OR = 0.52. Additionally, studies showed no significant difference between PPI and H2RA in treating epigastric pain after endoscopy (OR = 1.5; 95% CI: 0.43–5.26; p = 0.52). The mean change in wound size 28 days after EMR or ESD was 20.3 mm in the PPI group and 20.7 mm in the H2RA group, indicating no significant difference between the 2 groups (p = 0.86). Table 3 shows the frequency of intraoperative bleeding, delayed bleeding, epigastric pain, perforation, and wound healing rate in both the PPI and H2RA groups.

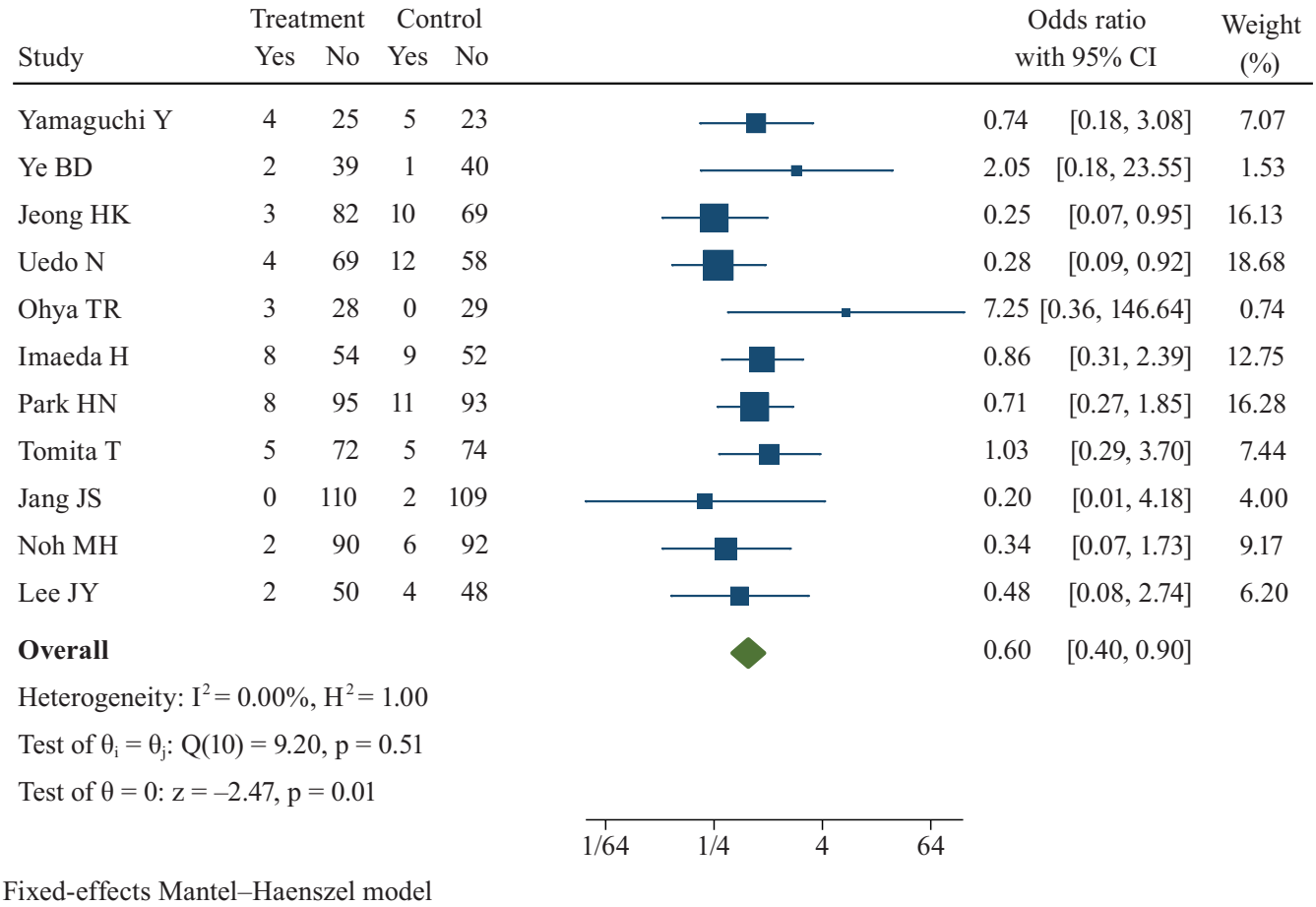

Fig. 6. The odds ratio (OR) for delayed bleeding between PPI and H2RA groups (p = 0.018). Each square shows the effect estimate of individual studies with their 95% confidence interval (95% CI). The size of the courts is proportional to the weight of each study in the meta-analysis. This plot shows studies in the order of publication date and first author's name (based on a fixed-effects model)

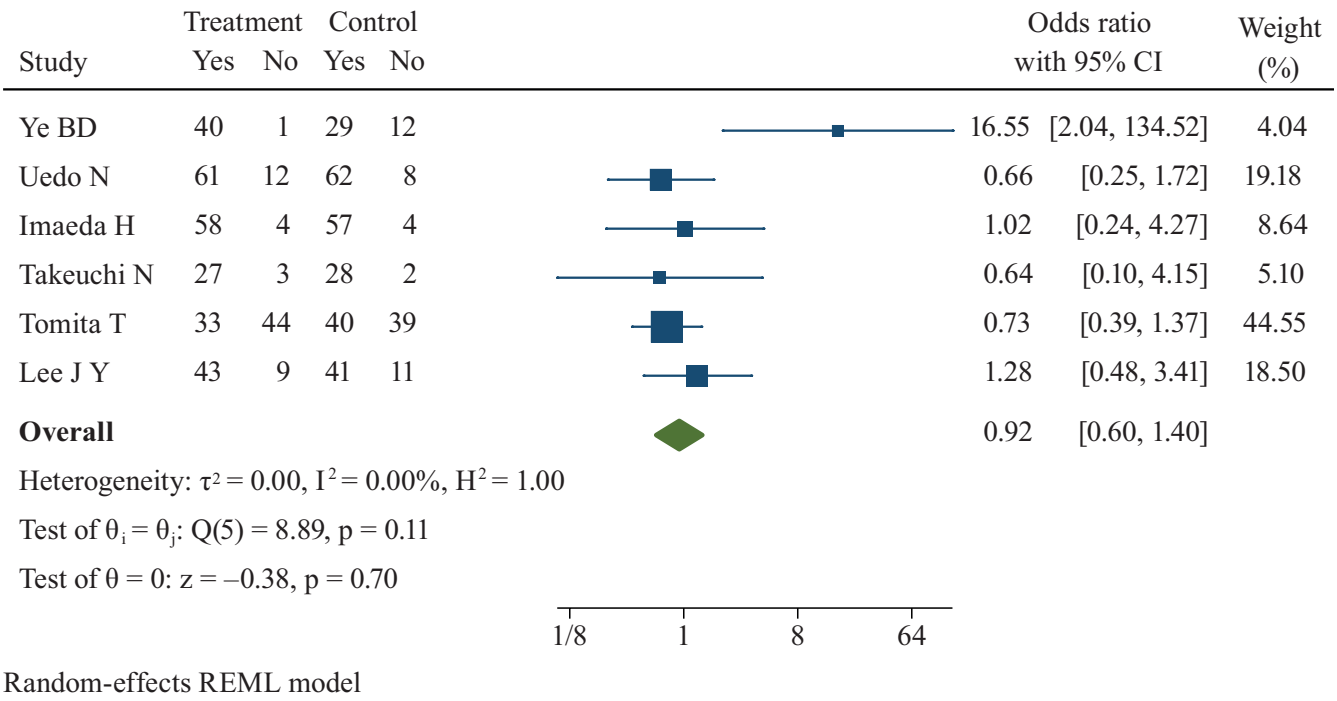

Fig. 7. The odds ratio (OR) for wound healing between PPI and H2RA groups (p = 0.7). Each square shows the effect estimate of individual studies with their 95% confidence interval (95% CI). The size of the courts is proportional to the weight of each study in the meta-analysis. This plot shows studies in the order of publication date and first author's name (based on a random-effects model)

Table 3. The average frequency of intraoperative bleeding, delayed bleeding, epigastric pain, perforation, and wound healing rate in PPI and H2RA groups

Frequency (%)		Outcome
H2RA	PPI	Medication
21	25	surgery bleeding
8.7	6.3	delayed bleeding
29	17	epigastric pain
78	81	wound healing
13	18	perforation

PPI – proton pump inhibitors; H2RA – histamine-2-receptor antagonists.

Discussion

Endoscopic therapy is considered for managing primary gastric malignancies.[18] An artificial pattern of acute gastric ulcer is created through EMR or ESD.[28] Acid-suppressive agents are prescribed to prevent bleeding and induce rapid wound healing caused by EMR or ESD. Since the blood coagulation system is sensitive to pH changes in the stomach, acid-blocking drugs can help stabilize blood clots by maintaining a neutral pH in the stomach, and inhibit frequent bleeding and rapid wound healing.[29,30]

Proton pump inhibitors and H2RA are both acid-inhibiting agents. Even though PPIs are more potent inhibitors of gastric acid secretion than H2RA, it has been reported that H2RAs act significantly faster than PPIs.[1,13] Several studies have compared PPIs with H2RAs in preventing delayed bleeding and wound healing caused by EMR and ESD, and the results were contradictory.[1,15,16,19,20,31] Some studies have reported that treatment with PPIs is superior to that of H2RAs,[1,16] while other investigations have shown no difference between the 2 treatments.

The aim of this study was to elaborate and combine the findings of previous studies and meta-analyses about evaluating the effect of PPI compared to H2RA on bleeding management and wound healing after EMR or ESD. This meta-analysis shows that PPI prevents delayed bleeding in patients after endoscopic treatment better than H2RA, particularly in patients receiving ESD treatment. However, PPI and H2RA were not significantly different from endoscopic therapy in intraoperative bleeding, epigastric pain, wound healing, and perforation. These findings conform with previous meta-analyses in this field.[32,33] Stomach pH affects blood coagulation and the accumulation of platelets at the bleeding site. Additionally, pepsin, which digests blood clots at the wound opening, is active at a pH of less than 5. In laboratory conditions, platelet function is severely impaired at low pH.[34] Therefore, reducing stomach acidity to neutral stabilizes the clotting mechanism and prevents bleeding.[35] Proton pump inhibitors are more effective in raising intragastric pH than H2RAs. It has been reported that intragastric pH was significantly higher in patients who had taken PPI the day before ESD than those who had taken H2RA.[16] This meta-analysis suggests that PPI is more effective than H2RA in preventing delayed bleeding in patients after endoscopic treatment, particularly in those receiving ESD treatment.

In the subgroup analysis of endoscopic treatment, PPI was more effective than H2RA in preventing bleeding only in patients treated with ESD and not those treated with EMR. The success rate of block removal is increased using ESD, a novel technique built on EMR, which enables the removal of larger lesions.[35] However, it causes a significant amount of mucosal detachment, which increases the risk of harm to submucous tissues and the superficial layer of the muscularis propria surrounding blood vessels. As a result, ESD produces an artificial wound that is wider and deeper than EMR.[16] Wound bleeding is more common in ESD than in EMR due to the large resected area because delayed bleeding is highly connected to the lesion size.[18] The findings of this study showed that PPI had a better effect on large gastric ulcers caused by ESD, while it had an equivalent impact on H2RA on small ulcers caused by EMR. This finding is consistent with the previous meta-analysis.[32]

The PPI subgroup analysis showed no discernible difference between the groups that received omeprazole and those that received rabeprazole. According to a meta-analysis by Zhang et al., the type of PPI used to manage upper gastrointestinal bleeding following endoscopy has little to no impact,[33] which is consistent with the results obtained in our study. It has also been highlighted that there is no significant difference between different PPI doses regarding wound healing and bleeding control after ESD.[36] Furthermore, we discovered that PPI prevented bleeding better than H2RA in 4- and 8-week treatments. It has already been shown that the duration of PPI treatment does not affect wound healing and bleeding.[30]

Studies have reported that PPIs are more effective than H2RA in preventing bleeding.[32,37] It has also been suggested that the combined treatment of PPI and cytoprotective agents, including rebamipide, may be effective in iatrogenic wound healing.[32] Prospective randomized trials showed that combined drugs could facilitate the speed of wound healing and improve treatment outcomes.[38–40] On the other hand, some trials have indicated limited or no effect of combined medication on bleeding and wound healing.[22–24] Our study showed that using PPI alone or with cytoprotective agents is more effective in preventing bleeding after ESD than H2RA.

Proton pump inhibitors or H2RAs promote fast healing of artificial gastric ulcers after EMR or ESD. Since PPIs are more potent than H2RAs in increasing intragastric pH, it can be hypothesized that PPIs promote faster wound healing and prevent bleeding episodes more effectively than H2RAs after EMR or ESD.[35] However, many studies have found no difference between PPI and H2RA in wound healing.[15,16,25,32,35] Our meta-analysis also did not show a significant difference between PPI and H2RA in the healing of gastric ulcers after EMR and ESD.

No severe side effects were reported in the included studies. It is crucial to evaluate both acute and chronic side effects of PPIs and H2RAs, as treatment duration may range from 3 days to several weeks. The findings indicated that the adverse effects of these 2 medication classes are minor and transient.[33] The study suggests that the adverse effects of PPIs and H2RAs are minor and transient, with a frequency of 1–3%.[41,42] These medications frequently cause nausea, vomiting, headaches, constipation, flatulence, diarrhea, stomach discomfort, and dizziness.[43] These are just a few of the common side effects. The most severe side effect following ESD or EMR, which was not statistically different in the 2 groups, was epigastric discomfort in the trials examined in this meta-analysis. This conclusion aligns with those of earlier studies.[16,32] According to studies by Esaki et al. and Uedo et al., lesion size and tumor site are associated with bleeding and wound healing.[15,16] However, it required much effort to categorize patients in the current meta-analysis based on these variables. Although *H. pylori* infection is a known contributor to the pathophysiology,[16,44,45] it has no influence on wound healing or bleeding following ESD.

Limitations

This meta-analysis has some limitations. First, there was no information on delayed bleeding, epigastric pain or wound healing in any of the included investigations. In addition, there may have been slight differences in assessing delayed bleeding and the wound healing process, although each study clearly defined outcomes. Second, different drugs were used in the studies included. Other types of PPI and H2RA may have introduced some bias. Third, all included trials were from Japan and South Korea, and no relevant data were published from Western countries. Additionally, it is important to consider the potential for publication bias, as the number of papers included and the variations in sample size may have an influence on the results.

Conclusions

According to this meta-analysis, PPIs are more effective than H2RAs in preventing delayed bleeding in patients undergoing endoscopic therapy, particularly those receiving ESD. The effects of endoscopic treatment on intraoperative bleeding, epigastric discomfort, wound healing, and perforation did not substantially differ between PPI and H2RA. However, more reliable evidence for PPI or H2RA treatment using various administration techniques in other regions worldwide must be obtained, particularly through large-scale randomized controlled studies.

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Effects of physical activity and sedentary behavior on serum vitamin D in patients with chronic obstructive pulmonary disease

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Abstract

Background. Chronic obstructive pulmonary disease (COPD) is a complex, heterogeneous disease with multiple extrapulmonary manifestations, among which vitamin D deficiency and insufficiency are very common in COPD and are associated with the health status and clinical outcomes of COPD patients.

Objectives. This paper aims to analyze the impact of leisure-time physical activity (LTPA) and daily sitting time (DST) and their interactions on serum vitamin D in patients with COPD.

Materials and methods. Participants aged ≥40 years from the National Health and Nutrition Examination Survey (NHANES) in the USA from 2007 to 2012 who had undergone pulmonary function tests and vitamin D tests were selected as the study participants. Participants' LTPA and DST were assessed using the General Practice Assessment Questionnaire (GPAQ). Multivariate logistic regression analysis was used to analyze the relationship between serum vitamin D, LTPA, DSA and the combination of the 2 in patients with COPD, and the results were expressed as odds ratio (OR) and 95% confidence interval (95% CI).

Results. This study included 1,448 samples. The mean vitamin D concentration of the samples was (68.27 ±26.78) nmol/L; 360 participants (24.86%) had vitamin D deficiency and 539 participants (37.22%) had vitamin D insufficiency. Vitamin D and 25(OH)D3 expression levels differed across the 4 groups (150 min/week and DST > 8 h revealed the highest vitamin D expression levels, while LTPA 8 h showed the lowest. Vitamin D was weakly correlated with FEV_1, FVC, BMI, age, and LTPA ($p < 0.01$), but not with DST. Body mass index (BMI) was weakly positively correlated with DST ($r = 0.142$, $p < 0.01$).

Conclusions. Serum physical activity and DST independently affect vitamin D levels in COPD patients; therefore, increasing physical activity and minimizing DST may help improve vitamin D levels and prevent vitamin D deficiency.

Key words: physical activity, sedentary behavior, vitamin D, chronic obstructive pulmonary disease

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Background

Chronic obstructive pulmonary disease (COPD) is a clinical syndrome characterized by chronic respiratory symptoms, abnormal lung structure and impaired lung function.[1] It is a prevalent condition globally and is associated with significant morbidity and mortality. Despite its high incidence, COPD is frequently underdiagnosed, resulting in delayed recognition until the disease has reached an advanced stage. Over the last 2 decades, the treatment options for COPD have expanded considerably, including new oral and inhaled medications and innovative surgical and bronchoscopic procedures.[2] External environmental factors (such as smoking, exposure to harmful particles, lower respiratory tract infection, and occupational exposure) and internal factors (such as genetic variation, epigenetic factors and population aging) lead to multiple mechanisms involved in the pathogenesis of COPD, which include oxidative stress, protease and antiprotease imbalance, immune inflammation, apoptosis, as well as systemic and lung damage and repair.[3,4] Owing to the initial oxidative and inflammatory events described above, COPD is linked to several changes in the respiratory airways, in particular the remodeling of alveolar and airways epithelium, mucoid plug formation, increased density of inflammatory cells, smooth muscle hyperplasia, and fibrosis.[5] These changes are considered to be the part of the tissue regeneration and repair processes that further increase mucus production and lead to emphysematous destruction of the gas-exchanging surface of the lung.[6] These airway changes manifest as clinical symptoms, including dyspnea and a persistent cough, with or without sputum production. Usually, spirometry is recommended as the subsequent diagnostic test, or a postbronchodilator FEV_1/FVC ratio of less than 0.70 is used for further confirmation.[7]

While COPD is curable and the cure varies from case to case, the dietary and lifestyle changes are also considered indispensable for a quick recovery. While there is a lack of definitive data, the existing scientific evidence suggests that certain foods and nutrients, particularly those with antioxidant and anti-inflammatory properties, as well as when consumed in a balanced dietary pattern, are linked to improved pulmonary function, slower decline in lung function and a decreased risk of COPD. Understanding the impact of diet on COPD can equip healthcare professionals with an evidence-based framework to provide patients with valuable guidance to improve their respiratory wellbeing.[8] It is also worth noting that nutritional deficiencies need to be assessed and replenished to achieve an effective treatment outcome either via dietary or pharmacological means.

Among several nutritional deficiencies, vitamin D has been extensively reported. Vitamin D deficiency and insufficiency are very common and are associated with the health status and clinical outcomes of patients with COPD.[9,10] Moreover, vitamin D is a potential modality for the treatment of COPD due to its anti-inflammatory and antioxidant properties, as well as its ability to modulate the immune response.[11,12] Although some recent studies suggest that vitamin D supplementation may benefit patients with COPD, the results are inconsistent[13,14] and the best way to prevent abnormal vitamin D metabolism in patients with COPD has not been determined.

Vitamin D plays a significant role in preventing inflammation and modulating molecular signaling pathways associated with tissue remodeling.[15] Inflammation is a complex immune response involving the activation of various signaling molecules and cellular processes. Vitamin D has been shown to have anti-inflammatory properties by modulating the expression and function of inflammatory mediators, such as cytokines and chemokines.[16,17] It can inhibit the production of pro-inflammatory cytokines while promoting the synthesis of anti-inflammatory cytokines, thereby helping to maintain a balanced immune response and dampening excessive inflammation. Moreover, vitamin D has been shown to influence molecular signaling pathways involved in tissue remodeling. Tissue remodeling refers to the process of structural changes in tissues that occur in response to injury, repair or chronic diseases. Vitamin D can regulate the expression of genes involved in extracellular matrix synthesis and degradation and cell proliferation and differentiation. It can also influence the activity of enzymes and signaling molecules implicated in tissue remodeling processes, such as matrix metalloproteinases (MMPs) and transforming growth factor-beta (TGF-β)[18] It has also been found to be effective in various lung inflammatory diseases.[19] By exerting anti-inflammatory effects and modulating molecular signaling related to tissue remodeling, vitamin D helps maintain tissue homeostasis and prevent excessive tissue damage. However, it is important to note that the specific mechanisms and effects of vitamin D in these processes are still an area of active research, and further studies are needed to fully understand its role in preventing inflammation and tissue remodeling.

The connection between physical activity, sedentary behavior and serum vitamin D levels in patients with chronic obstructive pulmonary disease (COPD) lies in the impact of lifestyle factors on vitamin D synthesis. Inactivity and prolonged sedentary behavior can contribute to reduced exposure to sunlight, which is essential for the body's production of vitamin D. This deficiency in physical activity and excessive sedentary time may consequently lead to lower serum vitamin D levels in COPD patients.[20,21] Recent studies have shown that physical activity in patients with COPD is affected by a variety of extrapulmonary factors (such as skeletal muscle dysfunction, depressive symptoms and nutritional status).[22,23] Regular exercise training and rehabilitation can alleviate the above extrapulmonary complications syndrome[24]; in the general population, serum vitamin D is related to physical activity, and adequate physical activity is a protective factor for maintaining normal vitamin D levels.[25,26] However, there is limited epidemiological

evidence on the effects of physical activity and sedentary behavior on serum vitamin D in patients with COPD. Therefore, there is an ongoing scientific effort to explore the role of vitamin D in COPD and reduce the disease burden.

Objectives

This study aims to analyze the impact of leisure-time physical activity (LTPA) and daily sitting time (DST), as well as their interactions, on serum vitamin D in patients with COPD. Additionally, the study suggests ways of preventing abnormal vitamin D metabolism in patients with COPD. The aim of this study is to propose the inclusion of vitamin D status in the diagnosis and treatment of COPD, and to pave the way for further research on need of vitamin D interventions and the development of physical activity standards for routine COPD treatment.

Materials and methods

Study design and setting

This study involves 3 cycles of data retrieved from the National Health and Nutrition Examination Survey (NHANES) conducted between 2007 to 2012 and published by the Centers for Disease Control and Prevention (CDC).

Participants

Among the 48,407 samples in 3 cycles, this study included participants older than 40 years and whose lung function met the diagnostic criteria for COPD,[17] while excluding those lacking data on vitamin D, physical activity time and DST. All NHANES protocols were approved by the National Center for Health Statistics Ethics Review Board. Written informed consent was obtained from all participants. The datasets used contained no personally identifiable information, so they were not subject to ethical review. Demographic data, spirometry, disease information, dietary data, laboratory and questionnaire data associated with disease definitions were collected and merged from different NHANES files.

Variables

According to the current studies, the following variables were chosen as confounding factors measured in the baseline survey. Demographic characteristics included age (40–60, >60), sex (male or female), race/ethnicity (Mexican American, other Hispanic, non-Hispanic White, non-Hispanic Black, and other race), education level (high school or less, some college and college graduate or higher), family poverty–income ratio (total family income divided by poverty line: <1.3, from 1.3 to <3.5 or ≥3.5); body mass index (BMI; calculated as weight [kg] divided by height [m^2] which was separated into 3 groups BMI <25, BMI range from 25.0 to 29.9 or BMI ≥30); lung function test[27]; smoking status (never, former, current)[28]; comorbidities included asthma, congestive heart failure, coronary heart disease, diabetes, cancer, and arthritis. Vitamin D intake was assessed based on the intake of vitamin D2 + D3 in the previous month. Then, LTPA (none, from 0 to <150 or ≥150) and DST (<4, 4 to 6, from 6 to 8 or >8) were chosen as categorical variables.

Experimental procedure

Recreational physical activity time, sedentary time and vitamin D determination

The Global Practice Assessment Questionnaire (GPAQ) was used to assess participants' LTPA and DST. The GPAQ is a validated questionnaire developed by the World Health Organization (WHO) to monitor physical activity. This questionnaire was applied in more than 100 countries worldwide via the WHO step-by-step surveillance approach. This questionnaire was used to gather information on daily physical activity, LTPA and sedentary behavior.[29] When answering the questionnaire, participants were asked to report their moderate- and high-intensity recreational activities in a week. Leisure-time physical activity is defined as the total time of conducting moderate-intensity recreational activity (min) plus twice the total time of conducting the vigorous-intensity recreational activity [min]. Based on the 2018 Physical Activity Guidelines for Americans, participants were classified as inactive (not participating in any physical activity), insufficiently active (more than 0 min but less than 150 min per week) and adequately active (more than 150 min active). Participants answered the question: "On a typical day, how much time do you usually spend sitting at school, at home, getting to and from places, or with friends, including time spent sitting at a desk, traveling in a car or bus, reading, playing cards, watching television, or using a computer?" to assess the participants' sedentary time. Study participants were divided into 4 groups based on their DST (h/day) (0 to <4, from 4 to <6, from 6 to 8, and ≥8 h/day).[30,31] Serum 25(OH)D2 and 25(OH)D3 concentrations were determined using standard liquid chromatography-tandem mass spectrometry (LC-MS/MS), following CDC recommendations. Participants' blood was drawn by qualified medical practitioners. Measurement of serum 25(OH)D levels was expressed as nmol/L.[32]

Statistical analyses

According to the vitamin D expression level defined by the relevant consensus, the enrolled patients were divided into vitamin D deficiency, vitamin D insufficiency and vitamin D sufficiency.[33] Vitamin D, 25(OH)3 and 25(OH)2 expression levels, vitamin D intake, age, BMI,

forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), FEV_1/FVC, LTPA, and DST were used as continuous variables with mean ± standard deviation (M ±SD). In this study, we employed a rigorous statistical approach to assess the relationship between vitamin D expression and 2 key lifestyle factors: LTPA and DST. To ensure the robustness of our analysis, we conducted several preliminary tests to examine the underlying assumptions of our chosen statistical methods.

We began by conducting tests of normality and homogeneity of variance on our data, as presented in Supplementary Table 1. The Kruskal-Wallis H test was utilized to assess variations in vitamin D expression based on 2 primary lifestyle factors: LTPA and DST.

Among the nonparametric tests, we used Spearman's rank correlation coefficient to obtain a correlation between vitamin D and its other dependencies. Vitamin D intake number, age (from 40 to 60, >60), BMI (<25, from 25 to 30 and ≥30), ethnicity, family poverty–income index, education level, smoking history, complications, and LTPA (none, from 0 to <150 and ≥150) and DST (<4, 4 to 6, from 6 to 8, and >8) as categorical variables. They were expressed as absolute numbers and constituent ratios, and the differences between groups were compared using χ^2 test. Logistic regression of the joint association of DST and with vitamin D was performed using the Wald χ^2 test. Furthermore, we also performed the calculation of odds ratios (ORs) based on control and treatment regimens using MedCalc v. 20.106 (MedCalc Software Ltd., Ostend, Belgium).

Spearman's correlation analysis was used to explore the correlation among vitamin D, FEV_1, FVC, BMI, age, DST, and LTPA. Multivariate logistic regression analysis was used to study the relationship between vitamin D and LTPA, DSA, and the combination of the two. Results were expressed as OR and 95% confidence interval (95% CI). In Model 1, there was no adjustment of confounding factors; in Model 2, age, sex, and race were adjusted as confounding factors; in Model 3, age, sex, race, BMI, family poverty–income index, education level, and smoking history were adjusted as confounding factors; in Model 4, age, sex, race, BMI, household poverty–income index, education level, smoking history, vitamin D intake, comorbidities, and FEV_1 were adjusted as confounding factors; in Model 5, age, sex, race, BMI, household poverty–income index, education level, smoking history, vitamin D intake, comorbidities, and FVC were adjusted for confounding factors. Data analysis was performed using IBM SPSS 26.0 (IBM Inc., Armonk, USA). A p-value <0.05 represented a statistical difference. GraphPad Prism 8 (GraphPad Software, San Diego, USA) was employed to generate comprehensive visualizations, including scatter plots and box plots (Fig. 2–10).

Results

This study included 1,448 samples for analysis (Fig. 1). Table 1 shows selected demographics and potential confounders according to vitamin D level.

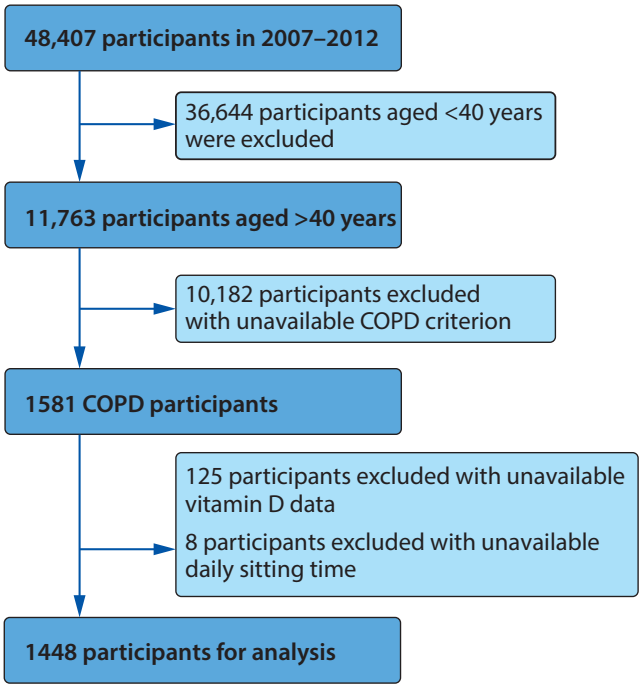

Fig. 1. Flow diagram of the overall procedure. A total of 1,448 samples were included for analysis

COPD – chronic obstructive pulmonary disease.

Table 1. Demographic and clinical characteristics of patients

Characteristic	All (1,448)	Vitamin D deficiency (360)	Vitamin D insufficiency (539)	Vitamin D sufficiency (549)	Statistical value	p-value
Vitamin D [nmol/L], mean ±SD	68.27 ±26.78	35.48 ±9.20	63.00 ±7.07	94.96 ±18.49	1271.28 (K–W)	0.000
25(OH)D3 [nmol/L], mean ±SD	63.65 ±26.30	33.28 ±9.37	59.15 ±9.53	87.98 ±21.47	1087.83 (K–W)	0.000
25(OH)D2 [nmol/L], mean ±SD	4.62 ±11.71	2.19 ±3.03	3.84 ±6.67	6.96 ±17.38	43.47 (K–W)	0.000
Number of patients with vitamin D intake (%)	343 (23.7)	21 (1.5)	115 (7.9)	207 (14.3)	124.81 (χ^2)	0.000
Vitamin D intake [μg], mean ±SD	21.49 ±134.56	12.38 ±5.62	33.91 ±232.06	15.51 ±11.11	12.81 (K–W)	0.002
Age [years], mean ±SD	62.06 ±10.35	60.93 ±9.87	61.68 ±10.71	63.19 ±10.20	12.72 (K–W)	0.002
40–60, n (%)	598 (41.3)	164 (11.3)	233 (16.1)	201 (13.9)	8.49 (χ^2)	0.014
>60, n (%)	850 (58.7)	196 (13.5)	306 (21.1)	348 (24.0)		

Table 1. Demographic and clinical characteristics of patients – cont.

Characteristic		All (1,448)	Vitamin D deficiency (360)	Vitamin D insufficiency (539)	Vitamin D sufficiency (549)	Statistical value	p-value
Sex, n (%)	men	913 (63.1)	222 (15.3)	365 (25.2)	326 (22.5)	8.51 (χ^2)	0.014
	women	535 (36.9)	138 (9.5)	174 (12.0)	223 (15.4)		
BMI [kg/m^2], mean ±SD		27.85 ±5.97	28.40 ±7.04	28.26 ±5.99	27.08 ±5.06	10.74 (K–W)	0.005
<25, n (%)		502 (34.7)	129 (8.9)	163 (11.3)	210 (14.5)	20.72 (χ^2)	<0.001
25–30, n (%)		522 (36.1)	105 (7.3)	211 (14.6)	206 (14.2)		
≥30, n (%)		422 (29.2)	126 (8.7)	163 (11.3)	133 (9.2)		
Race, n (%)							
Mexican American		105 (7.3)	34 (2.3)	50 (3.5)	21 (1.5)	148.79 (χ^2)	<0.001
Other Hispanic		23 (1.6)	41 (2.8)	36 (2.5)	100 (6.9)		
Non-Hispanic White		887 (61.3)	148 (10.2)	326 (22.5)	413 (28.5)		
Non-Hispanic Black		277 (19.1)	134 (9.3)	94 (6.5)	49 (3.4)		
Other		79 (5.5)	21 (1.5)	28 (1.9)	30 (2.1)		
Family poverty–income ratio*, n (%)							
<1.3		433 (33.7)	139 (10.8)	152 (11.8)	143 (11.1)	28.33 (χ^2)	<0.001
from 1.3 to <3.5		502 (39.1)	116 (9.0)	200 (15.6)	186 (14.5)		
≥3.5		350 (27.2)	59 (4.6)	133 (10.4)	158 (12.3)		
Educational attainment*, n (%)							
High school or less		793 (54.8)	219 (15.1)	302 (20.9)	272 (18.8)	18.82 (χ^2)	0.001
Some college		366 (25.3)	90 (6.2)	137 (9.5)	139 (9.6)		
College graduate or higher		288 (19.9)	51 (3.5)	100 (6.9)	137 (9.5)		
Smoking, n (%)							
Never		401 (27.7)	82 (5.7)	157 (10.8)	162 (11.2)	35.59 (χ^2)	<0.001
Former		548 (37.8)	109 (7.5)	207 (14.3)	232 (16.0)		
Current		400 (34.5)	169 (11.7)	175 (12.1)	155 (10.7)		
Comorbidities, n (%)							
Asthma		276 (19.1)	79 (5.5)	104 (7.2)	93 (6.4)	3.56 (χ^2)	0.169
Congestive heart failure*		69 (4.8)	19 (1.3)	22 (1.5)	28 (2.0)	0.92 (χ^2)	0.631
Coronary heart disease*		105 (7.3)	25 (1.7)	47 (3.3)	33 (2.3)	3.04 (χ^2)	0.218
Diabetes		229 (15.8)	72 (5.0)	80 (5.5)	77 (5.3)	7.32 (χ^2)	0.120
Cancer		244 (16.9)	52 (3.6)	80 (5.5)	112 (7.7)	7.92 (χ^2)	0.019
Arthritis		603 (41.6)	135 (9.3)	215 (14.8)	253 (17.5)	7.68 (χ^2)	0.021
FEV1 and FVC, mean ±SD							
FEV_1(L)		2.35 ±0.79	2.17 ±0.74	2.40 ±0.81	2.43 ±0.77	22.32 (K–W)	0.000
FVC(L)		3.72 ±1.12	3.48 ±1.06	3.79 ±1.14	3.80 ±1.11	18.58 (K–W)	0.000
FEV_1/FVC		0.63 ±0.07	0.62 ±0.08	0.63 ±0.07	0.64 ±0.07	10.18 (K–W)	0.006
LTPA [min/wk], mean ±SD		137.93 ±299.61	94.63 ±286.90	136.07 ±299.85	168.16 ±304.43	32.93 (K–W)	0.000
None (inactive), n (%)		859 (59.3)	251 (17.3)	325 (22.4)	283 (19.5)	34.78 (χ^2)	0.039
From 0 to <150 (insufficiently active), n (%)		231 (16.0)	49 (3.4)	90 (6.2)	92 (6.4)		
≥150 (active), n (%)		358 (24.7)	60 (4.1)	124 (8.6)	174 (12.0)		
DST [h], mean ±SD		5.45 ±3.29	5.84 ±3.45	5.37 ±3.39	5.27 ±3.07	6.47 (K–W)	0.023
<4, n (%)		700 (48.3)	154 (10.6)	270 (18.6)	276 (19.1)	10.252 (χ^2)	0.114
4–6, n (%)		317 (21.9)	82 (5.7)	106 (7.3)	129 (8.9)		
6–8, n (%)		204 (14.1)	57 (3.9)	77 (5.3)	70 (4.8)		
>8, n (%)		227 (15.7)	67 (4.6)	86 (5.9)	74 (5.1)		

BMI – body mass index; K–W – Kruskal–Wallis H test; FEV_1 – forced expiratory volume in 1 second; FVC – forced vital capacity; SD – standard deviation; LTPA – leisure-time physical activity; DST – daily sitting time; min/wk – minutes per week; *there was some missing data in these baseline characteristics.

Table 2. The expression of vitamin D according to LTPA

The expression of vitamin D	LTPA			Statistical value	p-value (Kruskal–Wallis H test)
	None (inactive)	from 0 to <150 (insufficiently active)	≥150 (active)		
Vitamin D [nmol/L]	65.11 ±27.02	69.23 ±24.99	74.93 ±26.07	39.51	<0.001
25(OH)D2 [nmol/L]	4.39 ±11.80	5.03 ±12.19	4.89 ±11.16	13.43	0.001
25(OH)D3 [nmol/L]	60.71 ±26.13	64.67 ±24.52	70.05 ±26.70	33.58	<0.001

SD – standard deviation; LTPA – leisure-time physical activity. Values are presented as mean ±SD.

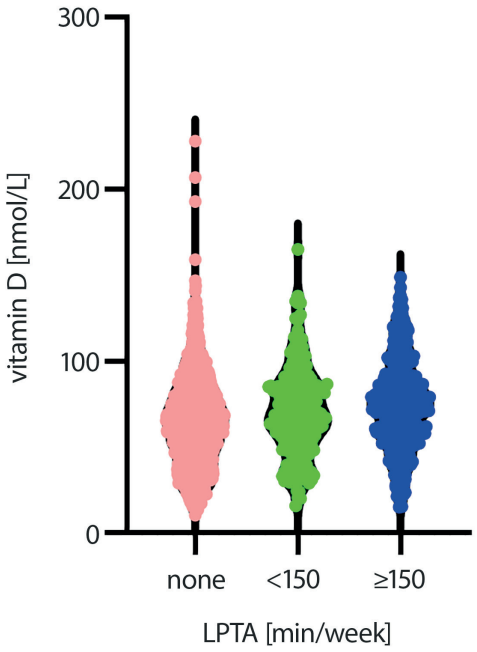

Fig. 2. The expression of vitamin D in different leisure-time physical activity (LTPA) levels

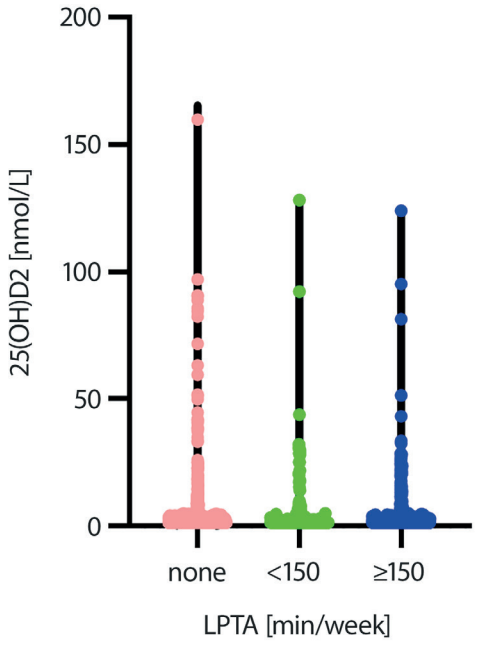

Fig. 3. The expression of 25(OH)D2 in different leisure-time physical activity (LTPA) levels

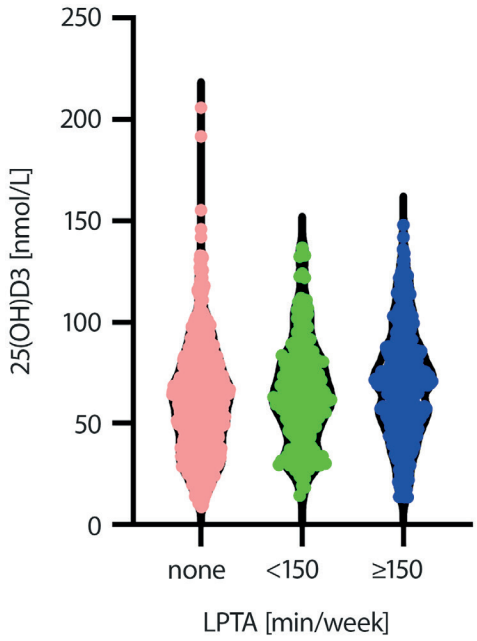

Fig. 4. The expression of 25(OH)D3 in different leisure-time physical activity (LTPA) levels

The average concentration of vitamin D in the samples was 68.27 ±26.78 nmol/L. Of the 1,448 participants, 360 (24.86%) had vitamin D deficiency, with an average concentration of (35.48 ±9.20) nmol/L, while 539 participants (37.22%) were insufficient in vitamin D, with an average concentration of (63.00 ±7.07) nmol/L. Participants with vitamin D deficiency were younger, more obese, smokers, had poorer lung function, and had lower education levels and household income. Additionally, these participants were mainly non-Hispanic White men, who were less likely to take vitamin D supplements, and the probability of combining tumors and rheumatic diseases was low. Only 4.1% of the patients in the vitamin D deficiency group conducted sufficient LTPA (≥150 min/week), while 17.3% of the participants did not carry out any LTPA, and their activity time was significantly shorter than that of vitamin D insufficient and vitamin D sufficient participants, with a statistical difference ($p < 0.001$). The Kruskal–Wallis H test was utilized to assess variations in vitamin D expression based on 2 primary lifestyle factors: LTPA and DST. Compared to the other 2 groups of participants, the vitamin D deficiency group had a longer sedentary time ($p < 0.05$), and 4.6% of the participants exceeded 8 h of sedentary

Table 3. The expression of vitamin D according with the level of daily sitting time

The expression of vitamin D	Daily sitting time [h]				Statistical value	p-value (Kruskal–Wallis H test)
	<4	4–6	6–8	>8		
Vitamin D [nmol/L]	68.80 ±25.92	69.78 ±26.20	67.51 ±29.19	65.23 ±27.83	6.18	0.103
25(OH)D2 [nmol/L]	4.15 ±9.23	4.37 ±9.92	5.82 ±16.67	5.33 ±15.00	0.07	0.995
25(OH)D3 [nmol/L]	64.66 ±25.89	65.39 ±26.53	61.69 ±28.58	59.89 ±24.76	8.58	0.036

SD – standard deviation. Values are presented as mean ±SD.

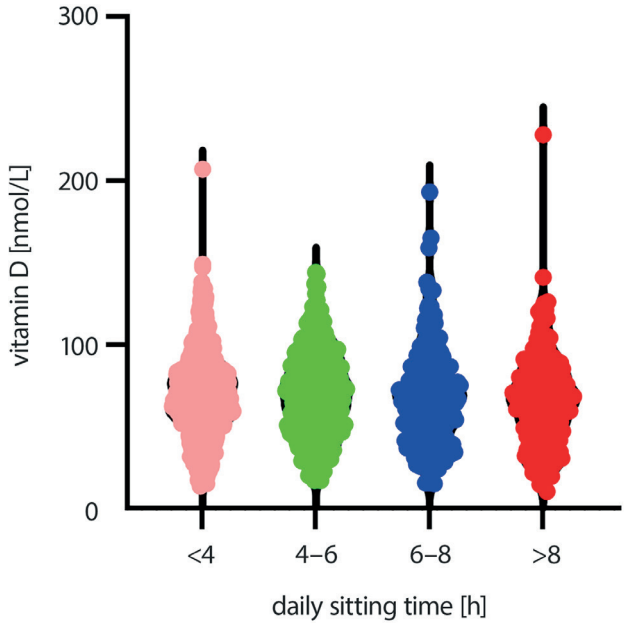

Fig. 5. The expression of vitamin D in different daily sitting time (DST) levels

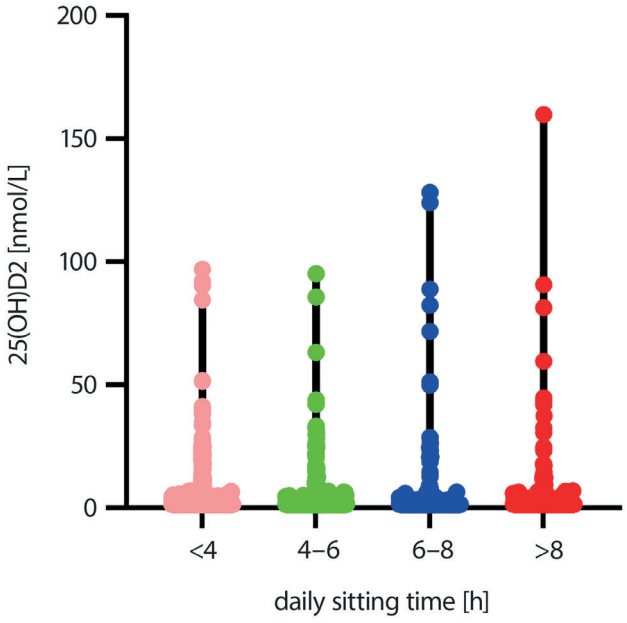

Fig. 6. The expression of 25(OH)D2 in different daily sitting time (DST) levels

time, with no statistical difference (t = 0.191, p = 0.848) found. The expression levels of vitamin D, 25(OH)D2 and 25(OH)D3 varied between different physical activity

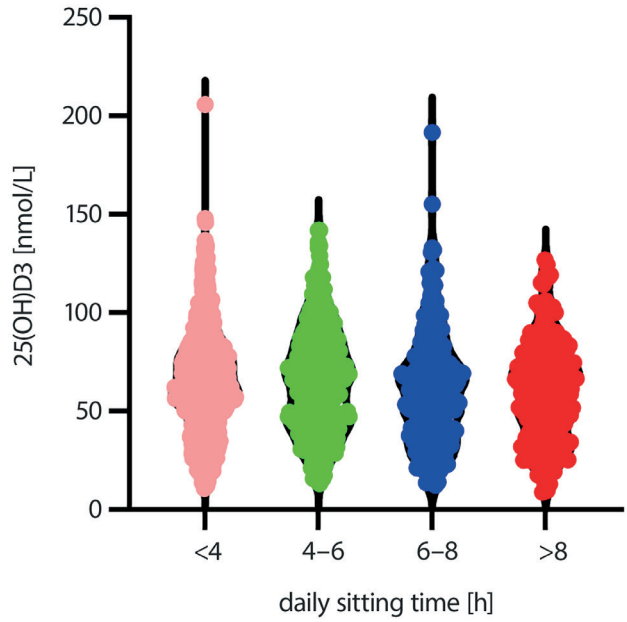

Fig. 7. The expression of 25(OH)D3 in different daily sitting time (DST) levels

times ($p < 0.05$): the expression level of vitamin D was the highest in the participants with LTPA ≥150 min/week, and the lowest in those who did not conduct any physical activity (Table 2, Fig. 2–4); between different sedentary times, only the expression level of 25(OH)D3 showed statistical difference ($p < 0.05$), and there was no statistical difference in the vitamin D and 25(OH)D2 expressions ($p > 0.05$) (Table 3, Fig. 5–7). The combined analysis of physical activity time and sedentary time found that the expression levels of vitamin D 25(OH)D2 and 25(OH)D3 were different among the 4 groups ($p < 0.05$). The participants with LTPA ≥150 min/week and sedentary time >8 h showed the highest vitamin D expression level, while the participants with LTPA <150 min/week and sedentary time >8 h had the lowest vitamin D expression level (Table 4, Fig. 8–10).

Table 5 showed the correlations among variables. Vitamin D had a weak correlation with FEV_1, FVC, BMI, age, and LTPA ($p < 0.01$), but had no correlation with sedentary time; BMI and sedentary time were weakly positively correlated ($r = 0.142$; $p < 0.01$); and LTPA was weakly positively correlated with lung function parameters (FEV_1, FVC; $p < 0.01$).

To further analyze the independent effects of physical activity and sedentary time, and their interaction

Table 4. The expression of vitamin D according with the joint associations of total sitting time and leisure-time physical activity (LTPA)

Activity time [h]	<150		≥150		Statistical value	p-value (Kruskal–Wallis H test)
Sedentary time [h]	>8	≤8	>8	≤8		
Vitamin D [nmol/L]	60.94 ±28.05	67.06 ±26.29	78.97 ±22.31	74.22 ±26.66	44.32	<0.001
25(OH)D2 [nmol/L]	5.24 ±15.63	4.39 ±11.04	5.61 ±12.90	4.76 ±10.84	8.21	0.042
25(OH)D3 [nmol/L]	55.6 ±23.70	62.66 ±26.08	73.34 ±23.42	69.4 ±27.24	39.94	<0.001

SD – standard deviation. Values are presented as mean ±SD.

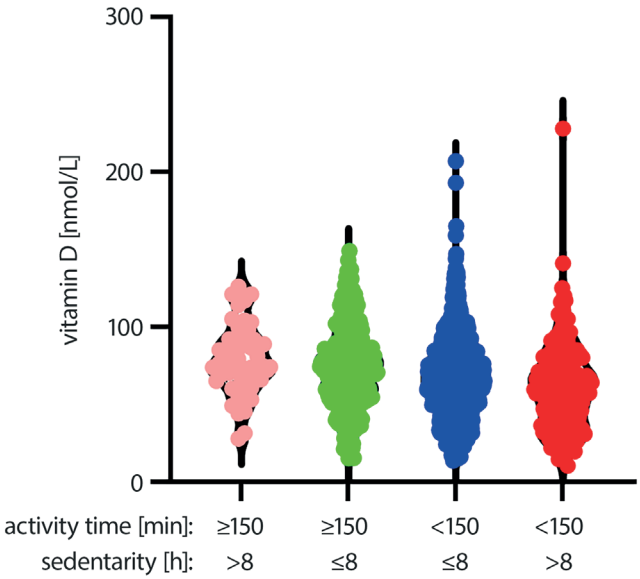

Fig. 8. The expression of vitamin D in combined analysis of physical activity time and daily sitting time (DST)

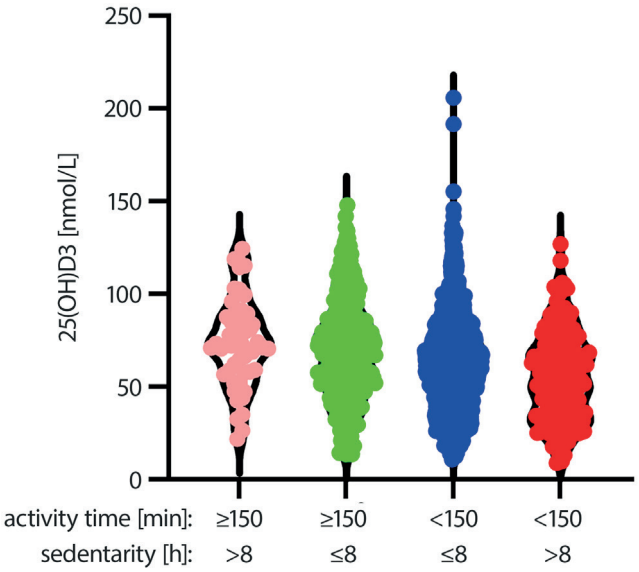

Fig. 10. The expression of 25(OH)D3 in combined analysis of physical activity time and daily sitting time (DST)

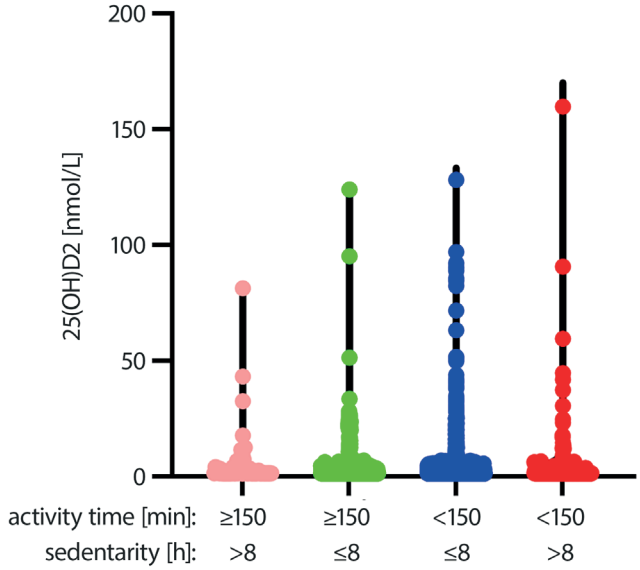

Fig. 9. The expression of 25(OH)D2 in combined analysis of physical activity time and DST

on vitamin D in patients with COPD, vitamin D status (vitamin D deficiency, vitamin D insufficiency and vitamin D sufficiency in order) was used as the dependent variable, and an ordinal logistic regression was conducted to provide the estimation and direction of the influence of physical activity, sedentary time and their interaction on vitamin D sufficiency. Table 6 and Table 7 show that LTPA and sedentary time were related to vitamin D status. Compared with vitamin D deficiency, increased LTPA was a protective factor for vitamin D insufficiency and vitamin D sufficiency (Table 6), while increased sedentary time was a high-risk factor for vitamin D insufficiency and vitamin D sufficiency (Table 7). These associations remained after adjustment for relevant covariates (both p-values for trend <0.05).

Table 8 shows the results of a joint analysis of LTPA and sedentary time on vitamin D status. The combined effect of sedentary time and LTPA still impacted vitamin D status. Compared with the sedentary time of more than 8 h and active time of less than 150 min, when the active time was less than 150 min, sedentary time of less than 8 h was a protective factor for maintaining sufficient vitamin D levels; when the active time was longer than 150 min, no matter how long of sitting time (even more than 8 h) was a protective factor for maintaining normal vitamin D levels, and this relationship remained even after relevant covariates adjustment (both p-values for trend <0.05).

Parallelism test results were analyzed according to ordered logistic regression (Supplementary Table 2–4). We used vitamin D status as a dependent variable to conduct a multi-categorical logistic regression to analyze

Table 5. Relationship between vitamin D and related factors

Variables	Vitamin D [nmol/L]	FEV_1 [L]	FVC [L]	BMI [kg/m^2]	Age [years]	DST [h]	LTPA [min/week]
Vitamin D [nmol/L]	1	0.095*	0.084*	–0.080*	0.096*	–0.036	0.160*
FEV_1 [L]	–	1	0.943*	–0.077*	–0.336*	0.002	0.185*
FVC [L]	–	–	1	–0.095*	–0.328*	0.025	0.157*
BMI [kg/m^2]	–	–	–	1	0.054#	0.142*	–0.035
Age [years]	–	–	–	–	1	0.018	–0.025
DST [h]	–	–	–	–	–	1	–0.008
LTPA [min/week]	–	–	–	–	–	–	1

Spearman correlation coefficient was used. *$p < 0.01$; #$p < 0.05$; BMI – body mass index; FEV_1 – forced expiratory volume in 1 second; FVC – forced vital capacity; LTPA – leisure-time physical activity; DST – daily sitting time.

Table 6. Ordered logistic regression models investigated the association between LTPA and serum 25(OH)D concentrations in chronic obstructive pulmonary disease (COPD) patients

Models	LTPA [min/week]			Wald χ^2	p-value for tend
	none (745, 59.1%)	from 0 to <150 (204, 16.2%)	≥150 (312, 24.7%)		
Model 1	Ref.	1.42 (1.08, 1.85)	1.97 (1.56, 2.49)	33.92	0.000
Model 2	Ref.	1.32 (1.01, 1.74)	1.89 (1.49, 2.39)	27.71	0.000
Model 3	Ref.	1.20 (0.89, 1.62)	1.67 (1.28, 2.20)	13.69	0.000
Model 4	Ref.	1.17 (0.86, 1.59)	1.49 (1.13, 1.98)	7.84	0.005
Model 5	Ref.	1.18 (0.87, 1.61)	1.51 (1.14, 2.00)	8.27	0.004

Model 1 – LTPA; Model 2 – LTPA, age, sex, race; Model 3 – LTPA, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking; Model 4 – LTPA, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FEV_1, vitamin D intake; Model 5 – LTPA, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FVC, vitamin D intake. LTPA – leisure-time physical activity; FEV_1 – forced expiratory volume in 1 second; FVC – forced vital capacity; OR – odds ratio; 95% CI – 95% confidence interval. In the logistic regression model, LTPA was treated as the predictor. Values are presented as OR (95% CI).

Table 7. Ordered logistic regression models investigated the association between DST and serum 25(OH)D concentrations in chronic obstructive pulmonary disease (COPD) patients

Models	DST [h]				Wald χ^2	p-value for tend	per 1 h/d increase
	<4	4–6	6–8	>8			
Model 1	Ref.	0.95 (0.75, 1.22)	0.77 (0.58, 1.03)	0.72 (0.54, 0.95)	6.98	0.008	0.97 (0.94, 0.99)
Model 2	Ref.	0.84 (0.65, 1.08)	0.69 (0.52, 0.93)	0.58 (0.44, 0.78)	15.84	0.000	0.94 (0.91, 0.97)
Model 3	Ref.	0.85 (0.65, 1.12)	0.70 (0.51, 0.96)	0.53 (0.38, 0.72)	16.89	0.000	0.93 (0.90, 0.96)
Model 4	Ref.	0.85 (0.64, 1.12)	0.68 (0.49, 0.95)	0.47 (0.34, 0.65)	21.11	0.000	0.92 (0.89, 0.95)
Model 5	Ref.	0.84 (0.63, 1.12)	0.67 (0.48, 0.94)	0.47 (0.34, 0.65)	21.42	0.000	0.92 (0.89, 0.95)

Model 1 – DST; Model 2 – DST, age, sex, race; Model 3 – DST, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking; Model 4 – DST, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FEV_1, vitamin D intake; Model 5 – DST, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FVC, vitamin D intake. DST – daily sitting time; OR – odds ratio; 95% CI – 95% confidence interval; in the logistic regression model, DST was treated as the predictor. Values are presented as OR (95% CI).

the effects of physical activity, sedentary time, and their interactions on vitamin D. Increased time spent on leisure physical activity after correction for relevant covariates was a protective factor for vitamin D sufficiency compared with vitamin D deficiency (Supplementary Table 5). Increased sitting time was a high factor for vitamin D adequacy (Supplementary Table 6). The joint analysis found that sitting for less than 8 h was a protective factor for maintaining adequate vitamin D levels when compared with sitting for more than 8 h and activity for less than 150 min (Supplementary Table 7). When the activity time was longer than 150 min, whether the sedentary time was more than 8 h was a protective factor to maintain the normal vitamin D levels.

Discussion

This study found that abnormal vitamin D levels were common in COPD patients, and that there was a significant association between physical activity and sedentary behavior

Table 8. Ordered logistic regression models investigated the joint association of daily sitting time (DST) and leisure-time physical activity (LTPA) with vitamin D levels among chronic obstructive pulmonary disease (COPD) patients

Activity time [h]	<150		≥150		Wald χ^2	p-value for tend
Sedentary time [h]	>8 (173, 11.9%)	≤8 (917, 63.3%)	>8 (54, 3.7%)	≤8 (304, 21.0%)		
Model 1	Ref.	1.57 (1.17, 2.13)	3.36 (1.87, 6.03)	2.57 (1.81, 3.65)	35.98	<0.001
Model 2	Ref.	1.82 (1.33, 2.49)	3.35 (1.85, 6.09)	2.86 (2.00, 4.10)	35.67	<0.001
Model 3	Ref.	2.04 (1.46,2.87)	3.28 (1.69, 6.34)	2.83 (1.91, 4.19)	25.27	<0.001
Model 4	Ref.	2.13 (1.50, 3.01)	2.74 (1.83, 4.12)	2.32 (1.18, 4.59)	16.89	<0.001
Model 5	Ref.	2.13 (1.50, 3.01)	2.76 (1.84, 4.14)	2.33 (1.18, 4.60)	17.21	<0.001

Model 1 – the joint of daily sitting time and leisure-time physical activity; Model 2 – the joint of daily sitting time and leisure-time physical activity, age, sex, race; Model 3 – the joint of daily sitting time and leisure-time physical activity, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking; Model 4 – the joint of daily sitting time and leisure-time physical activity, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FEV_1, vitamin D intake; Model 5 – the joint of daily sitting time and leisure-time physical activity, age, sex, race, BMI, family poverty–income ratio, educational attainment, smoking, comorbidities, FVC, vitamin D intake.
eFEV1 – forced expiratory volume in 1 second; FVC – forced vital capacity; OR – odds ratio; 95% CI – 95% confidence interval; BMI - body mass index.
Values are presented as OR (95% CI).

and vitamin D levels in patients with COPD. The lack of sufficient physical activity and exhibiting long-term sedentary behavior are high-risk factors for abnormal expression of vitamin D in patients with COPD. Adequate physical activity and proper control of sedentary behavior can effectively improve vitamin D levels in patients with COPD.

Sedentary behavior and decreased physical activity due to exertional dyspnea are one of the main life characteristics of patients with COPD, and this lifestyle is associated with poor outcomes in patients with COPD[34]: physical activity level and health status of patients with COPD,[35] the symptom burden,[36–38] prolonged sedentary time, and decreased physical activity levels can all affect the mortality rate and the number of exacerbations[39] in patients with COPD. A study with a mean follow-up of 2.7 years found that the number of daily steps in patients with COPD decreased periodically over time, and this change was significantly correlated with the degree of airflow limitation in patients with COPD. Regular physical activity can alleviate the decline in lung function in patients with COPD, reduce the deterioration of health status (symptom burden)[38,40–42] and limit the risk of hospital admission and mortality in patients with COPD.[43,44] Although physical activity is also beneficial for COPD extrapulmonary comorbidities, related research is limited.[22,23] Moreover, these studies did not analyze the effect of serum vitamin D levels in patients with COPD. Sedentary behavior or insufficient physical activity are the risk factors for vitamin D deficiency in the elderly patients.[45,46] An analysis based on the NHANES data showed that physical activity was associated with serum vitamin D expression levels and was not related to the exercise environment (indoor or outdoor environment).[47] However, Scragg et al. studied the NHANES III database and found that the association between outdoor exercise and vitamin D was stronger than that of indoor environments.[48] Ceolin et al. showed that moderate-to-severe physical activity was a protective factor for maintaining normal levels of vitamin D in the elderly,[46] and Scragg et al. found out that regular outdoor activities and activity frequency are associated with higher vitamin D levels, and not with activity intensity.[48] The above findings were based on the general population, and our findings showed that adequate activity time was related to high levels of vitamin D expression in patients with COPD, while prolonged sedentary behavior was related to lower vitamin D levels in patients with COPD. This observation confirmed the independent association of physical activity and sedentary time with serum vitamin D levels in patients with COPD.

Considering that high-intensity physical activity can alleviate the harmful effects of being sedentary, we conducted a joint analysis of the relationship between physical activity time and sedentary time and serum vitamin D levels in patients with COPD. A meta-analysis summarizing evidence from papers involving more than 1 million adults discovered that the risk of death was 59% higher in the least active group (8 h/day of sedentary activity, 2.5 MET h/week of physical activity (about 30 min/week of LTPA)) when compared to the most active group (4 h/day of sedentary activity, physical activity 35.5 MET h/week). The study showed that high levels of physical activity appear to lower the risk of death due to prolonged sitting.[49] Another study on cancer populations discovered that sedentary behavior was related to an increased risk of death among inactive cancer populations through a combined analysis of sedentary time and active time. Overall mortality (HR: 5.38; 95% CI: 2.99–9.67) and cancer (HR: 4.71; 95% CI: 1.60–13.9) mortality were the highest in inactive cancer patients who sat for more than 8 h/day.[30] Our joint analysis revealed that insufficient physical activity, coupled with a sedentary lifestyle of less than 8 h, emerged as a risk factor contributing to the challenge of maintaining optimal vitamin D levels in COPD patients.

Furthermore, to strengthen the statistical significance of the present study, we eveluated several recent studies on vitamin D intervention and analyzed the odds of COPD

occurrence.[50–52] Based on these papers, we developed a forest plot indicating a strong possibility that vitamin D deficiency and hence its intervention can help in limiting COPD prevalence (Fig. 2).

The impaired ability of the skin to synthesize vitamin D due to the toxic effects of smoking and age. Decreased activity and lack of outdoor exercise contribute to reduced sun exposure. Additionally, intake of glucocorticoids increases the metabolic breakdown of vitamin D. Furthermore, the natural course of COPD involves mechanisms such as inflammatory response, decreased liver potential activation function, decreased gastrointestinal absorption functions, and vitamin D being blocked in adipose tissue, all of which can lead to metabolic disorders of vitamin D in patients with COPD.[53,54] Physical activity may ameliorate the aforementioned disturbances through various mechanisms. High levels of inflammation in COPD patients, often associated with insufficient physical activity,[35] can be mitigated by regular exercise training. This can lead to reductions in immune cell counts and a transformation of the cytokine phenotype from pro-inflammatory to anti-inflammatory.[55,56] Furthermore, physical activity has the potential to counteract the damage caused by smoking by reducing oxidase activity, promoting antioxidation[57] and mitigating inflammation.[32] Additionally, it worth noting that physical activity can also stimulate the release of vitamin D from adipocytes through fat mobilization, further contributing to its beneficial effects.[58]

Limitations

This study has several limitations. First, we could not make causal inferences in this cross-sectional study, so the association between physical activity and serum vitamin D in patients with COPD needs further confirmation. Second, although our study used a validated physical activity questionnaire, measurement error was inevitable. In addition, non-leisure physical activities (such as work) may have confounded the results of the measurements. Third, we did not analyze whether these activities were carried out outdoors or indoors.

Conclusions

In patients with COPD, serum vitamin D levels are independently associated with physical activity and sedentary behavior, and increasing physical activity time and reducing sedentary time may play an important role in improving vitamin D levels in COPD patients and preventing vitamin D deficiency. Future studies should analyze the effects of activity environment (indoor or outdoor, exposure to sunlight, etc.), frequency and intensity on serum vitamin D concentrations in COPD patients. In addition, the optimal duration of activity and sedentary time needs to be further confirmed.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10124644. The package includes the following files:

Supplementary Table 1. Test of normality and homogeneity of variance.

Supplementary Table 2. Test of parallel lines of ordered logistic regression models investigate the association between the joint association of DST and LTPA and serum 25 (OH)D concentrations in COPD patients.

Supplementary Table 3. Test of parallel lines of ordered logistic regression models investigate the association between DST and serum 25 (OH)D concentrations in COPD patients.

Supplementary Table 4. Test of parallel lines of ordered logistic regression models were used to investigate the association between the joint association of DST and LTPA and serum 25 (OH)D concentrations in COPD patients.

Supplementary Table 5. Multivariate logistic regression models were used to investigate the association between LTPA and serum 25 (OH)D concentrations in COPD patients.

Supplementary Table 6. Multivariate logistic regression models were used to investigate the association between DST and serum 25 (OH)D concentrations in COPD patients.

Supplementary Table 7. Multivariate logistic regression models were used to investigate the association between association of DST and LTPA and serum 25 (OH)D concentrations in COPD patients.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

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Limited utility of salivary mineral content in prediction of fragility fractures among postmenopausal women

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Abstract

Background. Osteoporosis is a metabolic disease characterized by increased bone fragility. As it is characterized as a general skeletal disease, changes can also be seen in the stomatognathic system (edentulism, wrong fitting of dentures, etc.). The question is whether early changes in the salivary mineral content and acid-base balance may reflect skeletal status and risk of bone fracture.

Objectives. The objective of the study was to evaluate whether minerals in the saliva were associated with skeletal fractures in a population of postmenopausal women.

Materials and methods. In this observational study, dental examinations along with the collection of saliva were conducted in 117 randomly recruited women (mean age 64.6 ±5.9 years). The study group included 23 study participants with fractures, of which 10 had a history of osteoporotic fractures. Saliva samples for mineral content including copper (Cu), zinc (Zn), calcium (Ca), and phosphorus (P), as well as salivary pH were collected and analyzed to determine associations between salivary mineral content and fracture risk.

Results. As a result, the median pH value was 6.8, and the median levels for Cu (0.35 µmol/L), Zn (0.61 µmol/L), Ca (0.7 mmol/L), and P (6.64 mmol/L) were observed. No differences were noted in salivary mineral content and acid-basic balance between the fractured and non-fractured participants.

Conclusions. The results of our study suggest that salivary mineral content has limited usability in predicting skeletal fragility in postmenopausal women when used alone.

Key words: minerals, osteoporosis, saliva, dental status, fracture

Background

Osteoporosis is a metabolic disease characterized by low bone mass and deterioration of the bone microarchitecture and quality, usually leading to increased bone fragility, which in turn results in severe negative health outcomes.[1,2] There are numerous risk factors that can contribute to age-related decreases in bone mass in women. Hormonal disturbances, such as hypogonadism and estrogen deficiency, long-term glucocorticoid therapy, thyroid diseases, and a number of chronic endocrine diseases are essentially associated with bone loss and may be, indirectly, responsible for clinically significant fractures.[3–5] Women after menopause, due to a rapidly progressing estrogen deficiency, are exposed to the detrimental effects on bone and, therefore, are at a higher risk of osteoporosis. Along with hormonal insufficiency, they also experience various co-morbidities influencing calcium-phosphate and mineral metabolism that can increase bone resorption and negatively affect bone quality and strength. All those factors, along with a history of any previous fracture (including all sustained significant fractures beyond those related to osteoporosis), make postmenopausal women more susceptible to increased skeletal fragility.[6]

A generalized compromised skeletal status resulting from osteoporosis may be associated with alterations in other mineralized tissues, including the teeth, and the alteration of microelement levels, although the causal pathway remains unclear.[7–11] It has been reported that clinical manifestations of osteoporosis in the stomatognathic system may result in periodontal disease and a loss of teeth with all the consequences, like wrong fitting of dentures and several dental and oromandibular dysfunctions.[12–18] In the adult population, the number of natural teeth can be recorded in the assessment of osteoporosis, particularly to estimate the risk of hip fracture.[19] The connections through dental status, oral diseases, bone mass, bone loss, and risk of osteoporosis have been largely studied in adult and older populations. However, available data are inconsistent to some extent and cannot be generalized, mainly due to the variety of different research methods used.[20–25]

A question arises whether the aforementioned changes in the masticatory system may reflect any changes in salivary content and the acid-base balance. Another issue is whether salivary characteristics and other markers measured in the saliva may accurately indicate, or correspond to, general metabolic processes or measurable dysfunctions of the skeleton.

Objectives

This study aimed to evaluate the relationship between salivary mineral content, saliva characteristics, and the prevalence of fragility fractures in women after menopause.

Materials and methods

Patients

The study group was randomly selected from the population of Zabrze in Silesia, Poland. The recruitment part consisted of acquiring a list of 3,000 women aged over 55 years (which was 10% of the gender and age-specific population) and sending them invitations to participate. Of these women, 365 responded to the invitation. Out of them, only 117 women agreed to visit the dental office and adhered to the entire study protocol.

Specimen characteristics, assay methods

At the following stage, saliva was collected after a precise preparation of the standard to test salivary pH, calcium (Ca), copper (Cu), phosphorus (P), and zinc (Zn) content.[26–28] Prior to collection, all the participants were told to refrain from eating, drinking, gum chewing, tobacco smoking, and oral hygiene procedures (mouth rinses, flossing, brushing) for a minimum of 1 h before the collection, and to avoid any dental work within 24 h prior to sample collection. Visits occurred between 8:00 AM and 11:00 AM. At the start of the appointment, study participants were asked to rinse their mouth with deionized water for 1 min. Afterwards, they spit the water out. Five minutes after the oral rinse, unstimulated saliva was gathered into a sterile graduated test tube. The spitting method was conducted in accordance with standardized guidelines. The collection lasted 5 min. The samples were stored on ice and then refrigerated at –80°C immediately after the saliva collection.

A questionnaire-based direct interview, medical records and a clinical examination ascertained by the physician were used to obtain data on past history of fractures, nutritional habits, smoking, and long-term medical therapies. At least 1 non-accidental fracture event after the age of 40 years was included in the analysis, and all fractures were confirmed using routine radiographs. Standard anthropometric methods were used to evaluate weight and height and to calculate body mass index (BMI) based on a standard formula.

Study design

The research was a part of the Silesia Osteo Active Study, which was conducted in 2015.[26,29,30] A detailed description of the Ca, Zn, Cu, P, and pH assessment methods was published in our previous work.[26] The study obtained the approval from the Ethics Committee at the Medical University of Silesia (approval No. KNW/0022/KB1/22/III/14/15). Informed consent was collected from each participant prior to the examinations.

Statistical analyses

All statistical analyses were performed using Statistica v. 13.3 program (StatSoft, Tulsa, USA). The assumption of normality was tested with density functions and Shapiro–Wilk tests. Normally distributed descriptive data were presented as mean values ± standard deviations (M ±SD), whereas data with a non-normal distribution were presented using the median and quartiles (1st quartile (Q1) and 3rd quartile (Q3)). Data with non-normal distribution were analyzed using Mann–Whitney U and Spearman rank tests. Data with normal distribution were analyzed with the use of t-tests. Homogeneity tests were performed using the Levene and Brown–Forsythe tests. A p-value of less than 0.05 was considered statistically significant.

Results

The study group included 117 Caucasian women aged 64.6 ±5.9 years, all of whom were at least 1 year post-menopause, as self-reported. The mean BMI was 29.5 ±4.7 kg/m^2. Full demographic data with main fracture-related factors are presented in Table 1. The average number of teeth presented was 13.2 ±9.1 (min 0, max 30). Twenty-three participants (19.7%) experienced previous fractures that had occurred after the age of 40 years. In 10 women (8.5% of the whole study group), it was a clinically significant result of low-energy trauma (low-energy fracture (LEF)). In 16 participants (13.7%), the fracture location was the wrist (in 7 cases, the wrist, radius, and/or ulna fractures were classified as LEF), in 6 cases (5.1%), the ankle (including 3 cases of LEF), and in 1 woman, a vertebral fracture in the lumbar spine was found and assigned to a LEF.

The median value of salivary pH was 6.8 (Q1–Q3: 6.5–7.1), while the median Ca concentration was 0.7 (Q1–Q3: 0.53–1.19) mmol/L, Cu 0.35 (Q1–Q3: 0.19–0.57) µmol/L, Zn 0.61 (Q1–Q3: 0.15–1.22) µmol/L, and P 6.64 (Q1–Q3: 5.73–8.07) mmol/L.

Mean salivary pH did not correlate with salivary mineral content (Spearman's rank correlation test $p > 0.05$). A significant positive correlation was found between Cu and Zn ($r = 0.48$) and Cu with P ($r = 0.38$) content (Spearman's rank test, $p < 0.05$). Concise results of the Spearman's rank test analysis are presented in Table 2. Next, participants were stratified with regard to the history of any fractures that had occurred after the age of 40 years. To test for homogeneity of variance, a Levene's test was performed (Table 3). It confirmed the homogeneity assumption of the variance ($p > 0.05$) with respect to age, BMI, salivary pH, Cu, P, and Ca concentrations. As it comes to Zn, Levene's test indicated heterogeneity between the groups. Therefore, the comparison of the groups with respect to Zn

Table 1. Basic characteristics of the study group including demographic data and morbidity

Variable	Value	
Mean age [years]	64.6 ±5.9	
Mean age of menopause [years]	49.4 ±5.2	
variable	number of participants	percentage of group
BMI [kg/m^2]	29.5 ±4.7	
Underweight	0	0
Normal weight	21	17.9
Overweight	45	38.5
Obese	51	43.6
Past and current smoking	56	47.8
History of osteoporosis (based on a previous single DXA scan and interpretation)	27	23.1
Chronic kidney disease	7	6
Hypothyroidism	45	38.5
History of long-term glucocorticoid therapy (>3 months)	8	6.8
Hormone replacement therapy (>3 years)	43	36.8

BMI – body mass index; DXA – dual energy X-ray absorptiometry.

Table 3. Homogeneity test results (Levene's test)

Analyzed parameter	F	df	p-value
Age	2.6	115	0.111
Body mass index	0.18	115	0.669
Salivary pH	2.3	115	0.128
Salivary Ca concentration	1.2	115	0.277
Salivary Cu concentration	0.4	115	0.531
Salivary Zn concentration	18.2	115	<0.001
Salivary P concentration	0.6	115	0.432

Ca – calcium; Cu – copper; Zn – zinc; P – phosphorus; df – degrees of freedom.

Table 2. Correlation between salivary mineral content (Spearman's test), n = 117

Analyzed parameter	Salivary Ca concentration	Salivary Cu concentration	Salivary Zn concentration	Salivary P concentration
Salivary pH	r = –0.17, p = 0.106	r = –0.03, p = 0.768	r = –0.06, p = 0.627	r = –0.16, p = 0.202
Salivary Ca concentration	r = 1	r = 0.14, p = 0.360	r = 0.2, p = 0.171	r = 0.12, p = 0.319
Salivary Cu concentration	r = 0.14, p = 0.360	r = 1	r = 0.48, p < 0.001	r = 0.38, p = 0.034
Salivary Zn concentration	r = 0.2, p = 0.171	r = 0.48, p < 0.001	r = 1	r = 0.12, p = 0.501
Salivary P concentration	r = 0.12, p = 0.319	r = 0.38, p = 0.034	r = 0.12, p = 0.501	r = 1

Table 4. Comparison of the salivary mineral content between the subgroups according to fracture prevalence

Compared parameter	Without fracture	With fracture	Test used	Test value	df	p-value
Number and % of participants	94 (80.3% of the study group)	23 (19.7% of the study group)	–	–	–	–
Age (M ±SD (95% CI)) [years]	63.9 ±6.6 (62.6, 65.2)	66.3 ±7.7 (62.7, 69.4)	t-test	t = –1.39	115	0.256
BMI (M ±SD (95% CI)) [kg/m^2]	29.5 ±4.8 (28.5, 30.4)	29.9 ±4.3 (28.03, 31.8)	t-test	t = –0.43	115	0.648
pH (median; Q1–Q3)	6.85; 6.5–7.3	6.8; 6.5–7	U Mann–Whitney	U = 959.5	–	0.405
Cu (median; Q1–Q3) [µmol/L]	0.36; 0.2–0.62	0.33; 0.15–0.54	U Mann–Whitney	U = 326.5	–	0.417
Zn (median; Q1–Q3) [µmol/L]	0.61; 0.15–1.07	0.61; 0.1–1.38	U Mann–Whitney	U = 358	–	0.716
Zn (median; Q1–Q3) [mmol/L]	0.67; 0.53–1.13	0.81; 0.68–1.22	U Mann–Whitney	U = 561	–	0.533
P (median; Q1–Q3) [mmol/L]	6.7; 5.8–7.8	6.46; 5.6–8.06	U Mann–Whitney	U = 337	–	0.83

Cu – copper; Zn – zinc; P – phosphorus; BMI – body mass index; M – mean; SD – standard deviation; 95% CI – 95% confidence interval; Q1 – 1st quartile; Q3 – 3rd quartile; df – degrees of freedom.

concentration was performed with 2 independent sample comparisons of the means test with unequal variance (Welch's test), but the p-value remained greater than 0.5 (w = –0.95, df = 11.07, p = 0.363), as well. In respect to age and BMI, t-tests for independent groups were used, but no significant differences were found (p > 0.05). For group comparisons with respect to salivary pH, Cu, Ca, and P, the Mann–Whitney U test was performed (p > 0.05). No significant differences between fractured and non-fractured participants were found (Table 4).

Discussion

This study attempted to evaluate the relationship between salivary mineral characteristics and clinically significant fractures in the female population. Despite negative results, our report provides information on poorly studied (so far) associations indicating that further research, preferably prospective studies, is needed. The relationship between all fractures (osteoporotic and non-osteoporotic) was analyzed, assuming that the prognostic power of prior fractures as a risk of subsequent fractures is not affected by the nature of the fracture. This was based on a recent observation by Leslie et al.[6] There is still very little information available on mineral content concentrations in saliva, as well as the importance of selected individual minerals in postmenopausal osteoporosis. Furthermore, it is believed that the composition of saliva changes with age.[31]

The importance of Ca and P in bone turnover and the role of minerals such as Zn and Cu in bone metabolism are well known, although not all of their mechanisms of action in postmenopausal osteoporosis are fully understood. Zinc and Cu are important for bone integrity and elasticity, whereas Cu induces low bone turnover by suppressing both osteoblastic and osteoclastic functions. As a cofactor for lysyl oxidase, Cu is required in the cross-linking of collagen and elastin. Zinc is a constituent of about 300 enzymes. Moreover, it regulates the secretion of calcitonin from the thyroid gland and impacts bone turnover. Zinc deficiency causes a reduction in osteoblastic activity, collagen and chondroitin sulfate synthesis, and alkaline phosphatase activity.[32–34]

There is a growing interest in diagnostic procedures based on saliva testing. Saliva is increasingly used for the early diagnosis of osteoporosis and fracture risk assessment. The methods for collecting this biological material are simple, cost-effective, painless, and, most importantly, non-invasive and safe for both the patient and the operator.[27,28] Saliva is regarded as a "mirror of the body" and has a number of important roles, mainly in maintaining the health of the oral cavity, while it may also indirectly affect the entire human metabolism. One of the essential functions of saliva is to maintain and protect the hard tissues of the tooth by reassuring physicochemical balance and providing a unique source of Ca and phosphate ions.[28]

Saliva contains ingredients that can act as buffers when the pH is below or above their isoelectric point.[35] The buffers maintain the resting saliva pH between 5.7 and 6.2. However, to ensure the integrity of the tooth structure, the oral pH should be around 6.3 or higher. It has been well documented that the dissolution of enamel occurs when the pH falls below a critical pH, i.e., 5.5, and that the acidic environment promotes demineralization of the inorganic substance of the tooth. Median values obtained in the present study proved to be quite similar in both subgroups (6.8–6.85) and matched normal values.[36,37] Slightly lower pH values were found in study participants with fractures, but the difference was not significant. Our results were similar to those observed by Pereira et al., who compared postmenopausal women with low bone mineral density (BMD) to controls without bone loss and found a pH proportion of 7.1–7.2. However, a weak correlation has been shown between BMD and pH in the latter study.[11] An impact of hormone replacement therapy on an increase of salivary pH and, at the same time, the reduction of Ca levels in saliva in postmenopausal patients has been reported elsewhere.[38,39] Additionally, Tabor et al. conducted an epidemiological study of randomly recruited postmenopausal women. The study

demonstrated that a high calcium content in saliva, co-existing with a low pH, was associated with lower femoral and spinal bone density in postmenopausal women.[26] Contrary to our results, Wasti et al. demonstrated that Ca content in saliva may have been strongly indicative of the presence or absence of osteoporosis in postmenopausal women.[40] Saha et al. also found a significant increase in salivary Ca and alkaline phosphatase in study participants with osteoporosis and osteopenia compared to healthy controls.[9] Noticeably, the methodological approach and the design of a given study may, to a great extent, affect the interpretation of the results. Osteoporosis is commonly understood as bone loss and decreased BMD, according to the current practice guidelines and definition. However, the core element of the disease is low energy fractures, implying major clinical issues around menopause. Thus, the connection between BMD and other mineralized tissues, such as teeth, and the relationship between salivary minerals and bone minerals may be obscured or misleading. Our study was, therefore, focused strictly on the prevalence of fractures.

Limitations

The study has some limitations. Firstly, prospective observation was not performed, and the study population was limited only to women. Further, the number of study participants with fractures was relatively small, yet reflected a general rate of fragility in the population due to the randomized approach. It is also possible that a few silent fractures may have been omitted due to a lack of radiographic evidence. Nevertheless, this appears to be the first report evaluating salivary characteristics and postmenopausal fractures. Data from larger populations, using a prospective design, would be necessary to support or contradict our observations.

Conclusions

There was no association between salivary mineral content and the prevalence of fracture. The results of our study suggest that salivary mineral content has limited usability in predicting skeletal fragility in postmenopausal women, and should therefore be regarded with caution in routine practice.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10429621. The package contains the following files:

Supplementary Fig. 1. Scatterplots illustrating the relationships between the data analyzed by the Spearman correlation (salivary Ca, Cu, P, Zn, pH).

Supplementary Fig. 2. The violin plots illustrating data distribution in two compared groups (fractured compared to non-fractured).

Data availability

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

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What do we know about eligible organ donors? Analysis of data from a local Registry

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Abstract

Background. The imbalance between supply and demand for organ donations remains a hot topic for international debate. Brain-dead organ donors (DBDs) constitute the majority of organ donations in Poland.

Objectives. To identify the factors that guided intensivists in qualifying a brain-dead patient as a potential organ donor, and whether the factors that significantly influenced the decision to qualify constituted an actual contraindication.

Materials and methods. We performed a retrospective study based on data from the Silesian ICU Registry from 2010–2020 and publicly available information from Poltransplant. We compared the demographic and clinical characteristics of patients diagnosed with brain death who were identified as eligible and ineligible organ donors.

Results. Out of 25,465 patients enrolled in the Silesian ICU Registry, brain death was diagnosed in 385 (1.51%) study participants, and 61 of the records were excluded due to data incompleteness. In the remaining group (n = 324), there were 201 men and 123 women. Of them, only 180 study participants were reported as eligible donors (55.5%). Six patients had absolute contraindications to organ donation.

Conclusions. A relatively small number of patients diagnosed with brain death were qualified by intensivists as eligible organ donors, with a limited number of medical factors influencing this decision. This means that other non-medical factors may affect the qualification of DBDs for organ procurement.

Key words: brain death, tissue and organ procurement, tissue donors

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Background

The imbalance between supply and demand for organ donation remains a hot topic for international debate and continues to determine the prognosis of patients with end-stage organ dysfunction.[1] In 2020, 529 deceased organ donor applications were received in the Polish Transplant Coordinating Center (Poltransplant). In 74% of these cases, a successful organ procurement was performed, resulting in 1,183 organ transplantations.[2] Yet, by the end of the year 2021, the total number of patients awaiting transplantation was vastly larger, reaching a total of 5,741 cases. Indeed, Poland ranks 23rd out of 28 European countries in terms of deceased organ donors (per million population).[3]

Brain-dead organ donors (DBDs) make up the majority of organ donations and outnumber cardiac arrest organ donors and living donors.[2,4] The number of donations after cardiovascular death is low, but the retrieval programs seem promising.[5,6] Therefore, the patients eligible for donation after brain death are mainly diagnosed with a traumatic brain injury (TBI) or subarachnoid hemorrhage.[7] Such acute neurological states often require admission to the intensive care unit (ICU), which then becomes the main facility for DBD qualification and brain death management.[8,9] Therefore, based on medical and non-medical conditions, intensivists of brain-dead patients determine who qualifies and should be submitted to donor programs.

In this study, we compared the demographic and clinical characteristics of patients diagnosed with brain death who were and were not submitted by their attending physicians to Poltransplant as eligible organ donors.

Objectives

The purpose of this study was to identify the factors that influenced ICU physicians to not qualify a patient with confirmed brain death as an eligible organ donor, and whether the factors that significantly influenced the qualification decision constituted an actual contraindication for donation. In other words, we aimed to find out whether the process of qualifying a patient with confirmed brain death as an eligible donor is a deliberate process based on structured criteria, or whether it depends on the subjective judgment of the qualifying physician.

Materials and methods

Study design

In this study, we compared the demographic and clinical characteristics of patients diagnosed with brain death who were or were not submitted by their attending physicians to the Poltransplant as eligible organ donors.

Setting

This retrospective cross-sectional study was based on data from the Silesian Registry of Intensive Care Units, Poland, from the years 2010–2020. This Registry is a secured voluntary collection of demographic and medical data regarding over 25,000 adult patients hospitalized between 2010 and 2020 at ICUs in the urban region of southern Poland.[8]

Participants

Patients who were diagnosed with brain death were included in the study. We excluded patients with insufficient data regarding hospitalization or organ donation status (n = 61). After exclusions, the study group consisted of a total of 324 patients.

Variables

Demographic and medical data were retrieved, including sex, age, comorbidities, primary ICU admission cause, patient's condition on admission, and applied treatment and invasive procedures during the ICU stay. In the paper, we used definitions and categories applied a priori in the Registry.[10] Patient submission as an eligible organ donor was defined as the outcome. Submission meant that the patient was reported to the Poltransplant center as an eligible organ donor. An eligible donor is a patient with confirmed brain death in whom there are no known absolute contraindications to becoming a donor.

Data sources/measurement

All data were obtained from the Silesian ICU Registry. All data were analyzed employing units used in the Registry.

Bias

We excluded patients whose stay data were incomplete or unclear upon evaluation. Except for the excluded patients, every patient with a diagnosis of brain death was included. Potential bias was reduced due to the fact that we were working with a Registry in which the structure was standardized. The Registry was not focused on any outcome or purpose, only on collecting data, which may potentially reduce the risk of selection bias.

Study size

The study size was achieved by using all available data from the Registry from all years of its functioning.

Quantitative variables

The only quantitative variables analyzed in our study were age and length of stay. Quantitative variables were expressed as median and interquartile range (IQR).

Statistical analyses

Statistical analysis was performed using MedCalc Statistical Software v. 15.4 (MedCalc Software Ltd., Ostend, Belgium). Qualitative variables were expressed as absolute values and percentages. Between-group differences for quantitative variables were assessed using the Mann–Whitney U test. Their distribution was verified with the Shapiro–Wilk test, while χ^2 or Fisher's exact tests were applied for qualitative variables. A $p < 0.05$ was considered statistically significant.

Results

Of the 25,465 patients included, 385 (1.51%) were diagnosed with brain death. Sixty-one patients with a diagnosis of brain death registered in 2010 (n = 11), 2011 (n = 47) and 2020 (n = 3) were excluded due to incomplete data (Fig. 1).

Taken altogether, 324 (1.27%) of the registered study participants were diagnosed with brain death, and only about half of them (55.5%) were regarded by their attending physicians as eligible organ donors. The median age of brain-dead patients was 54 years (IQR: 43–64), and there were more male patients (n = 237; 61.5%). Considering the patients' chronic diseases, the most common were arterial hypertension (42.3%) and coronary artery disease (24.9%). A more detailed between-group comparison is presented in Table 1.

In most cases, patients were admitted to the ICU from the emergency department (32.2%), and in almost every case (96.4%), it was their first ICU admission. The most common cause of admission was acute respiratory failure (with additional complaints of acute heart failure and an acute altered neurological status, Table 2).

Regarding data at admission, patients who were submitted as eligible organ donors were more likely to be unconscious (95.0% compared to 88.1%) and less likely to need hemodynamic support with catecholamines than non-submitted patients (43.8% compared to 65.3%, Table 3).

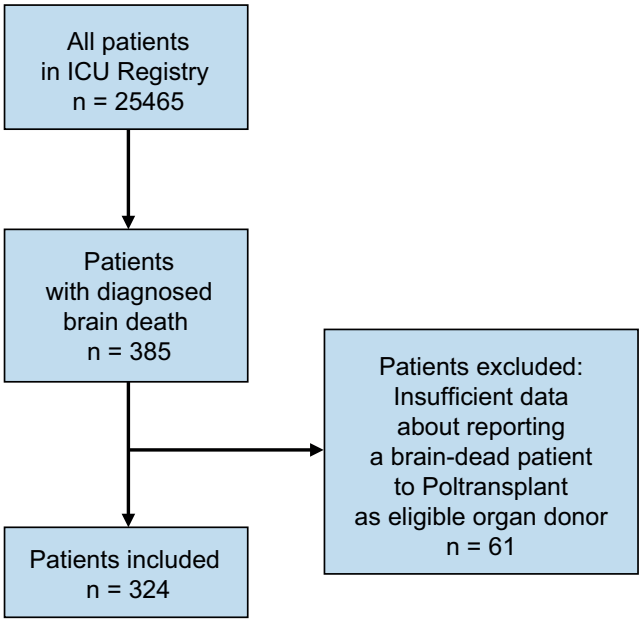

Fig. 1. Flow diagram for the patient selection process

ICU – intensive care unit; Poltransplant – Polish Transplant Coordinating Center.

Table 1. Comparison of demographics and comorbidities between brain-dead study participants who were submitted as eligible and ineligible organ donors

Variable	Patients not submitted as eligible organ donors (n = 144)	Patients submitted as eligible organ donors (n = 180)	Test value	df	p-value and test name
Female	52 (36.1%)	71 (39.4%)	0.249	1	0.617[a]
Age* [years]	60 (IQR 50–69)	51 (IQR 41.5–60)	–	–	<0.001[b]
Coronary artery disease	53 (36.8%)	31 (17.2%)	14.972	1	<0.001[a]
Chronic heart failure	39 (27.1%)	12 (6.6%)	23.627	1	<0.001[a]
Arterial hypertension	63 (43.8%)	79 (43.8%)	0.008	1	0.930[a]
Chronic respiratory failure	16 (11.1%)	2 (1.1%)	13.401	1	<0.001[a]
Alcohol abuse	9 (6.2%)	23 (12.7%)	3.132	1	0.076[a]
Diabetes mellitus	15 (10.4%)	17 (9.4%)	0.011	1	0.917[a]
Chronic renal failure	16 (11.1%)	1 (0.5%)	15.869	1	<0.001[a]
Previous cerebral stroke	13 (9%)	16 (8.8%)	0.023	1	0.878[a]
Chronic neurological disorders	10 (6.9%)	8 (4.4%)	0.536	1	0.464[a]
Malignancies	6 (4.1%)	0 (0%)	5.521	1	0.018[a]
HCV infection	1 (0.6%)	3 (1.6%)	0.079	1	0.778[a]

Continuous variables were expressed using medians and interquartile ranges (IQR). Qualitative variables were expressed as absolute values and percentages. *Normality should be rejected due to result of Shapiro–Wilk test (W-value = 0.9892; p = 0.016); HCV – hepatitis C virus; [a] – χ^2 test; [b] – Mann–Whitney U test; df – degrees of freedom.

Table 2. The primary reason for ICU admission*

Variable	Patients not submitted as eligible organ donors (n = 144)	Patients submitted as eligible organ donors (n = 180)	χ^2	df	p-value
Acute respiratory failure	114 (79.1%)	131 (72.7%)	1.442	1	0.229
Exacerbation of chronic respiratory failure	12 (8.3%)	1 (0.5%)	10.627	1	0.001
Acute heart failure	87 (60.4%)	87 (48.3%)	4.225	1	0.039
Sudden cardiac arrest	48 (33.3%)	30 (16.6%)	11.263	1	<0.001
Shock (any)	30 (20.8%)	12 (6.6%)	13.002	1	<0.001
Multiorgan failure	14 (9.7%)	14 (7.7%)	0.176	1	0.674
Sepsis	4 (2.7%)	1 (0.5%)	1.343	1	0.246
Acute pancreatitis	3 (2%)	0 (0%)	1.855	1	0.173
Post-surgical status	36 (25%)	30 (16.6%)	2.930	1	0.086
Traumatic brain injury with multiorgan failure	6 (4.1%)	12 (6.6%)	0.536	1	0.464
Non-traumatic brain injury	31 (21.5%)	80 (44.4%)	17.651	1	<0.001
Traumatic brain injury	17 (11.8%)	41 (22.7%)	5.828	1	0.015
Poisoning/intoxication	2 (1.3%)	3 (1.6%)	0.063	1	0.801
Severe metabolic disorders	20 (13.9%)	18 (10%)	0.823	1	0.364

*Patients could be classified in several causes of admission, e.g., a patient with respiratory failure may also suffer from circulatory failure, acute neurological or metabolic state, etc. Qualitative variables were expressed as absolute values and percentages. ICU – intensive care unit; df – degrees of freedom.

Table 3. The medical status at ICU admission

Variable	Patients not submitted as eligible organ donors (n = 144)	Patients submitted as eligible organ donors (n = 180)	χ^2	df	p-value
Lack of consciousness	127 (88.1%)	171 (95.0%)	4.140	1	0.041
Endotracheal intubation and mechanical ventilation	125 (86.8%)	148 (82.2%)	0.945	1	0.331
Catecholamine support (any)	94 (65.3%)	79 (43.8%)	13.860	1	<0.001

Qualitative variables were expressed as absolute values and percentages. ICU – intensive care unit; df – degrees of freedom.

Table 4. Medical support required during ICU stay

Variable	Patients not submitted as eligible organ donors (n = 144)	Patients submitted as eligible organ donors (n = 180)	χ^2	df	p-value
Catecholamine support	132 (91.6%)	169 (93.8%)	0.309	1	0.578
Need for tracheostomy	14 (9.7%)	3 (1.6%)	8.885	1	0.002
Need for RRT	15 (10.4%)	2 (1.1%)	12.125	1	<0.001
Antibiotics use	88 (61.1%)	117 (65%)	0.367	1	0.544
Surgery during ICU stay	11 (7.6%)	39 (21.6%)	11.012	1	<0.001

Qualitative variables were expressed as absolute values and percentages. RRT – renal replacement therapy; ICU – intensive care unit; df – degrees of freedom.

During the ICU hospitalization, the submitted patients were less likely to be dependent on ventilatory support with a need for tracheostomy (1.6% compared to 9.7%) and less likely to have qualified for renal replacement therapy (RRT; 1.1% compared to 10.4%) than the non-submitted individuals (Table 4). The median length of ICU stay did not differ between groups (non-submitted: 5 (IQR: 2.5–10) compared to submitted: 5 (IQR: 3–8); p = 0.136).

We selected 10 factors, considering primarily the clinical aspect, that could significantly negatively influence the intensivist's decision on the patient's qualification as an eligible organ donor, and reviewed how many patients had at least one of these factors (regardless of whether the factor was statistically significantly more or less frequent in patients who qualified or were not qualified to be eligible donors). The chosen factors included shock (any type), sepsis and sudden cardiac arrest as the primary cause of admission, comorbidities before admission (diabetes, chronic circulatory failure, chronic renal failure, chronic respiratory failure, and alcohol abuse), and a need

for continuous renal replacement therapy (CRRT) or need for tracheostomy during the ICU stay. Seventy-three of the 180 patients not qualified as eligible organ donors (40.55%) had at least one of these selected factors.

Discussion

The results of this study indicate the possible larger problem of qualifying too few patients as eligible organ donors despite confirmed brain death. The efficiency of the donor qualification for these patients appears to be unsatisfactory, as only half of brain-dead patients were regarded as eligible organ donors by their attending physicians. Only a few medical variables significantly influenced donor eligibility, which may indicate the presence of other factors affecting the organ donation process.[11]

The upper age limit for disqualifying a patient from being a donor is variable, with an increasing trend over the years,[12] and can vary from organ to organ. For example, for kidney donation, the limit is an age >70 in most cases. It is worth noting that for each organ, this is a relative contraindication, which is due to the statistically higher incidence of damage and reduced organ function in the elderly and, thus, reduced transplant survival.[13] However, this should not be applied to every case, as age should only be an auxiliary factor in assessing the suitability of a patient's organs for possible transplantation, not a determining factor. Despite this, the fact that younger patients were statistically more likely to qualify is clear in our study.

Chronic organ failure is not an absolute contraindication to organ donation. Intuitively, it seems obvious that a worse organ condition, as determined with biomarkers, imaging studies or clinical signs of failure, will negatively affect organ function in the prospective recipient. However, there is a lack of strong evidence to support this claim, so chronic organ failure in an eligible donor should not be considered a contraindication to donation in every case, nor should it be a factor that, in isolation, without being linked to the full clinical picture of the patient, influences the failure to qualify a person with a confirmed brain death as an eligible donor.

Both in Poland and internationally, the main cause of brain death is acute neurological conditions, often associated with TBI.[14,15] Thus, it is not surprising that patients admitted for the aforementioned conditions more often qualified as eligible donors. These conditions mostly caused disorders of consciousness, which may indirectly indicate that qualified patients were more often unconscious on ICU admission. It should be noted, however, that unconsciousness is a very broad concept that can result from many causes, not only those directly related to brain damage.

Our study lacks data on the type of procedures performed during the ICU stay. However, it can be assumed that the majority of these were neurosurgical procedures aimed at reducing intracranial pressure (ICP), such as craniotomy. Patients with increased ICP requiring decompression will be the vast majority of patients with severe brain damage, which can lead to brain death and qualify for organ donation. This may explain why they were qualified more often.

Neither the need for a tracheostomy during an ICU stay nor the initiation of RRT are absolute contraindications to organ donation from a donor with confirmed brain death. The fact that in our study, these factors were more common in the group of ineligible patients may be explained by a prolonged ICU stay, which is often associated with performing the above procedures (especially tracheostomy, which is most often performed when prolonged mechanical ventilation is required).[16] The length of ICU stay is indirectly impacted by the severity of the patient's condition, which may translate into the deterioration of organ function, regardless of the reason for the ICU stay, and thus may explain the increased incidence of tracheostomies in patients who were ultimately not reported as eligible donors. The Registry did not provide information on when or why RRT was initiated in a patient, but it should be assumed that in most cases, this information refers to the initiation of RRT prior to the determination of brain death. Although the use of RRT in eligible donors is beneficial in the presence of acute kidney injury (AKI),[17] the need for this procedure prior to the determination of brain death was likely dictated by the severity of the patient's condition and driven by the therapeutic indications specific to the patient, hence the higher number of patients who required RRT in the group that did not qualify as eligible donors is to be expected.

Donation from eligible DBDs is suboptimal, and the multidirectional attempts to improve retrieval rate are insufficient.[18,19] Considering absolute values of the individual variables, it should be noted that there were very few patients with absolute contraindications to organ donation, such as isolated cases of malignancies in our study (6 cases).[20]

Only 40.55% of the patients had at least 1 of 10 factors that, according to the authors, could have significantly influenced the patient's ineligibility as a donor. It should be noted that the selected factors are not absolute contraindications to organ donation. The qualification process is, of course, complex and should not be reduced to an assessment of single factors; however, it appears that a significant proportion of patients are disqualified from being an eligible donor due to other unspecified factors, which is an opportunity for improvement.

It is worth mentioning that, according to Polish law, an absolute contraindication to organ donation from a deceased donor is an objection expressed during life as defined by law.[21] As of December 31, 2020, a total of 37,728 people were registered in the Central Register of Objections.[2] This represents 0.09% of the Polish population. Therefore, this parameter can be considered irrelevant

in the context of this study as well as in the context of patient eligibility for organ donation.

Given the increasing need for organ transplantation, it is important to maximize the number of eligible organ donors. This can be achieved by increasing the qualification rate of confirmed brain-dead patients. To improve donation outcomes, it is crucial to provide thoughtful and critical care management to eligible organ donors, with a focus on meeting donor management goals.[22] This could contribute directly to an increase in the absolute number of transplants performed, thereby reducing mortality and improving the well-being of those waiting for a transplant. The results also suggest the need to ask oneself before deciding not to qualify a patient diagnosed with brain death as an eligible donor – is this patient unable to be a donor, or does he or she have any absolute contraindications to organ donation? It seems reasonable to consider the patient's condition on a case-by-case basis and try to qualify them for organ donation, as this can significantly increase donation rates.[23] Moreover, it has been documented that many declined donor livers have the potential to be evaluated by machine perfusion.[24]

Limitations

This study has several limitations. First, it was a registry-based analysis and therefore retrospective in design and with a limited amount of available data. This paper did not analyze the organ donation process as the Registry was not designed for that purpose. Therefore, certain variables were not included. Some variables were too vague to interpret and determine their impact on the qualification process of a patient diagnosed with brain death as an eligible donor.

Laboratory data, including inflammatory markers or biochemical indices, were not monitored. Additionally, 61 patients were excluded from the analysis due to incomplete data. It is important to note that ward participation in the Registry was voluntary, which may have limited the amount and representativeness of the data inputted. Furthermore, the Registry was run locally, exclusively in the Silesian Province of Poland. Although the study group seems representative, it is important to compare the results obtained with those from institutions across Poland.

Conclusions

In our study, a small number of patients diagnosed with brain death were considered eligible organ donors by their attending physicians. A significant proportion of patients did not have any factors that could have potentially influenced donor eligibility, which may indicate the presence of other factors affecting the whole organ donation process. This implies that the clinician's subjective judgment may play a significant role, which could result in disqualifying a considerable number of eligible donors.

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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Blindness of intentions and metacognitive deficits during moral judgements in schizophrenia

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Abstract

Background. Previous research has shown that moral judgments are affected by social cognitive abilities, such as theory of mind (ToM). This study examines how information about an actor's beliefs and the consequences of their actions affect the moral evaluation of the character's behavior in social events. Our research builds upon previous studies, which have shown that these factors contribute differently to moral judgments made by both adults and young children.

Objectives. This study aimed to explore how participants with schizophrenia and healthy controls read stories about social situations in the context of moral judgments.

Materials and methods. The study used the research procedure that included 4 variants of 16 scenarios describing social situations, and thus comprising 64 stories. After each story, participants evaluated their confidence level on a 4-point scale. To assess delusional beliefs, the Polish adaptation of the Peters Delusion Inventory (PDI) questionnaire and the Paranoia Checklist (PCh) were used. Respondents completed these questionnaires after completing the scenario test procedure.

Results. In social situations, patients with paranoid schizophrenia were found to evaluate actions of protagonists who attempted to harm another person more leniently than when it was an accident. Conversely, healthy individuals judged those actors who expressed intentions to hurt another person significantly more harshly than in an accident situation. Metacognition measures show that paranoid schizophrenia patients make moral judgments with high confidence, despite being based on an incorrect reading of the other person's intentions.

Conclusions. The study indicates that ToM has a significant impact on the moral judgment of others. Decreased moral cognition can result from both positive and negative symptoms. Deficits related to metacognition can also sustain such cognitive distortions.

Key words: paranoid schizophrenia, metacognition, theory of mind, moral judgments

Background

Paranoid symptoms are often observed among people diagnosed with schizophrenia.[1] Studies show that patients with schizophrenia, classified as paranoid and non-paranoid, process social information differently. For instance, patients with paranoid symptoms have difficulty recognizing negative emotions compared to healthy people.[2] Additionally, they tend to interpret ambiguous stimuli (e.g., neutral facial expressions) as threatening.[3] Moreover, studies on patients with paranoid schizophrenia show their increased attribution of hostility, blame and aggression in socially ambiguous situations.[4] Similar abnormalities of social cognition appear in the general population of those with a tendency to paranoid thinking.[5]

It seems that deficits in social cognition result from deficits in the theory of mind (ToM), which are commonly diagnosed among patients with schizophrenia[6–9] and, according to some researchers, are related to paranoid beliefs.[1,10] Moreover, people with schizophrenia exhibit many deficits in social cognition, including ToM,[6,7,11,12] which are related to the processes of moral cognition and together form an integral aspect of social functioning.[13–15]

Research indicates that moral judgment is a complex socio-cognitive process that requires ToM skills.[16] For instance, neurocognitive studies among children show that the observer's knowledge about the mental state of another person (actor) is integrated with their knowledge about the outcome of the actor's behavior.[16] When the scenarios of events describing the interaction between 2 people show a conflict related to information about the actor's behavior (the result of their behavior and intentions), the explanation of this behavior from a moral viewpoint will depend more on perceived intentions than the result of behavior.[16–18]

Current data from studies among patients with schizophrenia are insufficient to identify some problems with the ability to make moral judgments. Some studies indicate that there are no deficits in moral judgments, i.e., patients with schizophrenia have no observable difficulties in understanding the intentions of others in social situations and in evaluating the moral acceptability of the resulting behavior.[19] There are also studies conducted among adolescents indicating deficits in moral judgments.[15] In contrast, research by McGuire et al.[20] indicates a link between negative symptoms in schizophrenia and severe judgments regarding behavior that is commonly accepted as a minor offense. The inconsistency and incompleteness of these findings calls for this research gap to be filled. Moral cognition in schizophrenia may play an important role in better understanding aggressive behavior in this illness, which is often wrongly attributed to deficits in moral cognition.[21]

According to some researchers, metacognition may be another important factor influencing moral judgments, and it covers the processes of ToM that relate only to one's own thoughts and beliefs.[22] The term metacognition refers to thinking about one's thinking: thinking about thinking.[22] It is assumed that metacognition enables an individual to observe their mental processes (monitoring) and use knowledge about their processes to regulate behavior (control).[23,24] There is also a view that metacognition is used to evaluate one's own decisions. Therefore, if metacognitive processes are inappropriate, the individual ceases to adequately assess and perceive the functioning of their cognitive processes, including own decisions. In the case of mental disorders, there may be permanent metacognitive dysfunction manifested by a lack of adequate knowledge about the individual's cognitive processes. Therefore, abnormalities in metacognition may be a common cause of psychotic symptoms that also occur in schizophrenia.[25,26]

Metacognitive impairment has been found to contribute to the development and persistence of schizophrenia symptoms.[27] There are data indicating an association between metacognition and overall symptom severity, and between impaired metacognitive functioning and the severity of schizophrenia symptoms, i.e., positive symptoms,[28] negative symptoms[27,29,30] or disorganized symptoms.[31]

Judgments about social situations depend not only on inference about the intentions of the actor of an event, but also on understanding one's own knowledge of the subject. The most studied metacognition deficit is cognitive distortion in the form of overconfidence. This leads to a subjective level of confidence in one's own judgments, conclusions or predictions that is higher than objective criteria indicate. Recent studies have shown the presence of a strong overconfidence effect in schizophrenia, as well as in other mental disorders.[32,33]

Objectives

Previous studies indicate that dysfunctional metacognition has a significant effect on moral inference about the behavior and intentions of actors in social situations. In the present study, we hypothesized that assessment of the morality of actors' behavior depends on the confidence of the assessors in their moral judgments and their perception of the actors' intentions in determining their behavior. We expect that the perceived effect of the actors' behavior influences the assessment, from a moral perspective, of actions in various scenarios presenting social interactions. In the study, it was expected that there would be significant differences in the assessments made by healthy people and by those with schizophrenia in social situations that arouse a dissonance between the actor's intentions and the result of their behavior. It was assumed that people experiencing paranoid delusions would attribute negative intentions whenever the outcome was negative, regardless of the intentions of protagonists, as a result of the very nature of delusions. Based

on the results of previous research,[16,34,35] we explored the effect of dissonance between the perceived intention of actors and the outcome of their behavior. It is expected that such dissonance will result in "softer" judgments of the actors' behavior. This should be especially noticeable in situations where the actor attempted (attempt) to hurt someone rather than in situations where the other person was ultimately hurt by accident (accident). Such assessments would result from ToM deficits typical for people with schizophrenia.

Materials and methods

Participants

The study included 10 patients diagnosed with paranoid schizophrenia (5 women, 5 men) and 10 healthy controls (6 women, 4 men). Patients in the clinical group were recruited from the Day Care Psychiatric Unit in Lubin, Poland. Participants in the control group were recruited among students of the Faculty of Psychology at the SWPS University in Wrocław, Poland. The study was approved by the Commission for Research Ethics at the Second Faculty of Psychology at the SWPS University. The participants in the research procedure gave their informed consent to participate in the study. A diagnosis of schizophrenia was confirmed using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV Axis I Disorders (SCID-I)[36] and approved by a board-certified psychiatrist. To exclude individuals with mental or neurological disorders from the control group, we utilized the Mini-International Neuropsychiatric Interview (M.I.N.I.). Due to the small sample size and non-normal distribution of data, we used a non-parametric Mann–Whitney U test to compare variable distributions. The median age of the patients was 42 years (with the lower and upper quartiles being 36.25 and 47.75 years, respectively) and a range of 29–50 years. The median age of the healthy controls was 23.5 years with the lower and upper quartiles being 22 and 24 years, respectively and a range of 22–32 years.

Procedure

The study used the research procedure employes in other studies.[16,34,35] The Polish adaptation of the tool was carried out using the method of reverse translation. The research procedure included 4 variants of 16 scenarios that described social situations, resulting in a total of 64 stories. These stories were presented in 4 sequential parts: 1) background – information to set the scene (identical across all conditions); 2) foreshadow – information foreshadowing the outcome (negative or neutral); 3) belief – information stating the protagonist's belief about the situation (negative or neutral); 4) outcome – information about the protagonist's action and the resulting outcome. In each scenario, a 2 × 2 pattern was used: 1) the outcome was either negative or neutral, 2) the expectations of the protagonists regarding the outcome (negative or neutral). The 4 combinations of these 2 factors can be categorized as follows: 1) neutral (both the expectation and the outcome were neutral), 2) attempt (the expectation was negative, but the outcome neutral), 3) accident (the expectation was neutral, but the outcome negative), 4) intentional (both the expectation and the outcome were negative)

For example, in the scenario presented in Fig. 1, identifying the white powder in coffee as poison, rather than sugar, foreshadows the death of a person as a result of ingesting the poison. In each story used in the experiment, when a threat is actual (e.g., poison instead of sugar), action by the protagonist results in someone's death. Each possible belief was true for one outcome and incorrect for the other. The study participants independently switched individual slides in which a story was presented. The stories were then removed from the monitor screen and replaced with an instruction to assess the moral nature of the action on a scale of 1 (forbidden) to 7 (permissible) using the keyboard. This question, together with the scale, was then removed from the screen and replaced with an instruction to rate the participant's own confidence regarding this moral assessment on a scale from 1 (guessed) to 4 (100 percent sure) (Fig. 2). The study participants saw 4 variants of each scenario for a total of 64 stories. The scenarios were presented in a random order, and each respondent observed the stories in a different order. The text of the story was presented in 42-point font using the Calibri facetype in black on a white background. The stories were displayed on the screen of a laptop with a 15-inch computer matrix.

In addition, the study assessed the severity of delusional beliefs. For this purpose, the Polish adaptation of the Peters Delusional Inventory (PDI) questionnaires[37,38] and the Paranoia Checklist (PCh)[39,40] were used, which the respondents completed after completion of the scenario test procedure.

Statistical analyses

The statistical calculations was carried out using the R package (R Foundation for Statistical Computing, Vienna, Austria). Cronbach's alpha coefficient was used to assess the coherence of the moral assessments and recorded levels of confidence regarding the 16 scenarios for a fixed variant. A high value of the alpha coefficient indicates a high level of coherence in the answers. A coefficient of above 0.6 was regarded as acceptable, while a coefficient of above 0.7 was regarded as good. For both the moral assessments and the recorded levels of confidence, the alpha coefficient was at least 0.636 and was generally above 0.7 (it was always above 0.7 when the 2 study groups were combined). Hence, it was concluded that it is reasonable to summarize the reactions of a respondent to a given variant of the scenarios

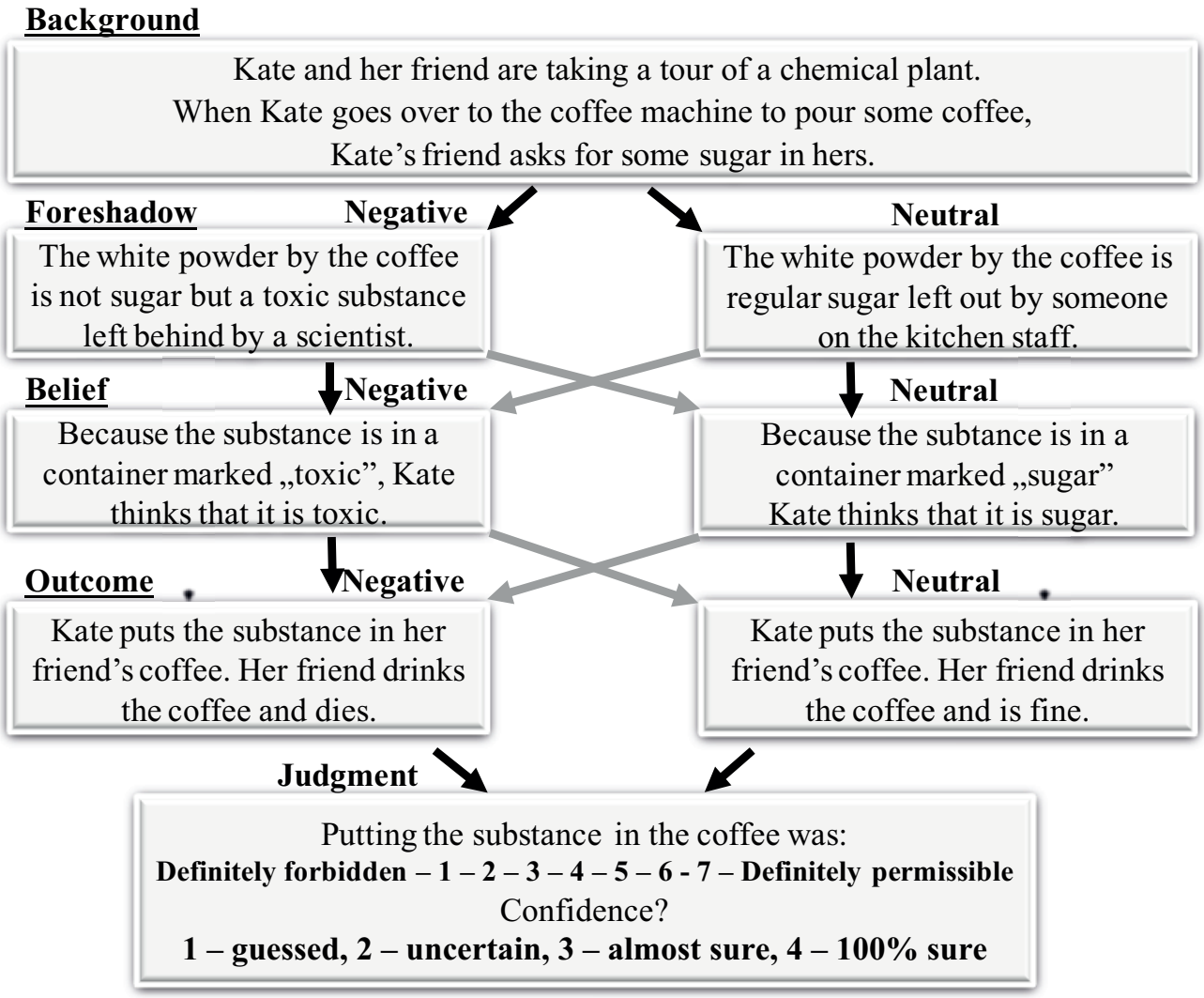

Fig. 1. Experimental stimuli and design

by taking the mean response over the 16 corresponding scenarios. The values of Cronbach's alpha coefficient for the moral assessments (level of permissiveness) according to scenario are given in Table 1. The corresponding values of Cronbach's alpha coefficient for the level of metacognitive confidence are given in Table 2.

Table 1. Cronbach's alpha coefficients for moral assessments values

Scenario	Overall	Patients	Controls
Neutral	0.743	0.774	0.663
Attempt	0.872	0.890	0.824
Accident	0.889	0.912	0.794
Intentional	0.875	0.892	0.809

Table 2. Cronbach's alpha coefficients for metacognitive confidence values

Scenario	Overall	Patients	Controls
Neutral	0.757	0.679	0.796
Attempt	0.835	0.888	0.765
Accident	0.803	0.636	0.858
Intent	0.824	0.766	0.840

Due to the small sample size and lack of normality, the nonparametric Mann–Whitney U test was used to compare responses from the patient and control groups. Wilcoxon's test for paired samples was used to compare the responses of members of a single group to different scenarios.

In order to compare the level of paranoid beliefs and the responses (the level of confidence and moral assessment) according to group, the nonparametric Mann–Whitney test was used (Table 3). A summary of the Scale for the Assessment of Negative Symptoms (SANS) and the Scale for the Assessment of Positive Symptoms (SAPS) diagnoses for patients is given in Table 4 (the control group was not diagnosed). The Mann–Whitney U test was used to compare the levels of metacognitive confidence and moral assessments reported by the groups for a given scenario (Table 5 and Table 6, respectively).

The Wilcoxon signed rank test for paired samples was used to compare differences between the levels

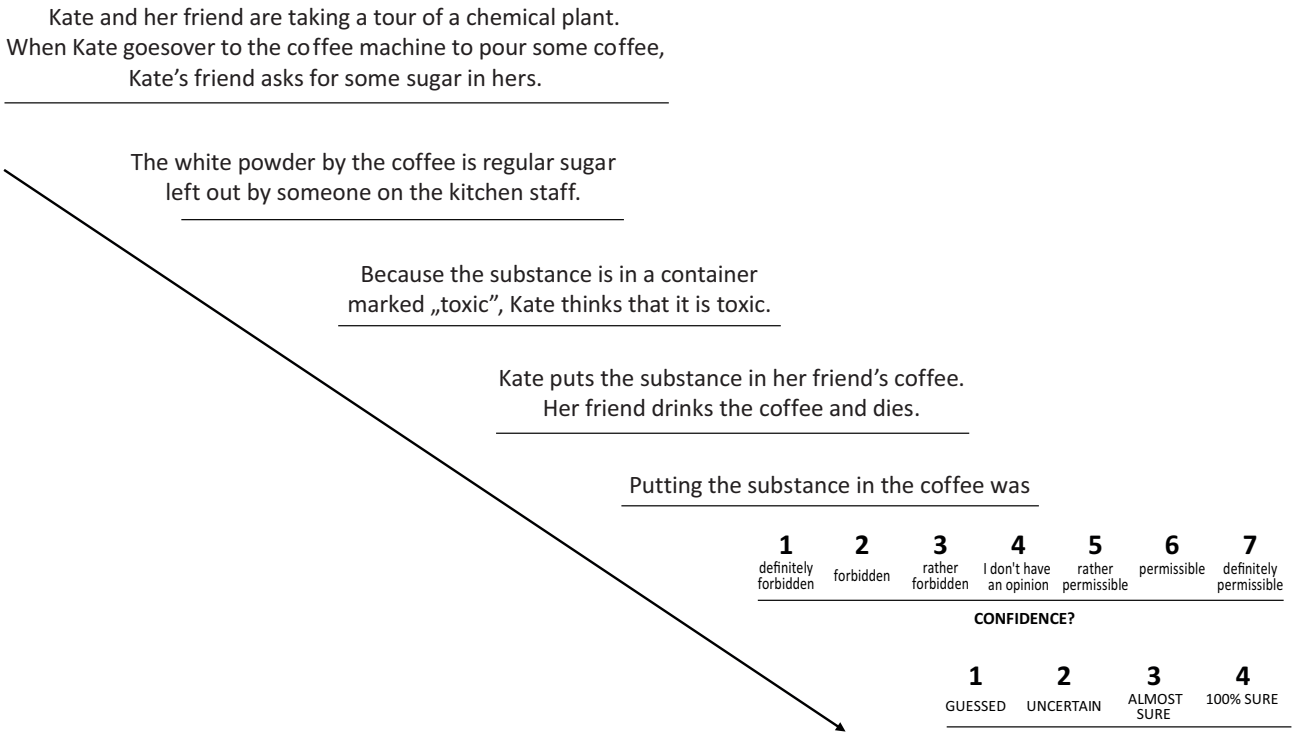

Fig. 2. Research procedure

Table 3. Summary of paranoid beliefs

Subscale	Patients			Controls			U	p-value
	Q1	Med	Q3	Q1	Med	Q3		
PDI (distress)	44.00	53.50	55.25	7.00	13.00	24.50	0.000	0.004
PDI (preoccupation)	35.50	44.00	56.50	8.25	13.50	22.50	6.500	0.003
PDI (convince)	41.25	48.00	53.50	11.00	20.50	26.75	9.000	0.004
PCh (frequency)	28.00	39.00	42.00	23.50	27.5	32.00	22.50	0.072
PCh (convince)	28.00	40.00	49.00	29.00	32.50	40.75	34.50	0.413
PCh (distress)	24.00	37.00	64.00	21.25	22.00	28.50	26.00	0.130

Q1 – 1st quartile; Q3 – 3rd quartile; PDI – Peters Delusional Inventory; PCh – Paranoia Checklist.

Table 4. Summary of SANS and SAPS among patients

Scale	Q1	Med	Q3
SANS			
Affective Flattening/Blunting	8.00	15.00	17.75
Alogia	0.00	5.00	7.50
Avolition/Apathy	2.25	4.00	5.50
Anhedonia/Asociality	3.50	8.00	11.00
Attention	2.25	3.50	4.00
General	19.75	34.00	45.75
SAPS			
Hallucinations	0.00	0.00	0.00
Delusions	0.00	1.00	4.75
Bizare Behavior	0.00	0.00	3.75
Positive Formal Tgought Disorder	0.00	3.00	12.00
General	48.50	76.00	96.00

Q1 – 1st quartile; Q3 – 3rd quartile; SANS – Assessment of Negative Symptoms; SAPS – Scale for the Assessment of Positive Symptoms.

of metacognitive confidence and moral assessments reported by a group for pairs of scenarios (for the patients: Table 7 and Table 8, respectively, for the controls: Table 9 and Table 10, respectively). The significance level used in all cases was 5% (i.e., a difference is inferred when the p-value is < 0.05).

Results

The Polish versions of the SANS, the SAPS,[41] PDI,[37] and PCh[39] questionnaires were used to measure paranoid beliefs and schizophrenia symptoms (Table 3,4). The results of the PDI and PCh on the frequency of delusional beliefs show statistically significantly higher results for patients on some subscales.

Intragroup analyses indicated significant differences between the moral assessments according to scenario (Table 6–8, Fig. 3). In both groups, the behavior

Table 5. Comparison between levels of metacognitive confidence for patients and controls. Each observation is the average of 16 measurements on a 4-point scale (1–4) representing the level of metacognitive confidence. The analysis employed the Mann–Whitney U test

Scenario	Patients			Controls			U	p-value
	Q1	Med	Q3	Q1	Med	Q3		
Neutral	3.328	3.470	3.643	3.515	3.630	3.735	36	0.306
Attempt	3.225	3.560	3.658	3.395	3.595	3.810	43.5	0.649
Accident	3.000	3.345	3.735	3.208	3.530	3.630	43.5	0.649
Intentional	3.140	3.595	3.953	3.548	3.750	3.925	43.5	0.648

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

Table 6. Comparison between moral assessments of patients and controls. Each observation is the average of 16 measurements on a 7-point scale (1–7) representing the assessment of the permissibility of an action. The analysis employed the Mann–Whitney U test

Scenario	Patients			Controls			U	p-value
	Q1	Med	Q3	Q1	Med	Q3		
Neutral	4.293	5.095	5.363	4.660	5.160	6.113	37	0.344
Attempt	2.380	2.750	3.750	2.343	2.690	3.015	57	0.623
Accident	2.033	2.815	3.420	2.440	3.815	4.390	26	0.076
Intentional	1.190	1.500	2.203	1.515	1.625	2.143	39.5	0.449

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

Table 7. Differences between level of metacognitive confidence according to the scenario type (patients). Each observation is the average of 16 measurements on a 4-point scale (1–4) representing the level of metacognitive confidence. The analysis employed Wilcoxon's test for paired samples

Scenario 1	Q1	Med	Q3	Scenario 2	Q1	Med	Q3	W	p-value
Neutral	3.328	3.470	3.643	Attempt	3.225	3.560	3.658	21.5	0.674
Neutral	3.328	3.470	3.643	Accident	3.000	3.345	3.735	26	0.293
Neutral	3.328	3.470	3.643	Intentional	3.140	3.595	3.953	21.5	0.674
Attempt	3.225	3.560	3.658	Accident	3.000	3.345	3.735	31.5	0.313
Attempt	3.225	3.560	3.658	Intentional	3.140	3.595	3.953	20	0.812
Accident	3.000	3.345	3.735	Intentional	3.140	3.595	3.953	8.5	0.207

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

Table 8. Differences between moral assessments according to the scenario type (patients). Each observation is the average of 16 measurements on a 7-point scale (1–7) representing the assessment of the permissibility of an action. The analysis employed Wilcoxon's test for paired samples

Scenario 1	Q1	Med	Q3	Scenario 2	Q1	Med	Q3	W	p-value
Neutral	4.293	5.095	5.363	Attempt	2.380	2.750	3.750	52.5	0.012
Neutral	4.293	5.095	5.363	Accident	2.033	2.815	3.420	55	0.002
Neutral	4.293	5.095	5.363	Intentional	1.190	1.500	2.203	55	0.002
Attempt	2.380	2.750	3.750	Accident	2.033	2.815	3.420	36	0.415
Attempt	2.380	2.750	3.750	Intentional	1.190	1.500	2.203	55	0.006
Accident	2.033	2.815	3.420	Intentional	1.190	1.500	2.203	40.5	0.038

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

of the protagonists in the stories was considered to be most acceptable in the neutral scenarios, in which negative intentions did not guide the protagonists, and their behavior was not a threat to the other person. Moreover, in both groups, the behavior in which the protagonist intentionally harms the other participant of a social event was judged to be the most unacceptable. While those diagnosed with schizophrenia gave statistically similar moral assessments of the actor's behavior in the "attempt" and "accident" scenarios, members of the control group judged attempt to harm another person more harshly than in situations in which harm resulted from an unintended act, i.e., an accident.

However, no significant differences were observed between the groups according to the level of metacognitive confidence (Table 5).

Table 9. Differences between level of metacognitive confidence according to the scenario type (controls). Each observation is the average of 16 measurements on a 4-point scale (1–4) representing the level of metacognitive confidence. The analysis employed Wilcoxon's test for paired samples

Scenario 1	Q1	Med	Q3	Scenario 2	Q1	Med	Q3	W	p
Neutral	3.515	3.630	3.735	Attempt	3.395	37595	3.810	13.5	1.000
Neutral	3.515	3.630	3.735	Accident	3.208	3.530	3.630	32.5	0.646
Neutral	3.515	3.630	3.735	Intentional	3.548	3.750	3.925	10	0.291
Attempt	3.395	3.595	3.810	Accident	3.208	3.530	3.630	25	0.813
Attempt	3.395	3.595	3.810	Intentional	3.548	3.750	3.925	11.5	0.114
Accident	3.208	3.530	3.630	Intentional	3.548	3.750	3.925	17	0.308

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

Table 10. Differences in moral assessments according to the scenario type (controls). Each observation represents the average of 16 measurements on a 7-point scale (1–7) representing the assessment of the permissibility of an action. The analysis employs Wilcoxon's test for paired samples

Scenario 1	Q1	Med	Q3	Scenario 2	Q1	Med	Q3	W	p
Neutral	4.660	5.160	6.113	Attempt	2.343	2.690	3.015	55	0.002
Neutral	4.660	5.160	6.113	Accident	2.440	3.815	4.390	55	0.002
Neutral	4.660	5.160	6.113	Intentional	1.515	1.625	2.143	55	0.002
Attempt	2.343	2.690	3.015	Accident	2.440	3.815	4.390	3	0.010
Attempt	2.343	2.690	3.015	Intentional	1.515	1.625	2.143	51	0.019
Accident	2.440	3.815	4.390	Intentional	1.515	1.625	2.143	55	0.038

Scale for the Assessment of Negative Symptoms (SANS). Q1 – 1st quartile; Q3 – 3rd quartile.

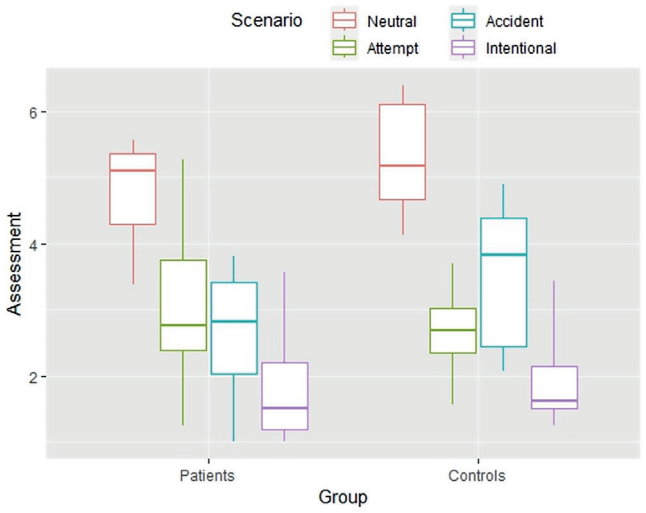

Fig. 3. Difference between groups in moral judgements according to scenario

Discussion

The results show a strong relationship between ToM and moral judgments. Empirical research has revealed the role of intention in moral judgments. For instance, the study by Cushman et al.[42] showed that the assessment of the harmfulness or permissibility of someone's behavior depended on the mental states of the observed study participants. Cushman proposed an explanation based on splitting moral judgments into 2 processes: the outcome of someone's action and the mental state that led to that action.

The patients made incorrect moral judgments about the characters' intentions, even though the actors in an incident showed a desire to hurt another person. The mild moral assessments by patients of the actors' behavior in the "attempt" scenarios probably result mainly from the lack of harmful effects that originate from the protagonists' actions. On the other hand, perceiving negative intentions can lead to the imputation of blame even in the absence of actual harm, similar to failed homicide attempts. Experiencing inappropriate desires is often enough to cause blame, even when causally detached from the harmful event.[43,44] We also blame people who benefit from someone else's misfortune, even if they are not the perpetrators themselves – we nevertheless consider such behavior to be morally inappropriate [43] Moral judgments are inherently related to ToM and many studies also point to neural correlates of the described relationship.[45,46]

The observer's consideration of the actor's lack of intention to harm the other person may lead the observer to assign less blame to the actor in an accident situation. In contrast, if the actor's behavior is driven by intentions to harm the other person, the observer may morally condemn the actor's behavior, even in a situation where there were no negative consequences for the other person. Research indicates that processes related to moral cognition mediate the relationship between the presence of specific psychotic symptoms and their significance for violence.[47]

The above data indicate a link between schizophrenia and a reduced ability to make appropriate moral judgments, resulting from a reduction in the ability to read the intentions of others, which is consistent with the findings

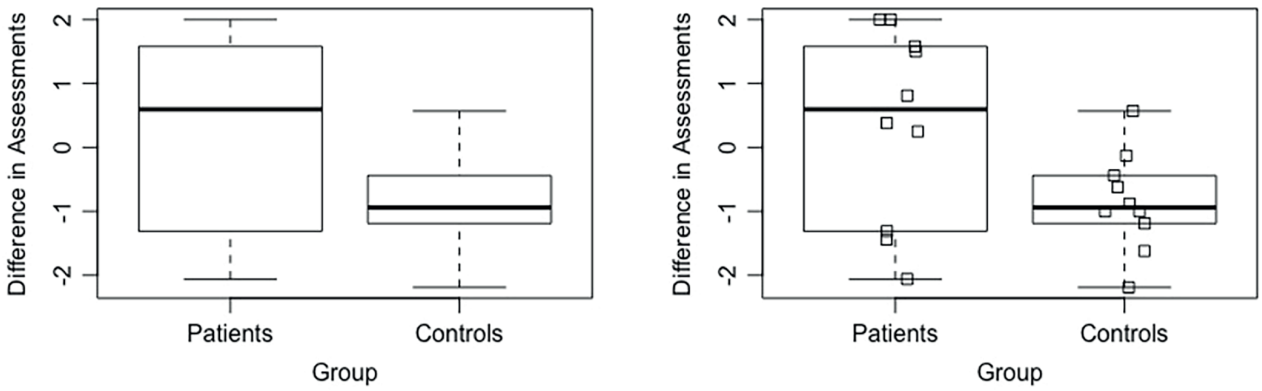

Fig. 4. Difference between groups in moral assessment of accidents and attempts

of the studies presented earlier.[19,20] The present results indicate a reduction in the level of moral cognition and its impact on the evaluation of the intentions and moral conduct of others.

Another mediator of the postulated relationships is social cognition, including mentalization skills, the dysfunctions of which will be reflected in moral judgments and thus in attitudes and behaviors, including aggressive ones.[48]

The present study also shows interesting results regarding the confidence expressed in moral judgments. The analysis showed no differences in metacognition for intergroup measurements (Fig. 3,4). The mean confidence level for both groups in each scenario is above 3 on a 4-point scale. This means that the respondents expressed almost complete confidence in their moral judgments. It also means that patients did not show any doubts about their assessment in those situations that mainly required understanding the intentions of others (in which they showed "blindness" to these intentions as opposed to healthy people). Although they assessed the behavior of the protagonist, who intentionally tried to hurt another person more leniently than those who harmed another by accident, they did not express any doubts about their judgment on a subjective scale of certainty. This means that there was a deficit at the level of reading intentions, which significantly influenced the moral judgments of patients and the metacognition component. This translated into the confidence with which the assessments were made and, thus, into their durability and consistency. In a situation where, despite a mild moral evaluation, someone expresses doubts about his assessment, it may mean that the assessor is reflecting whether the assessment should be harsher.

This type of research allows us to deepen the understanding of moral inference in people with schizophrenia, which may also allow for a better understanding of the mechanisms behind some behaviors, including violent ones. It is worth noting that people suffering from mental illness, particularly schizophrenia, are socially stigmatized due to common stereotypes about increased levels of aggressive behavior in this population.[49]

Limitations

The present work has limitations. Firstly, the patients found it difficult to read stories and make judgments. The task included 64 stories describing social situations. Additionally, the number of study participants was limited to 10 in both the patient and control groups due to the demanding nature of the task. To reduce cognitive load, it would be valuable to replicate the present study with fewer stimuli. This would allow for an increase in sample size.

Conclusions

Patients diagnosed with schizophrenia and experiencing severe symptoms during hospitalization tend to overlook the intentions of the actor towards the affected person when making moral evaluations of observed behavior in social situations. Limited moral reasoning can result from both positive and negative symptoms, and deficits related to metacognition can further sustain such cognitive distortions.

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Disability and emotional symptoms in women with lipedema: A comparison with overweight/obese women

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Abstract

Background. Lipedema is characterized by the painful abnormal deposition of adipose tissue in the lower limbs and is often misdiagnosed as obesity. Considering the numerous bothersome physical symptoms of lipedema, women with lipedema may have greater disability and emotional problems than women with lifestyle-induced obesity.

Objectives. Our study aims to assess disability, anxiety and depression symptoms in women with lipedema compared to women with overweight/obesity.

Materials and methods. Women with lipedema (n = 45, with a mean age of 41 years) and women who are overweight/obese (n = 43, with a mean age of 44.95 years) were asked to complete the following questionnaires: The World Health Organization Disability Assessment Schedule (WHO-DAS II), Beck's Depression Inventory – II (BDI-II), and The Hospital Anxiety and Depression Scale (HADS).

Results. Despite the higher BMI in the overweight/obesity group, the group with lipedema was more disabled in numerous domains of the WHO-DAS II questionnaire, including Life activities – domestic, work and school responsibilities and Participation in society When the influence of BMI was adjusted, a difference in the domain of Mobility was also present. The study groups did not differ in anxiety and depression symptoms.

Conclusions. We showed that behavioral impairment was the main factor affecting functioning in women with lipedema. Emotional symptoms did not differentiate the study groups. Leg volumes and adipose tissue pain intensity were associated with greater disability in women with lipedema, and should be considered in managing women with this condition and in future research estimating the effectiveness of lipedema treatment.

Key words: lipedema, obesity, disability, depression, adipose tissue

Background

Lipedema is a chronic and progressive condition that gradually leads to a disproportionate body. It occurs almost exclusively in women and is characterized by the painful abnormal deposition of adipose tissue in the lower extremities and, in $^1/_3$ of cases, also within the arms. The term "lipedema" was initially coined by Hines and Allen in 1940.[1] Women with lipedema typically have characteristic bilateral and symmetrical "column" legs with sparing of the feet with a cuff sign at the ankle and masses of nodular fat within the adipose tissue in later stages of the disease. In most cases, lipedema is accompanied by pressure-induced or spontaneous pain in the affected areas of adipose tissue and a tendency for easy bruising.[2–4]

The incidence of lipedema was estimated at 11%.[2,5] There are no known biomarkers for diagnosing lipedema. Therefore, the recognition of lipedema is clinical and based on established criteria.[5,6]

The etiology of lipedema is not well understood. The onset of lipedema is usually during hormonal changes, such as puberty, pregnancy or menopause. This suggests a link with female sex hormone fluctuations.[7] Genetic inheritance is also possible.[2,5] Women with lipedema often have first-line women relatives with lipedema.[8]

Histological examination of adipose tissue demonstrated that lipedema results from both adipocyte hypertrophy and hyperplasia.[9] Excessive accumulation of adipose cells probably leads to hypoxia, resulting in adipocyte necrosis and macrophage recruitment.[10] Moreover, adipose tissue in lipedema compared to control was demonstrated to be associated with greater fibrosis,[9,11] inflammation, angiogenesis, and microangiopathy.[12]

Lipedema is a relatively common disease, but is still underrecognized and often misdiagnosed by clinicians as lifestyle-induced obesity.[13] As a result, patients are recommended to follow a low-calorie diet and increase physical activity.[14] However, this treatment generally has little to no effect on leg volumes.[11] Even bariatric surgery does not significantly decrease the areas affected by lipedema despite weight reduction.[15] The lack of a diagnosis can make the patients feel helpless.[16] This contributes in a very significant way to the deterioration of the mental state of patients and to further psychological consequences, such as self-esteem issues, anorexia nervosa, bulimia, depression, self-harm, and suicidal thoughts.[13,14,16,17] The chronic pain of adipose tissue, easy bruising, and complications or comorbidities of lipedema such as obesity, secondary lymphedema (lipolymphedema), joint degeneration and, in extreme cases, mobility difficulties may also affect the mental state of the patient. According to the findings of the study conducted by Erbacher and Bertsch in a 150-person group of women with lipedema, 36.7% presented at least 1 symptom of psychological disorder, with depression and anxiety in 26% and 3.3% of patients, respectively.[18] The incidence of depression in women with lipedema in other studies was estimated to be 31–59%.[19,20] Each of these aspects contributes to a poor quality of life (QoL) in patients with lipedema, as was demonstrated in numerous studies.[19,21–23] Moreover, depressive and anxiety symptoms often coexist, resulting in more severe impairment.[24]

In light of recent findings, there might be a biochemical connection between neural and endocrine mechanisms associated with the adipose tissue and emotional state of women with lipedema. The adipose tissue is rich in nociceptive neurons,[25] and it was indicated that chronic pain may result in changes in brain activity involved in emotional regulation.[26,27] Chronic pain as a repeating negative stimulus on the central nervous system may also lead to the generation of neurovisceral hyperactivity.[28] It was also demonstrated that adipose tissue is an active endocrine organ capable of producing various signaling molecules, including cytokines and proinflammatory hormones – the adipokines, such as leptin, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, resistin, and visfatin, in addition to many others.[29] These molecules can have an effect both locally and throughout the body, with a potentially wide range of impact.[29] Considering that depression and anxiety can be caused by neural mechanisms associated with signaling molecules,[30] adipose tissue potentially might directly impact a person's emotional state. Interestingly, neuroimaging and brain stimulation represent a promising new approach to diagnosing and individualizing the treatment of emotional disorders in the future.[31] A recently published study on mice revealed that essential oils could benefit neuroprotection and decrease depressive symptoms.[32] So far, no studies have evaluated the use of essential oils in treating lipedema. However, it should be noted that the low-carbohydrate high-fat diet (LCHF), which is rich in vegetable oils, has been shown to not only decrease leg volume in lipedema but also alleviate pain in adipose tissue.[33,34] Therefore, the addition of further essential oils may enhance the effects of the LCHF diet. Searching for new methods of treating lipedema is especially desirable since there is no cure for this entity.

Lipedema management is still symptom-based and aims to reduce patient discomfort, disability and disease progression.[11] Treatment includes a conservative approach and surgical interventions. The conservative treatment consists of a diet, especially a LCHF diet,[33,34] physical activity, complex decongestive therapy, and intermittent pneumatic compression.[11,35–37] Surgical treatment involves the removal of excess fatty tissue through liposuction.[38,39]

The co-occurrence of lipedema and obesity and the influence of one on the deterioration of the other has long been known.[40] Increased body weight is also associated with disability, depression and reduced QoL.[41–43] However, to our knowledge, no study has compared a woman's physical and emotional status with lipedema to those with lifestyle-induced obesity.

Objectives

Our study was designed to assess the differences in disability, anxiety and depression symptoms in women with lipedema compared to women with lifestyle-induced overweight/obesity. Considering the numerous bothersome physical symptoms of lipedema (above all, body disproportion and pain in the affected areas of adipose tissue), we hypothesized that women with lipedema may have a greater disability in many life areas and emotional problems compared to women with lifestyle-induced overweight/obesity.

Materials and methods

Study design

Our study was a part of the project in which we implemented the LCHF diet for 7 months in women with lipedema and women with lifestyle-induced overweight/obesity to assess the diet's laboratory and clinical (both physical and emotional) effects. Most of the project results are yet to be published, except for 1 study on the lymphoscintigraphic alterations in the lower limbs[44] and 1 paper on the impact of the LCHF diet on blood parameters.[34]

In this study, women diagnosed with lipedema (n = 45, with a mean age of 41.00 ±13.32 years) and women with lifestyle-induced overweight/obesity (n = 43, with a mean age of 44.95 ±13.90 years) who agreed to complete self-administered questionnaires evaluating disability, anxiety and depression symptoms were included. The women in both study groups were recruited from January 2021 to May 2022, and each woman's participation in the study was voluntary. The woman's consent to use the LCHF diet was not an inclusion criterion for this study. The LCHF diet was not implemented by 11 women (24%) in the group with lipedema and by 13 women (30%) in the overweight/obese group. The questionnaires were completed before starting the LCHF diet.

Women with lipedema were the patients from the Outpatient Angiology Clinic. Lipedema was recognized in women by an angiologist, based on established clinical criteria of lipedema,[1] and was classified into 4 clinical stages and 5 types of disease.[5,11,45]

The overweight/obese women were, in part, patients from the Outpatient Angiology Clinic, but the majority were volunteer employees of the hospital where the study was conducted.

The patients in both study groups were assessed in medical and demographical aspects and completed self-administered questionnaires. Women with lipedema were additionally asked about the intensity of pain within the lower legs on a graduated scale from 0 (no pain at all) to 10 (full of pain).[26]

Ethical approval was provided by the Local Bioethical Committee (approval No. KB-690/2017). All women gave informed written consent before inclusion in the study, following the principles outlined in the Declaration of Helsinki.

Inclusion and exclusion criteria

Diagnosis of lipedema was stated according to the criteria established by Wold et al.[1] The minimal inclusion criteria to fulfill for the group with lipedema was a body disproportion, i.e., excessive accumulation of adipose tissue in the lower limbs compared to the upper body and the presence of at least 1 of 2 clinical symptoms: 1) pressure-induced or spontaneous pain of adipose tissue and 2) tendency for easily bruising.

The inclusion criterion for the overweight/obesity group was a body mass index (BMI) above 25 kg/m^2. The exclusion criteria for both study groups were the presence of lymphedema, edema in the course of other diseases, such as chronic venous insufficiency, heart failure, chronic kidney disease and hepatic insufficiency, abnormal thyroid-stimulating hormone (TSH) level, diabetes, depression, neoplasms, pregnancy, a period of at least 6 months after pregnancy, and diagnosis of depression or anxiety in the past.

Questionnaires

The self-administered questionnaires listed below were used to assess disability, anxiety and depression symptoms in both study groups.

WHO-DAS II

The World Health Organization's Disability Assessment Scale II (WHO-DAS II) is a generic assessment instrument developed by the WHO to provide a standardized method for measuring health and disability.[46] Importantly for our study, the questionnaire provides a metric of the impact of any health condition in terms of disability related to many life areas and interactions between the person and the environment.[46]

The WHO-DAS II questionnaire covers 6 disability domains: 1) Cognition - understanding and communicating (6 questions), 2) Mobility – moving and getting around (5 questions), 3) Self-care – attending to one's hygiene, dressing, eating and staying alone (4 questions), 4) Getting along – interacting with other people (5 questions), 5) Life activities – domestic, work and school responsibilities (8 questions), and 6) Participation-joining in community activities, participating in society (8 questions). In addition, the WHO-DAS II questionnaire includes a question about the subjective assessment of health (domain H1) and questions about the impact of disability on everyday activity (domains H2–H5). The questionnaire evaluates the last 30 days preceding the survey. The questionnaire can be self-administered or conducted by an interviewer. It comprises 36 items and uses a 5-point Likert scale for responses, ranging from "none" (0 points) to "extreme" (4 points).[46] The results of the domains were summed up and converted into a percentage value of disability for each domain separately, according to the formulas given below:

Domain 1 = (D1.1+D1.2+D1.3+D1.4+D1.5+D1.6)/24*100
Domain 2 = (D2.1+D2.2+D2.3+D2.4+D2.5)/20*100
Domain 3 = (D3.1+D3.2+D3.3+D3.4)/16*100
Domain 4 = (D4.1+D4.2+D4.3+D4.4+D4.5)/20*100
Domain 5.1 = (D5.1+D5.2+D5.3+D5.4)/16*100
Domain 5.2 = (D5.5+D5.6+D5.7+D5.8)/16*100
Domain 6 = (D6.1+D6.2+D6.3+D6.4+D6.5+D6.6+D6.7+D6.8)/32*100

The higher calculated scores indicate a more elevated level of disability in each domain. The questions H1 and H2 were calculated directly according to the 5-point Likert-type scale of answers, as written above (0–4 points for each question). The answers to the questions H3–H5 represented a given number of days and constituted a direct value in the calculations.

Beck Depression Inventory-II

Beck Depression Inventory-II (BDI-II) is one of the most widely used psychometric questionnaires designed to measure depression symptoms and their severity in persons aged ≥13 years.[47,48] The questions are related to cognitive, somatic, affective, and vegetative symptoms of depression in the past 2 weeks. Each item has a set of 4 responses, ranging in intensity.[47,48]

Beck Depression Inventory-II is a self-report and consists of 21 questions that must be answered on a scale from 0 (not at all) to 3 (very much). The number of points for all answers should be added, and the total score is used to determine the severity of depression symptoms.[47,48]

The Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is a questionnaire commonly used by doctors in a variety of settings, including primary care, hospital and psychiatry. It was designed to initially identify patients with anxious and depressive states who need further psychiatric evaluation.

The Hospital Anxiety and Depression Scale is a self-report questionnaire consisting of 14 items, divided into 2 subscales: HADS-A (anxiety) and HADS-D (depression), with a set of 4 responses for each question ranging from 0 (no impairment) to 3 (severe impairment). The questionnaire estimates the past week. The total score ranges from 0–21 for anxiety and 0–21 for depression, with a greater score indicating more severe symptoms. According to Pais–Ribeiro et al., the interpretation of scores 0–7 represents "normal', 8–10 – "mild", 11–14 – "moderate", and 15–21 "severe" symptoms.[49] The HADS does not include somatic symptoms of emotional distress that may be caused by the illness itself.[50]

Statistical analyses

Results are presented as mean values ± standard deviations (M ±SD) or median and quartiles Q1 and Q3 when the data distribution was normal or non-normal, respectively. The conformity of the distribution in the given variable to the normal distribution was verified using the Shapiro–Wilk test and homogeneity of variances with Levene's test (Supplementary Table 1). If the distribution was statistically significantly different from normal, a non-parametric Mann–Whitney U test was applied, otherwise – the student's t-test. For categorical variables, Fisher's exact test was used when the variable had only 2 categories and the χ^2 test when there were more categories for the variable. As the distribution of many variables differs from a normal one, a robust analysis of covariance (ANCOVA) test (using a function 'rlm' from the R-package "MASS") was used to compare the results of WHO-DAS II, BDI-II and HADS questionnaires between the study groups adjusted for the influence of BMI. For the same reason, the relationships between variables from the questionnaires and leg volume and adipose tissue pain intensity (both defined as predictors) were analyzed using the 'rlm' function for each parameter from the questionnaire separately. As the 'rlm' is resistant to skewed data distribution and the presence of outliers, no assumptions were checked. Differences were considered statistically significant when $p < 0.05$.

All statistical analyses were performed using GraphPad Prism 9 for Windows (GraphPad Software, Inc., San Diego, USA), Statistica v. 13 (TIBCO Software Inc., Palo Alto, USA) and using the R-package "MASS" (Venables and Ripley 2002; Springer, New York, USA) in the R-environment 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Medical and demographic parameters

The basic medical and demographic characteristics of the study participants are presented in Table 1. The study groups did not differ statistically significantly in age. However, the overweight/obesity group had higher weight, BMI, waist circumferences, and waist-to-hip ratio (WHR).

Differences in the parameters from the questionnaires between the study groups

The WHO-DAS II questionnaire was filled in by all the study participants, which means that there were no missing data. The BDI-II questionnaire was not completed by 2 women from the lipedema group (4.4%) and 1 woman from the overweight/obesity group (2.3%). The HADS questionnaire was not filled in by 2 women from the overweight/obesity group (4.7%). The reason for not completing all the questionnaires by the study participants was probably a lack of time, absent-mindedness or fatigue with filling in many forms. The data were missing completely

Table 1. Medical and demographic characteristics of the study participants

Parameter		Lipedema group (n = 45)	Overweight/ obesity group (n = 43)	Statistics	
				Z/X^2/t; df	p-value
Age [median (Q1, Q3)]		38.00 (31.50, 49.50)	45.00 (33.00, 54.00)	–1.56	0.120*
Number of years spent studying at school, college or university [median (Q1, Q3)]		17.00 (14.00, 19.00)	17.00 (14.00, 19.00)	–0.19	0.856*
Current marital status	single	15 (33.33%)	10 (23.26%)	5.50; 5.00	0.358**
	currently married	17 (37.77%)	21 (48.84%)		
	in separation	0	1 (2.33%)		
	divorced	4 (8.89%)	7 (16.28%)		
	widowed	4 (8.89%)	1 (2.22%)		
	cohabiting with a partner	5 (11.11%)	3 (6.98%)		
Current main work status	employed	25 (55.56%)	33 (73.33%)	9.37; 7.00	0.227**
	own business	4 (8.89%)	4 (8.89%)		
	voluntary work	2 (4.45%)	0		
	student	3 (6.67%)	2 (4.44%)		
	home maker	2 (4.45%)	0		
	retired	5 (11.11%)	4 (8.89%)		
	disabled	0	0		
	no occupation	1 (2.22%)	0		
	other	3 (6.67%)	0		
Height [cm], M ±SD		166.28 ±7.32	165.5 ±6.15	0.51; 86.00	0.614***
Weight [kg], M ±SD		84.08 ±16.73	94.57 ±16.39	2.97; 86.00	**0.004***
BMI [kg/m^2], M ±SD		30.53 ±6.24	34.41 ±5.05	3.19; 86.00	**0.002***
Age at the onset of lipedema/obesity [median years (Q1, Q3)]		23.00 (15.00, 33.50)	28.00 (12.00, 39.00)	0.00	1.000*
Arterial hypertension, yes (%)		9 (20.0%)	11 (25.58%)	–	0.615****
Insulin resistance, yes (%)		11 (24.44%)	16 (37.21%)	–	0.249****
Hypothyroidism (compensated with supplementation, yes (%)		8 (17.78%)	10 (23.26%)	–	0.602****
BMI [kg/m^2]	normal	11 (24.44%)	0	14.38; 4.00	**0.006****
	overweight	8 (17.78%)	10 (23.26%)		
	1st class obesity	17 (37.78%)	15 (34.89%)		
	2nd class obesity	5 (11.11%)	11 (25.58%)		
	3rd class obesity	4 (8.89%)	7 (16.28%)		
	extreme obesity (>50 kg/m^2)	0	0		
Waist circumference [cm], M ±SD		95.33 ±12.86	108.48 ±11.07	5.13; 86.00	**<0.001***
Hip circumference [cm], M ±SD		113.41 ±11.36	115.4 ±11.15	0.83; 86.00	0.411***
WHR (mean waist-to-hip ratio)		0.8403 ±0.08	0.9423 ±0.08	6.28; 86.00	**<0.001***
Legs volume [median mL (Q1, Q3)]	right leg	12204 (10448, 14785)	11690 (9639, 13263)	–1.43	0.155*
	left leg	12541 (10613, 14725)	11742 (9767, 13054)	–1.77	0.078*
	mean volume of both legs	12428 (10492, 14806)	11735 (9486, 13315)	–1.55	0.123*
Stage of lipedema	I	22 (48.89%)	–	–	–
	II	21 (46.67%)			
	III	2 (4.44%)			
Type of lipedema	1 (buttocks)	0	–	–	–
	2 (buttocks, hips and thighs)	6 (13.33%)			
	3 (from hips to ankles)	21 (46.67%)			
	4 (arms and legs)	10 (22.22%)			
	5 (calves)	0			
Family history of lipedema, yes (%)		32 (71.11%)	–	–	–
Pain of adipose tissue (spontaneous or on pressure, yes (%)		41 (91.11%)	0	–	**<0.001****
Intensity of pain of adipose tissue (from 1 (minimal pain) to 10 (maximal pain) M ±SD		4.64 ±2.69	–	–	–
Easy bruising, yes (%)		44 (97.78%)	11 (24.44%)	–	**<0.001****

Statistically significant results ($p < 0.05$) are in bold; *Mann–Whitney U test; **χ^2 test; ***student's t-test; ****Fisher's exact test. M ±SD – mean ± standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile.

at random and consequently did not affect the obtained results.

There were statistically significant differences between the lipedema group and the overweight/obesity group in the WHO-DAS II questionnaire regarding domain 5.1 (Life activities – domestic responsibilities, leisure), 5.2. (Life activities – work and school), 6 (Participation – joining in community activities, participating in society), H2 (the impact of difficulties DAS1-DAS6 on life), and H3 (number of days in the previous 30 days in which difficulties DAS1-DAS6 were present).

Since the groups differed regarding BMI, the calculations were done after adjusting the effect of BMI. With the BMI adjustment, the differences between the groups in domain 2 (Mobility – moving and getting around), 5.1 (Life activities – domestic responsibilities, leisure), 6 (Participation – joining in community activities, participating in society), H2 (the impact of difficulties DAS1-DAS6 on life), and H3 (number of days in the previous 30 days in which difficulties DAS1-DAS6 were present) could be observed. The difference between the study groups in domain 5.2. (Life activities – work and school) after adjusting for the effect of BMI was close to statistical significance (p = 0.071; Robust ANCOVA test).

The study groups did not differ in anxiety and depression symptoms evaluated with the BDI-II and HADS questionnaires. Study group comparisons with the questionnaires used in the study are presented in Table 2.

Table 2. Study groups comparison with questionnaires WHO-DAS II, BDI-II and HADS

Scale		Lipedema group (n = 45)		Overweight/obesity group (n = 43)		Statistics		Statistics adjusted for the influence of BMI***	
		Me (Q1, Q3)/ M ±SD	Std. err.	Me (Q1, Q3)/ M ±SD	Std. err.	Z/t; df	p-value	Z	p-value
Domain 1	Cognition – understanding and communicating	12.50 (4.17, 25.00)	1.90	8.33 (0.00, 25.00)	2.29	–1.22	0.224*	1.48	0.139
Domain 2	Mobility – moving and getting around	15.00 (0.00, 32.50)	2.78	5.00 (0.00, 20.00)	2.64	–1.71	0.088*	2.58	**0.010**
Domain 3	Self-care – attending to one's hygiene, dressing, eating and staying alone	0.00 (0.00, 6.25)	1.39	0.00 (0.00, 6.25)	0.96	–0.04	0.969*	0.13	0.898
Domain 4	Getting along – interacting with other people	10.00 (5.00, 22.50)	2.28	0.00 (5.00, 20.00)	2.32	–1.66	0.097*	1.48	0.138
Domain 5.1	Life activities – domestic responsibilities	18.75 (0.00, 28.13)	3.34	0.00 (0.00, 18.75)	3.10	2.01	**0.044***	2.54	**0.011**
Domain 5.2	Life activities – work and school	12.50 (0.00, 25.00)	3.42	0.00 (0.00, 18.75)	2.86	–1.99	**0.047***	1.81	0.071
Domain 6	Participation in society – joining in community activities	21.88 (12.50, 31.25)	2.22	12.50 (3.13, 21.88)	1.81	–3.18	**0.001***	3.15	**0.002**
Domain H1	Overall health assessment in the last 30 days	1.00 (1.00, 2.00)	0.14	1.00 (1.00, 2.00)	0.10	–0.05	0.966*	0.54	0.586
Domain H2	The impact of difficulties DAS1–DAS6 on life	1.00 (1.00, 2.00)	0.14	1.00 (0.00, 1.00)	0.14	–3.24	**0.001***	2.98	**0.003**
Domain H3	Number of days in the past 30 days in which difficulties DAS1–DAS6 were present	5.00 (4.50, 17.50)	1.55	0.00 (0.00, 4.00)	1.30	–4.30	**<0.001***	4.13	**<0.001**
Domain H4	Number of days in the past 30 days in which there was a total inability to carry out usual activities or work because of any health condition	0.00 (0.00, 1.50)	0.44	0.00 (0.00, 0.00)	0.64	–0.96	0.342*	1.14	0.254
Domain H5	Number of days in the past 30 days in which there was a reduction in usual activities or work because of any health condition	0.00 (0.00, 4.50)	1.04	0.00 (0.00, 2.00)	0.85	–1.23	0.222*	1.52	0.128
BDI-II		11.00 (7.00, 16.00)	1.02	8.00 (3.00, 14.25)	1.17	–1.52	0.130*	1.51	0.132
HADS-A		9.16 ±3.59	0.53	8.10 ±3.35	0.52	1.41; 84	0.162**	0.98	0.327
HADS-D		5.00 (3.00, 8.00)	0.50	6.00 (3.00, 8.00)	0.48	–0.72	0.474*	0.04	0.970

*Mann–Whitney U test; **student's t-test; ***robust analysis of covariance (ANCOVA) test (see the "Statistical Analysis" section for detail). Statistically significant results (p < 0.05) are in bold. Missing data: in WHO-DAS II: no missing data; in BDI-II: in the lipedema group – 4.4%, in the overweight/obesity group – 2.3%; in HADS-A and HADS-D: in the lipedema group – no missing data, in the overweight/obesity group – 4.7%. M ±SD – mean ± standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile.

Statistically significant relationships in the lipedema group

Statistically significant relations between leg volume/pain intensity and parameters from the questionnaires used in the study of the group with lipedema were as follows: 1) between leg volumes and domain 3 (Self-care – attending to one's hygiene, dressing, eating and staying alone), 5.1 (Life activities – domestic responsibilities), 5.2 (Life activities – work and school), H1 (Overall health assessment in the last 30 days), and H5 (Number of days in the past 30 days in which there was a reduction in usual activities or work because of any health condition); 2) between pain intensity and domain 2 (Mobility – moving and getting around) and H5 (Number of days in the past 30 days in which there was a reduction in usual activities or work because of any health condition). Relationships between leg volume and pain intensity and parameters from the questionnaires in the group with lipedema are presented in Table 3.

Discussion

Our study is the first to compare disability, anxiety and depression symptoms in women with lipedema to women with lifestyle-induced overweight/obesity.

Despite the lower BMI in the lipedema group than in the overweight/obesity group, the lipedema group reported worse functioning in numerous domains of the WHO-DAS II questionnaire, including domestic, work and school responsibilities and participation in society. Both groups did not differ statistically significantly in depression or anxiety symptoms. After adjusting for BMI, the differences between the study groups were observed in domestic responsibilities, participation in society and mobility. Therefore, our study strongly indicates that lipedema is a much more disabling condition than overweight/obesity. However, obesity is also a limiting condition in many aspects of life. In the study of Sirtori et al., BMI values in patients with obesity have been shown to correlate with the severity of public distress as measured by the impact of weight on the QoL (IWQoL-Lite) questionnaire and, to a lesser extent, with the participation domain of the WHO-DAS II questionnaire.[43]

Appropriate physical activity and diet are essential medical recommendations in lipedema treatment.[11] However, in simple overweight/obesity, a reduced diet usually results in weight loss. In lipedema, more aggressive management is needed to decrease leg volumes, including using a ketogenic diet[33,34] or liposuction.[38,39]

The disproportionate distribution of fat between the upper and lower body, as seen in lipedema, can cause sensations of heaviness, fatigue and discomfort in the lower limbs. Most women with lipedema also suffer from pressure-induced or spontaneous pain in the affected adipose tissue areas.[2–4] Understandably, such legs can cause difficulty in standing, moving and walking long distances. It was evidenced in our study by the difference in scores of domain 2 in the WHO-DAS II questionnaire (Mobility – moving and getting around) between the lipedema and the overweight/obesity groups after adjusting for BMI. Mobility difficulties may explain the reduced ability to perform domestic, work and school responsibilities in women with lipedema compared to women with overweight/obesity, as was also shown in our study (difference in the domain 5.1 and 5.2 scores of the WHO-DAS II questionnaire).

The recommendation to increase physical activity in the treatment of lipedema in many women with severe lipedema may be challenging to implement. Moreover, many women with lipedema also suffer from orthostatic edema, which worsens during warm weather and exercise.[51] Therefore, when recommending increased physical activity, the type of physical activity should be adapted to the degree of edema.[11] Compression therapy is effective in preventing orthostatic edema and should be recommended to be worn during physical activity.[11]

The worse functioning of women with lipedema in domain 6 of the WHO-DAS II questionnaire (Participation in society) and also in 5.2 (Life activities – work and school) compared to the overweight/obesity group may indicate that women with lipedema do not accept their appearance and disease. Our results may also suggest that women with lipedema do experience a lack of acceptance and understanding from other people, including medical personnel.[14] Despite the growing awareness of lipedema diagnosis in society and among doctors, patients with lipedema still face a lack of understanding. They are often unfairly accused of being lazy in their efforts to achieve a healthy body weight. A study by Dudek et al. indicates that the appearance-related distress of patients with lipedema contributes to a deterioration in QoL.[52] In the other research by Dudek et al., it was demonstrated that women who were more open to experiencing both pleasant and painful emotions and were more engaged in their lives reported higher QoL scores.[22] Therefore, it seems that in caring for women with lipedema, it is essential to pay attention to how much the disease isolates an individual patient from society. It can be assumed that social isolation not only decreases the QoL of women with lipedema but also worsens the course of the disease in the aspects of self-care (including body weight), as well as compliance with medical recommendations, i.e., proper diet maintenance and regular use of compression therapy.

As we expected, women with lipedema and a greater volume of legs and adipose tissue pain were more disabled. Leg volume was associated with worse physical functioning, less self-care, and disability in domestic and professional activity. Adipose tissue pain was associated with greater difficulties in mobility. Previous studies demonstrated that the pain of adipose tissue is the main parameter affecting QoL in women with lipedema.[3,19] The etiology of pain in lipedema is unclear. Histological studies suggest that

Table 3. Robust multivariable linear models of relationships between variables from questionnaires and their predictors (legs volume and adipose tissue pain intensity) in the group with lipedema

Explained variable		Predictor	Coeff.	SE	Z	p-value
Domain 1	Cognition – understanding and communicating	intercept	0.07	7.31	0.01	0.992
		leg volume	0.00	0.00	1.52	0.128
		pain intensity	0.96	0.74	1.31	0.191
Domain 2	Mobility – moving and getting around	intercept	–12.27	8.32	–1.47	0.141
		leg volume	0.00	0.00	1.76	0.078
		pain intensity	3.75	0.84	4.47	**<0.001**
Domain 3	Self-care – attending to one's hygiene, dressing, eating and staying alone	intercept	–6.87	2.47	–2.78	0.005
		leg volume	0.00	0.00	3.29	**0.001**
		pain intensity	0.40	0.25	1.61	0.108
Domain 4	Getting along – interacting with other people	intercept	4.07	7.51	0.54	0.588
		leg volume	0.00	0.00	1.11	0.268
		pain intensity	0.14	0.76	0.18	0.856
Domain 5.1	Life activities – domestic responsibilities	intercept	–15.64	10.47	–1.49	0.135
		leg volume	0.00	0.00	2.65	**0.008**
		pain intensity	1.95	1.05	1.85	0.064
Domain 5.2	Life activities – work and school	intercept	–17.76	10.51	–1.69	0.091
		leg volume	0.00	0.00	2.63	**0.009**
		pain intensity	2.02	1.06	1.91	0.056
Domain 6	Participation in society – joining in community activities	intercept	8.82	8.49	1.04	0.299
		leg volume	0.00	0.00	1.20	0.229
		pain intensity	1.07	0.85	1.25	0.212
Domain H1	Overall health assessment in the last 30 days	intercept	0.02	0.51	0.04	0.971
		leg volume	0.00	0.00	2.31	**0.021**
		pain intensity	0.07	0.05	1.46	0.145
Domain H2	The impact of difficulties DAS1–DAS6 on life	intercept	0.42	0.46	0.91	0.365
		leg volume	0.00	0.00	1.75	0.080
		pain intensity	0.05	0.05	1.11	0.268
Domain H3	Number of days in the past 30 days in which difficulties DAS1–DAS6 were present	intercept	1.09	5.85	0.19	0.853
		leg volume	0.00	0.00	1.07	0.284
		pain intensity	0.69	0.59	1.18	0.239
Domain H4	Number of days in the past 30 days in which there was a total inability to carry out usual activities or work because of any health condition	intercept	0.00	0.00	–0.48	0.631
		leg volume	0.00	0.00	0.91	0.361
		pain intensity	0.00	0.00	0.85	0.398
Domain H5	Number of days in the past 30 days in which there was a reduction in usual activities or work because of any health condition	Intercept	–4.50	1.82	–2.48	0.013
		leg volume	0.00	0.00	2.91	**0.004**
		pain intensity	0.42	0.18	2.31	**0.021**
BDI-II		intercept	7.13	3.67	1.94	0.052
		leg volume	0.00	0.00	0.68	0.496
		pain intensity	0.27	0.37	0.73	0.467
HADS-A		intercept	11.88	2.20	5.40	<0.001
		leg volume	0.00	0.00	-1.14	0.254
		pain intensity	-0.08	0.22	-0.38	0.702
HADS-D		intercept	1.11	1.95	0.57	0.570
		leg volume	0.00	0.00	1.85	0.065
		pain intensity	0.18	0.20	0.90	0.366

Statistically significant results ($p < 0.05$) are in bold. Coeff. – coefficient; SE – standard error of the coefficient; Z – test value.

the inflammatory process and hypoxia may be responsible for this symptom.[11] Another potential mechanism involves nerve compression in the septa, surrounding the growing fatty lobules within adipose tissue.[53]

However, while the volume of lipedema and the pain intensity influenced daily functioning in our study, surprisingly, they did not affect the severity of depressive and anxiety symptoms. Similarly, the lower limbs' circumference and pain intensity were not associated with the severity of emotional symptoms. These consistent findings suggest that behavioral impairment is the primary factor influencing functioning in women with lipedema compared to women with overweight/obesity, and emotional symptoms do not play a significant role. However, these results are puzzling, as other studies have shown significantly higher levels of depressive and anxiety symptoms in groups of patients with lipedema.[18–20]

Considering that no published study assessing depressive and anxiety symptoms in women with lipedema has used the HADS or BDI-II questionnaire, it is impossible to directly compare the results of our research with those from other centers. Nevertheless, Dudek et al. demonstrated that more than half of the women with lipedema (59.2%) reported a heightened level of depressive symptoms,[21] and the greater intensity of depressive symptoms had a significant impact on women's QoL.[52] Al-Wardat et al. showed that women with lipedema had significant difficulties with emotion regulation associated with anxiety symptoms.[54] Clarke et al. revealed that women with lipedema in stages 3–4 compared to stages 1–2 were more likely to report depression, emotional lability, eating disorders, as well as feeling lonelier, more fearful, more likely to stay at home and less likely to have visited a psychologist.[55] Similarly, in the study by Erbacher and Bertsch, the percentage of women with lipedema and mental health disorders (such as depression, anxiety disorders, eating disorders, or post-traumatic stress disorder (PTSD)) was significantly greater in the subgroup with a BMI ≥ 40 kg/m^2 than with a BMI < 40 kg/m^2 (49.3% compared to 25.9%, respectively).[18]

The lack of difference between our study groups in severity of anxiety and depressive symptoms can be explained by the fact that in the previous studies, the lipedema group was only compared to healthy individuals,[54] while our results demonstrate the comparison of lipedema to lifestyle-induced overweight/obesity group. Moreover, the majority of women in our lipedema group (96%) were in the 1st and 2nd stages of the disease. It might also be suspected that the long-term lack of diagnosis of enlarged, heavy and painful legs may result in the symptoms of depression and anxiety. Women with lipedema in our study had the diagnosis of lipedema before participation in this study and most of them hoped that the condition of their legs would improve after implementing the LCHF diet. Additionally, most women in the study groups who decided to participate in our study were ready to follow a rigorous LCHF diet. The hypothetical presence of depression or anxiety symptoms might be associated with reduced motivation and might make it difficult to take on such a challenge. Therefore, perhaps the planned intervention in our study prompted to participate in our research women who did not have symptoms of depression or anxiety.

Our findings have not only theoretical but also practical implications for women with lipedema for healthcare providers. Leg volumes and adipose tissue pain intensity were the factors associated with worse functioning of women with lipedema, and they should be taken into account in the management of women with this condition. The experience of the authors of this publication indicates the high effectiveness of the LCHF diet in reducing not only BMI but also leg volumes and pain of adipose tissue,[33,34] i.e., the impact of the LCHF diet on parameters in our study that were demonstrated to be disabling to the functioning of women with lipedema. Complete decongestive therapy (CDT) and physical exercises have also been demonstrated to significantly improve leg volume, alleviate pain and positively affect function of patients with lipedema.[56] Another treatment of proven effectiveness in this regard is liposuction.[57,58]

Leg volumes and adipose tissue pain intensity should also be estimated in further regular control visits of women with lipedema after intensive treatment. Maintaining body weight and leg volume in the long term may be difficult for women with lipedema. Therefore, it is necessary to educate and motivate them to undertake appropriate diet and physical activity.

Limitations

The limitations of this study may result from the difficulty in distinguishing lipedema from obesity, especially in patients with poorly expressed symptoms of lipedema or with greater obesity. However, the group selection criteria used in our study and the examination of all women by angiologists experienced in the diagnosis and treatment of women with lipedema significantly reduced the possibility of incorrect classification into the study groups.

It is also possible that the planned implementation of the LCHF diet in women enrolled in our study may have an impact on depressive and anxiety symptoms. Firstly, by decreasing these possible symptoms and giving hope for improvement in women in both study groups, and secondly, by the effect on the recruitment of women who were mainly ready to follow a rigorous LCHF diet, i.e., probably without signs of depression or anxiety.

Additionally, the number of women in both study groups was too small to distinguish the subgroups of patients according to the stage and type of lipedema or degree of obesity with BMI or additional symptoms, especially pain of adipose tissue. Our study evaluated the relationship between leg volumes and adipose tissue pain intensity and parameters from questionnaires in the lipedema group. However, the direct comparison of specific subgroups of patients might be very informative.

Conclusions

Our study indicates that behavioral impairment is the main factor affecting functioning in women with lipedema, and emotional symptoms did not differentiate patients with lipedema and lifestyle-induced overweight/obesity. Despite the lower BMI in the lipedema group compared to the overweight/obesity group, the lipedema group reported worse functioning.

Legs volume and adipose tissue pain intensity were the most disabling factors in women with lipedema, and they should be taken into account when planning treatment for women with this condition. It is important to consider these factors in future research assessing the effectiveness of lipedema treatment.

Lipedema requires a complex and multidirectional treatment. In light of our findings, it seems that patient education and social support might improve QoL of women with lipedema. Further research in this field should be conducted on a larger patient cohort to identify subgroups based on the stage and type of lipedema, degree of obesity and accompanying clinical symptoms.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10427200. The package includes the following files:

Supplementary Table 1. The results of normal data distribution and variance homogeneity tests.

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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Mesoderm/mesenchyme homeobox I may promote tumor progression in human hepatocellular carcinoma

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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. The clinical response rate for molecularly targeted medications is limited despite significant advancements in molecularly targeted therapy for hepatocellular carcinoma (HCC). Therefore, it is necessary to find new and robust therapeutic targets for the treatment of HCC. Recent research has shown that mesoderm/mesenchyme homeobox gene 1 (*Meox1*) is closely associated with cancer progression.

Objectives. The aim of this study was to evaluate the clinical relevance as well as biological function of *Meox1* in HCC.

Materials and methods. *Meox1* protein expression level was identified through immunohistochemistry (IHC) examination of pathological tissues from 25 HCC patients. The aim of the analysis was to investigate the relationship between clinicopathological traits and *Meox1* expression. Biological function assays of *Meox1* in HCC, including proliferation, colony formation, migration, and invasion, were performed with Huh7 and Hep3B cells.

Results. In this study, *Meox1* expression in HCC tissues was significantly higher ($p < 0.05$) compared to paracancerous tissues. Especially in HCC tissues of patients with cirrhosis, the level of *Meox1* expression was significantly elevated when compared to HCC tissues of patients without cirrhosis ($p < 0.05$). High *Meox1* expression was significantly associated with tumor-node-metastasis (TNM) stage ($p < 0.05$) and the Barcelona Clinic Liver Cancer (BCLC) stage ($p < 0.05$). Moreover, *Meox1* silencing suppressed the proliferation, colony formation, migration, and invasion of Huh7 and Hep3B cells.

Conclusions. Our data reveal that *Meox1* may play a crucial role in the development of HCC, and given the function of *Meox1* in proliferation and metastasis, targeting *Meox1* may offer a promising approach for combined and adjuvant therapeutics of HCC.

Key words: progression, proliferation, hepatocellular carcinoma, metastasis, mesoderm/mesenchyme homeobox gene I

Background

Hepatocellular carcinoma (HCC), which accounts for about 75–85% of primary liver cancer cases in China, is the leading cause of cancer-related deaths.[1,2] The main therapeutic strategies for HCC patients with early-stage disease are surgery and radiofrequency ablation.[3–5] However, most HCC patients are at an advanced stage at the first diagnosis and are treated with transcatheter arterial chemoembolization (TACE).[5] Molecularly targeted therapy includes various kinase inhibitors,[6] such as sorafenib,[7] lenvatinib[8] and apatinib,[9] and immune checkpoint inhibitors, such as nivolumab[10] and pembrolizumab.[11] Despite significant advancements in molecularly targeted therapy for HCC, these medications have a limited clinical response rate,[12] which emphasizes the need to explore novel and effective therapeutic targets for HCC treatment.

A key transcription factor called mesoderm/mesenchyme homeobox l (*Meox1*) is necessary for cell proliferation and differentiation, and organ formation throughout embryonic development.[13,14] To date, many studies linked aberrant expression of *Meox1* to the development of cancer. The *Meox1* protein was markedly upregulated in human non-small cell lung cancer (NSCLC) tissues and linked to unfavorable prognosis. The inhibition of *Meox1* expression suppressed lung cancer cell proliferation and mammosphere formation in vitro.[15] The *Meox1* protein was also abnormally expressed in ovarian cancer, which promotes cell growth through interaction with *PBX1*.[16] Moreover, *Meox1* was crucial in breast cancer stem cell (CSC) maintenance and epithelial–mesenchymal transition, and it was linked to unfavorable survival outcomes, breast cancer stage and lymph node metastasis in trastuzumab-resistant *PTEN*-deficient breast cancer.[17] In addition, *Meox1* knockdown inhibited the proliferation of triple-negative breast cancer cells in vitro and tumor growth in vivo.[18] However, the clinical significance and biological role of *Meox1* in HCC have not been investigated.

During our research, we not only investigated the expression level of *Meox1* in human HCC tissues and evaluated the relationship between *Meox1* expression and poor progression in patients with HCC, but also elucidated the role of *Meox1* in HCC cell malignancy in vitro for the first time.

Objectives

The aim of this study was to assess the clinical significance and biological role of *Meox1* in HCC.

Materials and methods

Collection of clinical samples

Pathological specimens were collected from 25 patients with HCC who underwent surgical operations at the 980th Hospital of People's Liberation Army (PLA) Joint Logistics Support Force (Shijiazhuang, China) between January 2018 and November 2022. The clinicopathological parameters, including age, gender, pathogeny, cirrhosis, tumor size, tumor number, vascular invasion, lymph node metastasis, tumor-node-metastasis (TNM) stage,[19] the Barcelona Clinic Liver Cancer (BCLC) stage system,[20] and results of biochemical tests, including alpha-fetoprotein (AFP), carcinoma embryonic antigen (CEA), prothrombin time (PT), albumin, as well as total bilirubin, were collected. Radiotherapy, chemotherapy or other biological therapies were not administered to any of the patients. Furthermore, no occurrences of malignant tumors, cardiovascular or cerebrovascular ailments, diabetes, pulmonary fibrosis, or kidney disease were observed among the patients. The Ethics Committee of the 980th Hospital of the PLA Joint Logistics Support Force gave its approval to this study (approval No. 2022-KY-127). All patients signed written informed consent.

Immunohistochemistry analysis

The protein level of *Meox1* was analyzed using immunohistochemistry (IHC) staining. Briefly, after deparaffinization in xylene and in various concentrations of alcohol, antigen retrieval was conducted using ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0), followed by treatment with 3% hydrogen peroxide for 25 min in the dark at room temperature. After that, the specimens were incubated with primary antibodies against *Meox1* (1:500; cat. No. ab105349; Abcam, Cambridge, UK) overnight at 4°C after being blocked with 3% bovine serum albumin (BSA) for 30 min.

A semiquantitative scoring system was used to assess *Meox1* expression in accordance with the percentage of positive cells and staining intensity. Pathologists scored the expression in a blinded manner.[15,21] Four categories were established for the percentage of positive cells: 0 (0~5%), 1 (6~25%), 2 (26~ivided into 4 levels: 0 (no staining), 1 (faint staining), 2 (moderate staining), and 3 (strong staining). The multiplication of both factors determined a positive grade that could be negative (0), weakly positive (from 1 to 4), moderately positive (from 5 to 8), or strongly positive (from 9 to 12). *Meox1* expression was classified into 2 categories: low expression (negative and weakly positive) or high expression (moderately and strongly positive).

Cell lines and culture

The Huh7 and Hep3B cell lines were stored at the Hebei Key Lab of Laboratory Animal Science of Hebei Medical University (Shijiazhuang, China) and cultured as previously described.[21,22]

RNA interference and stable-knockdown cell screening

Recombinant lentivirus carrying specific short hairpin RNA (shRNA) for *Meox1* or a negative control was packaged by GenePharma Co., Ltd. (Shanghai, China). Two different *Meox1* shRNAs were used to perform the experiment. The sequence for *Meox1* shRNA 1 was 5'-GAA ATC ATC CAG GCG GAG AAA-3'; *Meox1* shRNA 2 was as follows: 5'-CTG CCA ATG AGA CAG AGA A-3';[23] The negative control shRNA sequence was as follows: 5'-TTC TCC GAA CGT GTC ACG T-3'.

To conduct *Meox1* inhibition experiments, we infected HCC cells (Huh7 and Hep3B) with recombinant lentivirus carrying its specific shRNA (sh-*Meox1*) and negative control shRNA (sh-NC). Stable cell clones with *Meox1* knockdown were established via 0.6 μg/mL puromycin selection for Huh7 cells and 0.3 μg/mL puromycin for Hep3B cells.

Western blotting

Radioimmunoprecipitation (RIPA) lysis buffer (Solarbio, Beijing, China) containing freshly added phenylmethyl sulphonyl fluoride (PMSF; Solarbio) was used to extract total protein from cells based on the manufacturer's instructions. A bicinchoninic acid assay (BCA) protein assay kit (Solarbio) was used to test the concentrations of protein according to the manufacturer's guidelines. The protein samples underwent separation utilizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then moved onto a nitrocellulose membrane (MilliporeSigma, St. Louis, USA), followed by blocking with 5% non-fat dry milk. Primary antibodies, including anti-*Meox1* (1:1,000; cat. No. ab105349; Abcam) and anti-*GAPDH* (1:10,000; cat. No. ab181602; Abcam), were used to conduct the experiments. The secondary antibody used was horseradish peroxidase (HRP)-conjugated Affinipure goat anti-rabbit immunoglobulin G (IgG) (H+L) (1:5,000; cat. No. SA00001-2; Proteintech, San Diego, USA). The immunoblots were detected as previously described.[22]

Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo Lab, Kumamoto, Japan) was employed to assess cell proliferation. The analysis was conducted according to the previously described methods.[22]

Colony formation assay

Six-well plates containing 500 cells/well were used to seed cell lines, which were then routinely cultivated for 2 weeks. The plates were discarded after a visible cell colony formed, rinsed with phosphate-buffered saline (PBS) and treated with methyl alcohol for 30 min, followed by staining with 0.5% crystal violet for 20 min to count the cell number. The colony formation efficiency was calculated using the following formula: colony number divided by seeded cell number multiplied by 100%.

Cell migration and invasion assay

Eight-micrometer Transwell® chambers containing polycarbonate filters (Corning Company; Corning, USA) were used to conduct the cell invasion and migration assays. For the invasion assay, the chambers were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, USA), and the migration assay was carried out without Matrigel. The analysis was conducted as previously described.[22]

Statistical analyses

Statistical analysis was performed using IBM SPSS 26.0 software (IBM Corp., Armonk, USA). Pathological specimens were collected from 25 patients with HCC undergoing surgical operations at the 980th Hospital of PLA Joint Logistics Support Force. Three separate independent runs of each cell function experiment (cell proliferation, colony formation, cell invasion and migration assay) were conducted. The frequency or medians (interquartile range (IQR)) was used to represent the values. The Fisher's exact test or non-parametric Mann–Whitney U test or Kruskal–Wallis H test were utilized for examining the data, as appropriate. The Spearman's correlation analysis was utilized for examining the relationship between the 2 variables. Survival curves for HCC were produced using the Kaplan–Meier method and the log-rank test. All comparisons were 2-tailed, and a $p < 0.05$ denoted statistical significance.

Results

High expression of *Meox1* in HCC tissues

To evaluate the expression level of *Meox1* in human HCC tissues, we conducted an IHC analysis. The results revealed that *Meox1* was primarily localized in the nucleus (Fig. 1A). In the 25 HCC tissues, the *Meox1* showed negative expression in 2, while weak, moderate and strong positive expression in 6, 14 and 3, respectively (Table 1). Among the 25 paracancerous tissues, 4 were negative and 13 and 8 were weakly and moderately positive, respectively, and there was no strongly positive expression (Table 1). *Meox1* expression was significantly higher in HCC tissues compared to paracancerous tissues ($p < 0.05$, Mann–Whitney U test). Furthermore, we compared the percentage of positive cells expressing *Meox1* in hepatocellular carcinoma (HCC) and paracancerous tissues (Fig. 1B). The median (IQR) of positive cells percentage of *Meox1* in HCC was 0.7318 (0.2904–0.8486), while in paracancerous tissue it was 0.3607 (0.0932–0.663); a statistically significant distinction existed ($p = 0.048$, Mann–Whitney U test). The result indicate that *Meox1* is highly expressed in HCC tissues.

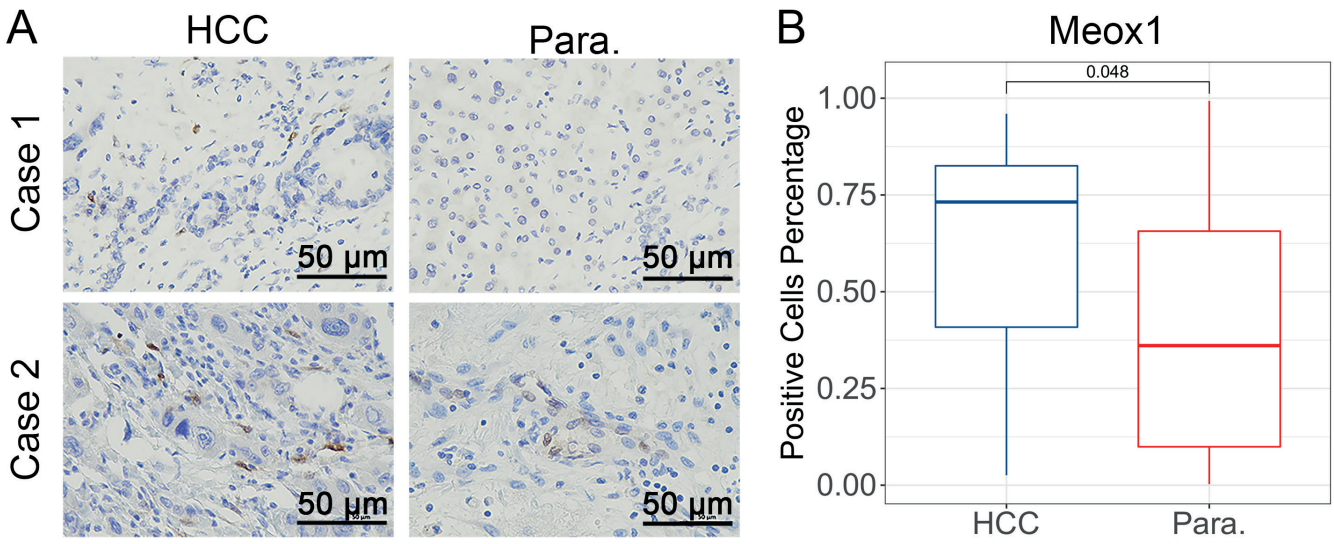

Fig. 1. The expression of *Meox1* in HCC and paracancerous tissues was determined using immunohistochemistry (IHC). A. Representative staining in HCC tissues and paracancerous tissues at ×400. *Meox1* protein was mainly localized in the nucleus. The brown signal is the *Meox1* protein, and the blue signal is the nucleus; B. The median (interquartile range (IQR)) of *Meox1*-positive cells percentage in HCC was 0.7318 (0.2904–0.8486), while in paracancerous tissue it was 0.3607 (0.0932–0.663). Mann–Whitney U test was used to examine the difference of *Meox1*-positive cells percentage between HCC and paracancerous tissues (p = 0.048, Mann–Whitney U test)

HCC – hepatocellular carcinoma; Para. – paracancerous tissue.

Table 1. The expression of *Meox1* in HCC and paracancerous tissues

Immunohistochemical grade	HCC tissues (n = 25, 100%)	Paracancerous tissues (n = 25, 100%)	p-value*
Negative (–)	2 (8%)	4 (16%)	0.011
Weakly positive (+)	6 (24%)	13 (52%)	
Moderately positive (++)	14 (56%)	8 (32%)	
Strongly positive (+++)	3 (12%)	0 (0%)	

*The non-parametric Mann–Whitney U test was used to examine the difference of *Meox1* expression between HCC and paracancerous tissues.

Table 2. The expression of *Meox1* in hepatocellular carcinoma (HCC) tissues with or without cirrhosis

Cirrhosis	Number of cases (%)	Low expression	High expression	p-value*
No	7 (28%)	5	2	0.017
Yes	18 (72%)	3	15	

* The Fisher's exact test was used to examine the data.

High expression of *Meox1* in HCC patients with cirrhosis

We further observed the expression level of *Meox1* in HCC tissues of patients with and without cirrhosis. The results indicated that among 18 HCC patients with cirrhosis, *Meox1* expression was low in 3 HCC tissues and high in 15. In the following 7 patients without cirrhosis, *Meox1* expression was low in 5 HCC tissues and high in 2 (Table 2). The expression of *Meox1* was markedly elevated in HCC tissues from patients with cirrhosis compared to patients without cirrhosis ($p < 0.05$, Fisher's exact test).

We also observed the expression level of *Meox1* in paracancerous tissues of patients with and without cirrhosis (Table 3). In 18 HCC patients with cirrhosis, 10 patients had low *Meox1* expression while 8 had high expression, respectively. However, in 7 HCC without cirrhosis, *Meox1* was expressed at low levels in 7 patients, and there was no high expression. The results show that there is no significant difference in *Meox1* expression between paracancerous tissues of HCC with cirrhosis and HCC without cirrhosis ($p = 0.057$, Fisher's exact test).

Meox1 expression level linked to poor progression in HCC

Statistical analysis revealed a significant relationship between *Meox1* expression and BCLC stage ($p < 0.05$, Fisher's exact test) as well as TNM stage ($p < 0.05$, Fisher's exact test). However, *Meox1* expression did not show

Table 3. The expression of *Meox1* in paracancerous tissues with or without cirrhosis

Cirrhosis	Number of cases (%)	Low expression	High expression	p-value*
No	7 (28%)	7	0	0.057
Yes	18 (72%)	10	8	

* The Fisher's exact test was used to examine the data.

Table 4. The relationship between the expression of *Meox1* and clinical characteristics in hepatocellular carcinoma (HCC) patients

Variables	Number of cases (%)	Low expression	High expression	p-value*
Gender Male Female	25 22 (88%) 3 (12%)	8 6 2	17 16 1	0.231
Age [years] ≤60 >60	25 12 (48%) 13 (52%)	8 5 3	17 7 10	0.411
Pathogeny HBV HCV unknown	25 17 (68%) 3 (12%) 5 (20%)	8 6 1 1	17 11 2 4	0.828
Tumor size [cm] ≤5 >5	25 14 (56%) 11 (44%)	8 5 3	17 9 8	0.695
Tumor number Single Multiple	25 20 (80%) 5 (20%)	8 8 0	17 12 5	0.140
Vascular invasion No Yes	25 18 (72%) 7 (28%)	8 8 0	17 10 7	0.057
Lymph node metastasis No Yes	25 22 (88%) 3 (12%)	8 8 0	17 14 3	0.527
TNM stage I–II III–IV	25 15 (60%) 10 (40%)	8 8 0	17 7 10	0.008
BCLC stage 0+A B+C	25 16 (64%) 9 (36%)	8 8 0	17 8 9	0.022

* The Fisher exact test was used to examine the data; HBV – hepatitis B Virus; HCV – hepatitis C virus; TNM – tumor-node-metastasis; BCLC – Barcelona Clinic Liver Cancer staging.

any statistically significant relationships with other clinicopathological traits such as age, sex, tumor size, tumor number, vascular invasion, and lymph node metastasis (Table 4). In addition, *Meox1* expression and clinical parameters (Table 5), such as alpha-fetoprotein (AFP), carcinoma embryonic antigen (CEA), CA125, CA199, prothrombin time (PT), albumin, as well as total bilirubin, did not significantly correlate, according to Spearman's analysis. These results showed that abnormal expression of *Meox1* may be correlated with the progression of HCC.

Gene expression of *Meox1* in HCC

We further investigated the gene expression of *Meox1* in HCC using the Gene Expression Profiling Interactive Analysis (GEPIA) database. The analysis revealed that the expression of the *Meox1* gene was notably increased in 369 HCC tissues than in 160 normal tissues (Fig. 2A).

Table 5. Spearman's analysis of the correlation between the expression of *Meox1* and clinical parameters

Clinical parameter	*Meox1* expression	
	Spearman's correlation	p-value*
AFP	0.188	0.369
CEA	0.23	0.303
CA125	–0.272	0.246
CA199	0.097	0.683
PT	–0.327	0.171
Albumin	0.206	0.384
Total bilirubin	0.114	0.633

* The correlation between *Meox1* expression and clinical parameters were examined using Spearman's analysis; AFP – alpha-fetoprotein; CEA – carcinoma embryonic antigen; CA125 – cancer antigen 125; CA199 – cancer antigen 199; PT – prothrombin time.

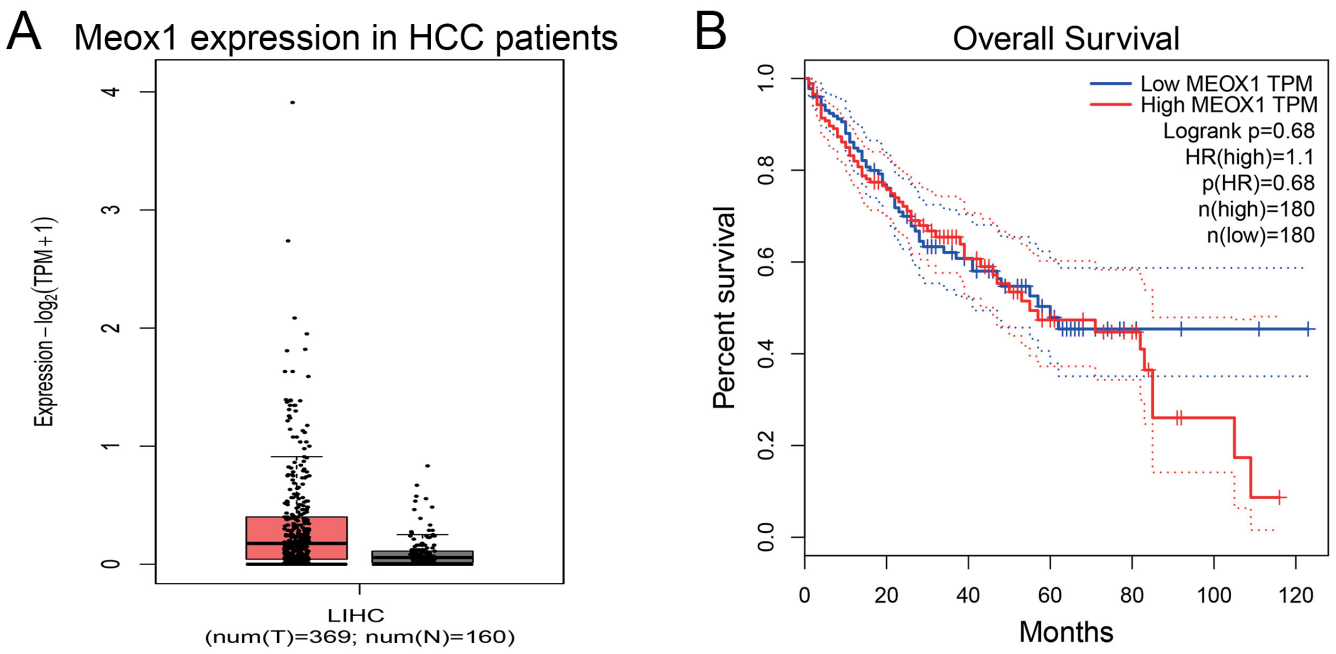

Fig. 2. The expression of *Meox1* in patients with liver hepatocellular carcinoma (LIHC) and overall survival (OS) curves. A. Data from the Gene Expression Profiling Interactive Analysis (GEPIA) database showed that the expression of *Meox1* was higher in hepatocellular carcinoma (HCC) tissues than in normal tissues; B. Survival curves for LIHC patients from the GEPIA database [p = 0.68, Kaplan–Meier method, log-rank test].

However, the survival analysis suggested that high *Meox1* expression may not be significantly correlated with overall survival (OS) in HCC (Fig. 2B).

Meox1 silencing suppressed HCC cell malignancy in vitro

To explore the role of *Meox1* in HCC cell malignancy, *Meox1* expression was reduced by infecting HCC cells (Huh7 and Hep3B cells) with recombinant lentiviruses containing its particular shRNA (sh-*Meox1*). As shown in Fig. 3A and 3B, HCC cell lines with stable *Meox1* knockdown were successfully constructed, and the knockdown efficiency was confirmed. Initially, we observed the function of *Meox1* in cell proliferation using CCK-8 assay. The results indicated that the growth rate of Huh7 cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to those in sh-NC and the blank control at 72 h, 96 h and 120 h (Fig. 3C, $p < 0.05$, respectively, Kruskal–Wallis H test). The same phenomenon was observed in Hep3B cells (Fig. 3D, $p < 0.05$, Kruskal–Wallis H test), indicating that *Meox1* silencing suppressed the proliferation of HCC cells. In addition, colony formation assays revealed that the size and the number of colonies were markedly decreased in both Huh7 and Hep3B cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups (Fig. 4, $p < 0.05$, Kruskal–Wallis H-test). The data suggested that *Meox1* silencing inhibited colony formation of HCC cells and may exert a negative effect on cell self-renewal function.

Cell migration and invasion are important characteristics of malignancy.[24,25] Therefore, we assessed the contribution of *Meox1* in HCC cell migration and invasion using a transwell (An experimental method used for the study of biological processes such as cell migration and invasion) assay (Fig. 5). The findings demonstrate that the migration of cells significantly decreased when *Meox1* was knocked down in both Huh7 and Hep3B cells, compared to the sh-NC and blank control groups. In Huh7 and Hep3B cells with *Meox1* knockdown, the number of invading cells notably decreased, indicating that *Meox1* regulates both migration and invasion properties in HCC cells. Based on the data, silencing *Meox1* reduced the malignancy of HCC cells and may be critical for HCC development.

Discussion

The discovery of novel and effective therapeutic targets for HCC treatment is crucial for clinical application. Accumulated evidence has shown that *Meox1* is closely related to the progression of several cancers, including lung,[15] breast[17,18] and ovarian cancer.[16] Studies have confirmed that *Meox1* expression in tumors was increased. This study aimed to evaluate *Meox1* expression in HCC and investigate how it affected the biological functions of HCC cells.

We first assessed the expression level of *Meox1* in HCC through IHC and bioinformatics analysis. The findings demonstrated a notable upregulation of *Meox1* in HCC tissues, aligning with previous studies conducted on lung, breast and ovarian cancer. Importantly, our observations indicated a notable increase in *Meox1* expression levels in HCC tissues from patients with cirrhosis compared

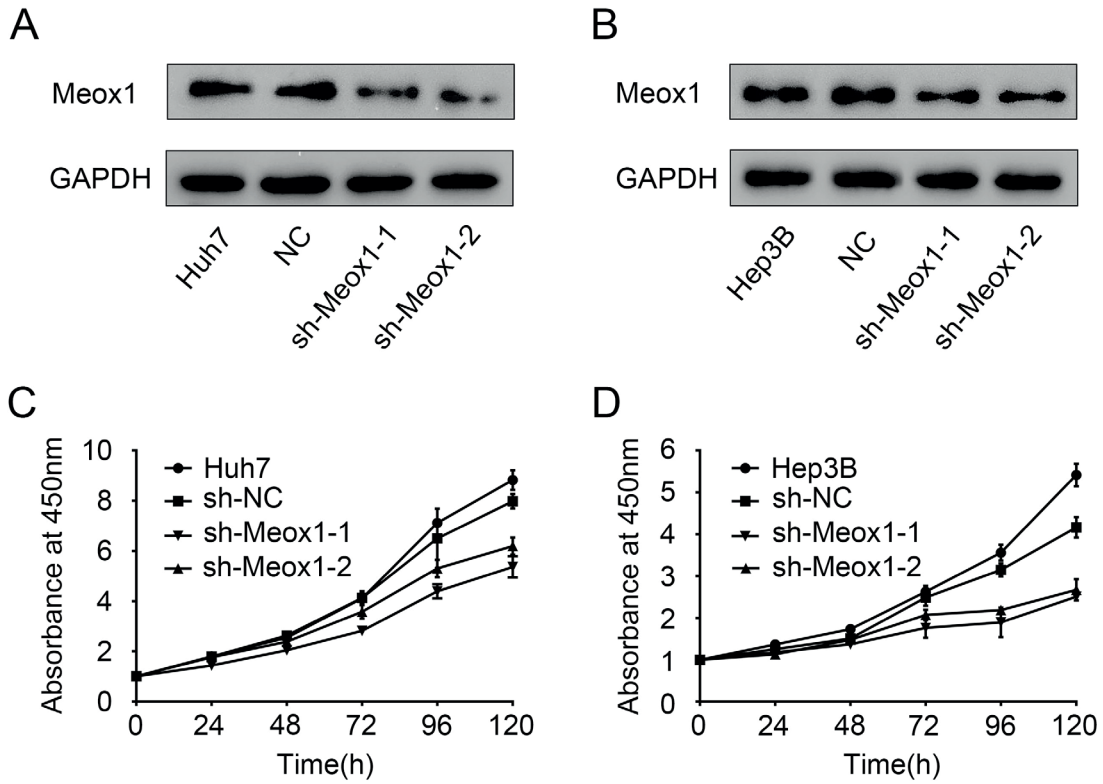

Fig. 3. *Meox1* gene silencing inhibits the proliferation of hepatocellular carcinoma (HCC) cells. Stable cell clones with *Meox1* knockdown were established by infecting HCC cells (Huh7 and Hep3B) with recombinant lentivirus carrying its specific shRNA (sh-*Meox1*) and negative control shRNA (sh-NC) with puromycin selection. A. The expression of *Meox1* in *Meox1* knockdown Huh7 cell lines was detected using western blot analysis (n = 3); B. The expression of *Meox1* in *Meox1* knockdown Hep3B cell lines was detected with western blot analysis (n = 3). Cell lines in the logarithmic growth phase were resuspended and seeded into 96-well plates at a density of 1×103 cells/well. Ten microliters of Cell Counting Kit-8 (CCK-8) were added to each well at the indicated times (0, 24, 48, 72, 96, and 120 h) and then incubated for 2 h at 37°C in a 5% humidified CO_2 incubator. The optical density (OD) value was measured at a wavelength of 450 nm every 24 h using a microplate reader; C. Proliferation effect of *Meox1* in Huh7 cells. The growth rate of Huh7 cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to that with sh-NC and the blank control at 72 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test); the same phenomenon was also found at 96 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test) and 120 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test); D. Proliferation effect of *Meox1* in Hep3B cells. The growth rate of Hep3B cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to that with sh-NC and the blank control at 72 h ($n = 3$, $p < 0.05$, the Kruskal–Wallis H test); the same phenomenon was also found at 96 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test) and 120 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test)

to those without cirrhosis. Advanced liver fibrosis and cirrhosis are principle risk factors for HCC, and up to 90% of cases are based on this background.[26,27] The tumor microenvironment (TME) has a vital function in progression of HCC. Liver fibrosis can promote HCC progression by regulating the TME, including cytokine secretion, immune surveillance, tumor angiogenesis, and extracellular matrix synthesis.[28,29] According to numerous research, *Meox1* is crucial in organ fibrosis.[23,30–33] More recently, 1 study found that *Meox1* is a central regulator in the transformation of fibroblasts to profibrotic myofibroblasts and is necessary for tumor growth factor beta (*TGFβ*)-induced fibroblast activation.[33] In addition, *Meox1* may promote hepatic stellate cell (HSC) activation, which is a crucial event during liver fibrosis.[33] We speculated that abnormal *Meox1* expression might be closely linked to liver fibrosis as well as HCC and involved in the TME, but this hypothesis needs to be further explored.

Previous data from breast cancer have shown that elevated *Meox1* is linked to an advanced tumor stage and poor OS.[17] Another study on lung cancer also indicated that increased *Meox1* promoted tumor progression and contributed to shorter OS, increased lymph node metastasis, and advanced stage of HCC, and was an independent poor prognosis predictive factor identified with Cox multivariate regression analysis.[15] We further evaluated the potential implication of *Meox1* in HCC, and clinical relevance analysis showed that high *Meox1* expression was positively correlated with advanced tumor stage. However, the survival analysis from GEPIA databases indicated that high *Meox1* expression may not be significantly correlated with OS in HCC. We speculate that this variation may be brought about by *Meox1*'s various tumor-specific actions.

Meox1 is considered an important transcription factor that promotes tumor cell growth in ovarian cancer.[16] Inhibition of *Meox1* expression also effectively suppressed the proliferation of lung cancer[15] and breast cancer cells.[17,18] We constructed *Meox1* stable knockdown HCC cell lines to examine *Meox1*'s role in HCC. It was found that inhibiting *Meox1* expression significantly suppressed the proliferation and colony formation of 2 different HCC cell lines. These results imply that *Meox1* may be crucial

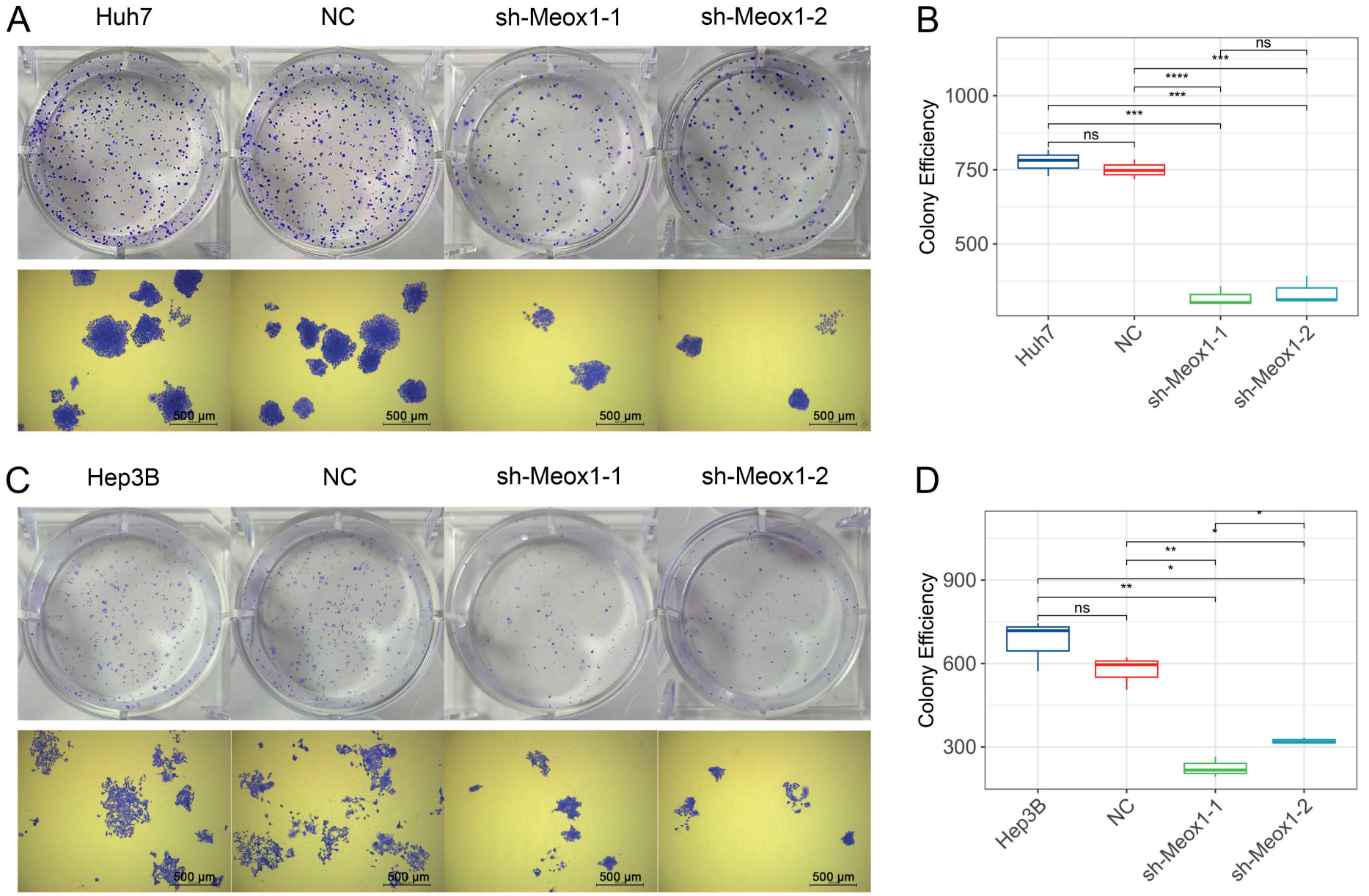

Fig. 4. *Meox1* gene silencing inhibits the colony formation of hepatocellular carcinoma (HCC) cells. A. *Meox1* gene silencing inhibits colony formation of Huh7 cells; B. Quantification of results from colony formation in A; the number of colonies were markedly decreased in Huh7 cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups (n = 3, $p < 0.05$, Kruskal–Wallis H test); C. *Meox1* gene silencing inhibits colony formation of Hep3B cells; D. The quantification of results from colony formation in C; the number of colonies were markedly decreased in Hep3B cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups (n = 3, $p < 0.05$, Kruskal–Wallis H test)

ns – no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

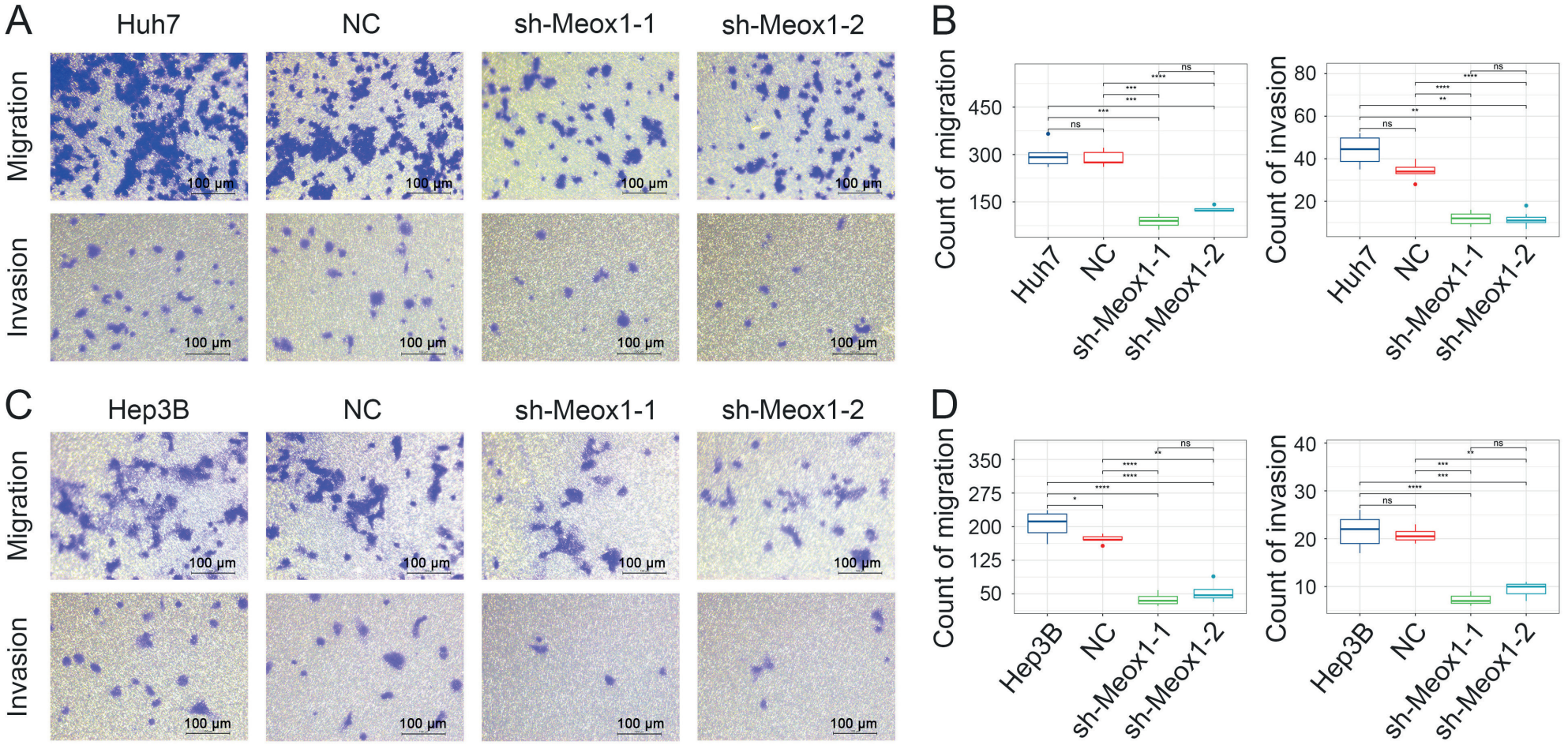

Fig. 5. *Meox1* gene silencing inhibits the migration and invasion of hepatocellular carcinoma (HCC) cells. The cell lines were seeded into the Transwell (an experimental method used for the study of biological processes such as cell migration and invasion) chamber at a density of 1×104/well and incubated for 24 h for the migration assay and at a density of 3×104/well, and then incubated for 48 h for the invasion assay. After the indicated time, the cells were fixed with methyl alcohol for 30 min, followed by staining with 0.5% crystal violet for 20 min. The stained cells were counted under an inverted microscope in 5 randomly selected fields at a magnification of ×100. A. Effect of *Meox1* knockdown on the migration and invasion of Huh7 cells (n = 3); B. Quantification of results from migration in A; the migration of cells was significantly decreased when compared to the negative control shRNA (sh-NC) and blank control groups when *Meox1* was knocked down in Huh7 cells (left, $p < 0.05$, Kruskal–Wallis H test). The quantification of results from invasion in A; the invasion of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Huh7 cells (right, $p < 0.05$, Kruskal–Wallis H test); C. Effect of *Meox1* knockdown on the migration as well as invasion of Hep3B cells (n = 3); D. The quantification of results from migration in C; the migration of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Hep3B cells (left, $p < 0.05$, Kruskal–Wallis H test). The quantification of results from invasion in C; the invasion of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Hep3B cells (right, $p < 0.05$, Kruskal–Wallis H test)

ns – no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

in regulating cell proliferation. Moreover, experiments with shRNA knockdown showed that downregulation of *Meox1* expression reduced migration and invasion in 2 different HCC cell lines, indicating that *Meox1* could regulate HCC cell metastasis. These results suggest that targeting *Meox1* not only decreases the rapid proliferative behavior, but also suppresses the aggressive metastatic potential to reduce the malignancy of HCC cells, and in consideration of the significant correlation between the *Meox1* and vascular invasion and advanced tumor stage in HCC, it is plausible that abnormal expression of *Meox1* may accelarate the progression of HCC. Future investigations should involve HCC clinical research with large samples, and HCC animal experiments are warranted to verify the role of *Meox1* in HCC. The detailed molecular mechanism of the role of *Meox1* in HCC progression deserves further research.

Limitations

The study may have been conducted in a specific HCC model or population, which means that the results may not be applicable to other types of cancers or populations.

Conclusions

Our studies found that *Meox1* was highly expressed in HCC tissues, especially in HCC with cirrhosis, and was closely correlated with advanced stage of HCC. Moreover, *Meox1* silencing suppressed the proliferation, colony formation, migration, and invasion of HCC cells. Given the physiological significance of *Meox1* in proliferation and metastatic features and the implications of these findings for the progression of HCC, targeting *Meox1* may offer a possible strategy for adjuvant and combination therapies of HCC.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10090681. The package includes the following files:

Supplementary Fig. 1 Kruskal-Wallis H test was utilized for examining the data from colony formation of Huh7 cells.

Supplementary Fig. 2 Kruskal-Wallis H test was utilized for examining the data from colony formation of Hep3B cells.

Supplementary Fig. 3. Kruskal-Wallis H test was utilized for examining the data from migration of Huh7 cells.

Supplementary Fig. 4. Kruskal-Wallis H test was utilized for examining the data from invasion of Huh7 cells.

Supplementary Fig. 5. Kruskal-Wallis H test was utilized for examining the data from migration of Hep3B cells.

Supplementary Fig. 6. Kruskal-Wallis H testwas utilized for examining the data from invasion of Hep3B cells.

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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Integrated analysis of a competing endogenous RNA network reveals a ferroptosis-related 6-lncRNA prognostic signature in clear cell renal cell carcinoma

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Conflict of interest
None declared

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* Qing Zheng and Zhenqi Gong contributed equally to this work.

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Abstract

Background. Establishing a robust signature for prognostic prediction and precision treatment is necessary due to the heterogeneous prognosis and treatment response of clear cell renal cell carcinoma (ccRCC).

Objectives. This study set out to elucidate the biological functions and prognostic role of ferroptosis-related long non-coding RNAs (lncRNAs) based on a synthetic analysis of competing endogenous RNA networks in ccRCC.

Materials and methods. Ferroptosis-related genes were obtained from the FerrDb database. The expression data and matched clinical information of lncRNAs, miRNAs and mRNAs from The Cancer Genome Atlas (TCGA) database were obtained to identify differentially expressed RNAs. The lncRNA-miRNA-mRNA ceRNA network was established utilizing the common miRNAs that were predicted in the RNAHybrid, StarBase and TargetScan databases. Then, using progressive univariate Cox regression, least absolute shrinkage and selection operator (LASSO) and multivariate Cox regression analysis of gene expression data and clinical information, a ferroptosis-related lncRNA prognosis signature was constructed based on the lncRNAs in ceRNA. Finally, the influence of independent lncRNAs on ccRCC was explored.

Results. A total of 35 ferroptosis-related mRNAs, 356 lncRNAs and 132 miRNAs were sorted out after differential expression analysis in the TCGA-KIRC. Subsequently, overlapping lncRNA-miRNA and miRNA-mRNA interactions among the RNAHybrid, StarBase and TargetScan databases were constructed and identified; then a ceRNA network with 77 axes related to ferroptosis was established utilizing mutual miRNAs in 2 interaction networks as nodes. Next, a 6-ferroptosis-lncRNA signature including PVT1, CYTOR, MIAT, SNHG17, LINC00265, and LINC00894 was identified in the training set. Kaplan–Meier analysis, PCA, t-SNE analysis, risk score curve, and receiver operating characteristic (ROC) curve were performed to confirm the validity of the signature in the training set and verified in the validation set. Finally, single-sample gene set enrichment analysis (ssGSEA) and ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) analysis showed that the signature was related to immune cell infiltration.

Conclusions. Our research underlines the role of the 6-ferroptosis-lncRNA signature as a predictor of prognosis and a therapeutic alternative for ccRCC.

Key words: bioinformatics analysis, ferroptosis-related lncRNA, competing endogenous RNA network, prognostic signature, clear cell renal cell carcinoma

Background

Based on the data from the American Cancer Society, an estimated 79,000 new cases of kidney cancer occurred in the USA in 2022, with nearly 13,920 deaths attributed to this disease.[1] Histologically, renal cell carcinoma (RCC) constitutes the vast majority (90%) of kidney cancer cases, primarily comprising clear cell RCC (ccRCC; 70%), papillary RCC (10–15%) and chromophobe RCC (5%).[2] The incidence of RCC has been gradually increasing due to population aging, obesity and environmental pollution.[1] According to the latest global data, about 4.4 per 100,000 people are diagnosed with RCC every year. The incidence of RCC is twice as common in men as in women, which poses a significant health risk to men.[3,4]

Early surgical intervention is currently the main treatment method for clear cell RCC (ccRCC), with an overall 5-year survival rate of >90% for most patients who undergo early resection surgery.[3,4] However, about 30% of patients experience metastatic recurrence after nephrectomy, which significantly affects postoperative survival.[5] Additionally, ccRCC demonstrates high resistance to chemotherapy and radiotherapy, leading to a poor prognosis.[6] According to reports, the 5-year survival rate for patients with advanced ccRCC is only 11.7%.[7] The survival outcomes of ccRCC patients vary greatly, making it difficult for clinicians to accurately predict patient prognosis. Traditionally, the clinical and pathological features of patients have been used to evaluate the risk of recurrence and predict prognosis. Recently, researchers have explored molecular biomarkers to reliably predict the prognosis of ccRCC.[8] In 2018, a validated prognostic molecular signature, ClearCode34, was introduced. Based on the expression of 34 genes, ClearCode34 demonstrated satisfactory prediction performance.[9] Moreover, a study published in 2022 by Helmink et al. utilized deep RNA sequencing to construct B-cell-related gene signature, aiming to predict the impact of anti-PD-1 therapy on the efficacy and prognosis of ccRCC patients.[10] It can be seen that by integrating RNA sequencing data with survival information, molecular-based characteristics provide new options for assessing the prognosis of ccRCC patients. Therefore, considering the high incidence and mortality rate of ccRCC, it is crucial to explore molecular features with prognostic value that affect ccRCC patients.

Cell death induction, including apoptosis, necroptosis, pyroptosis, and autophagy, is the core mechanism of antitumor drugs. Approximately 10 years ago, the concept of ferroptosis was introduced as a new form of programmed cell death.[11] Ferroptosis is an iron-dependent form of regulatory cell death caused by excessive lipid peroxidation and is involved in the development and progression of different tumors, including ccRCC.[12] Currently, inducing ferroptosis in ccRCC is a promising strategy. Miess et al. has proved that inhibiting the synthesis of glutathione can make ccRCC sensitive to ferroptosis and finally prevent tumor growth.[13] In addition, a study by Yang et al. also confirmed that the Hippo pathway effector TAZ can regulate the ferroptosis sensitivity of RCC.[14] Therefore, modulating ferroptosis may have a number of important implications for future RCC therapeutic practices.

Long non-coding RNAs (lncRNAs) are non-coding transcripts containing more than 200 nucleotides.[15] Accumulating studies have shown that lncRNAs play a significant role in the occurrence, development and metastasis of ccRCC, and can serve as reliable prognostic factors.[16,17] Instead of using a single lncRNA to analyze its prediction of disease, it is more effective to comprehensively analyze the expression profile of certain pathway-related lncRNAs. However, only a few studies have screened ferroptosis-related lncRNAs in ccRCC. Moreover, very little information is available on lncRNA signatures to explain the relationship between lncRNAs and ferroptosis-related genes using the competing endogenous RNA (ceRNA) network.

Objectives

Our objective was to establish a ferroptosis-related lncRNA signature for predicting the prognosis of ccRCC patients. Furthermore, by constructing ceRNA networks and analyzing the immune microenvironment, we sought to elucidate the underlying mechanisms of ferroptosis-related lncRNAs in ccRCC.

Materials and methods

Data acquisition and differentially expressed gene analysis

Overall, 112 ferroptosis-related genes (Supplementary Table 1) were obtained from the FerrDb Database (http://www.zhounan.org/ferrdb) concerning the following screening conditions: validated in human and protein-coding. Gene expression data and matched clinical profiles (including lncRNAs, microRNAs (miRNAs) and messenger RNAs (mRNAs)) of the KIRC cohort were downloaded from The Cancer Genome Atlas (TCGA; https://www.cancer.gov/ccg/research/genome-sequencing/tcga) database using the "GDCRNATools" package of R software,[18] duplicate samples were removed, and only samples with sample_type of "PrimaryTumor" and "SolidTissueNormal" were retained. Then, the "GDCRNATools" package of R software was applied to analyze the differential expression of lncRNA, miRNA and ferroptosis-related mRNA based on the conditions: method: DESeq2, Normalization: Voom, $p = 0.05$ and $\log|\text{Fold Change}| = 2$.

Construction of the ceRNA network

The miRNA can regulate gene expression by targeting the 3'UTR of mRNA. The ceRNA network refers to non-coding RNA, such as lncRNA, that can competitively

bind to miRNAs and reduce their inhibition on mRNA. The interactions of differentially expressed lncRNA and miRNA, as well as the interactions of miRNA and ferroptosis-related mRNA, were predicted utilizing TargetScan (www.targetscan.org), StarBase (https://starbase.sysu.edu.cn) and RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) databases. The intersection between the 3 databases was identified using the "vennR" package of R software. Then, the ceRNA network was established using the common miRNAs in the 3 databases that connect lncRNAs and mRNAs. Following the mechanism of the ceRNA network, we only retained the relationships as upregulated lncRNA\downregulated miRNA\upregulated mRNA and downregulated lncRNA\upregulated miRNA\downregulated mRNA, which were used to construct the ceRNA network, and then the results were imported into Cytoscape 3.7.1 (https://cytoscape.org) for visualization.

Identification and validation of a prognostic lncRNA signature based on the ceRNA network

Using the survival data in TCGA and excluding the incomplete survival time data, we constructed the prognostic signature using lncRNAs in ceRNA. KIRC patients were randomly assigned into the training and test cohorts in a 1:1 ratio, utilizing the "caret" package in R 4.1.1. In the training set, univariate Cox regression analysis of overall survival (OS) was performed to explore the ferroptosis-related lncRNAs with prognostic values ($p < 0.01$) in the ceRNA network. Then, to minimize the risk of overfitting, least absolute shrinkage and selection operator (LASSO) regression analysis was utilized to construct a prognostic signature in the R package "glmnet". The following formula:

$$\text{risk score} = (\beta1\times G1+\beta2\times G2+\beta3\times G3+...+\beta n\times Gn)$$

was used to calculate the risk score for each patient and to predict the prognosis of the patient according to the score, where β stands for the coefficient of each lncRNA, G means each lncRNA expression value, and n represents the number of lncRNAs.

Based on the median risk score of the training set, we stratified patients into 2 groups. The "Pheatmap" package was applied to draw a scatter diagram to describe the distribution pattern of risk scores and the corresponding survival time of each patient in the training and validation set. The "Stats" package was used for principal component analysis (PCA) to describe the gene expression distribution in the signature. Then, the "Rtsne" package was used for t-distributed stochastic neighbor embedding (t-SNE) analysis to describe the distribution of survival status in different risk groups. Receiver operating characteristic (ROC) curves, drawn by the "timeROC" package, were used to verify whether the signature could be considered a useful biomarker in the training and validation set. Finally, the "survival" package was used for survival analysis for each lncRNA in the signature to verify its prognostic value in the KIRC cohort. The mRNAs competitively inhibited by lncRNAs in the ceRNA network were verified in the Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) database (based on the TCGA and Genotype-Tissue Expression (GTEx) data) in ccRCC.

Independent prognostic analysis of the ferroptosis-related lncRNA signature

To further estimate the independent prognostic value of the ferroptosis-related lncRNA signature, univariate and multivariate Cox regression analyses were used to find out whether it was influenced by other clinical features. Available clinical characteristics including sex, age and tumor-node-metastasis (TNM)-based staging were converted into dichotomous variables and used to calculate OS-based hazard ratios (HRs) and 95% confidence intervals (95% CI). A $p < 0.05$ was considered statistical significance.

Functional enrichment analysis

The "limma" R package was performed to select the risk-related differentially expressed genes (DEGs) in the KIRC cohort based on | log2 Fold Change |≥1.2 and false discovery rate <0.05 as a standard between the high- and low-risk groups. The "clusterProfiler" R package was applied to conduct Gene Ontology (GO; https://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) analyses for risk-related DEGs based on the criterion: gene count >5 and p-value < 0.05. The infiltrating scores of 16 immune cells and the activities of 13 immune-related pathways were quantified with single-sample gene set enrichment analysis (ssGSEA) using the "GSVA" R package. Using the annotated gene set provided in Supplementary Table 2, we quantified the immune infiltration enrichment scores of different immune cells and immune-related functions to further study the correlation between risk scores and immune status.

Statistical analyses

Statistical analysis was performed using R software (R v. 4.1.1; R Foundation for Statistical Computing, Vienna, Austria). The key R-scripts used in this study were presented in the Supplementary material – R-scripts. Continuous variables were analyzed with the Wilcoxon test (Mann–Whitney test), whereas categorical data was analyzed using the χ^2 test or Fisher's exact test. Univariate and multivariate Cox regression analysis was used to identify the related factors affecting the OS of KIRC patients. The results of the proportional hazards assumption for Cox regression were conducted based on Schoenfeld's global and individual test (Supplementary Table 3, Supplementary

Fig. 2,3). Kaplan–Meier analysis and the log-rank test were performed to calculate the survival difference. For all statistical analyses, a p-value < 0.05 was considered statistically significant without special explanation.

Results

Identification of DEGs in the KIRC cohort

After filtering the sample types of Additional-NewPrimary (1 transcriptome and 1 miRNA) and duplicate samples (8 transcriptomes and 28 miRNAs), a total of 583 transcriptome samples, and 583 miRNA samples, including 512 KIRC patients and 71 normal controls were obtained from the KIRC cohort of TCGA database. Through differential expression analysis, we screened out 356 lncRNAs (286 upregulated and 70 downregulated), 132 miRNAs (62 upregulated and 70 downregulated) and 35 ferroptosis-related mRNAs (18 upregulated and 17 downregulated) (Fig. 1A–C).

Establishment of a ferroptosis-related ceRNA network in the KIRC cohort

To understand the regulatory crosstalk between different RNA molecules, we constructed a ferroptosis-related ceRNA network in the KIRC cohort. RNAHybrid, StarBase and TargetScan databases were utilized to predict the network of lncRNAs, miRNAs and mRNAs. In total, 8658,1263,160426 lncRNA-miRNA interactions and

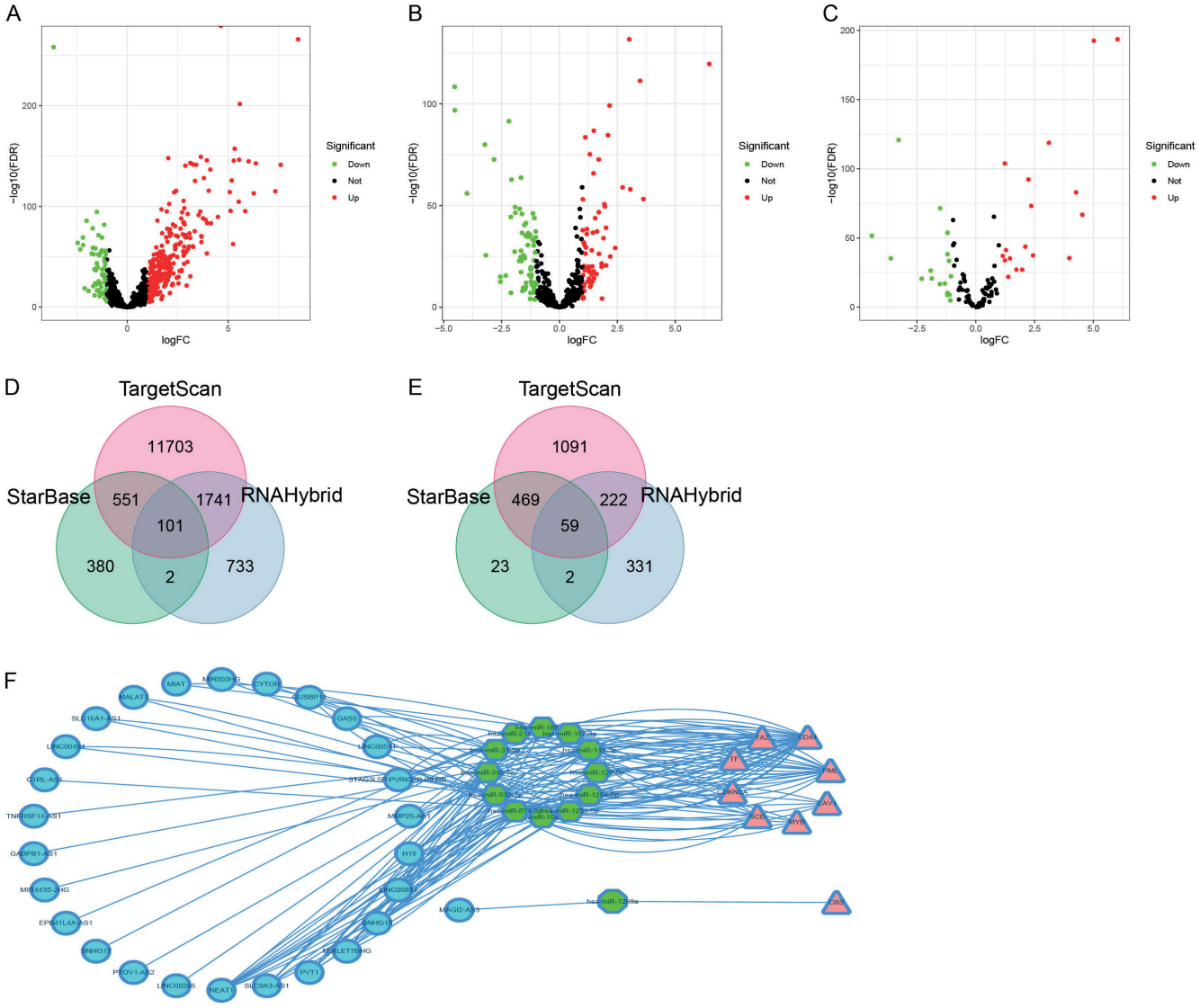

Fig. 1. A screen of the differentially expressed ferroptosis-associated lncRNAs, miRNAs and mRNAs in KIRC. Volcano plot representing the differentially expressed (A) lncRNAs (286 upregulated and 70 downregulated), (B) miRNAs (62 upregulated and 70 downregulated) and (C) mRNAs (18 upregulated and 17 downregulated) between the normal and the KIRC groups. The upregulated and downregulated lncRNAs, miRNAs and mRNAs are highlighted in red and green, respectively. Venn diagrams of (D) lncRNA-miRNA interactions and (E) miRNA-mRNA interactions predicted in RNAHybrid, StarBase and TargetScan databases; F. The ceRNA network consists of 27 lncRNAs, 13 miRNAs and 9 mRNAs

KIRC – kidney renal clear cell carcinoma; RNA – ribonucleic acid; lncRNAs – long non-coding RNAs; miRNAs – microRNAs; mRNAs – messenger RNAs; ceRNA – competing endogenous RNA.

1594,913,10067 miRNA-ferroptosis-related mRNA interactions were predicted, respectively (Fig. 1D,E). Taking miRNA as the connecting point, we constructed a ceRNA network with 182 axes, according to the expression of genes in the network. The intersection of target miRNAs for downregulated lncRNAs, downregulated mRNAs with upregulated miRNAs, and target miRNAs for upregulated lncRNAs, upregulated mRNAs with downregulated miRNAs were selected and finally, a ceRNA network with 77 axes was obtained (Fig. 1F). Among them, there were 27 lncRNAs (26 upregulated and 1 downregulated), 13 miRNAs (12 downregulated and 1 upregulated), and 9 mRNAs (8 upregulated and 1 downregulated). The regulatory relationships disclosed by these networks may provide an idea for exploring the molecular mechanism of the ferroptosis-related lncRNAs.

Construction of lncRNA signature based on the ferroptosis-related ceRNA network

Given the potential of ferroptosis-related ceRNA in regulating patient prognosis, we focused on 27 lncRNAs within the ceRNA network. A total of 508 eligible ccRCC patients (4 samples with missing survival time were removed) with integrated information were incorporated in the TCGA-KIRC dataset and randomly assigned to the train set (254 samples) and validation set (254 samples). Primarily, 27 lncRNAs in the ferroptosis-related ceRNA network were extracted to identify the prognostic risk model. Then, we performed the Cox proportional hazard assumption test based on Schoenfeld's residuals. All the lncRNAs in the univariate Cox regression model satisfied the proportional hazards assumptions ($p > 0.05$) (Supplementary Table 3). The Schoenfeld's residual plots did not suggest a violation of the proportional hazard assumption (Supplementary Fig. 2). Next, 14 lncRNAs significantly related to OS were selected in the univariate Cox regression analysis, which was considered as potential predictors. Finally, a LASSO regression algorithm was performed for feature selection. When the partial likelihood binomial deviation reaches the minimum value, the most appropriate tuning parameter λ for LASSO regression is 0.055. According to the penalty parameter (Lambda) in the model, we established a prognostic signature of KIRC patients consisting of 6-ferroptosis related lncRNAs including PVT1, CYTOR, MIAT, SNHG17, LINC00265, and LINC00894 (Fig. 2A–C). A risk score for each patient was calculated according to the following risk formula:

$$\begin{aligned}\text{risk score} = {} & \beta \times \text{expression value of PVT1} \\ & + \beta \times \text{expression value of CYTOR} \\ & + \beta \times \text{expression value of MIAT} \\ & + \beta \times \text{expression value of SNHG17} \\ & + \beta \times \text{expression value of LINC00265} \\ & + \beta \times \text{expression value of LINC00894}\end{aligned}$$

We evenly categorized patients into a high-risk group (n = 127) and a low-risk group (n = 127) based on risk scores (Fig. 3A). As depicted in Fig. 3B,C, PCA analysis and t-SNE analysis were used to reduce the dimension of the data and observe the significant difference between the 2 groups. Our results indicated that patients in the high- and low-risk groups showed a significant 2-way distribution. As displayed in Fig. 3D, there were significantly more deaths in high-risk patients than in low-risk ones. Additionally, the Kaplan–Meier curve revealed that patients in the high-risk group have corresponded to a worse OS in the training set (Fig. 4A, logrank test, $\chi^2 = 32.6$, $p = 1.136e{-}08$). Time-dependent ROC curves were performed to evaluate the predictive accuracy of the risk score for OS, and the area under the curve (AUC) was 0.791, 0.740 and 0.890 in predicting 1-, 5- and 10-years OS in KIRC, respectively (Fig. 4B). More significantly, the signature shows superior prognostic prediction efficiency than tumor-node-metastasis (TNM) staging with the AUC of risk score of 0.78 on multi-factor ROC (Fig. 4C).

Validation of the 6-lncRNA signature in the validation set

Subsequently, we performed the same analysis in the validation set. Once more, 254 patients in the validation set were divided into high-risk (n = 127) and low-risk (n = 127) groups based on the median risk values in the train set (Fig. 5A). As predicted, the results of PCA analysis and t-SNE analysis indicated that the patients of the validation set in the high- and low-risk group also showed 2-way distribution, and patients in the high-risk group have corresponded to more death cases (Fig. 5B–D). Moreover, like the training set, the Kaplan–Meier curve showed that the mortality of patients in the high-risk group was significantly higher than that in the low-risk group (Fig. 6A, log-rank test, $\chi^2 = 17.9$, $p = 2.371e{-}5$). The AUC reached 0.723, 0.714 and 0.780 in predicting 1-, 5- and 10-year OS, and the risk score in muti-ROC was 0.721 (Fig. 6B,C). These results indicated that the prognostic signature can be regarded as a qualified prognostic evaluation.

Survival analysis, univariate Cox regression analysis and expression comparative analysis for the signature in the ceRNA network

Moreover, to further evaluate the signature, survival analysis was performed for each lncRNA in the signature (PVT1, CYTOR, MIAT, SNHG17, LINC00265, and LINC00894). As shown in Fig. 7, the results showed that all 6 lncRNAs were significantly negatively correlated with OS. Then, to explore the regulatory relationship of the signature on ferroptosis-related genes, Cytoscape 3.7.1 software was used to show the specific regulation relationships of 6 lncRNAs in the signature and 7 ferroptosis-related genes (Fig. 8A). By comparing ccRCC samples with normal kidney samples in TCGA and GTEx databases, we analyzed the expression of 7 lncRNA-regulated mRNAs (including CD44, PML, TAZ, CDKN2A, SCD, MYB, and CAV1) in the ceRNA network. The cutoff

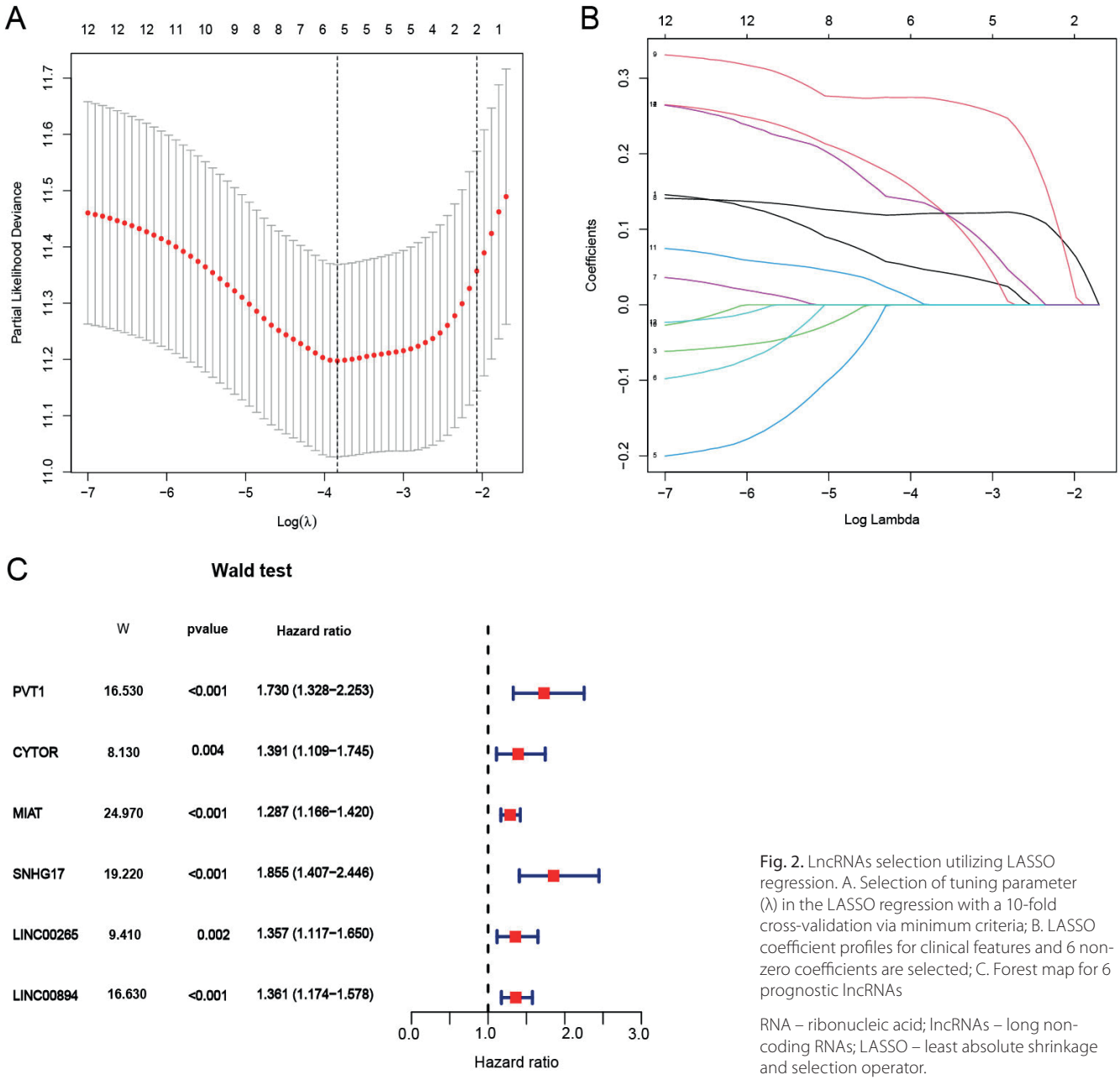

Fig. 2. LncRNAs selection utilizing LASSO regression. A. Selection of tuning parameter (λ) in the LASSO regression with a 10-fold cross-validation via minimum criteria; B. LASSO coefficient profiles for clinical features and 6 non-zero coefficients are selected; C. Forest map for 6 prognostic lncRNAs

RNA – ribonucleic acid; lncRNAs – long non-coding RNAs; LASSO – least absolute shrinkage and selection operator.

value was set to |log2 Fold Change | > 1, $p < 0.05$, and the results showed that all 7 ferroptosis-related genes were highly expressed in ccRCC, among which 5 were statistically significant, except TAZ and MYB (Fig. 8B). Besides, in the FerrDb database, we discovered that these 7 genes all regulate ferroptosis with the help of lipid reactive oxygen species (ROS), except PML, and could be divided into 2 categories: ferroptosis suppressors (CAV1, CD44, PML, and SCD) and ferroptosis drivers (CDKN2A, TAZ and MYB) (Supplementary Fig. 1).

Prognostic independence analysis of ferroptosis-related lncRNA signature and clinical features

Univariate and multivariate Cox regression analyses were performed among the available variables (including age, gender and TNM staging) to determine whether the signature was an independent prognostic predictor for OS. All the covariates in the multivariate Cox regression model satisfied the proportional hazards assumptions and the global Schoenfeld's test (Supplementary Fig. 3). Univariate Cox regression analysis showed that both in training set and validation set, TNM staging (training set: HR = 1.946, 95% CI = 1.604–2.361, Wald test, W = 45.650, $p < 0.001$; validation set: HR = 1.858, 95% CI = 1.533–2.252, Wald test, W = 39.900, $p < 0.001$) and risk signature (training set: HR = 3.208, 95% CI = 2.199–4.678, Wald test, W = 37.620, $p < 0.001$; validation set: HR = 3.600, 95% CI = 2.274–5.698, Wald test, W = 29.880, $p < 0.001$) were significantly correlated with OS (Fig. 9A,C). After independent adjustment for other clinical features in multivariate Cox regression, risk signature remained a reliable prognostic predictor of OS

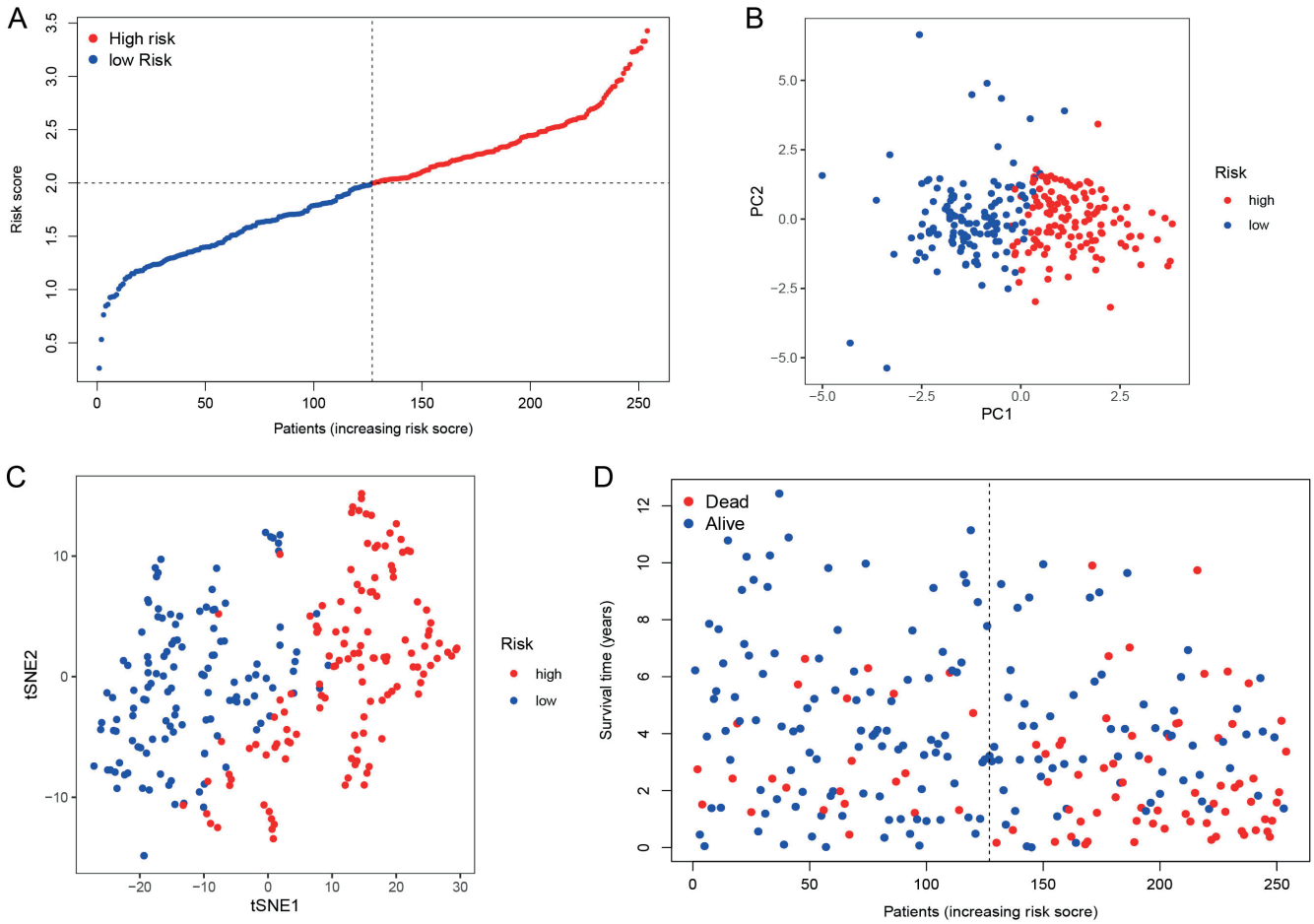

Fig. 3. The validation of the ferroptosis-related signature in the training set. A. The distribution of risk scores per patient in the training set; B. PCA and (C) t-SNE analysis revealed that the expression levels of ferroptosis-related lncRNAs involved in model construction could distinguish patients in the high- and low-risk groups in the training set; D. The distributions of OS status in the training set. The red dots represent dead patients and the blue dots represent living patients

PCA – principal component analysis; t-SNE – t-distributed stochastic neighbor embedding; RNA – ribonucleic acid; lncRNAs – long non-coding RNAs; OS – overall survival.

in both cohorts (train set: HR = 2.612, 95% CI = 1.793–3.805, Wald test, W = 25.000, $p < 0.001$; validation set: HR = 2.416, 95% CI = 1.522–3.835, Wald test, W = 14.200, $p < 0.001$) (Fig. 9B,D). The detailed Cox regression results can be found in Supplementary Table 4. Moreover, we examined the linear relationship between the log hazard and the continuous variable age. The results showed an overall linear trend in the relationship between the continuous covariate age and log hazard (Supplementary Fig. 4).

GO and KEGG enrichment analyses

To evaluate the biological functions and pathways associated with the prognostic signature, GO enrichment and KEGG pathway analyses were performed using DEGs between high- and low-risk groups. In total, 1,168 DEGs were significantly enriched in 367 biological processes, 51 cellular components, 52 molecular functions, and 10 KEGG entries. The top 10 enriched items of GO and KEGG were selected and demonstrated. As expected, GO analysis revealed that there were several molecular function entries related to lipid metabolism: these functions may be related to lipid peroxidation of ferroptosis. The KEGG analysis indicated that glycerophospholipid metabolism and phospholipase D signaling pathway were enriched. In addition, some immune-related items were also found to be significantly enriched in the 2 cohorts, including T cell activation, T cell differentiation, regulation of T cell activation, positive regulation of leukocyte cell adhesion, positive regulation of T cell activation, regulation of T cell differentiation, regulation of lymphocyte differentiation positive regulation of cell-cell adhesion, regulation of leucocyte cell adhesion, lymphocyte differentiation, cytokine receptor activity in molecular functions, immune receptor activity, cytokine binding, and so on (Fig. 10A–D).

ssGSEA and ESTIMATE analysis of immune infiltration

The results from GO and KEGG suggest that the ferroptosis-related signature may be involved in the regulation of the immune microenvironment. Therefore,

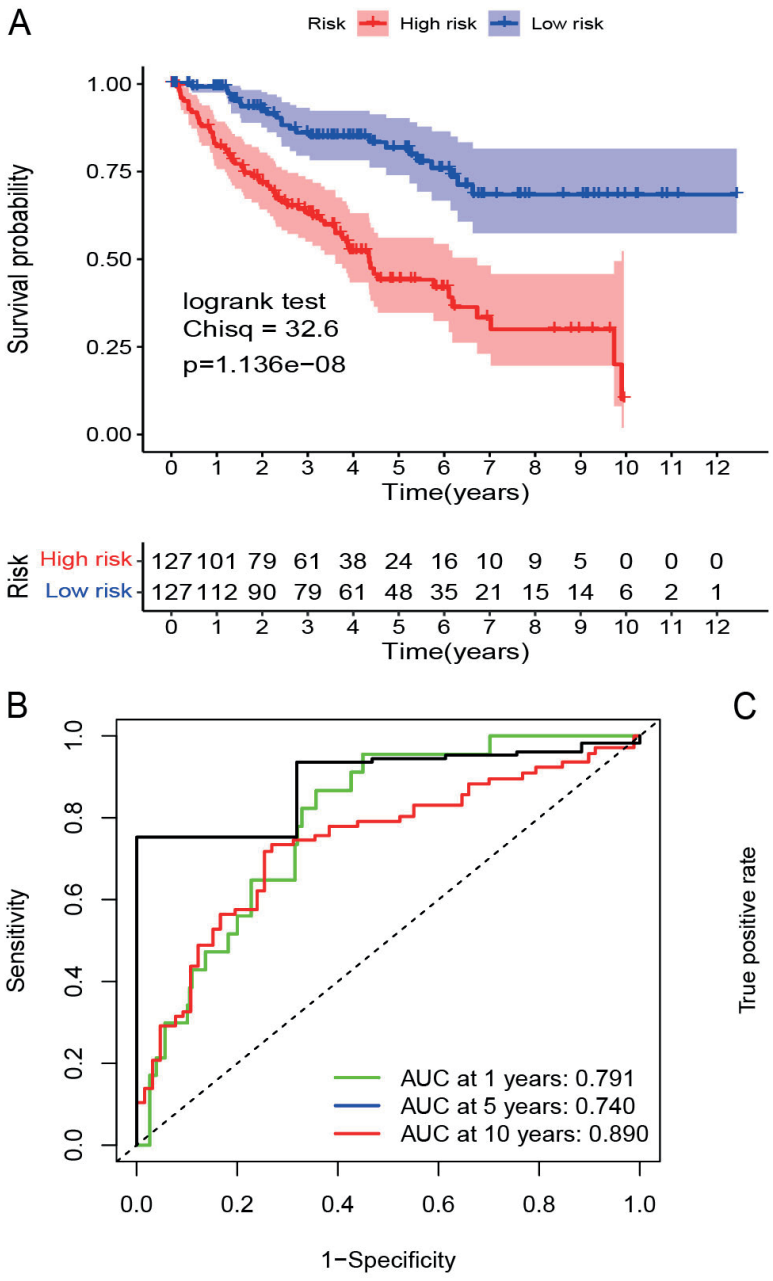

Fig. 4. The ferroptosis-related signature predicting overall survival (OS) in patients with KIRC in the training set. A. Kaplan–Meier curve to predict the OS of patients in the high- and low-risk cohorts; B. ROC curve analysis to evaluate the prognostic performance of the signature at 1 year (AUC = 0.791), 5 years (AUC = 0.740) and 10 years (AUC = 0.890); C. Time-dependent ROC analysis of the accuracy of the signature in the training set

KIRC – kidney renal clear cell carcinoma; ROC – receiver operating characteristic; AUC – area under the curve.

we explored its potential role in the TME. The ssGSEA approach was utilized to further evaluate the correlation between the risk score and immune cell infiltration status, and the results revealed that 9 of the 16 immune cells were found to have significantly high infiltration enrichment scores in the high-risk group, including $CD8^+$ T cells, pDCs, T helper cells, Th1 cells, TIL, aDCs, B cells, and Th2 cells (Fig. 11A). In terms of immune function, except for type II immune interferon response, the high-risk group was positively correlated with other immune-related functions (Fig. 11B). Then, we performed the ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) algorithm to further analyze the differences in immune components of TME in the high- and low-risk groups, and our results indicated that the ESTIMATE score and immune score were increased significantly in the low-risk group (Wilcoxon rank sum test, W = 38063, $p < 0.001$; Wilcoxon rank sum test, W = 41373, $p < 0.001$) (Fig. 11C), while stromal score was no significant difference between the 2 groups (Wilcoxon rank sum test, W = 31739, $p > 0.05$) (Fig. 11C).

Discussion

Clear cell renal cell carcinoma displays considerable heterogeneity in both its molecular characteristics and biological behavior, thereby presenting substantial challenges for clinicians involved in the treatment of cancer patients.[19] In clinical practice, grading and staging are commonly used clinical pathological parameters to assess the prognosis of ccRCC and guide treatment decisions.[20] However,

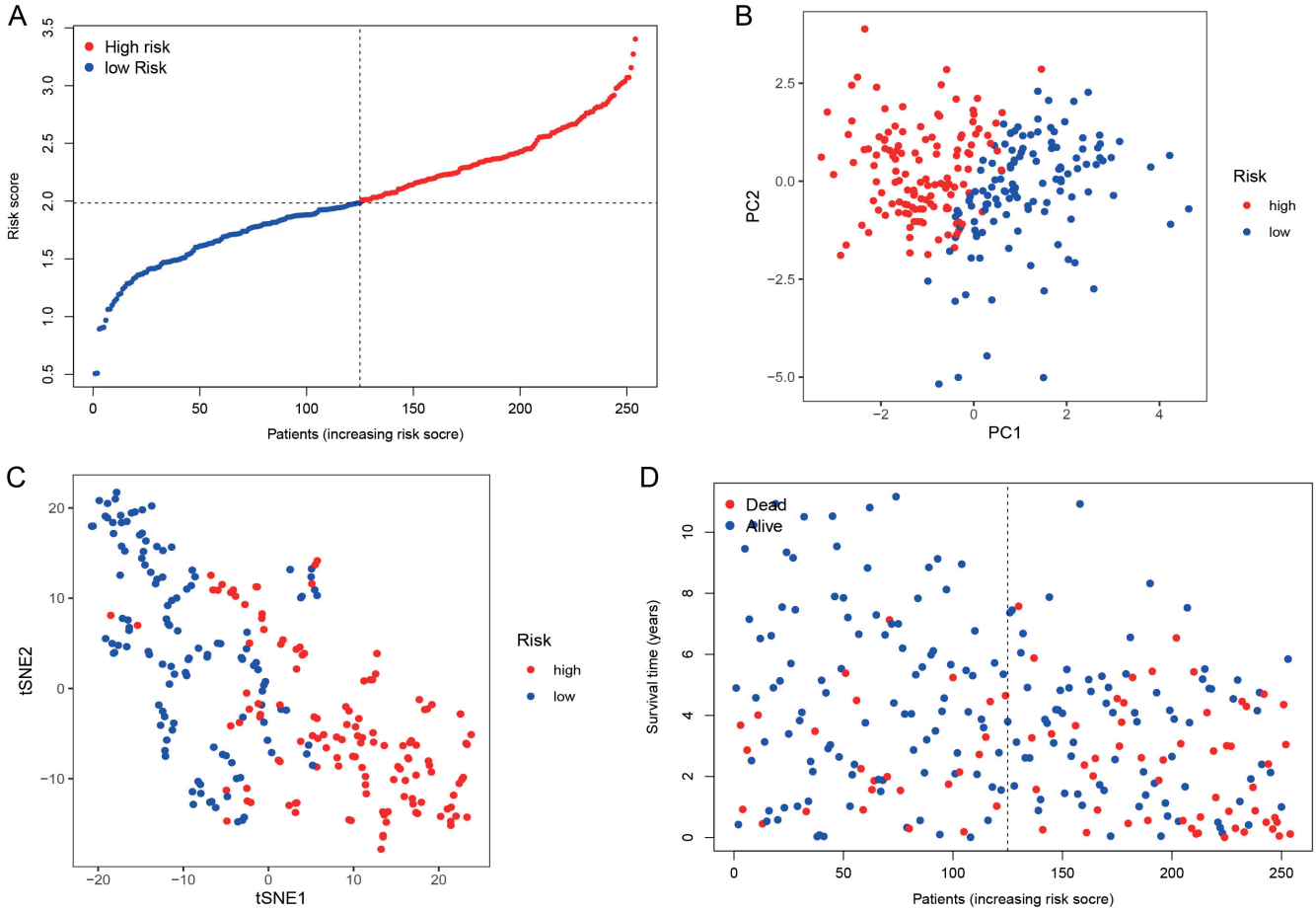

Fig. 5. The validation of the ferroptosis-related signature in the testing set. A The distribution of risk scores per patient in the testing set; B,C. PCA (B) t-SNE (C) analysis revealed that the expression levels of ferroptosis-related lncRNAs involved in model construction could distinguish patients in the high- and low-risk groups in the testing set; D. The distributions of OS status in the testing set. The red dots represent dead patients and the blue dots represent living patients

PCA – principal component analysis; t-SNE – t-distributed stochastic neighbor embedding; RNA – ribonucleic acid; lncRNAs – long non-coding RNAs; OS – overall survival.

patients with the same cancer staging often exhibit significant differences in prognosis and response to treatment.[21] Specifically, conventional histopathological measures and similar treatment strategies may result in curing patients, while others may die prematurely. This indicates the limitations of clinical pathological parameters in describing the survival outcomes of ccRCC patients.[21] Therefore, it is necessary to surpass the prognostic features of the current staging system in order to accurately identify patients who may develop aggressive diseases with poor survival rates and provide better guidance for clinical therapy. In the current study, we first identified lncRNAs, miRNAs and ferroptosis-related mRNAs through differential expression analysis of 583 samples downloaded from the KIRC cohort of the TCGA database. Then, through RNAHybrid, StarBase and TargetScan databases, 27 lncRNAs, 13 miRNAs and 9 mRNAs conforming to the ceRNA mechanism were predicted and selected. Through a series of analyses, including univariate Cox regression analysis, LASSO regression analysis and multivariate COX regression analysis, 6 lncRNAs significantly associated with OS were identified and used to establish a ferroptosis-related signature. To explore the distribution of each case with different risk values, PCA and t-SNE analyses were performed in the training and validation cohorts, and our results showed that KIRC patients with low-risk scores could be distinguished from those with high-risk scores. Moreover, the death probability distribution analysis also found that the number of deaths in KIRC patients with high-risk scores was significantly higher than that in KIRC patients with low-risk scores. Through Kaplan–Meier curve analysis and Cox regression analysis, we identified that the ferroptosis-related signature could stratify the risk for ccRCC patients based on OS and serve as an independent prognostic factor for clinical outcomes. Furthermore, the ROC curve analysis demonstrated that the ferroptosis-related signature could accurately predict short- and long-term survival in ccRCC patients, with significantly higher predictive accuracy compared to most clinical features. Taken together, these results indicate that the ferroptosis-related signature could be a stable and robust tool for clinicians to predict the prognosis of ccRCC patients.

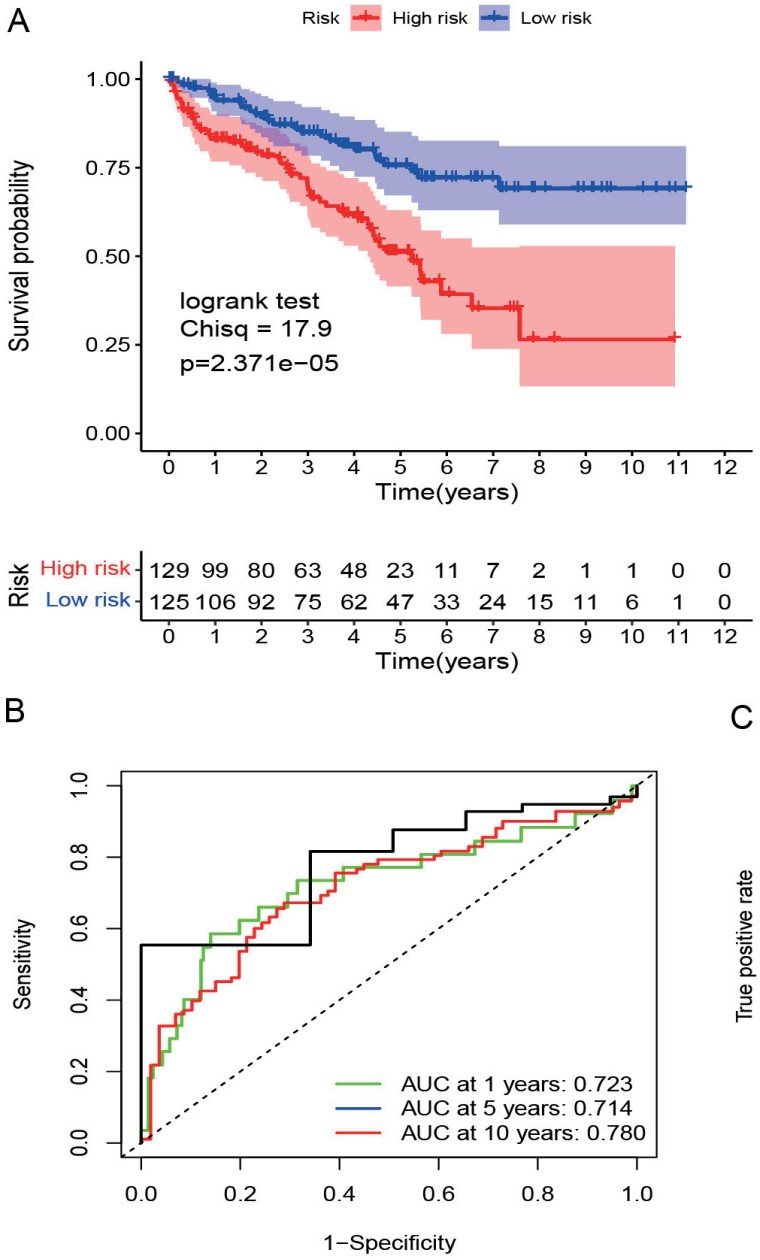

Fig. 6. The ferroptosis-related signature predicts overall survival (OS) in patients with KIRC in the testing set. A. Kaplan–Meier curve to predict the OS of patients in the high- and low-risk cohorts; B. ROC curve analysis to evaluate the prognostic performance of the signature at 1 year (AUC = 0.723), 5 years (AUC = 0.714) and 10 years (AUC = 0.780); C. Time-dependent ROC analysis of the accuracy of the signature in the testing set

KIRC – kidney renal clear cell carcinoma; ROC – receiver operating characteristic; AUC – area under the curve.

Within the ferroptosis-related signature that we constructed, a higher risk indicated a poor prognosis. To further understand the underlying molecular mechanisms, we initially analyzed the effects of 6 lncRNAs on OS in different risk subgroups. Not surprisingly, the 6 lncRNAs (SNHG17, CYTOR, LINC00894, LINC00265, MIAT, and PVT1) included in the signature were significantly associated with the unfavorable prognosis of ccRCC. Among the 6 lncRNAs in the signature, some have previously been reported to be associated with ccRCC. Research has confirmed that lncRNA PVT1 is significantly overexpressed and can act as a ceRNA in the context of RCC.[22] Additionally, Yan et al. identified that lncRNA MLAT is upregulated in ccRCC tissues and cell lines and can act as a sponge for miR-29c, increasing the expression of ZEB1.[23] Recently, a study identified that SNHG17 was significantly upregulated in ccRCC and could be used as a prognosis predictor.[24] Deng et al. uncovered that LINC00894 was upregulated and significantly related to the prognosis in ccRCC patients.[25] Also, the function of CYTOR has been identified to be an oncogene in many cancers. However, as well as the LINC00265, it has not been elucidated in the previous studies and our research uncovered their potential roles in KIRC. To further investigate the role of ferroptosis-related lncRNAs in gene regulation in ccRCC, we constructed a ceRNA network. According to the regulatory network in the FerrDb database, all 7 genes were somehow connected with lipid ROS to regulate ferroptosis, except that PML was a straight ferroptosis suppressor. The high lipid ROS connection was in accord with the lipid metabolism relevancy found in GO analysis, which revealed that the signature was highly associated with the lipid peroxidation process in ferroptosis.

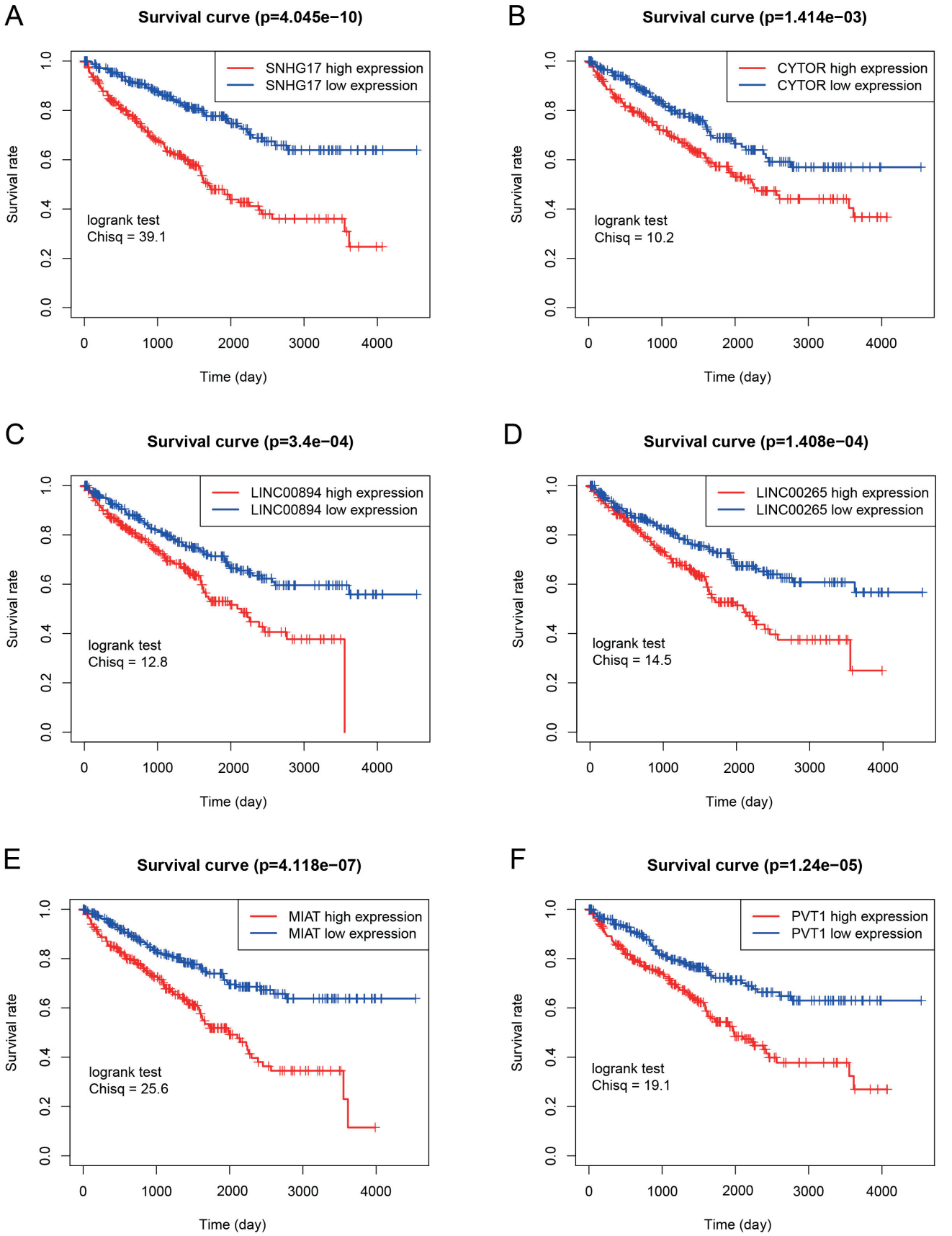

Fig. 7. The Kaplan–Meier curves of 6 prognostic ferroptosis-related lncRNAs in the signature. A Kaplan–Meier curve showing the overall survival (OS) of SNHG17 in the high- and low-risk group; B. Kaplan–Meier curve showing the OS of CYTOR in the high- and low-risk group; C. Kaplan–Meier curve showing the OS of LINC00894 in the high- and low-risk group; D. Kaplan–Meier curve showing the OS of LINC00265 in the high- and low-risk group; E. Kaplan–Meier curve showing the OS of MIAT in the high- and low-risk group; F. Kaplan–Meier curve showing the OS of PVT1 in the high- and low-risk group

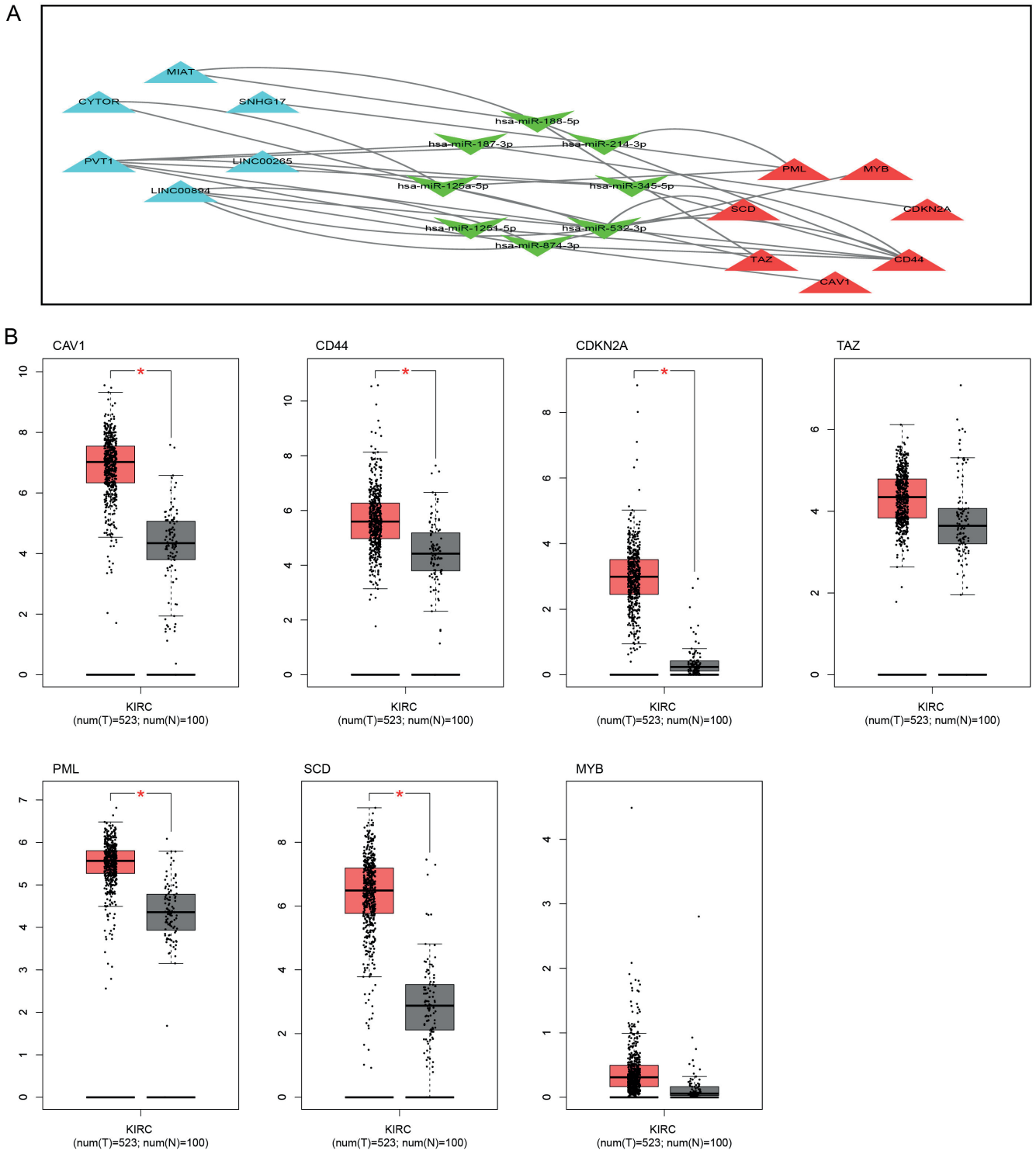

Fig. 8. The ferroptosis-related ceRNA network and the ferroptosis-related gene expression. A. ceRNA network: the light blue triangles indicate lncRNAs, the green triangles mean miRNAs, and the red triangles represent mRNAs; B. Expression of 7 ferroptosis-related genes between KIRC tumor and normal tissues

RNA – ribonucleic acid; lncRNAs – long non-coding RNAs; miRNAs – microRNAs; mRNAs – messenger RNAs; ceRNA – competing endogenous RNA; KIRC – kidney renal clear cell carcinoma; *p < 0.05.

Moreover, we uncovered that CAV1, CD44, PML, and SCD could act as a suppressor of ferroptosis, while CDKN2A, MYB and TAZ were drivers of ferroptosis, which may explain the double-sided effect of ferroptosis on tumor inhibition and tumor growth. CAV1, CD44, PML, and SCD have been confirmed to be highly expressed in tumor tissues and promote tumor progression by inhibiting ferroptosis.[26–29] CDKN2A has been identified to play a vital role in tumorigenesis by enhancing p53-dependent transactivation and ferroptosis,[30] the hypermethylation of CDKN2A might be a predictor of poor prognosis for cancer.[31] MYB, a vital transcription factor in solid tumors, can regulate

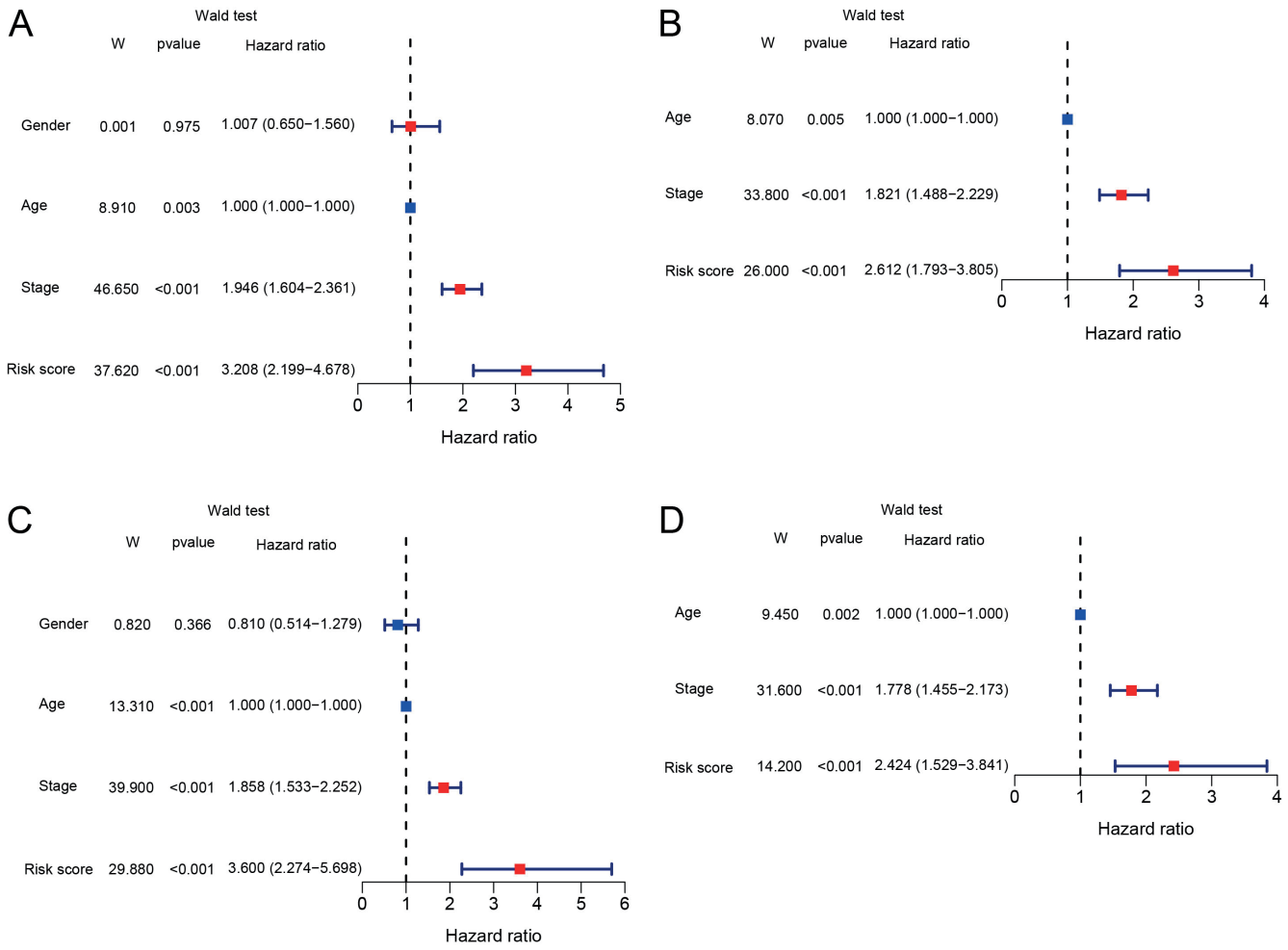

Fig. 9. Univariate and multivariate Cox regression analyses regarding overall survival (OS) in the training and testing set. A. Univariate Cox regression analysis in the training set; B. Multivariate Cox regression analysis in the training set; C. Univariate Cox regression analysis in the testing set; D. Multivariate Cox regression analysis in the testing set

the expression of CDO1; then, CDO1 can convert cysteine into taurine, reduce the utilization of cysteine, limit the synthesis of glutathione, inhibit the antioxidant capacity of cells, and eventually cause ferroptosis.[32] A recent study showed that TAZ is highly expressed in RCC and can mediate cell density-regulated ferroptosis.[14] These findings revealed potential regulatory mechanisms of ferroptosis-related molecules in the occurrence and development of ccRCC, providing potential biomarkers for the identification and personalized treatment of this cancer.

To better understand the transcriptional regulatory mechanisms of ferroptosis-related lncRNAs in ccRCC, we conducted a comprehensive exploration of their functions and pathways involved. The GO functional enrichment analysis revealed that these differentially expressed genes were involved in lipid metabolism-related functions, which may be associated with lipid peroxidation of ferroptosis, and the KEGG analysis revealed that several signaling pathways related to cancer and ferroptosis were enriched, such as glycerophospholipid metabolism, phospholipase D signaling pathway and lipase activity pathway. As previously reported, these pathways have been found to be abnormally overactivated in multiple types of cancers, which drives the proliferation, invasion and metastasis of cancer cells, and is often associated with poor clinical prognosis.[33–35] Hence, these oncogenic pathways may explain the impact of the ferroptosis-related signature on the prognosis of ccRCC patients. Drugs targeting these abnormally overactivated pathways may have the potential to inhibit the progression of this cancer.

It has been reported that the mechanism by which ferroptosis cells trigger potent immune responses may have some similarities with traditional immunogenic cell death.[36] Considering the close relationship between ferroptosis and tumor immunity, we utilized 2 algorithms (ESTIMATE and ssGSEA) to investigate the immune microenvironment associated with the ferroptosis-related signature in ccRCC. In the TME component analysis, the high-risk group had higher ESTIMATE scores and immune scores. Some studies have demonstrated that the abundance of immune cell components serves as an independent prognostic factor that is crucial to the prognosis of ccRCC.[37,38] Therefore, we hypothesized that the influence of 6 lncRNAs (SNHG17, CYTOR, LINC00894, LINC00265, MIAT, and PVT1)

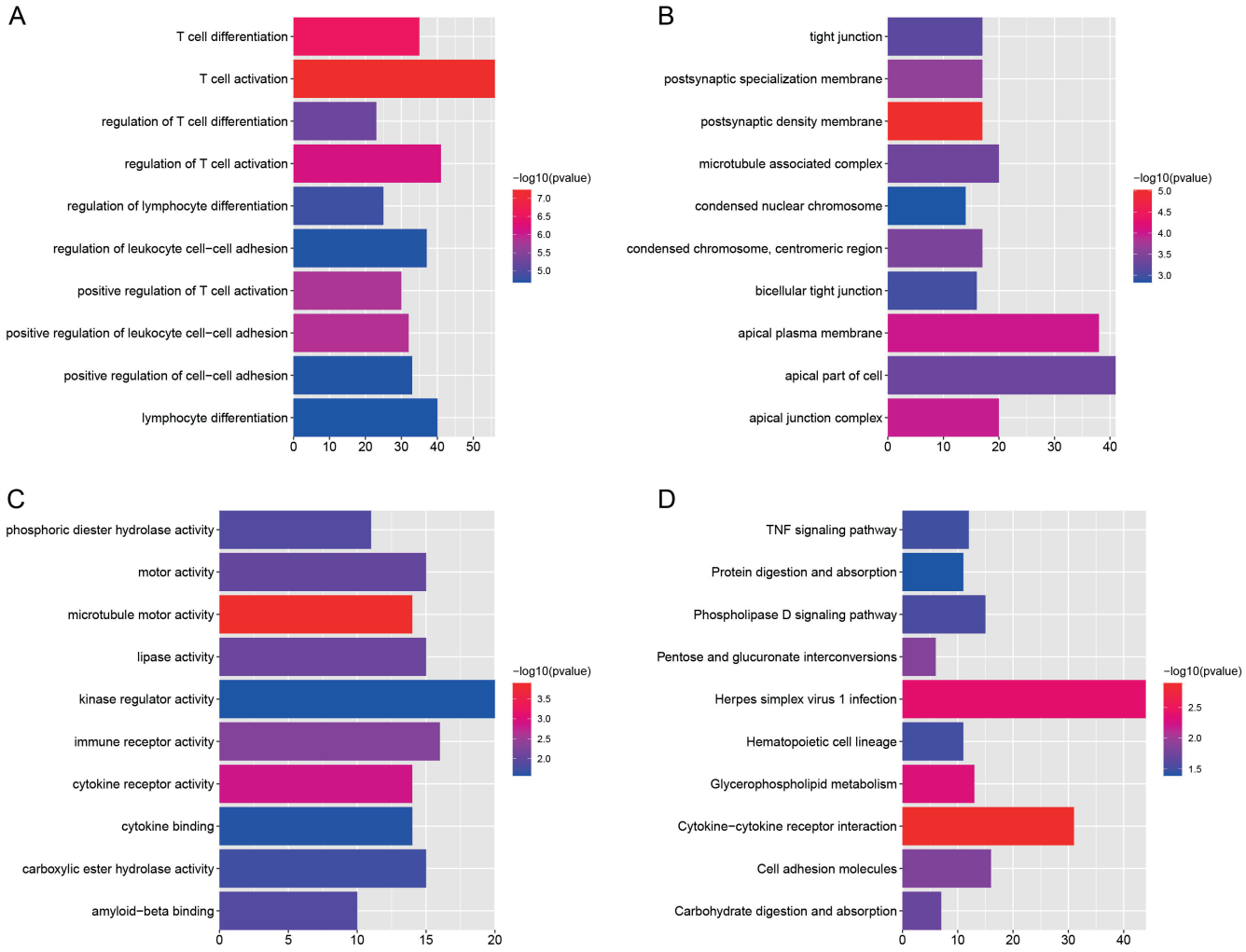

Fig. 10. GO and KEGG analyses using DEGs between high- and low-risk groups. A. BP items in GO analyses; B. CC items in GO analyses; C. MF items in GO analyses; D. KEGG pathways

GO – Gene Ontology; KEGG – Kyoto Encyclopedia of Genes and Genomes; DEGs – differentially expressed genes; BP – biological process; CC – cellular component; MF – molecular function.

on unfavorable survival among patients with ccRCC might be associated with the remodeling of immune components within the TME. This finding provides indirect evidence for the close association between the ferroptosis-related signature and the survival of ccRCC patients. The analysis of TME cell types from ssGSEA also fully supported this hypothesis. High-risk individuals had an active presence of immune cells, such as $CD8^+$ T cells and Th1 cells, indicating enhanced anti-tumor immune activity. Overall, these discoveries provided novel insights into the mechanisms of signature lncRNAs regulating the immune microenvironment of ccRCC patients.

Limitations

Our study has some limitations. For instance, the ferroptosis-lncRNA signature was developed according to the TCGA public database, but it would be better to have strong external data to confirm its validity and practicality. Furthermore, our study is mainly based on integrated bioinformatics and lacks confirmation of these findings from valid clinical studies.

Conclusions

In the present study, we developed a ferroptosis-related lncRNA signature exhibiting high accuracy and stability, which shows potential as a prognostic prediction tool for ccRCC patients. Moreover, by constructing a ceRNA network and conducting immune infiltration analysis, we elucidated the potential mechanisms by which ferroptosis-related lncRNAs regulate ccRCC, and explored their role within the TME, offering novel insights for precise treatment based on ferroptosis signaling. Our study provides a potential option for prognosis prediction and personalized treatment in ccRCC patients.

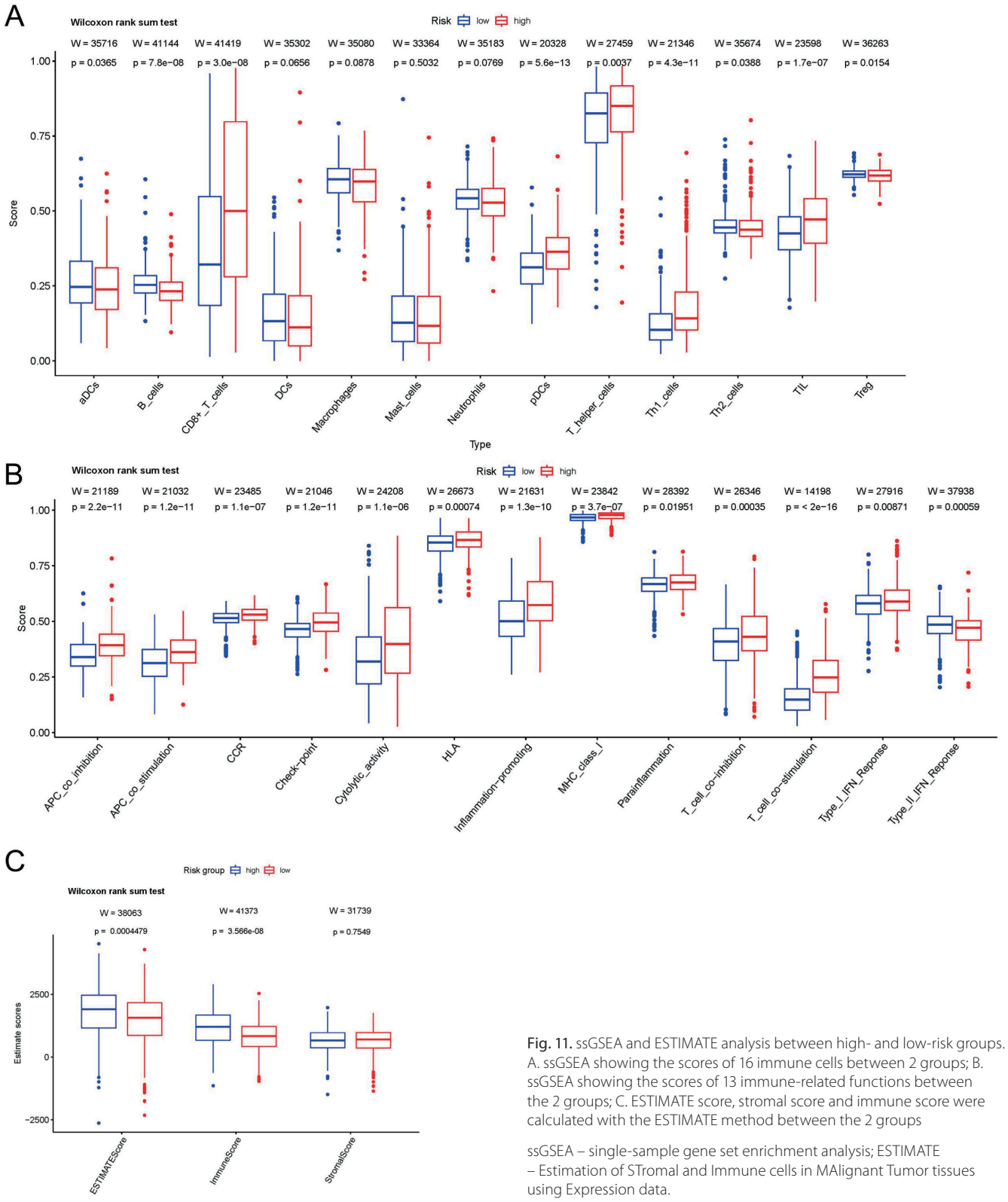

Fig. 11. ssGSEA and ESTIMATE analysis between high- and low-risk groups. A. ssGSEA showing the scores of 16 immune cells between 2 groups; B. ssGSEA showing the scores of 13 immune-related functions between the 2 groups; C. ESTIMATE score, stromal score and immune score were calculated with the ESTIMATE method between the 2 groups

ssGSEA – single-sample gene set enrichment analysis; ESTIMATE – Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data.

Data availability

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

Ethics approval and consent to participate

This article did not involve any studies with human or animal participants conducted by the authors, and therefore, ethical approval or consent was not required.

The study did not require any administrative permission or licenses to access the original data used in the research.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.8032200. The package includes the following files:

Supplementary Fig. 1. Seven ferroptosis-related genes were divided into 2 categories in the FerrDb database. Ferroptosis suppressor: CAV1, CD44, PML and SCD. Ferroptosis driver: CDKN2A, TAZ and MYB.

Supplementary Fig. 2. The results of the proportional hazards assumption (lncRNAs selection using univariate Cox regression) based on Schoenfeld's global and individual test.

Supplementary Fig. 3. The results of the proportional hazards assumption (selection of lncRNA signature and clinical features) based on Schoenfeld's global and individual test.

Supplementary Fig. 4. The linear relationship between the log hazard and the continuous variable age.

Supplementary Table 1. Ferroptosis-related genes (n = 112) validated in humans and protein coding were obtained from the FerrDb database.

Supplementary Table 2. Annotated gene sets to quantify the immune infiltration enrichment scores of different immune cells and immune-related functions.

Supplementary Table 3. The results of the proportional hazards assumption for Cox regression based on Schoenfeld's global and individual test.

Supplementary Table 4. The detailed Cox regression results, including HR, coefficient, 95% CI, Wald test, and likelihood ratio (LR) test

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Dental ceramic damage associated with incorrect laboratory procedures

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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

Ceramic is a commonly used material in dentistry for reconstructing missing teeth or their tissues due to its biocompatibility, durability and excellent esthetic properties. Despite these advantages, the ceramic restoration damage remains a significant clinical problem. Its causes can be divided into clinical and laboratory factors. The most known include uneven occlusion, improper preparation, trauma, or parafunctions. This study focuses on characterizing less known laboratory causes of ceramic restoration damage. We reviewed the current literature available in the PubMed and Scopus databases. On the basis of 63 selected studies, 3 basic causes of damage were identified: excessive stresses between the framework and ceramic veneering, poor quality of the connection between the facing layer and the substructure, and defects resulting from the nature of the ceramic material such as defects in the ceramic layer, brittleness and lack of flexibility. The stages of the manufacturing process of various permanent ceramic restorations were presented. By controlling these procedures, we can eliminate the errors, resulting in long-term effective functioning of the ceramic restorations.

Key words: ceramics, crowns, zirconium oxide, dental restoration failure, metal–ceramic alloys

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Introduction

Ceramic materials are commonly used in dentistry. Metal–ceramic restorations have been considered the "gold standard" in prosthetic rehabilitation of damaged tooth structures since the late 1950s. They integrate the high strength of metal substructure with the ceramic veneering esthetics.[1] Over the past 30 years, the growing demand for highly esthetic and natural-looking prosthetic restorations has led to the development of new ceramic materials with excellent mechanical strength and a high degree of biocompatibility, which enabled metal base elimination.[2] However, despite continuous material and technological progress, the survival rates of ceramic restorations invariably depend on the correctness of clinical and laboratory procedures.

Ceramic restorations are an effective and long-term prosthetic reconstruction. A systematic review assessing the durability of prosthetic crowns over a 5-year period has shown that conventional metal–ceramic restorations show a similar success rate (95.7%) to lithium disilicate all-ceramic restorations, leucite-reinforced glass ceramics (96.6%), glass-infiltrated alumina (94.6%), and zirconium oxide (93.8%). However, the survival rate of felspathic crowns was lower (90.7%), particularly in the posterior region (87.8%).[3]

In addition, a systematic review demonstrated a 94.4% survival rate for metal–ceramic bridges over the 5-year observation period. It was higher than that of all-ceramic bridges made of reinforced glass ceramics (85.9%), glass-infiltrated aluminum oxide (86.2%) or zirconium oxide (90.1%).[4]

Despite many unquestionable advantages, ceramic restorations may deteriorate over time. The main cause of distant complications is ceramic veneering damage, so-called chipping. The authors noted that zirconium oxide (3.1%) and metal–ceramic (2.6%) crowns showed a higher incidence of ceramic chipping, while crowns made of leucite ceramics and lithium disilicate showed a higher frequency of framework fracture (2.3%) over the 5-year period.[2] On the other hand, in the case of bridges after the same period, the frequency of ceramic chipping was the highest for ceramic restorations on glass-infiltrated alumina (31.4%) and densely sintered zirconium oxide (20.4%). All-ceramic restorations made of reinforced ceramics glass (10.1%) and alumina infiltrated with glass (12.9%) also showed the highest fracture frequency. It is important to note that these are objective findings and not subjective evaluations.[4]

There are many classifications of ceramic damage available in the literature.[1,5,6] One of them is the classification of Michalakis and Agustín, which divides them into 3 damage types: cohesive, adhesive and adhesive-cohesive. Cohesive damage is characterized by chipping within the veneering ceramic layer, adhesive damage is characterized by chipping with the prosthetic restoration base exposed, while adhesive-cohesive damage is a combination of 2 types of ceramic damage.[1,5]

Heintze and Rousson classified the damage according to its size and reparability. Grade 1 refers to superficial damage. It is a small chip, which can be fixed just by polishing the ceramic restoration surface. Grade 2 is a moderate chipping of the veneering ceramic. It requires intraoral repair with a composite resin. Grade 3 is an extensive damage of veneering ceramic, which requires the replacement of the damaged fixed prosthesis for both functional and esthetic reasons.[6]

In addition, we can also include cracking of the substructure and span of the bridge as a type of damages. A study by Saito et al. showed that zirconia-based ceramic restorations failures are most often cohesive (88.8%).[7] Also, Agustín et al. observed that the most common type of failure for zirconia-core ceramic restorations was cohesion (71.66%), compared to metal–ceramic restorations, all of which showed adhesive failure.[1]

On the other hand, literature reviews conducted by Heintze and Rousson, Raigrodski et al. and Anusavice show that the most common types of permanent dentures chipping are grades 1 and 2, which are esthetic defects often unnoticed by the patient and not associated with the failure of prosthetic reconstruction.[6,8,9]

Unlike clinical issues, which are directly controlled by the dentist, problems related to the laboratory process are not part of everyday practice. The purpose of this work is to identify errors that may occur during the laboratory stage of execution, which can result in premature loss or damage of permanent restorations such as crowns and bridges.

Materials and methods

This review is based on a literature search conducted in the PubMed, Embase and Scopus databases. Article published between 2002 and 2021 were included. We performed a combined free text term and medical-subject heading (MeSH) search. Our inclusion criteria were based on a PICO (Patient, Intervention, Comparison, Outcome) strategy. The search strategy was developed in stages, incorporating the type of patients who use fixed partial

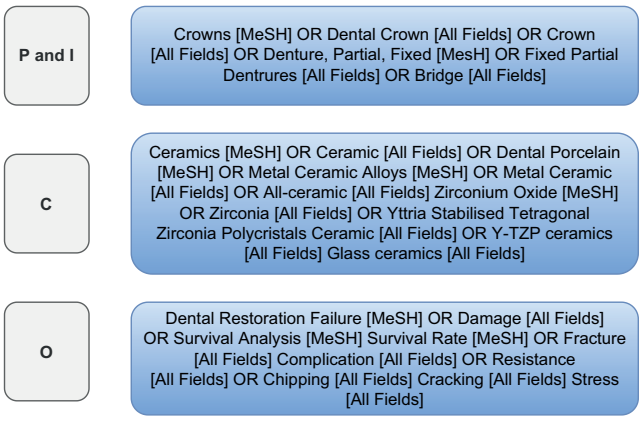

Fig. 1. Search strategy

dentures (crowns and bridges), the type of materials used (all-ceramic, metal–ceramic, zirconia, and glass–ceramic), and the presence of complications or restoration failure. The full strategy is presented in Fig. 1.

The combination in the builder was set as "P & I AND C AND O". Exclusion criteria were defined as follows: hybrid material restorations, case studies and languages other than English.

Two independent researchers performed the selection of the studies. In the 1st step, titles and abstracts were screened for relevant articles. In the 2nd step, full texts were assessed. The results are summarized in Fig. 2.

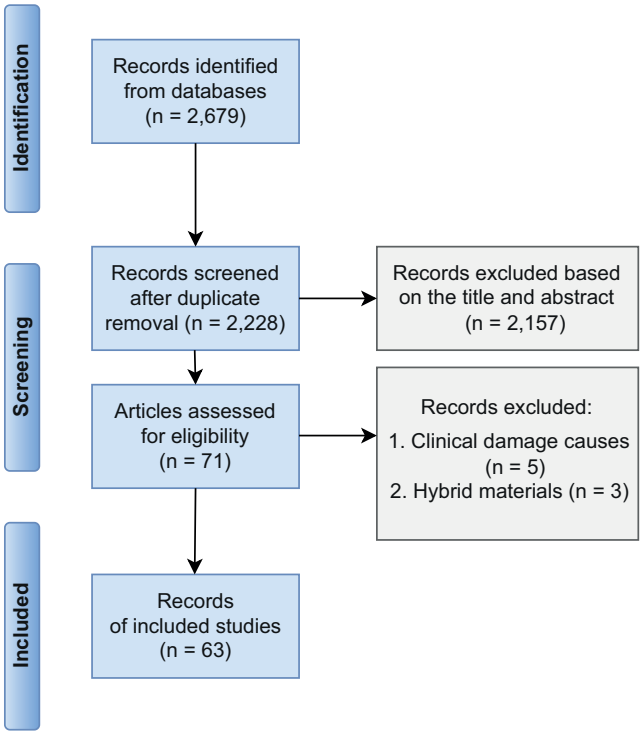

Fig. 2. Article selection process

Results

The evaluation included 63 articles after final eligibility assessment, selected from 2,679 papers that met the keyword criteria during the literature review.

Residual stress occurring during laboratory procedures is a crucial factor in the damage of ceramic restorations. The factors affecting the residual stresses of veneers include the functional stresses, thermal expansion coefficients of the framework and ceramic veneering, firing temperature and cooling time, geometry of the ceramic restoration, processing technique of the zirconia framework, and choice of veneering method and framework material for the fixed denture. It is important to consider all of these factors when designing and fabricating dental restorations. The bonding quality of veneering ceramic to the framework and the poor properties of the veneering ceramic are important factors that influence the survival rates of restorations.[10–13]

Based on the literature review, the following laboratory factors leading to ceramic damage were distinguished and presented in Fig. 3.

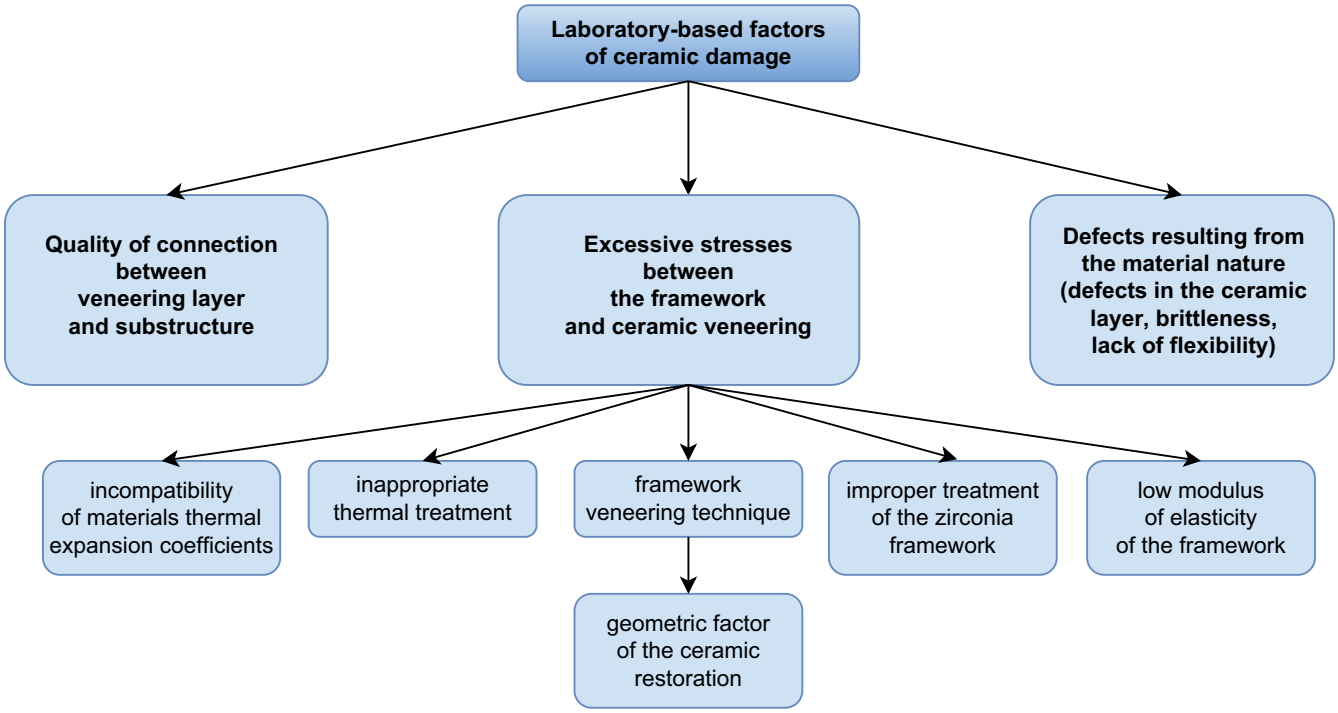

Fig. 3. Laboratory causes of ceramic damage

Excessive stresses between the framework and ceramic veneering

Incompatibility of thermal expansion coefficients of materials

Studies have found that compatibility of thermal expansion coefficients (coefficient of thermal expansion (CTE)) between veneering porcelain and the metal or ceramic substructure is critical to avoid formation of cracks after the firing process.[14] Ceramic coefficient of thermal expansion should be slightly lower than the CTE of the core material. As the weaker veneering ceramic cools, a small compressive stress, known as residual compression, is generated. This compensates for the tensile stresses that arise from the mechanical load of the prosthetic restoration.[14,15]

A study by Juntavee and Dangsuwan evaluating the effect of a veneering ceramic type with different CTE on a zirconia framework showed that a veneering ceramic CTE 0.77–0.87 × 10^{-6}/°C lower than the CTE of zirconia resulted in the desired residual compressive stress. It provided favorable bonding strength between the fired ceramic and the zirconia.[16] In the case of metal–ceramic restorations, the CTE of the metal alloy lower by 0.5 × 10^{-6}/°C than the thermal expansion coefficient of the fired ceramic was found to be the most favorable.[17,18] Much higher substructure CTE than CTE of the ceramic veneering layer can result in a compressive stress during cooling process. These stresses run parallel to the framework and may lead to delamination of the veneering ceramic from the framework. However, when the CTE of the framework is definitely lower than that of the veneer, the tensile stresses increase, which initiates the formation of cracks running on the surface of the veneering ceramics.[14]

A mismatch of the CTEs of the veneering ceramic and the zirconia framework above 10% has been proven to cause porcelain fracture.[14] The coefficients of thermal expansion discrepancies may be more critical for all-ceramic restorations compared to metal–ceramic restorations. The former, due to the higher stiffness and brittleness of the ceramic substructure, do not tolerate tensile stresses.[19] Additionally, the study by Swain showed that the higher rates of veneer ceramic chipping in zirconia ceramic restorations may be due to residual stresses resulting from a greater thermal mismatch between the zirconia core and the veneer ceramic. This phenomenon may be caused by the poor thermal conductivity of zirconium compared to the metal alloy.[14]

Inappropriate thermal treatment

In order to obtain the correct anatomy and esthetics of ceramic restorations, the manufacturing process consists of several stages, during which successive layers of ceramics are applied. Each layer undergoes sintering cycles at a temperature well above the glass transition temperature of the veneering ceramic.[19] In order to obtain satisfactory results, it is extremely important to follow the manufacturer's guidelines regarding time, temperature, number of firings, and the recommended porcelain cooling protocol.

Saini et al. demonstrated on the basis of experimental studies that the firing temperature of dental ceramics lower than recommended by the manufacturers causes superficial and deep porosity. The ceramic inhomogeneity reduces the strength of the material.[20]

It is equally important to follow the correct cooling protocol. According to Swain, the rate of cooling after each firing cycle affects the amount of residual stresses developing in the ceramic restoration.[14] Restorations that are cooled by immediately opening the furnace after firing are exposed to thermal shock. The outer surface of the porcelain solidifies and shrinks earlier, while the inner part is still at a higher temperature. After cooling, the internal temperature drops, and the solid outer surface of the porcelain prevent the shrinkage of the inner layers. It results in residual tensile stresses locked into the material layers.[21] Also, an excessively prolonged cooling protocol from sintering temperature to room temperature may weaken the bond strength between the veneer and the framework. It generates residual tensile stresses resulting from the elastic relaxation of the glass phase contained in the veneering ceramic.[22]

Slow cooling protocol after the last firing program is particularly important in the fabrication of zirconia-based ceramic restorantions because unlike metal alloys (40–200 W/mK) and aluminum oxide (30 W/mK), glass ceramics (3–4 W/mK) and zirconia (1–4 W/mK) have a low thermal conductivity.[19] The ceramic framework retains heat for a longer period instead of transferring it to the veneering ceramic. During the firing and cooling process, unfavorable temperature distribution occurs. Internal stresses arise in the facing material, which initiate the formation of cracks with extensive propagation. In the case of a thick zirconia framework, test results suggest a slow cooling protocol below the glass transition temperature of the fused ceramic to compensate for the slow temperature transition through the zirconia. This procedure prevents the formation of large temperature gradients that generate residual stresses in the porcelain layer.[14,23,24] Tang et al. based on statistical analysis showed that slow cooling does not increase the average failure load in the case of porcelain crowns on a thin zirconia framework.[25]

Framework veneering technique

In the case of ceramic crowns, various methods of veneer production can be used – the conventional technique of layering, pressing, as well as the latest technique of making a ceramic veneer in CAD/CAM technology.[12,26]

Och et al. suggest that the method of veneering has a more significant impact on fracture toughness than the thickness of the framework or the material used in its execution. They investigated the effect of the metal and zirconia core veneering technique on ceramic fracture. The hot pressing technique (55.27 MPa) showed a higher fracture toughness than the conventional layer method (41.52 MPa).[12] Pressed ceramics have a more homogeneous structure, with fewer defects (pores, micro-cracks, scratches) due to the more controlled method of production from ready-to-use blocks. Traditional layer firing is a more sensitive technique where unintended errors may occur. Incorrect powder/liquid ratio, introduction of air bubbles during mixing the suspension, overdrying or firing of over-soaked ceramics, as well as time and temperature fluctuations during successive firings layers can be such errors. They can result in formation of porosity and microcracks in sintered ceramics, which lead to material damage.[12,27]

Consistent with a previous study, Christensen reported that hot-pressed fixed prostheses had a lower fracture rate over a 2-year observation period for both zirconia and metal–ceramic restorations compared to the traditional method.[28] In vitro studies by Beuer et al. showed better mechanical properties of restorations based on zirconium oxide with lithium disilicate ceramic veneers made in the CAD/CAM technology compared to conventionally veneered crowns and pressing technology.[26]

Improper treatment of the zirconia framework

Due to its high flexural and fracture strength, zirconia ceramics are one of the most popular materials used for substructures in all-ceramic restorations.[29] Zirconium oxide used in prosthetics is in form of sintered or pre-baked blocks/discs that are milled in the CAD/CAM system. Zirconia is a polymorphic material. It occurs in 3 allotropic forms: monoclinic (below 1,170°C), which changes with the temperature increase into the tetragonal form (1,170°C – 2,370°C) and to regular (cubic) at 2,680°C, corresponding to the melting point of zirconium oxide. The tetragonal form is the most advantageous in terms of biomechanics. To stabilize it during cooling to room temperature, 3 mol% yttrium oxide (Y_2O_3) is usually added.[30]

The mechanical properties of yttria-stabilized zirconia (3Y-TZP) depend on the grain size (0.2–1 um) and their size depends on the sintering temperature. Compared to other ceramic materials, zirconia ceramics exhibit excellent mechanical properties due to the strengthening transformation mechanism. This phenomenon occurs through the local transformation of the tetragonal phase into the monoclinic form under the influence of the spreading microcrack. During phase transformation, grain volume increases (4.5%). Compressive stresses arise around the transformed particles, which effectively prevent further propagation of the microcrack, increasing the fracture toughness of the material by closing the cracks.[31]

Despite good mechanical properties and biocompatibility, the unfavorable feature of zirconia is its susceptibility to the so-called "aging", also referred to as "low temperature degradation" (LTD). The essence of this phenomenon lies in the spontaneous transformation of the tetragonal form into the monoclinic, stable at low temperatures. The cause of premature "aging" and the loss of the ability of the material to prevent the spread of cracks is the oral cavity pH environment, moist and variable temperature connected with external stresses.[32]

Such stresses can be a result of abrasive blasting with aluminum oxide (used to improve the bond strength between veneer and the framework), or of the final framework correction with thick diamond coating grinding and polishing tools with an inadequate water cooling. Improper treatment of the core triggers stresses that lead to a spontaneous phase change from tetragonal to monoclinic at the point of overheating. Phase transformation is accompanied by an increase in grain volume (4%), which leads to loosening of the microstructure and degradation of the material surface to a depth of 80–110 nm. In addition, there is a change in the coefficient of thermal expansion (the tetragonal phase has a CTE of 10.8×10^{-6}/°C, the monoclinic phase – of 7.5×10^{-6}/°C) in the vicinity of overheating. Tensile stresses arise which weaken the bond between the veneering ceramic and the zirconia core. The described mechanism can led to veneer chipping but also cracking of the zirconia framework.[11,30,32] Some studies show that zirconia framework sandblasting does not improve the bonding strength between veneer and the base[33,34] and may reduce its strength by up to 30%.[35]

Low modulus of elasticity of the framework

The parameter that influences the long-term clinical success of porcelain restorations is the Young's modulus (E) of the substrate supporting the ceramic. Materials have their constant, strictly defined coefficient of elasticity, which is a measure of resistance to elastic deformation. In other words, the higher the modulus of elasticity of the porcelain support structure, the stiffer the support for brittle ceramics and the greater the resistance to deformation under load. For compound crowns, a low-modulus glass–ceramic veneering porcelain (E ~70 GPa) is supported by a stiffer metal substructure or a ceramic core (E 200–300 GPa) that can withstand high occlusal loads.[36,37] The use of an alloy substructure with a low modulus E will cause deflexion even under a small load due to the easy elastic deformation of the metal. Tensile stresses develop in the veneer layer, resulting in a greater tendency for the ceramic veneer to crack and chip.[38,39]

Single-layer (monolithic) ceramic restorations are adhesively bonded to a less rigid material such as dentine (E 18 GPa) or dentin replacement composite (E 15–20 GPa), which flexes under load, providing poor ceramic support. It is generally believed that low-strength glass–ceramics

require considerable thickness (typically 1.5–2 mm) to withstand the tensile stresses on the inner surface of the cementation caused by crowns bending under occlusal loading. In general, ceramic fracture may start at the upper occlusal surface, the concave cementation surface or at the margins of the ceramic restoration. It has been noted that in the case of monolithic ceramic restorations, the type of ceramic fracture is determined by the ceramic layer thickness. In the thin ceramic layers (below 1 mm), the stiffness of the substrate plays a significant role and radial cracks predominate under destructive loads. This damage type starts on the inner surface of the ceramic (cementation surface), where the tensile strength is greatest, and then spreads through the material to the outer surface, eventually leading to a critical fracture of the restoration. With the increase of the ceramic thickness above 2 mm, the dominance of the radial crack begins to disappear. Cracks that appear on the occlusal surface are responsible for ceramic chipping. Unlike radial fracture, contact stress damage on the occlusal surface is not dependent on the modulus of elasticity of the substrate supporting the ceramic.[36,40]

Geometric factor of the ceramic restoration

Crowns

The spatial structure of the crown affects the distribution of stresses that occur during chewing and thus plays a significant role in the ceramic resistance. It is a well-known fact that the occlusal forces acting along the long axis of the teeth are most favorable. Therefore, prosthetic restorations should be designed to minimize the lateral forces. Horizontal forces can be eliminated by locating the cusp tips on the occlusal surface in the central sulcus and not on the marginal ridges.[41] It is recommended to avoid sharp cusps near the edges of the crowns to prevent their damage.[42] They create stronger contact in axial loading and distribute forces over a smaller area. Increased local stresses predispose to the initiation of cracks and subsequent chipping of the ceramic.[43] However, the geometric factors above the crown are limited by the spatial constraints imposed by opposing and adjacent teeth.

Bridges

Geometric features of the prosthetic bridge, such as connector size, shape, pontic spread, and the curvature radius at their intersection, have a significant impact on the stresses concentration arising from the occlusal load. Occlusal loading creates a bending moment along the entire length of the bridge.[44] The connector between the crown placed on the abutment tooth and the pontic is most exposed to damage.[44–50] This is due to the relatively small thickness of the connector compared to other elements of the bridge.[47] Therefore, tensile stress concentration occurs in the gingival region of the connector, which leads to chipping of the brittle veneering ceramics and fracture of the prosthetic structure in this place.[50] This fact is confirmed by the results of experimental in vitro tests and factual analysis of damaged prosthetic bridges.[45,49,51] Therefore, it is advisable to keep the minimum diameter of the connector, which, according to the literature, may reduce the probability of damage to less than 5% over 20 years of its operation.[44] Furthermore, with increase in bridge length, the greater the size of the connecting surface should be introduced. In the case of bridges on a metal foundation, the minimum diameter of the connector is 6.25 mm^2.[48] On the other hand, for the majority of all-ceramic systems, connector areas of 9 mm^2 and 16 mm^2 have been proposed.[47,49,50,52]

However, the height of the connector (in the occlusal–gingival dimension) should be as large as possible, taking into account anatomical limitations (interstitial space, height of clinical crowns) and esthetic considerations.[49] In addition, it has been found that the fracture load values of permanent partial dentures increase with the size and with the radius of connectors curvature.[49,51] Oh and Anusavice confirmed this in their study, where smaller connector radii increased stress concentration. They showed that with the increase of connector curvature radius in the gingival fissure from 0.25 mm to 0.90 mm, the average breaking load of the prosthetic restoration increased by 140%. Thus, the likelihood of breakage can be reduced by using a connector with a radius of curvature of about 0.9 mm.[51]

Furthermore, the shape of the connector affects the number of stresses generated during the occlusal load. It has been found that a circular or oval cross-section of the connector shows less stress and better reinforcement than a square one.[46] From the biomechanical point of view, short bridge pontics are advantageous. Under occlusal loads, pontics bend minimally. Deflection increases with length and may lead to ceramic chipping or connector breakage. Large-span bridges, especially in the posterior region, are more prone to clinical failure.[53,54]

Thickness of the veneering ceramic

The thickness of the veneer affects the occurrence of stress between the framework and the ceramic veneer.[14] From the clinical point of view, a ceramic veneer thickness from 0.8 mm to 1.2 mm is considered optimal. Its variation depends on the opposing tooth, the occlusal space, preparation, and the complex anatomy of the ceramic crown, which has areas such as cusp and axial walls of varying thicknesses of porcelain.[55,56] Ceramics with inhomogeneous thickness over the entire veneered surface, exceeding 2 mm, have been associated with reduced strength of the prosthetic restoration due to the susceptibility of the ceramic to subsurface residual

stresses.[14,57] This was confirmed by Figueiredo et al., who studied the flexural strength and crack propagation in zirconia samples veneered with fluorapatite leucite glass ceramic of different thicknesses (1, 2 and 3 mm). The samples with the thinnest veneer (1 mm) presented the highest bending strength. As the thickness increased from 1 mm to 3 mm, the gradients of thermal stresses between the zirconia and the veneer increased significantly. In the samples with 2-mm veneering porcelain, chipping was observed within the veneering ceramic layer. The most serious damage and chipping of the veneering ceramic with exposure of the zirconia core was observed in the 3-mm veneering samples.[10] This finding is in line with the Swain's study that thick layers of low diffusion veneering ceramics, such as Y-TZP, cause high tensile stresses generated during firing and cooling of the porcelain.[14] In contrast, Badran et al. showed that the fracture toughness of 1.5-mm incisal veneer crowns was significantly higher than 3-mm incisal veneer crowns for both zirconia and metal alloys.[2] Cohesive spalling in the veneering ceramic layer is the dominant type of damage when using an uneven, thick layer of porcelain for veneering prosthetic restorations, both on zirconia and metal substructures.[2,58]

Ceramic restoration framework design

One of the causes of ceramic fractures is an improper framework design. It is important to prepare the framework so that it is in oval in shape, without undercuts and sharp edges that create stress points during chewing and subsequent fracture of the ceramic.[36]

Another factor affecting the success of a ceramic restoration is the thickness of the framework. According to the recommendations, thickness of the metal framework cannot be less than 0.3 mm, and the thickness of the Y-TZP ceramic should not be less than 0.5 mm.[59] As shown by the research results of Oh et al., the base material, but also its thickness, affect the flexural strength of the ceramic restoration. The tested samples with a metal core of 1-mm thickness showed a higher fracture toughness of the veneering ceramics (61.87 MPa) than the samples with a metal core of 0.5-mm (47.11 MPa) and 1-mm zirconia (49.97 MPa). It was noted that in the case of zirconia core samples, increasing the minimum recommended thickness of 0.5 mm (46.82 MPa) to 1.0 mm (49.97 MPa) did not significantly change the fracture toughness. In contrast to the metal-core samples, it was found that an increase in the thickness of the metal substructure increases its stiffness, reducing the bending and tensile stresses of the porcelain veneer under occlusal loads.[12]

The geometry of the framework is another factor that affects the strength of ceramic restorations. The substructure, both metal and ceramic, should take into account the anatomical structure of the future tooth crown. Ceramic layer applied both in the area of cusp and fissures should have a comparable thickness. A substructure with a non-anatomical shape and uniform thickness will lead to uneven support of the cusps or the incisal edge, which transfers the masticatory loads to the veneering ceramic instead of the substructure.[60,61]

Research indicates that masticatory stresses may be greater at the gingival margin than at the occlusal surface.[62] Therefore, an anatomic framework without anatomical support may not be a sufficient support for the veneering porcelain in the case of zirconia-based ceramic restorations. It was suggested that the anatomical framework should be modified by adding buccal and/or lingual support structures and increasing the thickness in the proximal area to reduce the amount of porcelain veneering in the non-visible area.[58,63] In contrast, other studies have shown that the modified framework design did not improve the fracture toughness of the restorations.[64,65]

Metal–ceramic restorations traditionally have a narrow metal margin. The construction of the restoration margin creates a grayish shadow in the gingival area, known as the "umbrella effect". In order to improve the esthetics, restorations with a ceramic margin were introduced. When designing a crown with a ceramic gingival margin, it should be remembered that the substructure should rest on the shoulder of the tooth and not on its veneer. The crown substructure should be designed to reach the inner edge of the shoulder. In this way, functional support of the substructure on the abutment can be achieved. An unsupported ceramic step may not be able to withstand the stresses that occur during cementation and mastication.[66] Yoon et al. found that the increase in unsupported porcelain caused by the reduction of the metal margin reduces the restoration breaking strength. The fracture toughness of metal–ceramic restorations with a ceramic shoulder and reduced substructure structure are influenced by the number of ceramic firings increasing with the weight of the edge porcelain (possibly increasing the thickness of the metal oxide layer), microcracks or porosity of the porcelain edge, marginal leakage, and loss of ceramic support through the framework.[66]

Quality of connection between veneering layer and substructure

The durability of ceramic restorations depends on the quality of the bond between the substructure and the ceramic. The bonding mechanism between veneering ceramics to the ceramic substructure is not fully understood, as is the bonding mechanism between ceramics and the metal alloy.[67] The bond strength between zirconia and porcelain is weaker than that between metal and porcelain.[33] This is confirmed by Fischer et al. who showed that mechanical surface treatment by sandblasting does not improve the adhesion between veneering ceramics and zirconia frameworks.[34] At the same time, the viscoelastic

properties of the veneering ceramics during sintering and the appropriately selected coefficients of thermal expansion can affect the adhesion between the ceramic and the zirconia core.[68]

When metal alloys are veneered with ceramics, the following factors are responsible for the bond between these materials: compressive stress resulting from the difference in material shrinkage, mechanical bond (microretention) and chemical bond (oxide layer formed on the metal surface).[69] According to ISO 9693 standards, the durability of a metal–ceramic restoration is sufficient when the metal–ceramic shear stress is greater than 25 MPa. To achieve a good mechanical bond, the metal substructure is initially prepared with carbide cutters and then sandblasted with aluminum oxide according to the manufacturer's recommendations. The purpose of the above treatment, apart from developing microretention and increasing the wettability of the metal with porcelain, is to clean it. Therefore, improperly performed multidirectional grinding instead of the recommended unidirectional grinding, as well as improper abrasive blasting, will result in the retention of impurities and air on the substructure surface. The impurities remaining in the metal layer are decomposed during firing, creating gas bubbles at the metal–ceramic interface, which reduces the strength of the porcelain.[70] In addition, before applying the first layer of porcelain, it is recommended to clean the metal surface with a steam jet. As indicated by Lahori et al., regular inspection of the steam generator is important, as impurities in the steam can cause a reduction in the bond strength between metal and ceramics.[67]

The most important mechanism affecting the ceramic–metal bond is the chemical bond between the ceramic and the oxide layer on the surface of the metal substructure.[70] An oxide layer is formed on the metal surface by heat treatment before firing the first ceramic layer (opaquer). The oxidation process initiates the formation of oxides, but also removes impurities from the metal framework. The thickness of the metal oxide layer is extremely important for the quality of the metal–ceramic bond. Their lack or too thin layer due to improperly conducted oxidation process for a given alloy, as well as too thick layer of oxides resulting from the application of too thick first layer and improper technique of opaquer firing cause adhesive type chips with exposure of the metal surface.[71] Sandblasting of a substructure need to match type of alloy used to its creation. Research has shown that nickel- and cobalt-based alloys tend to form a thick layer of oxides. Before applying porcelain, the metal framework should be sandblasted to remove excess oxides. Failure to do this may result in separation of the ceramic from the metal. In contrast, alloys based on a noble metals, such as gold alloys or palladium, form thinner oxide layers. Therefore, it is a mistake to sandblast them after the oxidation process.[67,70,71]

Defects resulting from the material properties

One of the main factors contributing to the clinical problem of veneer chips is the low strength of the veneer ceramics. Extremely important parameters describing the mechanical properties of ceramics are the modulus of elasticity, fracture toughness and bending strength. Fracture toughness, expressed by the critical stress intensity factor (K_{IC}) at which the current defect begins to increase, indicates the intrinsic ability of the material to resist rapid crack propagation and consequent critical failure. The values (K_{IC}) are variable and depend on the size, number and location of material defects, and environmental factors, such as humidity and temperature.[72]

A study by Borba et al. has shown that the microstructure of a ceramic significantly influences its mechanical properties. The glassy phase of the ceramic has the typical properties of glass. It makes the ceramic translucent, but it is also the cause of its brittleness and non-directional cracks pattern. In contrast, the crystalline phase, depending on the size, number and geometry of the crystals, provides the ceramic material with strength, stability during firing and resistance to stress. Thus, it should be emphasized that the higher the percentage of crystals in the ceramic structure, the greater the difficulty of defect propagation (slow crack growth) and its flexural strength. It is well known that ceramic and metal alloy substructures for crowns or bridges require the use of veneering ceramics to achieve excellent esthetics.

Borba et al. found that feldspar-based and leucite-reinforced veneering ceramics exhibited low fracture toughness (0.7 MPa $m^{½}$) and flexural strength (154 MPa and 160 MPa, respectively) compared to polycrystalline ceramics of yttrium oxide stabilized zirconia (Y-TZP) (6.5 MPa $m^{½}$, 700–1,200 MPa), glass infiltrated zirconia-based alumina ceramic (IZ) (3.6 MPa $m^{½}$, 440–620 MPa) and polycrystalline alumina (AL) (3.6 MPa $m^{½}$, 500 MPa). The susceptibility of veneering ceramics to damage occurring at low loads can be attributed to their microstructure, as they consist mainly of a glass phase (55–65% in feldspar ceramics) which is susceptible to crack propagation. The low porosity of polycrystalline ceramics of 0.1–0.2% compared to glass ceramics of 2.6–2.7% has an impact on good mechanical strength. The homogeneity and lower porosity of alumina and zirconium dioxide ceramics can be related to the high content of crystals in the structure. Their production from ready to use blocks in the CAD-CAM technology, in which there are no errors appearing in the ceramic sintering technology, also decreases their damage rates.[13,73]

Conclusions

Despite the favorable clinical prognosis, damage to ceramic restorations is a major problem in everyday clinical practice. Ceramic chipping in the anterior part of the dental

arch is a serious esthetic problem, while in the lateral a functional one. Management of damaged restorations requires knowledge of the etiology of this phenomenon. Based on the analyzed literature, ceramic damage is associated with errors that may occur at any stage of the laboratory process. In order to avoid them, it is recommended to select the right materials and, above all, to control the precision and quality of the technological process.

New technologies and ceramic materials are being developed to increase the strength of ceramic restorations and reduce complications. The latest achievement in the fabrication of ceramic restorations is the use of highly translucent zirconium dioxide (HT), as it allows the fabrication of esthetic, monolithic restorations without the need for veneering. Monolithic zirconia restorations are easier to fabricate than traditional ones. The limited number of steps reduces the possibility of laboratory errors. A single-material structure eliminates many of the problems mentioned in this article, such as the quality of the bond between the veneering layer and the substructure or excessive stress between different materials. At this point, according to the literature, we can distinguish problems related mostly to excessive reduction of the thickness of the restoration and to insufficient esthetic properties.

There are limited data describing long-term survival rate and complication types of monolithic zirconia restorations. Due to their relative novelty and the different types of materials, further long-term in vitro observations are needed before general conclusions can be drawn.[74–76] Another material worth mentioning is hybrid ceramics, which combines the advantages of glass ceramics and composites, and whose undeniable advantage is the possibility of repairing the prosthetic restoration directly in the patient's mouth.[77,78] Technological and material advances make it possible to reduce the number of errors made during laboratory procedures. However, it does not exempt the dentist from knowledge of basic laboratory procedures and the associated damage to ceramics. Knowledge of these processes may allow the clinician to control and eliminate them more precisely, resulting in a better long-term prognosis of the fabricated restorations.

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Mechanisms of resistance to venetoclax in hematologic malignancies

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Abstract

Venetoclax, a BH3 mimetic, is a novel targeted anti-cancer drug with a unique mechanism of action leading to the execution of apoptosis through inhibition of the Bcl-2 protein. The development of venetoclax has revolutionized the treatment paradigm of several hematologic malignancies, including treatment-naïve and relapsed or refractory chronic lymphocytic leukemia (CLL) as well as acute myeloid leukemia (AML) in unfit patients. However, despite the high effectiveness of venetoclax in these diseases, some patients, as in the case with other targeted therapies, develop primary or secondary resistance to the drug. Various mechanisms contributing to the resistance to venetoclax have been elucidated, including selection of mutations in the BCL-2 binding groove which decrease affinity to venetoclax, or compensatory overexpression of anti-apoptotic proteins such as MCL-1. Moreover, alterations in cell metabolism and signaling pathways like MAPK or ERK activation have also been reported, suggesting the resistance to venetoclax is highly complex and involves multiple pathways. This review aimed to describe the mechanisms of resistance to venetoclax in AML, CLL, multiple myeloma, and other hematologic malignancies, as well as to propose a perspective to circumvent it.

Key words: acute myeloid leukemia, resistance, chronic lymphocytic leukemia, apoptosis, venetoclax

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Introduction

Venetoclax is a novel targeted anti-cancer drug with a unique mechanism of action involving the execution of apoptosis of malignant cells through the selective inhibition of the B-cell lymphoma-2 (BCL-2) protein.[1] The BCL-2 family proteins are a pivotal point in the intrinsic pathway of programmed cell death (apoptosis).[2] This heterogeneous group of proteins regulates apoptosis by promoting either the cell's survival or its death.

The BCL-2 family proteins contain specific regions of homology called B-cell 2 homology (BH) domains, including BH1, BH2, BH3, and BH4 domains, which implicate the BCL-2 protein's functions in cells.[3] According to the function and number of BH domains, BCL-2 family proteins may be subclassified as proapoptotic and anti-apoptotic proteins.[3] BH3-only proteins (BID, BIM, PUMA, Noxa, HRK, BIK, BMF, and BAD) are proapoptotic proteins that share only a short BH3 region with other groups of the BCL-2 family proteins.[3] Proapoptotic BH-3-only proteins may exhibit their proapoptotic function as activators by directly activating BAX or BAK, or as sensitizers by neutralizing the anti-apoptotic function of BCL-2 proteins.[3] BAX and BAK are proapoptotic effectors that have multiple BH domains. In contrast, BCL-2, B-cell lymphoma-extra-large (BCL-xL), mantle cell lymphoma-1 (MCL-1), A1, BCL-B, and BCl-w are anti-apoptotic proteins and contain 4 domains, BH1–BH4.[3] The BH1, BH2 and BH3 domains of anti-apoptotic proteins form a hydrophobic cleft that can interact with the BH3 domain of proapoptotic proteins.[3] This interaction is an important regulatory mechanism of apoptosis (Fig. 1).

Various stimuli such as oncogenic stress, DNA damage or uncontrolled proliferation lead to the upregulation of proapoptotic BH3-only proteins, which contribute to the oligomerization of the effector proteins, BAX and BAK, in the mitochondrial outer membrane. Permeabilization of the mitochondrial membrane leads to leakage of cytochrome c, which binds to the adaptor molecule apoptotic protease activating factor 1 (APAF-1) in the cytosol. APAF-1 oligomerizes, forming an apoptosome that initiates the cascade of caspases. Finally, caspase proteases cleave dozens of proteins, resulting in rapid cell death.[3,4] In cancer cells, the balance between survival and cell death is disrupted. Resistance to apoptosis and persistent viability of malignant cells is one of the hallmarks of cancer.[5] Overexpression of BCL-2 was recognized in multiple hematologic malignancies, including chronic lymphocytic leukemia (CLL), follicular lymphoma (FL) and Waldenström macroglobulinemia.[6–8] Thus, BCL-2 was suggested to be a rational and potent target of novel therapies. Overexpression of anti-apoptotic BCL-2 proteins prevents malignant cells from undergoing apoptosis by blocking mitochondrial outer membrane permeabilization.[3] BH3 mimetics constitute a novel class of drugs that act as proapoptotic BH3 sensitizers. By binding to the BH3 domain groove of anti-apoptotic proteins such as BCL-2 or MCL-1, they enable the sequestration of BH3-only activator proteins, like BIM, and trigger apoptosis.[9]

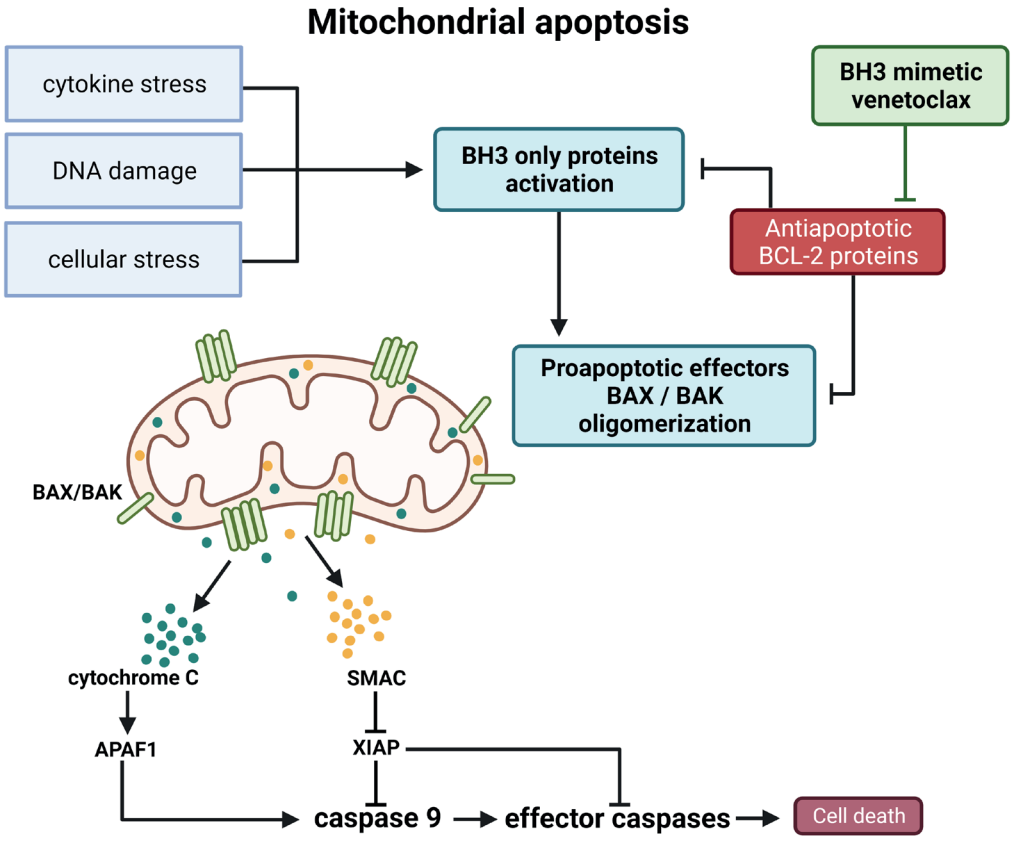

Fig. 1. Intrinsic apoptosis and venetoclax's mechanism of action. Created with BioRender.com

APAF1 – apoptotic peptidase activating factor 1; BCL-2 – B-cell lymphoma 2; BH3 – B-cell homology domain 3; XIAP – X-linked inhibitor of apoptosis protein.

The introduction of BH3 mimetics has enabled significant progress in the development of targeted therapies for several hematological tumors.[9–13] Despite initial difficulties with the first-in-class BH3 mimetic drug, navitoclax, which bound to both BCL-2 and MCL-1 and resulted in severe thrombocytopenia,[10,11] a highly selective BCL-2 inhibitor, venetoclax (ABT-199) has revolutionized current therapies in hematologic malignancies.[9] Based on the high efficacy shown in the MURANO trial investigating venetoclax in combination with anti-CD20 monoclonal antibody rituximab, the drug was approved for the treatment of relapsed or refractory (RR) CLL. The cohort treated with venetoclax had a 2-year rate of progression-free survival (PFS) of 84.9% (95% confidence interval (95% CI): 79.1–90.6) and an overall response rate of 92.3% (compared to 72.3% in the control bendamustine-rituximab treatment arm by a difference between the groups of 20.0 percentage points; 95% CI: 12.4–27.6).[12] In the VIALE-A study,[13] untreated AML patients were randomized to receive azacitidine with venetoclax or in monotherapy. Patients receiving venetoclax had a median overall survival (OS) of 14.7 months compared to 9.6 months in the control group (hazard ratio (HR) for death: 0.66; 95% CI: 0.52–0.85; $p < 0.001$). In this group, complete remission (CR) was obtained in 36.7% of patients (compared to 17.0% in the control group; $p < 0.001$).[13]

However, it has been noted that treatment with venetoclax does not lead to a cure for AML or CLL, and gradual resistance to the drug develops in most patients.[13,14] Pathways underlying the mechanism of intrinsic primary resistance of malignant cells to different anti-cancer therapies, including venetoclax, are complex and not well understood. When it comes to long-term exposure to venetoclax, the majority of patients would become refractory despite an initial response (secondary or acquired resistance).[15] Importantly, the main determinants of venetoclax resistance recognized in the literature are mutations in the BCL-2 binding groove and the upregulation of other anti-apoptotic proteins such as BCL-XL and MCL-1.[16,17] However, the mechanisms responsible for resistance to venetoclax are more complex and comprise changes in the metabolism of leukemic cells or the tumor microenvironment.[18–21] A better understanding of these mechanisms may allow pre-treatment identification of patients with a high probability of primary resistance to venetoclax or to develop venetoclax combinations preventing secondary resistance. Moreover, the prediction of sensitivity to venetoclax and risk stratification by assessment of potential risk factors such as the Gly101Val mutation may be crucial to address other tailored therapies for patients who do not respond to venetoclax.[22,23]

Objectives

This review aims to summarize mechanisms of resistance to venetoclax in hematologic malignancies and propose possible ways to counteract them.

Chronic lymphocytic leukemia/small lymphocytic lymphoma

Chronic lymphocytic leukemia is the most prevalent leukemia in the adult population in the Western world. It is characterized by the clonal expansion and accumulation of typically CD5-positive B-cells within the blood, bone marrow, lymph nodes, and spleen.[24] Long-lasting survival of malignant cells, which are highly dependent on BCL-2 and in the majority arrested in the G0/G1 cell cycle, is the key element in the pathophysiology of CLL.[25,26] In CLL, loss of miR-15a/16-1 results in overexpression of anti-apoptotic BCL-2 proteins and allows leukemic cells to escape apoptosis.[8] This entity remains incurable. However, significant progress in the prognosis of CLL has been achieved during the last decade due to the development of novel targeted therapies.[24] This included venetoclax, which showed high efficacy even in patients with poor risk due to genetic alterations, including *TP53* and *del(17p)*, or with comorbidities who did not qualify for the usual chemoimmunotherapy regimens.[14]

In CLL, venetoclax is administered in fixed-duration regimens when combined with anti-CD20 antibodies or Bruton tyrosine kinase (BTK) inhibitors or continuously in monotherapy. Despite the high effectiveness of the drug in general patient populations, a minority of patients experience primary or secondary resistance. Moreover, some patients may develop adverse events such as autoimmune cytopenias, which may result in venetoclax suspension or dose reduction and, thus, decreased drug efficacy, leading to CLL progression.[27–30]

Multiple studies have been conducted so far that identified a variety of mechanisms leading to venetoclax resistance in CLL (Fig. 2). One of the first reports concerning mechanisms of venetoclax resistance was performed by Tahir et al.[4] Analysis of venetoclax-resistant cell lines comprising activated B-cell-like (ABC) subtype diffuse large B-cell lymphoma (DLBCL) cell lines (HBL1 and U2932), 2 follicular/germinal center B-cell (GCB) lymphoma cell lines (OCI-Ly1 and SC-1), 2 mantle cell lymphoma (MCL) cell lines (HBL2 and Granta-519), as well as 1 leukemia line (RS4;11) revealed that there are multiple alterations in gene expression levels or in post-translational modifications that contribute to venetoclax resistance. This involved an increase in the anti-apoptotic proteins BCL-XL or MCL-1.[4] Interestingly, it was associated with a significant decrease in proapoptotic proteins such as BAX in the venetoclax-resistant leukemia cell line (RS4;11).[4] This suggests that the process is more complex and involves various pathways.[4]

Chronic lymphocytic leukemia is a genetically heterogeneous disease; thus, identification of secondary mutations driving resistance to venetoclax before clinical relapse may be practical to assess predictive factors. Interestingly, in a study performed by Herling et al.,[31] a group of 9 patients with either del(17p) or *TP53* mutations who clinically

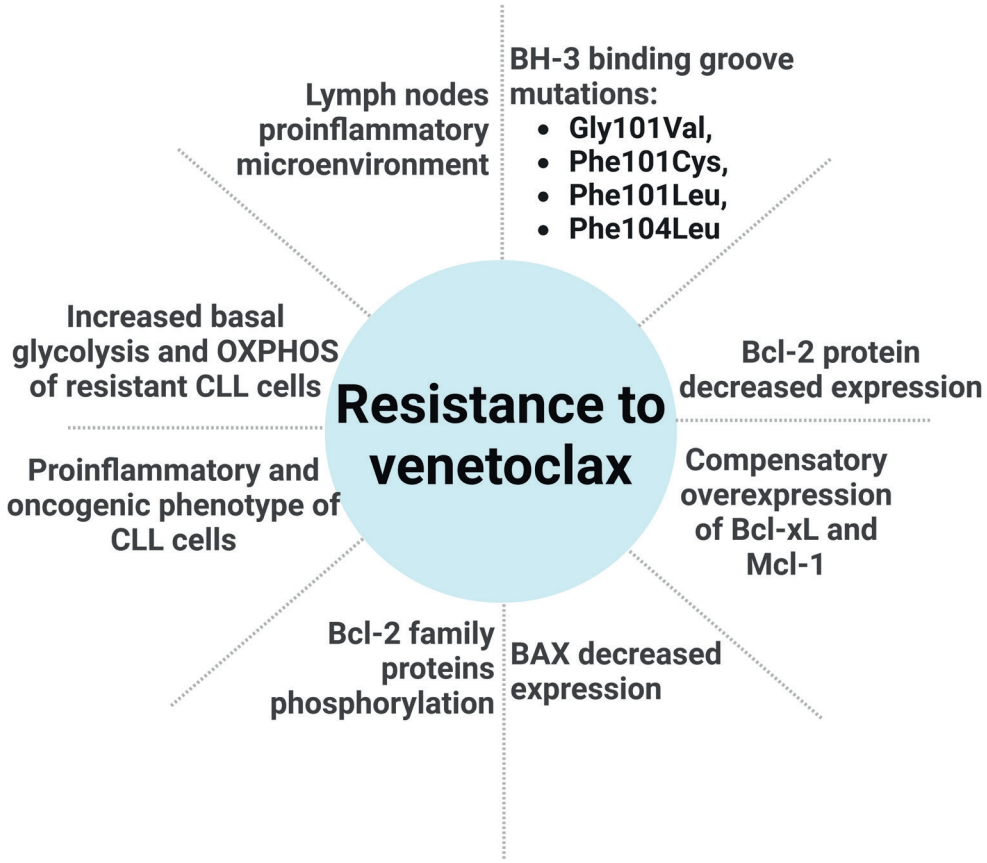

Fig. 2. Mechanisms of resistance to venetoclax in chronic lymphocytic leukemia. Created with BioRender.com

Bcl-2 protein – B-cell lymphoma-2; Bcl-xL – B-cell lymphoma-extra large; BH-3 – B-cell 2 homology 3; CLL – chronic lymphocytic leukemia; Mcl-1 – mantle cell lymphoma-1; OXPHOS – oxidative phosphorylation.

progressed or relapsed after treatment with venetoclax (median time 15.4 months), the accumulation of genome alterations was revealed. By performing whole-exon sequencing (WES), the authors showed multiple recurrent mutations that were not seen before treatment commencement.[31] In the majority of samples, alterations in genes associated with oncogenesis, such as *CD274* (*PD-L1*), *NOTCH1*, *RB1*, *SF3B1*, and *TP53*, were detected. Interestingly, in 2 patients, missense mutations of B-cell translocation gene 1 (*BTG1*) emerged during venetoclax treatment.[31] This phenomenon was reported to drive lymphomagenesis in Bcl-2 overexpression models of aggressive lymphomas.[32] In a singular case, a *BRAF* mutation was also revealed.[31] Additionally, *BRAF* mutations were detected in 2 RR CLL patients in a similar study.[33] Supposedly, BRAF inhibitors may be used along with venetoclax to postpone the time to resistance.

Interestingly, a study performed in RR CLL/SLL patients treated with venetoclax as monotherapy (median time on therapy 20.1 months (range: 1.2–60.3)) revealed genetic abnormalities associated with refractoriness to venetoclax that were not reported before.[33] Samples of patients were collected prior to and after discontinuation of venetoclax due to an unacceptable toxicity or disease progression. Of the 43 patients, 15 acquired mutations in BCL-2 and progression of the disease was observed in 14 patients.[33] Multiple novel BCL-2 mutations were obtained, including the most common Asp103 (11 occasions) and Gly101 (8 occasions). Interestingly, WES and targeting sequencing revealed a significant increase in expression of other anti-apoptotic protein genes comprising MCL1, BCL-XL and BCL2A1 (BFL-1, false discovery rate (FDR) <0.2), which are known for apoptosis inhibition in the presence of venetoclax.[33] Furthermore, loss-of-function mutations in the *PMAIP1*-encoding NOXA regulatory protein were identified in patients who developed resistance to venetoclax monotherapy with a variant allele frequency (VAF) >20% in 3 of 4 cases.[33] Hypothetically, *PMAIP1* may be a key driver of venetoclax resistance.[33]

Mutations of genes encoding protein binding sites of drugs are well-known mechanisms of resistance to any drug. Similarly, BCL-2 binding groove mutations, which decreased affinity to venetoclax, were also observed. For example, CLL cells cultured to become refractory to venetoclax had alterations in the BH3 binding groove, such as Phe101Cys, Phe101Leu and Phe104Leu, which contributed to venetoclax-binding disruption of BCL-2.[4,34] One of the first mutations discovered clinically in RR CLL at disease progression was Gly101Val, which was detected after the median time of treatment with venetoclax 36 months (range; 6.5–73 months).[17] Substitution of glycine to valine at amino-acid-position 101 of the BCL-2 protein resulted in poor binding of venetoclax to the binding groove.[17] Recent studies have also reported the presence of Gly101Val mutations in patients who became refractory to venetoclax, supporting this hypothesis.[22,23,35,36] A study

performed outside clinical trials revealed that Gly101Val and/or Asp103Tyr mutations were present in 16.7% of patients treated with venetoclax as monotherapy or combined with rituximab.[37] Ninety percent of them further relapsed (median time of follow-up of 26 months (7–32 months)).[37] All of the changes were detected using standard-of-care droplet digital polymerase chain reaction (ddPCR) tests. Hypothetically, screening for Gly101Val and Asp103Tyr mutations may be implemented in daily practice to identify resistance before clinical progression.[37] Interestingly, Gly101Val was present after cessation of venetoclax treatment for over 6 months[17] and in 3 out of 7 patients with CLL who failed to clear minimal residual disease (MRD) after 1 year of treatment with venetoclax.[35] Consequently, further studies are essential to evaluate Gly101Val in the context of re-treatment with venetoclax. Furthermore, in 10 out of 11 RR CLL patients resistant to venetoclax with the Gly101Val mutation, additional mutations were harbored.[22] The most common was the Asp103 codon with a substation of amino acids (6/11 patients); however, Val156Asp and in-frame insertion (Arg107_Arg110dup) were also reported.[22] Therefore, many BCL-2 gene protein mutations may develop during venetoclax administration.[22]

As presented, there is no single mutation that drives resistance to venetoclax in CLL cells. Interestingly, this issue was investigated by Thomalla et al.[38] A study performed on high-risk RR CLL patients revealed methylation of the PUMA promoter, which resulted in the downregulation of PUMA.[38] Importantly, an increase in DNA methylation from 10% to 30% was observed clinically in 5 out of 6 patients patients who acquired resistance toward venetoclax.[38]

In some cases, despite refractoriness to venetoclax, BCL-2 mutations are undetectable, and a novel mechanism was investigated comprising the overproduction of antiapoptotic proteins such as BCL-XL or MCL-1.[17,38] This was a compensatory way to overcome BCL-2 blockade favoring CLL survival, and was confirmed in vitro and further elucidated in clinical practice.[4,17] Interestingly, much attention has been drawn to the mechanisms of the overproduction of MCL-1 and BCL-XL. A few heterogenous metabolic pathways were identified which contribute to the process.

Interestingly, BCL-2 family proteins may undergo post-translational modifications, including phosphorylation, which alter their activity and may influence mitochondrial apoptosis.[39] Importantly, in the study performed by Guieze et al.,[37] patients refractory to venetoclax had detected growing subclones of CLL cells with an amplified region on chromosome 1q23.[40] This region encodes MCL-1 protein and PRKAB2 with the regulatory subunit of AMP-activated protein kinase (AMPK) genes.[40] Further studies performed on the samples revealed that MCL-1 and AMPK were expressed at higher levels. Overexpression of AMPK correlated with relapse ($p \leq 0.0062$), and a high-pretreatment MCL-1 expression (>10% of positive cells) was associated with shorter PFS ($p = 0.017$).[40] However, this was studied in a small number of patients and requires a larger cohort to confirm. Importantly, Chong et al.[41] also analyzed the impact of phosphorylation in regard to intrinsic or acquired resistance to venetoclax in lymphoid malignancies. They demonstrated that hyperphosphorylation of BCL-2 family proteins resulted in MCL-1 dependence instead of BCL-2 dependence in cells. Moreover, hyperphosphorylation of BCL-2 led to a stronger connection between BCL-2 and BAX, whose separation is crucial to initiate apoptosis.[41] Chronic lymphocytic leukemia samples at the time of progression during venetoclax treatment had increased hyperphosphorylated BCL-2, MCL-1 and BAD proteins compared to pre-treatment levels.[41] In this process, a few kinases were involved, comprising AKT, extracellular-signal-regulated kinase (ERK) and GSK3b kinases, suggesting a distinct pattern of kinase activity in resistance to venetoclax.[41] Importantly, the use of fingolimod, a protein phosphatase 2A-activating drug, reversed the resistance by switching the dependence from MCL-1 to BCL-2.[41]

Increased expression of receptor tyrosine kinase-like orphan receptor (ROR1) was detected in multiple hematologic malignancies, including CLL. Receptor tyrosine kinase-like orphan receptor activates the non-canonical WNT signaling pathway and, by activating cell survival signaling events, leads to oncogenesis.[42] Moreover, ROR1 expression was elucidated as a prognostic factor in CLL.[43] Receptor tyrosine kinase-like orphan receptor was investigated in terms of resistance to venetoclax. Ghia et al.[35] reported a significant rise in ROR1 expression in CLL patients after 1 year of treatment with venetoclax compared to values prior to treatment. In their study, samples of patients with CLL who did not eradicate MRD during continuous treatment with venetoclax were compared before and 1 year after therapy.[35] A significant correlation between the high level of expression of ROR1 prior to treatment and further failure to eradicate MRD was noted ($p = 0.00006$).[35] Importantly, the levels of ROR1 were higher after 1 year of the therapy. Furthermore, ROR1 induced the WNT pathway and, consequently, *BCL2L1* gene expression encoding the BCL-XL protein.[35] Interestingly, WNT5a is a ligand of ROR1, which is present in higher levels in CLL patients, contrary to the general population.[43] WNT5a may activate the nuclear factor-κB (NF-κB) pathway in CLL cells and, therefore, upregulate BCL-XL and MCL-1.[43]

Moreover, MCL-1 overproduction as a way of induction of resistance to venetoclax was associated with higher levels of NF-κB.[44] The enhanced activity of NF-κB was present in CLL cells after venetoclax therapy.[44] Supposedly, monitoring of NF-κB may serve as a biomarker indicative of relapse after venetoclax and the need for venetoclax re-administration or initiation of other drugs.[44] Moreover, elevated levels of protein kinase RNA-like endoplasmic reticulum kinase (pERK) were also observed in CLL cells resistant to venetoclax.[45] Other studies demonstrated that overexpression of MCL1 was a result of altered

phospho-p38 signaling.[38] Interestingly, WES in patients who relapsed to venetoclax revealed acquired loss of (8p), which affects the *MCL-1* gene.[45]

Cytogenetic abnormalities of CLL are important prognostic factors, and the trisomy 12 mutation chromosome, which often harbors Notch1, was linked to more rapid progression, bulky lymphadenopathy and increased risk of Richter transformation.[46,47] The CLL cells with trisomy 12 are characterized by reduced interferon regulatory factor 4 (IRF4) compared to other cytogenetic groups.[48] Importantly, a study performed by Fiorcari et al.[49] presented decreased sensitivity to venetoclax in CLL cells with trisomy 12.[49] The possible mechanism involved a reduction in the expression of IRF4. This significantly affects the functions of immune cells associated with the increased activity of the Notch2 protein, a part of the Notch signaling pathway.[49] Moreover, Notch-signaling exerts pro-survival effects on CLL cells due to the enhancement of MCL-1.[49]

Under normal "therapeutic" conditions, venetoclax affects the metabolism of CLL cells. Chronic lymphocytic leukemia cells resistant to venetoclax have higher basal levels of glycolysis.[40] Moreover, the loss of PUMA resulted in higher oxidative phosphorylation (OXPHOS) and adenosine triphosphate (ATP) production in mouse models.[38] Chronic lymphocytic leukemia cells in lymph nodes are more resistant to venetoclax and have increased expression of genes linked to glycolysis, OXPHOS, the tricarboxylic acid cycle, and amino acid metabolism.[50] The main substrate for the tricarboxylic acid cycle in lymph nodes for CLL cells was glutamine.[50] The BCR stimulation induced resistance to venetoclax, and the refractoriness was attenuated by inhibition of glutamine uptake.[50] Hypothetically, changes in metabolic pathways and blockade of glutamine import may decrease the resistance to venetoclax in lymph nodes.

The CLL microenvironment, which serves as an important site of activation and proliferation of CLL cells, was proven to drive resistance to venetoclax.[25] Tumor microenvironments, especially in lymph nodes, are suggested to maintain higher expression of Notch2 and MCL-1, and, therefore, may additionally contribute to venetoclax resistance.[49] Additionally, a higher concentration of anti-apoptotic BCL-XL in the lymph node environment was also present. This may explain the low efficacy of venetoclax in clearing nodal disease.[51] Interestingly, ibrutinib, a first-generation BTK inhibitor that mobilizes CLL cells from lymph nodes and other lymphoid niches, enhanced the cytotoxic activity of venetoclax in preclinical models.[52] Moreover, the synergistic feature of combining venetoclax and ibrutinib was assessed clinically in the CAPTIVATE study, which presented CR rates of 55% (95% CI: 48–63) in the overall population and of 31% (95% CI: 18–44) in patients with bulky disease >5 cm.[53] Nevertheless, cases of resistance to both venetoclax and ibrutinib were demonstrated in the literature with the presence of BCL-2, BTK and phospholipase Cγ2 (PLCG2) mutations. Interestingly, aside from the well-described Gly101Val mutation, novel genetic BCL-2 abnormalities were detected comprising the variant Ala113Gly (VAF, 31.7%) and in-frame mutations resulting in p.Arg107_Arg110dup detected in 3 patients (VAFs 0.4% and 5%).[54]

A study performed by Thijssen et al.[55] highlighted that pro-survival BCL-2 family members, which were aforementioned as leading to resistance to venetoclax, were upregulated by environmental signals. Stimulation of CLL cells with CD40 ligand in vitro, which mimicked activated T-cell-mediated signaling, contributed to an increase in BCL-2 family members: MCL-1, BCL-XL and Bfl-1. This resulted in a complete resistance to venetoclax.[55] Interestingly, the resistance was altered not only by rituximab, an anti-CD20 antibody, but also by c-Abl tyrosine kinase inhibitors imatinib and dasatinib.[55] Furthermore, CD40 stimulation led to the activation of the non-canonical NF-κB pathway in both normal B-cells and CLL and correlated with the higher expression of BCL-XL.[21] Nevertheless, an association of MCL-1 and NF-κB pathway remains contradictory in the literature. Upregulation of MCL-1 induced by CD40 was suggested to be dependent on PI3K-AKT-mTOR, not on NF-κB activation.[56]

Interestingly, overexpression of anti-apoptotic proteins, such as BCL-2, BCL-XL and MCL-1, associated with the lymph node environment, was assessed in the study performed by Jayappa et al.[20] Chronic lymphocytic leukemia cells from the peripheral blood and lymph nodes of both naïve and refractory to multiple drug treatment patients were compared. Chronic lymphocytic leukemia cells recently activated in lymph nodes, which were characterized with the same phenotype as resident cells ($CD69^+$/$CXCR4^{Low}$), presented increased expression of anti-apoptotic proteins comprising of MCL-1, BCL-XL and BCL-2 induced by the NF-kB pathway.[20] Moreover, this drug-resistant phenotype was present in the treatment-naïve patients' CLL cells.[20] Furthermore, venetoclax-resistant CLL cells expressed the phenotype $CD69^+$ Ki67 $CXCR4^-$, which was the same pattern as present in lymph nodes resident cells.[20] Importantly, previous studies substantiated the belief that the $CD69^+$ $CD38^+$ $CD49d^+$ phenotype was associated with refractoriness to venetoclax and demonstrated its linkage with unfavorable outcomes.[20,57] This indicates the possibility of intrinsic refractoriness to venetoclax.[20] Hypothetically, this may be a reason for the low OS in this group of patients.[20] Thus, the $CD69^+$ $CD38^+$ $CD49d^+$ phenotypes may be used in clinical practice to find patients who may relapse on therapy with venetoclax. Nevertheless, further studies are essential.[20] Moreover, CLL cells from lymph nodes expressed higher Ki-67.[58] Interestingly, an assessment of RR CLL cells revealed that survival in venetoclax positively correlated with Ki-67 expression, suggesting that proliferative potential is linked to resistance to venetoclax.[59] Importantly, the suggestion was made that resistance to venetoclax selects the most activated CLL cells with the highest proliferative capacity.[59]

Nurse-like cells (NLCs) are significant players in the leukemic environment. They produce chemokines such as CXCL12 and CXCL13 as well as secrete proteins, such as B-cell activating factor (BAFF) and proliferation-inducing ligand (APRIL), therefore being responsible for CLL cell survival.[60] WNT5a was presented in higher levels in CLL patients contrary to general populations.[61] However, a minority of CLL cells produce WNT5a, suggesting other sources of WNT5a production.[61] A study performed by Guo et al.[62] revealed that NLCs may serve as a source of WNT5a in patients with CLL. Furthermore, WNT5a induced survival and migration of CLL cells. Interestingly, further research revealed that CLL cells cultured with both NLCs and venetoclax were less susceptible to venetoclax compared to a group devoid of NLCs.[62] The postulated mechanism of NLCs-induced resistance is the activation of the NF-κB pathway in CLL cells and, subsequently, upregulation of MCL1 and BCL-XL.[62] Importantly, the upregulation of immune pathways, which are linked to resistance to venetoclax, was described in a recently published study with the 5-year results of the MURANO clinical trial.[63] The transcriptomic profile was evaluated prior to re-treatment and after therapy. Differences in transcriptomic profiles according to MRD status were revealed.[63] In non-responders, patients with detectable MRD and patients who relapsed after time, the overexpression of the *ABCB1* gene was associated with resistance and inflammatory genes, such as an inflammatory response, IFNγ response and IL2/STAT5, were detected.[63]

Multiple mechanisms leading to resistance to venetoclax have been described; however, more studies are essential to improve the outcomes of refractory to venetoclax patients with CLL.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in the elderly, as the median age at diagnosis is 70 years.[64] Unfortunately, due to comorbidities and advanced age, this subset of patients usually does not qualify for intensive induction chemotherapy, which is the standard of care in younger AML patients.[64] Therefore, venetoclax is a breakthrough in the therapy for patients ineligible for chemotherapy with a dismal prognosis. In clinical trials, venetoclax, especially combined with hypomethylating agents, such as azacitidine or low-dose cytarabine, presented a high overall response rate with an acceptable toxicity profile.[13,65]

Despite promising results in clinical trials, 24.7% of patients do not respond to the upfront treatment with venetoclax.[66] A retrospective analysis performed by Stevens et al.[18] outlined previous therapies in RR AML correlated with resistance and were predictive of response to venetoclax ($p = 0.0036$). Niu et al.[67] conducted a study on AML cell lines with intrinsic resistance to venetoclax in which treatment with venetoclax resulted in increased stability of the MCL-1 protein. Moreover, the increased stability of the MCL-1 protein contributed to the sequestration of BIM, which is an apoptosis-regulating protein.[67] One of the proposed theories that explain this event involves the displacement of BIM from the BCL-2 protein by venetoclax and, consequently, its sequestration by MCL-1.[67] Furthermore, the enhanced stability of MCL-1 may be an effect of ubiquitin ligase displacement or deubiquitinases; however, this requires further research to assess the mechanism.[67] A retrospective analysis performed by Zhang et al.[68] revealed that in the cohort of patients with AML refractory to venetoclax, none of the BCL-2 mutations were detected. Hypothetically, this may be due to the short time of exposure to venetoclax in this group (median time of exposure: 5 months (3–9 months)).[68]

Indeed, clonal mutations such as *FLT3-ITD*, one of the most common genetic alterations, or the newly emerged *TP53* mutation, were reported to be hypothetically associated with resistance to venetoclax.[68] Interestingly, this becomes coherent with other studies. Both *FLT3-ITD* and *PTPN11* mutations, which are associated with a substantially poorer prognosis in AML, were recognized before treatment in patients who did not respond to venetoclax therapy.[66,69–71] Supposedly, this may be an intrinsic mechanism of resistance to venetoclax caused by the enhanced expression of BCL-XL and MCL-1.[66] Studies performed in vitro presented that the FLT3-ITD inhibitor, quizartinib, together with venetoclax yielded durable remissions.[69] Nevertheless, further studies performed on larger cohorts are essential to evaluate the combined therapy of venetoclax and FLT3 inhibitors in clinical practice to overcome primary resistance.

Furthermore, sequencing of RNA derived from AML patients revealed additional genetic mutations associated with the immune system and inflammation-related responses, which were considered to be associated with primary resistance to venetoclax.[72] A strong correlation with resistance to venetoclax was seen with the overexpression of s100 family genes: *s100a6*, *s100a8* and *s100a9* (false discover rate <0.05), suggesting *s100a8* and *s100a9* genes as potential predictors of venetoclax resistance ($p < 0.05$).[72] Moreover, mutations of genes associated with the spliceosome *sf3b1* were correlated with lower response rates to venetoclax-based therapy and a lower OS (HR: 2.5; 95% CI: 1.1–5.65; $p = 0.02$).[65] Prior investigations have reported the detection of the *SF3B1* mutation in RR CLL patients resistant to venetoclax.[31] Interestingly, well-known mutations in *IDH1/2* or *NPM1*, which are both associated with a good prognosis in AML, were detected in a cohort of patients treated with venetoclax and correlated with favorable responses.[65,66,73]

Over the decades, the French-American-British (FAB) classification was used to classify AML, but nowadays, it does not have any clinical significance. This classification distinguishes blasts blocked at various differential stages

according to their morphologic similarity to hematopoietic cells.[74] Interestingly, in a study performed by Pei et al.,[75] 62% of patients with mature AML cells of the monocytic subtype FAB-M5 were refractory to venetoclax in combination with azacitidine.[75] This may be due to the loss of BCL-2 during the development of M5 cells. Moreover, a monocytic subtype of AML has an increased level of MCL1, with a lower BCL2/MCL1 ratio, as well as a higher expression of BCL2A1, BCL2L11 (BIM), BID, and JAK2.[75,76]

Interestingly, primitive AML displays a more prevalent regulation of OXPHOS through BCL-2.[75] Significantly, FAB-M5 was a predictor of response to venetoclax ($p = 0.0066$) with a median OS of 89 days compared to the OS of 518 days for non-FAB-M5 patients ($p = 0.0039$).[75] Moreover, further studies demonstrate the importance of JAK/STAT and/or MAPK pathways in blasts that are resistant to venetoclax.[76] The use of JAK/STAT and/or MAPK inhibitors, ruxolitinib or trametinib, respectively, may overcome resistance to venetoclax.[76]

Leukemic stem cells (LSCs) play a central role in the pathogenesis of AML.[77] Interestingly, LSCs have an altered baseline energy metabolism and cannot use basic sources such as glucose or fatty acids.[78] Thus, the metabolism of amino acids is an essential way to maintain OXPHOS and is the most important mechanism of energy to maintain the viability of LSCs.[78] Moreover, OXPHOS depends on BCL-2.[79] Inhibition of OXPHOS emerged as a mechanism of action for venetoclax.[79] The combination of venetoclax with azacitidine affects the metabolism of LSCs by disrupting the tricarboxylic acid cycle, decreasing the catabolism of amino acids, and consequently decreasing OXPHOS.[19,80] However, RR LSCs are metabolically distinct from de novo LSCs.[19] Contrary to de novo LSCs, RR LSCs differ by compensatory upregulation of additional metabolic pathways that exploit fatty acids. Subsequently, this may lead to resistance to venetoclax with azacitidine.[19] Interestingly, RR LSCs had enhanced nicotinamide metabolism and consequently increased metabolism of nicotinamide adenine dinucleotide (NAD^+), which is a crucial component in energy metabolism pathways. Increased energy metabolism is one of the mechanisms associated with resistance to venetoclax in RR AML and may explain the poor response to venetoclax in AML RR patients.[78]

Not only is the amino acid metabolism altered in refractory to venetoclax AML cells – fatty acids catabolism is also altered.[18] Additionally, resistant to venetoclax AML cell lines relied on glycolysis through upregulation of the PI3K/AKT pathway.[81] Interestingly, the *TP53* mutation, which correlates with a worse prognosis in AML, resulted in the upregulation of fatty acids in AML cell lines, which drives resistance to venetoclax.[66,82] Additionally, the upregulation of fatty acids was a result of MCL-1 overexpression through the RAS/MAPK pathway.[18] Furthermore, targeting fatty acid metabolism may be a potent way to circumvent resistance to venetoclax.[18]

Aside from alteration in BCL-2 or MCL-1 expression, a mutation in proapoptotic BAX was also reported as carrying resistance to venetoclax in AML.[83] Seventeen percent of patients who relapsed after venetoclax were carrying BAX variants, which were not reported before in patients with AML treated with chemoimmunotherapy.[83] The mutations of BAX, such as frameshift abnormalities or missense variants, disrupted BAX protein expression. This resulted in a deficiency of the proapoptotic function of BAX and was associated with resistance to venetoclax.[83] Moreover, preclinical studies revealed that the upregulation of MCL-1 is due to RAS/MAPK pathway activation.[84] Coherently, clinical data reports that patients with AML refractory to venetoclax harbor a *PTPN11* mutation which is one of the components of the RAS/MAPK signaling pathway.[69] Interestingly, a *PTPN11* mutation was strongly associated with FAB-M5,[85] which was mentioned as expressing lower levels of BCL-2 and being more resistant to venetoclax.[75] This finding was supported by another study performed by Pollea et al.[80] in which the presence of the *PTPN11* mutation along with other RAS pathway components was a predictor of shorter responses ($p = 0.0019$) to venetoclax.[80]

Multiple myeloma

Multiple myeloma (MM) is a plasma cell dyscrasia in which cytogenetic heterogeneity dictates prognosis. Of all the translocations in MM, t(11;14) is the most common and is regarded as a standard-risk cytogenetic aberration.[86] Multiple myeloma cells with t(11;14) have higher expression of the anti-apoptotic protein BCL-2 and lower expression of MCL-1 compared to normal MM cells. Higher expression of BCL-2 was demonstrated in MM cell lines, which was associated with resistance to dexamethasone.[87] Moreover, in RR MM with t(11;14) treated with venetoclax in combination or as monotherapy, favorable outcomes were observed.[88] Supporting studies presented initial promising results of the treatment of RR MM harboring t(11;14) with venetoclax combined with carfilzomib and dexamethasone.[89] Additionally, a combination of venetoclax with bortezomib was also presented.[90] The newly developed targeted therapies of BCL-2 inhibitors identify t(11;14) as the first predictive marker in MM.[91] Nevertheless, due to the increased risk of fatal infections and, therefore increased overall mortality,[90] venetoclax was not approved in the therapy of MM by the U.S. Food and Drug Administration (FDA).[92] Interestingly, a study performed by Leblay et al.[93] revealed that MM cells with t(11;14) have upregulation of B cell markers such as PTPRC (CD45), PIK3AP1, MS4A1 (CD20), CD79A, CCR7, IRF8, CIITA, CXCR4, BEND5, and VPREB3, whereas MM cells without t(11;14) have a "plasma cell" phenotype. Loss of the B-cell phenotype and selection of the plasma cell phenotype were associated with acquiring resistance to venetoclax.[93] Moreover, the resistant MM samples had decreased

expression of the regulatory protein NOXA, which was expressed in sensitive t(11;14) patients.[93] The loss of function mutation in NOXA was also observed in CLL resistant to venetoclax.[33]

Other possible mechanisms of resistance were studied by Chakrabroty et al.[94] In MM cell lines resistant to venetoclax, a significant increase in the anti-apoptotic proteins MCL1 and BCL-XL was observed. Moreover, resistant cells had significantly upregulated PKA-ERK-CREB pathways and downregulated apoptotic genes compared to parental cells.[94] As mentioned above, CLL is characterized by overexpression of BCL-2. Therefore, CLL cells are very sensitive to highly selective BCL-2 inhibitors such as venetoclax.[25] In comparison to MM, the expression of anti-apoptotic proteins is heterogenous, and MM is dependent on MCL-1 or BCL-2 or co-dependent on either BCL-2 MCL-1 or BCL-XL/MCL-1.[95] However, the majority of patients are MCL-1-dependent, and therefore, in this subset of patients, the MCL-1 inhibitor S63845 would be more efficacious. The use of venetoclax should be limited only to the minority of patients who are dependent on BCL-2.[96]

The bone marrow microenvironment plays a significant role in the pathogenesis of MM.[97] For example, mesenchymal stromal cells interact with MM cells through cell-cell adhesion, soluble factors and extracellular vesicles, leading to dysregulation of key metabolic pathways in MM cells and ultimately to drug resistance.[98] Interestingly, preclinical studies performed by Algarin et al.[99] demonstrated that resistance to venetoclax in MM was an effect of the enhanced interactions of MCL-1 and BIM by mesenchymal stromal cells. Moreover, this was avoided through combined use of venetoclax with an MCL-1 inhibitor (S63845), suggesting the possible role of double blockade of anti-apoptotic proteins to avoid resistance in MM.[99] The important role of the bone marrow microenvironment in MM in resistance to drugs was demonstrated in another study performed by Gupta et al.[96] They revealed that in the bone marrow microenvironment, interleukin (IL)-6 signaling and the Ras/MAPK pathway led to greater dependence on MCL1 rather than BCL-2 by MM cells.[96] This implies another possible reason for resistance to venetoclax in MM.

Other lymphoproliferative disorders

Due to its high efficacy in CLL and AML, venetoclax was studied in vitro to evaluate its effects on other hematologic malignancies. Follicular lymphoma is characterized by t(14;18), which juxtaposes the *BCL-2* gene to the immunoglobulin heavy chain gene promoter region, resulting in overexpression of BCL-2 and making venetoclax a rational target.[6] Nevertheless, venetoclax in FL was not as effective as it was reported in CLL.[100] Moreover, data regarding the mechanisms of resistance to venetoclax in FL remain scarce. Interestingly, a single case with the novel mutation *Phe104Ile* was reported in a sample of patients who developed resistance to venetoclax.[101] The mutation contributed to decreased affinity of the binding site of the BCL-2 protein to venetoclax and resistance to venetoclax in FL.[101] The mutations in the binding site of the BCL-2 are similar to those observed in the case of *Gly101Val* mutations in resistant CLL.[22,101] Moreover, preclinical studies suggested that resistance to venetoclax in FL may be due to the activation of ERK1/2 and AKT pathways, resulting in lower levels of BIM.[102] Nevertheless, further studies on this matter are essential to elucidate the cause of venetoclax failure in FL.

Resistance of MCL to venetoclax is linked to non-BCL-2 genes.[103] The genetic characteristics of patients' refractory to venetoclax were proposed and did not encompass mutations in *BCL-2* genes. Mutations that were suggested to be responsible for resistance to venetoclax comprised of *TP53*, *SMARCA4*, *CELSR3*, *CCND1*, and *KMT2D*.[103] However, due to the small number of patients, the data are limited, and study outcomes should be taken with caution. Similar to CLL, CD40 stimulation in lymph nodes resulted in the activation of the NF-kB pathway, a rise in BCL-XL expression, and, consequently, resistance of MCL cells to venetoclax.[104]

Considering the overexpression of BCL-2 in hairy cell leukemia (HCL), venetoclax was studied in vitro to analyze its efficacy toward HCL cells.[105] Venetoclax leads to apoptosis of HCL cells. The same study indicated the role of microenvironmental signals in the resistance to venetoclax in HCL.[105] Moreover, similarly to other presented studies, resistance to venetoclax in DLBCL was also demonstrated to be mediated by an increase in the BCL-XL protein.[106] Venetoclax may be a potent drug in other lymphoid malignancies such as DLBCL or HCL, but further studies, particularly clinical trials, are needed.

Future perspectives

Until now, resistance to venetoclax in hematological cancers has been considered an inevitable event involving multiple processes. The academic community is extensively exploring possible ways to overcome this problem. In line with this, identification of sensitivity biomarkers prior to starting venetoclax may be theoretically useful for the individualization of treatment with BH3 mimetics in clinical practice. One such biomarker may be the presence of an IDH1/2 mutation in AML, which was correlated with better response to venetoclax and was even a predictor for longer remission duration ($p = 0.042$).[69,73,80] Additionally, the identification of biomarkers for resistance during therapy with venetoclax, such as NF-κB in CLL or CD14 and/or CLEC7A in AML, may be a practical tool to elucidate the subset of patients who are refractory to venetoclax prior to clinical signs.[44,107]

An additional way to overcome resistance to venetoclax may be the administration of venetoclax in combination with active drugs having different mechanisms of action,

such as combination therapy with the BTK inhibitor, ibrutinib, for CLL.[104] In the treatment of CLL following resistance to venetoclax, administration of a BTK inhibitor may be recommended.[108] Interestingly, the blockade of the JAK1/2 pathway by ruxolitinib may also overcome resistance to venetoclax.[109] Moreover, pairing with other BH3 mimetics, such as MCL-1 inhibitors, or alvocidib, a cyclin-dependent kinase 9 (CDK9) inhibitor that downregulates MCL-1, may also be a possible way to counteract resistance.[110–112] Other combinations presented in vitro are comprised of high activity of SYK inhibition by entospletinib, as well as the phosphoinositide 3-kinase delta (PI3Kδ) inhibitor, idelalisib, in refractory to venetoclax CLL cells.[59] Eventually, novel BCL-2 inhibitors such as lisaftoclax may also be administered in patients with a secondary resistance to venetoclax.[113] Finally, cell lines resistant to venetoclax were sensitive to extrinsic apoptosis.[38] Drugs that could influence tumor necrosis factor alpha (TNF-α) or TNF-related apoptosis-inducing ligand (TRAIL) that modify external apoptosis may potentially be used in case of resistance to venetoclax, independently of intrinsic apoptosis.[38] More options to counteract resistance to venetoclax are suggested in the literature; nevertheless, their description is beyond the scope of this paper.

Limitations

This study may have several limitations, which are typical of literature review methodology. The review comprises only papers which were published in English. Therefore, the data may be incomplete. Moreover, due to the novelty of the topic, the mechanisms of resistance to venetoclax are now being studied extensively, and original papers are being published frequently. Therefore, it is not possible to review all of the newly published research.

Conclusions

Resistance to venetoclax in hematologic malignancies is a complex process that comprises plenty of mechanisms, including alterations in cellular metabolism and genetic mutations. Novel agents in the class of BH3 mimetics may be more effective in patients who developed resistance to venetoclax. Moreover, the administration of venetoclax combined with other agents may prevent or delay the development of resistance. Finally, further research is essential to identify and evaluate plausible targeted drugs that could reverse the resistance to venetoclax or novel BCL-2 inhibitors.

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