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Utilizing laser therapy to manage oral potentially malignant disorders in older adults at the primary care level

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Abstract

Oral lesions are a significant concern among older adults because they can progress to oral cancer if not diagnosed and treated promptly and effectively. Transportation to the hospital is a major barrier to oral healthcare for many older adults. The purpose of this editorial is to address the challenges of managing oral potentially malignant disorders in the older population, highlighting the barriers they face in accessing healthcare services, the potential use of laser therapy for management, and the direction of research in this area. Due to the limited access of the older to healthcare services, primary healthcare facilities within communities serve as their primary providers. Laser therapy is recommended for the management of oral potentially malignant disorders due to its favorable outcomes. This approach has been tested in several primary healthcare centers in Thailand. In our project, laser therapy was used to treat oral potentially malignant disorders in primary and secondary healthcare services. This includes photodynamic therapy for older patients with extensive lesions, as well as individuals with oral leukoplakia and erythroplakia who have declined curative surgery. It has also been used in cases of recalcitrant lichen planus to steroid or photobiomodulation therapy. This approach has been well accepted by both oral healthcare providers and patients. To expand access to these treatment options in such settings, it is critical to empower healthcare professionals, particularly dentists and dental nurses, to integrate laser techniques into geriatric care and oral cancer screening. Establishing a network foundation for orofacial laserology would also enhance the potential of such settings.

Key words: primary care, laser therapy, oral cancer, cancer prevention, older

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Introduction

The increasing proportion of the aging population globally has had a substantial impact on healthcare due to their compromised physical, mental and functional wellbeing. An observational study revealed a bidirectional association between weight loss and mental health in older adults, persisting even after 3 months of observation.[1] A recent systematic map of the systematic review included 39 articles indicating that the decline in oral function is primarily attributed to the frailty of older individuals. This review pointed out specific areas such as caries, periodontal disease, orofacial pain and temporomandibular disorders, mucosal lesions, dry mouth, and oral motor function as crucial gaps of knowledge for diagnosing, preventing, assessing risks, and treating non-operative or operative procedures. Among these domains, there was a significant gap in knowledge when it came to treating oral mucosal lesions in older people. Halitosis was also identified as an area where dentists need to improve their communication skills and knowledge of treatment due to its complex nature. A systematic review demonstrates that laser therapy and photodynamic therapy are effective treatments for this condition.[2,3] This issue requires a multidisciplinary approach from healthcare professionals.[4] In addition, a cross-sectional survey of 300 dentists working in private practice, dental clinics and universities found that 85% had difficulty diagnosing oral lesions.[5] Insufficient knowledge in managing oral mucosal lesions was also compounded by the discovery that potentially malignant disorders in the oral cavity were observed in 4.47% of the global population, 10.54% in Asia and 3.07% in Europe. These disorders exhibited unpredictable rates of progression to severe epithelial dysplasia and oral squamous cell carcinoma.[6]

The multicenter study involving 76,045 biopsy records from 7 centers in Asia and North America, with 11,346 older adults participating, revealed a 14.93% prevalence of geriatric oral lesions. The majority of these lesions were categorized as reactive or inflammatory oral lesions (46.58%), followed by malignant tumors, mostly squamous cell carcinoma (15.71%), odontogenic cysts (8.87%), benign tumors (7.41%), allergic or immunological disorders (6.65%), oral potentially malignant disorders (6.27%), and other categories (5.22%). Infections were the least prevalent at 3.18%. The study indicated the critical need for systematic screening for oral potentially malignant disoders and oral malignancy in the older population to enable early detection and intervention.[7] Similarly, a study of oral cancer screening programs conducted by general dentists in the USA highlighted the significance of screening for oral potentially malignant disoders. Skipping an oral cancer screening program can result in severe health, financial and social consequences for individuals and society as a whole. For instance, the disease may be more advanced, leading to limited or less effective treatment options, increased health risks and the need for complex and costly procedures.[8] The provided information emphasizes the crucial need for developing innovative treatments for oral potentially malignant disorders or epithelial dysplasia.

In addition to the challenges associated with maintaining fragile general and oral health, the socioeconomic status of older adults can serve as a significant barrier to accessing advanced treatments. Consequently, it is essential to select a suitable model for managing oral potentially malignant disorders and preventing oral cancer that is customized to a specific population. This selection should consider factors such as disease incidence, available resources and the healthcare system of the respective country.[9] In many Asian countries, the older in remote areas depend on primary healthcare systems. Therefore, the development of accessible and user-friendly healthcare technology for primary care workers is a valuable endeavor.

Objectives

This editorial aims to highlight the challenges of treating the older population, focusing on oral lesions, the barriers older adults face in accessing healthcare services, the critical role of primary care, and the potential use of laser therapy for managing oral potentially malignant disorders. Furthermore, we present our project, which is supported by the National Science Research and Innovation Fund (NSRF) in Thailand. This project concerns the development of laser therapy for the treatment of oral potentially malignant disorders in primary and secondary healthcare services. Additionally, we explore future research directions in these areas, emphasizing the need for multidisciplinary approaches.

The challenges of treating oral lesions in older population

The diagnosis of oral lesions often necessitates histopathological investigation through biopsy. Older individuals may exhibit reduced capacity to tolerate surgical procedures, including minor oral surgeries. A study of 23,217 newly diagnosed oral cancer patients revealed that, besides medical conditions such as poor general health, low body mass index (BMI) and advanced tumor stage, aging was found to be one of the significant reasons for not undergoing curative surgical treatment.[10] In our project on the development of laser therapy for treating oral potentially malignant disorders in primary and secondary healthcare services, we found that older people tended to refuse surgical treatment, including incisional biopsy under local anesthesia. This was mainly due to their inability to tolerate pain and bleeding during and after the surgery. According to a systematic review, there is strong evidence that frailty in older and oldest patients undergoing major surgery predicts postoperative mortality, complications and prolonged hospital stay.[11]

Using medication such as topical steroids to treat oral lesions in older adults without a definite diagnosis from a biopsy may only be effective for treating certain lesions, such as oral lichen planus. However, oral candidiasis, bad taste, nausea, dry mouth, sore throat, and swollen mouth were commonly found in older people after taking topical steroids.[12,13] The aforementioned side effects affect the oral health of older individuals.

Based on a recent systematic review, non-invasive methods for diagnosing oral potentially malignant disorders are in the developing phase, with histological investigation via surgical biopsy being the only method to obtain a definite diagnosis.[14] There was also a strong relationship between oral epithelial dysplasia and the malignant transformation of oral potentially malignant disorders. Therefore, the difficulty of undergoing surgery due to general health or attitude limits the effectiveness of oral cancer screening, prevention and treatment in the older individuals. Surgical techniques that are minimally invasive and cause fewer postoperative complications are highly beneficial for the management of oral potentially malignant disorders and the prevention of oral cancer in older individuals.

The barriers of older adults to accessing healthcare services and the critical role of primary care

The review of global access to oral care revealed that older individuals encounter challenges in receiving proper oral health treatment due to limitations in transportation. Consequently, this has led to an increase in the severity of oral disorders such as periodontal disease resulting in tooth loss and oral potentially malignant disorders transforming into oral cancer.[15]

To illustrate the local situation, we may consider the case of Thailand which is currently facing a growing concern with its aging population, as almost 20% of its 68.2 million inhabitants are older people.[16] This demographic shift increases the risk of chronic diseases among the aging population. To address this issue, sub-district health-promoting hospitals, which serve as the primary care units closest to the people, are providing comprehensive health services that encompass health promotion, disease prevention, medical treatment, and rehabilitative care, all aimed at ensuring continuous and accessible care for older adults.

A significant portion of Thailand's older population, 65.7%, lives in rural areas, with 12% living alone.[16] Many of these individuals need caregivers to help with daily activities. Access to oral care remains a major barrier to dental treatment for older adults. The most recent survey in Thailand indicates that 66.2% of older adults have not utilized dental services. Among those residing in rural areas, 32.2% received dental treatment at the primary healthcare level.[17] The lack of a caregiver to accompany them to the community hospital is a significant barrier to accessing dental services.

While dental treatment for older adults in Thailand is covered by national health insurance, additional costs, such as caregiver fees or transportation expenses, pose further challenges. The common oral disorders in the older people have been dental caries and periodontal disease, 60.0% had untreated dental decay teeth, and 48.7% had periodontal diseases.[17] The potentially malignant disorder has gradually increased in this group of people as a global situation.

To overcome this barrier, primary healthcare is considered necessary in ensuring access to overall wellbeing care, including oral care, for older people. The establishment of a robust primary care system in Thailand since 1970 has enabled the entire country to achieve universal healthcare coverage with efficiency and equity.[18] It is clear that primary healthcare can encompass not only health promotion but also disease prevention and control. Therefore, it is essential to introduce advanced technology and telehealth services to primary care providers. This will undoubtedly benefit the community and improve the overall health of older people who lack opportunities to seek healthcare services.

The significance of primary care was globally further emphasized during the COVID-19 pandemic, as the effectiveness of primary care and the community played a crucial role in stopping the outbreak of the disease.[19]

Using laser therapy for managing oral potentially malignant disorders

According to review articles published from 2011 to 2021,[20–22] the understanding and explanation of the etiology of oral potentially malignant disorders is influenced by multifactorial factors and genetic instability of keratinocytes, primarily due to oral mucosa inflammation. The progression of oral disorders to oral cancer exhibits varying temporal patterns based on the types of oral potentially malignant disorder involved. For instance, oral lichen planus presents a lower risk of progression to oral cancer than erythroplakia. Notably, the increased risk of progression is closely linked to oral epithelial dysplasia, necessitating conventional histopathological scrutiny via tissue biopsy.

Laser therapy can be used for a wide range of applications in managing oral potentially malignant disorders, including laser surgical biopsy, reduction of oral mucosa inflammation, promotion of healing, and treatment of oral squamous cell carcinoma in situ. Using high-intensity laser therapy for surgically managing oral potentially malignant disorders has been continuously reported. The conventional CO_2 laser, neodymium-doped yttrium aluminium

garnet (Nd:YAG) laser, potassium titanyl phosphate (KTP) laser, or the newer 445 diode laser demonstrated positive clinical results in terms of hemostasis control, less postoperative pain and short-term remission of the lesions.[23–25] It should be noted, however, that the recurrence and progression rates, especially in the case of oral epithelial dysplasia, were not lower than with other conventional procedures, especially when the laser was used in the vaporization technique. The authors highly recommend utilizing the excisional technique of laser surgery for oral epithelial dysplasia. In our project of treating oral potentially malignant disorders in primary and secondary healthcare services, we found that the older individuals tolerated well the use of laser for incisional and excisional biopsies under local anesthesia of lesions in which the size was smaller than 2 cm^2. The specimens were fully processed for histopathological investigation.

A recent systematic review of 26 clinical trials[26] and a recent clinical trial[27] demonstrated that photodynamic therapy, a non-thermal effect of laser therapy that generates reactive oxygen species (ROS), was an effective treatment in achieving complete-to-partial remission in the majority of oral lesions. This suggests that photodynamic therapy could be a promising treatment for oral potentially malignant disorders with epithelial dysplasia, and even early invasive squamous cell carcinoma. In our project of treating oral potentially malignant disorders in primary and secondary healthcare services, we used photodynamic therapy for the older patients with extensive lesions, as well as for individuals with oral leukoplakia and erythroplakia who declined curative surgery, and for cases of lichen planus that did not respond to steroid or photobiomodulation therapy. A systematic review revealed that photodynamic therapy is an effective treatment for oral lichen planus, offering the same efficacy as steroid use.[28]

According to systematic reviews, low-intensity photobiomodulation therapy provides effective results without side effects compared with steroids in patients with oral lichen planus.[13,29] This treatment is suitable for older patients with lesions that do not respond to steroid treatment or who experience side effects such as candidiasis.

Research direction and application of using laser therapy for managing oral potentially malignant disorders in primary care for older people

Minimally invasive laser surgery for oral lesions allows the older adults to have an incisional biopsy for an accurate diagnosis. Photodynamic and photobiomodulation therapies are noninvasive alternatives for older individuals who cannot take local or systemic steroids due to the risk of candidiasis. Photodynamic therapy appears to be "surgery without cutting" to maintain or control the lesions with oral epithelial dysplasia in the older individuals who are not able to undertake curative surgery.

The use of these therapies in older adults requires multiple treatment sessions. If these treatment options could be offered in primary care settings, the older patients would benefit directly. Our research on introducing laser therapy into primary care showed that the local healthcare personnel needed the proper laser machine to use in addition to the knowledge of how to apply laser therapy to the patients.[30] We found that the fulfillment of knowledge and proper laser equipment provided equivalent clinical efficacy with laser therapy in the older individuals across all levels of oral healthcare.[31] Therefore, research in this area focuses on developing user-friendly and precise techniques and specific laser machines to achieve this goal. Additionally, the preparation of photosensitizers for photodynamic therapy with ready-to-use options is being studied. In order to make these treatments available in primary care settings, healthcare professionals, especially dentists and dental nurses, need to be provided with knowledge of integrated laser techniques for geriatric care and oral cancer screening. A network foundation for orofacial laserology should also be established to provide further care for older adults.

Limitations

The operative definition of primary healthcare depends on the public healthcare system of an individual country.

Conclusions

The use of laser surgery, photodynamic therapy and photobiomodulation therapy allows for the treatment of oral potentially malignant disorders in older individuals, overcoming the limitations posed by their overall health. The introduction of laser therapy at the primary care level will directly benefit the older population in the control of potentially malignant oral conditions, thereby helping to prevent oral cancer.

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References

1. Payne ME, Porter Starr KN, Orenduff M, et al. Quality of life and mental health in older adults with obesity and frailty: Associations with a weight loss intervention. *J Nutr Health Aging*. 2018;22(10):1259–1265. doi:10.1007/s12603-018-1127-0
2. Grzech-Leśniak Z, El Mobadder M, Grzech-Leśniak K. Diagnosis, management and knowledge of halitosis among Polish and Lebanese dentists: Questionnaire-based survey. *Adv Clin Exp Med*. 2023;32(11):1257–1264. doi:10.17219/acem/161813

3. Woźniak A, Matys J, Grzech-Leśniak K. Effectiveness of lasers and aPDT in elimination of intraoral halitosis: A systematic review based on clinical trials. *Lasers Med Sci*. 2022;37(9):3403–3411. doi:10.1007/s10103-022-03656-3
4. Dibello V, Zupo R, Sardone R, et al. Oral frailty and its determinants in older age: A systematic review. *Lancet Healthy Longev*. 2021;2(8):e507–e520. doi:10.1016/S2666-7568(21)00143-4
5. Ergun S, Özel S, Koray M, Kürklü E, Ak G, Tanyeri H. Dentists' knowledge and opinions about oral mucosal lesions. *Int J Oral Maxillofac Surg*. 2009;38(12):1283–1288. doi:10.1016/j.ijom.2009.07.004
6. Mello FW, Miguel AFP, Dutra KL, et al. Prevalence of oral potentially malignant disorders: A systematic review and meta-analysis. *J Oral Pathol Med*. 2018;47(7):633–640. doi:10.1111/jop.12726
7. Dhanuthai K, Rojanawatsirivej S, Somkotra T, et al. Geriatric oral lesions: A multicentric study. *Geriatr Gerontol Int*. 2016;16(2):237–243. doi:10.1111/ggi.12458
8. Psoter WJ, Morse DE, Kerr AR, et al. Oral cancer examinations and lesion discovery as reported by U.S. general dentists: Findings from the National Dental Practice-Based Research Network. *Prevent Med*. 2019;124:117–123. doi:10.1016/j.ypmed.2019.03.034
9. Warnakulasuriya S, Kerr AR. Oral cancer screening: Past, present, and future. *J Dent Res*. 2021;100(12):1313–1320. doi:10.1177/00220345211014795
10. Wang CP, Liao LJ, Chiang CJ, et al. Patients with oral cancer do not undergo surgery as primary treatment: A population-based study in Taiwan. *J Formos Med Assoc*. 2020;119(1):392–398. doi:10.1016/j.jfma.2019.06.011
11. Lin HS, Watts JN, Peel NM, Hubbard RE. Frailty and post-operative outcomes in older surgical patients: A systematic review. *BMC Geriatr*. 2016;16(1):157. doi:10.1186/s12877-016-0329-8
12. Thongprasom K, Dhanuthai K. Steriods in the treatment of lichen planus: A review. *J Oral Sci*. 2008;50(4):377–385. doi:10.2334/josnusd.50.377
13. Leong XY, Gopinath D, Syeed SM, Veettil SK, Shetty NY, Menon RK. Comparative efficacy and safety of interventions for the treatment of oral lichen planus: A systematic review and network meta-analysis. *J Clin Med*. 2023;12(8):2763. doi:10.3390/jcm12082763
14. Khong B, Ferlito S, Quek S, et al. Past, present, and future diagnostic methods for the early noninvasive detection of oral premalignant lesions: A state of the art and systematic review [published online as ahead of print on May 2, 2024]. *Ear Nose Throat J*. 2024. doi:10.1177/01455613241245204
15. Peres MA, Macpherson LMD, Weyant RJ, et al. Oral diseases: A global public health challenge. *Lancet*. 2019;394(10194):249–260. doi:10.1016/S0140-6736(19)31146-8
16. National Statistical Office, Ministry of Digital Economy and Society. *The 2021 Survey of The Older Persons in Thailand* [in Thai]. Bangkok, Thailand: National Statistical Office, Ministry of Digital Economy and Society; 2022:193. https://www.nso.go.th/nsoweb/storage/survey_detail/2023/20230731140458_61767.pdf.
17. Bureau of Dental Public Health. *Report of the 9th National Oral Health Survey, Thailand 2023* [in Thai]. Bangkok, Thailand: Bureau of Dental Public Health; 2023. ISBN:978-616-11-5236-9. https://dental.anamai.moph.go.th/th/national-oral-health-survey-report/4952#wowbook/13. Accessed August 20, 2024.
18. Tangcharoensathien V, Witthayapipopsakul W, Panichkriangkrai W, Patcharanarumol W, Mills A. Health systems development in Thailand: A solid platform for successful implementation of universal health coverage. *Lancet*. 2018;391(10126):1205–1223. doi:10.1016/S0140-6736(18)30198-3
19. Frieden TR, Lee CT, Lamorde M, Nielsen M, McClelland A, Tangcharoensathien V. The road to achieving epidemic-ready primary health care. *Lancet Public Health*. 2023;8(5):e383–e390. doi:10.1016/S2468-2667(23)00060-9
20. Scully C. Oral cancer aetiopathogenesis: Past, present and future aspects. *Med Oral*. 2011;16(3):e306–e311. doi:10.4317/medoral.16.e306
21. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: Risk of progression to malignancy. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2018;125(6):612–627. doi:10.1016/j.oooo.2017.12.011
22. Warnakulasuriya S, Kujan O, Aguirre-Urizar JM, et al. Oral potentially malignant disorders: A consensus report from an international seminar on nomenclature and classification, convened by the WHO Collaborating Centre for Oral Cancer. *Oral Dis*. 2021;27(8):1862–1880. doi:10.1111/odi.13704
23. Mogedas-Vegara A, Hueto-Madrid JA, Chimenos-Küstner E, Bescós-Atín C. Oral leukoplakia treatment with the carbon dioxide laser: A systematic review of the literature. *J Craniomaxillofac Surg*. 2016;44(4):331–336. doi:10.1016/j.jcms.2016.01.026
24. Cloitre A, Rosa R, Arrive E, Fricain J. Outcome of CO_2 laser vaporization for oral potentially malignant disorders treatment. *Med Oral*. 2018;23(2):e237–e247. doi:10.4317/medoral.21984
25. Meisgeier A, Heymann P, Ziebart T, Braun A, Neff A. Wound healing after therapy of oral potentially malignant disorders with a 445-nm semiconductor laser: A randomized clinical trial. *Clin Oral Invest*. 2023;28(1):26. doi:10.1007/s00784-023-05438-9
26. Gondivkar SM, Gadbail AR, Choudhary MG, Vedpathak PR, Likhitkar MS. Photodynamic treatment outcomes of potentially-malignant lesions and malignancies of the head and neck region: A systematic review. *J Invest Clin Dent*. 2018;9(1):e12270. doi:10.1111/jicd.12270
27. Jing Y, Shu R, Wu T, et al. Clinical efficacy of photodynamic therapy of oral potentially malignant disorder. *Photodiagnosis Photodyn Ther*. 2024;46:104026. doi:10.1016/j.pdpdt.2024.104026
28. He Y, Deng J, Zhao Y, et al. Efficacy evaluation of photodynamic therapy for oral lichen planus: A systematic review and meta-analysis. *BMC Oral Health*. 2020;20(1):302. doi:10.1186/s12903-020-01260-x
29. Wang B, Fan J, Wang L, Chai L. Photobiomodulation therapy/photodynamic therapy versus steroid therapy for oral lichen planus: A systematic review and meta-analysis. *Photobiomodul Photomed Laser Surg*. 2021;39(3):145–154. doi:10.1089/photob.2020.4930
30. Sattayut S, Tanya S, Patcharanuchat P. Low-intensity laser therapy inducing photobiomodulation for oral health promotion of older people in primary health care unit: Case study [in Thai]. *J Gerontol Geriatr Med*. 2021;20(3):112–124. https://journalggm.org/view-article-79/. Accessed August 15, 2024.
31. Tanya S, Patcharanuchat P, Srisilapanan P, Sattayut S. O7: Transferring laser therapies for oral health care of older people: A multicenter study. *J Gerontol Geriatr Med*. 2023;23(1):32–32. https://www.journalggm.org/article_pdf-PA24011.pdf/. Accessed August 15, 2024.

Survival time in Alzheimer's disease: An overlooked measure of safety and efficacy of disease-modifying therapies

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Abstract

It is of vital importance to patients and physicians, as well as administrators and drug regulators, that the treatment for a disease has been shown to be safe and clinically meaningful in long-term use. Recent literature has highlighted 3 major categories of arguments for and against modification of the underlying disease process in Alzheimer's disease (AD): pathophysiology, biomarkers and data from clinical trials. We argue that the Alzheimer's arena is over-reliant on theories of disease modification based solely on brain positron emission tomography (PET) imaging and blood biomarkers of tau and Aβ peptides. Here, we instead focus on a historically-grounded empirical criterion from other fields of medicine to overcome the weak interpretations of short Alzheimer's trials: survival time (ST). Our analysis has identified 3 key points. First, if anti-amyloid therapies are AD-modifying treatments, then we argue that they should increase ST more than the standard "symptomatic" care with memantine and acetylcholinesterase inhibitors. Second, we question memantine and cholinesterase inhibitors being labeled simply as "symptomatic" Alzheimer's drugs since long-term use of them can produce disease modification, that is, increase ST. Third, we make a case for memantine or cholinesterase inhibitors being used as controls in clinical trials with amyloid-lowering and other drugs, and argue against their current under-use in care of Alzheimer's patients.

Key words: clinical trials, survival time, amyloid-β, Alzheimer's disease, anti-amyloid antibodies

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Introduction

It is of vital importance to patients and physicians, as well as administrators and drug regulators, that the treatment for a disease has been shown to be safe and clinically meaningful in long-term use. Recent 12–18 month clinical trials in early Alzheimer's disease (AD) patients with anti-Aβ monoclonal antibodies have demonstrated significant reduction of amyloid plaques in the brain as seen in amyloid positron emission tomography (PET) scans. However, a clinically meaningfull effect on slowing disease progression and cognitive decline has not been demonstrated.

On June 7, 2021, the U.S. Food and Drug Administration (FDA) approved aducanumab (Aduhelm®; Biogen, Cambridge, USA), an anti-Aβ monoclonal antibody, 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".[1] On January 31, 2024, Biogen announced that it would stop manufacturing Aduhelm and would also stop the ENVISION clinical trial, a confirmatory trial requested by the FDA to determine whether Aduhelm could actually slow disease progression and cognitive decline in AD patients. In the words of Christopher Viehbacher, CEO of Biogen: "When searching for new medicines, one breakthrough can be the foundation that triggers future medicines to be developed. Aduhelm was that groundbreaking discovery that paved the way for a new class of drugs and reinvigorated investments in the field."[2] Ultimately, this rhetoric of revolution has often been brandished instead of rigor when discussing the clinical benefits of aducanumab and other anti-amyloid antibodies.[3]

On July 6. 2023, the FDA approved lecanemab (Leqembi®; Eisai, Tokyo, Japan), an anti-Aβ monoclonal antibody which reduced amyloid in the brain, did not slow cognitive decline in women, and enhanced the decline of study participants with 2 *APOE4* genes. The claim of 27% of slowing cognitive decline in 18 months with lecanemab over placebo is due to a misinterpretation of data and trivial miscalculation; the real number is 9.3%.[4]

On July 2, 2024, the FDA approved donanemab (Kisunla™; Eli Lilly and Company,[5] Indianapolis, USA), an anti-Aβ monoclonal antibody for people with early symptomatic AD. The claim of 36% of slowing cognitive decline in 18 months with donanemab over placebo is due to a misinterpretation of data and trivial miscalculation; the real number is 9.6%.[6] On July 26, 2024, the European Medicines Agency (EMA) did not approve lecanemab, saying that "the benefits of lecanemab did not counterbalance the risk of serious side effects, especially bleeding and swelling in the brain".[7] Lecanemab, now being approved in China, Israel, Japan, and the USA, but nowhere else, raises the obvious question: How is it possible the same clinical trial data can be interpreted so differently, as providing experimental evidence to argue both for and against the efficacy and safety of lecanemab in human use?

The debate around disease modification in Alzheimer's disease

Indeed, there is no consensus on what we mean by the benefit/risk measure of anti-amyloid immunotherapies. Planche and Villain recently summarized 3 kinds of arguments for and against disease modification, pathophysiology, biomarker evidence, and clinical trial data, and argued that "With currently available data, one can therefore argue that anti-amyloid immunotherapies are [disease-modifying therapies (DMTs)] and are not".[8] One's position on the "disease-modifying" debate naturally depends on how one defines both AD (i.e., disease) itself, and what kind of modification is considered to be important for regulatory decision-making. It is not accidental that the largely USA-based definition of AD[9] as brain amyloidosis separate from cognitive symptoms led to regulatory approval of anti-amyloid antibodies, whereas the more European definition of AD[10] requiring symptoms before diagnosis, also led to more skepticism about benefit for patients.[11]

Concerning Planche and Villain's 3 classes of arguments for disease modification,[8] we consider that the AD field in general has been over-reliant on theoretical criteria, i.e., the amyloid hypothesis and amyloid-centric biomarkers including PET scans, to evaluate treatments.[12] We argue that the field should go beyond theoretical criteria to consider empirical and statistical criteria that historically were at the heart of decision making at the FDA.[13] We think it is important to consider objective, clinical criteria for claims of disease modification. Importantly, none of the recently approved monoclonal antibodies for AD reach thresholds for minimal clinically important difference (MCID)[14] in various cognitive domains.[15] However, the clinical level is not reducible to the cognitive level. It is important to consider functional and neuropsychiatric symptoms of AD when discussing disease modification.[16] A positive disease-modifying treatment should not worsen neuropsychiatric[17] or functional symptoms. Even though AD is a complex disease with different pathways,[18] we argue that the worsening of non-cognitive symptoms would be an argument against modification of those underlying disease processes leading to abnormal functioning.

In the context of these considerations of clinical benefit, it should be noted that an acetylcholinesterase inhibitor, donepezil (Aricept; Eisai) provides comparable, if not better, benefit in cognition and behavior than the anti-Aβ antibodies as measured by CDR-SB.[19] However, because of the fame of the amyloid hypothesis of AD,[20] and since donepezil does not reduce amyloid in the brain,[21] it is by definition not considered to be a disease-modifying treatment. What is important for the disease modification debate is whether objective measures in cognition with anti-amyloid treatments are maintained or even increase over time as compared to donepezil.[22]

However, these data for anti-amyloid treatments are not yet available. Evidence suggests that long-term use of donepezil leads to reduced functional decline[23] and mortality[24] in AD patients and also in nursing home residents with mixed dementia.[25] Interestingly, a causal inference study using data from electronic health records suggests that combination use of donepezil and memantine (an NMDA receptor inhibitor) creates "a significant beneficial additive drug-drug interaction" leading to significantly improved (ST) in AD patients.[26] In American veterans, memantine was associated with increased ST compared to donepezil.[27]

Survival time in Alzeimer's disease

Alzheimer's disease is a slowly progressing irreversible disorder of the brain and mind, and ST, after diagnosis at age 65 and over, is 4–20 years. There is a general consensus that earlier age of AD onset leads to a more aggressive form of the disease with a shorter ST.[28] To our knowledge, there has been no discussion of ST as a criterion for disease modification in AD. This is perhaps related to the ambiguity about whether AD is fatal, since in most cases, patients with severe AD die of secondary complications resulting from inadequate personal care and immobility, including pneumonia.[29] We can nevertheless draw on literature from oncology that uses ST as an important measure of disease modification.[30] From a public health perspective, improving ST is of vital importance, since AD is cited as a leading "cause of death," at 7th place in the USA.[31]

If the anti-amyloid therapies are AD modifying treatments in slowing disease progression and cognitive decline, then we argue that they should increase ST more than supposedly "symptomatic" treatments such as acetylcholinesterase inhibitors (donepezil, rivastigmine and galantamine) and/or memantine. Survival time is likely to be the most solid estimate for clinically meaningful benefit of putative treatments for AD, including current and future anti-Aβ monoclonal antibodies, since it has high value to public health and a low chance of bias compared to current measures used to evaluate treatments for AD, which may be sensitive to unblinding and not translate into clinical benefit.[32]

We also consider that over-reliance on anti-Aβ treatments as sources of disease modification has led to under-use of acetylcholinesterase inhibitors and memantine in both clinical trials and care. For instance, a recent longitudinal study of over 25,000 French nursing home residents found that over 80% of residents had received no exposure to either acetylcholinesterase inhibitors or memantine.[25] The authors of this study concluded that "use of conventional anti-dementia drugs is associated with a lower mortality in nursing home residents with dementia and should be widely used in this population".[25] We agree with this conclusion and argue that there is a solid case to be made for this to be applied to the research context of clinical trials. In other words, acetylcholinesterase inhibitors and/or memantine should be used as the "standard of care" control instead of placebo in amyloid-lowering and other clinical trials in Alzheimer's patients (dependent on disease stage).[33] We consider that provision of such care should be an ethical duty of trial sponsors, the absence of which would amount to withholding treatment, which would require further ethical justification.[34] We also consider that over-reliance on the amyloid hypothesis to find a disease-modifying treatment may also have led to neglect for studying the possible positive long-term consequences of acetylcholinesterase inhibitors and/or memantine. We consider that it is not appropriate to reduce the effects of these treatments to the merely "symptomatic" such as when the National Institute of Aging claims that they are "FDA-approved medications to manage symptoms".[33]

We argue that following up current patients and analyzing historical data on ST for AD trials should be a priority to establish the value of anti-amyloid and other therapies in the treatment and prevention of AD. Following up cases of autosomal dominant early-onset AD (ADAD) would provide the highest evidence of disease modification.[35] Survival time analyses have been performed in ADAD, finding that age of symptom onset is a stronger predictor of ST than different mutant variants of APP or PS1 responsible for ADAD.[36] However, the few cases of ADAD and the limited trial data on treatments[37] reduce statistical power and limit the amount of evidence available. Thus, follow up of the ST of tens of thousands of individuals having volunteered in historical and ongoing prevention trials in cognitively normal subjects at risk of developing AD with lecanemab (AHEAD3 and AHEAD45 studies) and donanemab (TRAILBLAZER-ALZ 3 study) will provide crucial analyses of disease modification. Importantly, our long-term follow-up proposal overcomes the current problem of the trial, i.e. disease mismatch, since current 12–18 month trials are only a snapshot of the long AD process.[8]

Overcoming limitations

However, there are limits to our position. Historical data on the first anti-amyloid therapy suggest that immunization with Aβ peptide as a treatment for AD provided "no evidence of improved survival".[38] However, historical data may be confounded by the fact that approx. 30% of people enrolled in amyloid-lowering trials were not amyloid positive and therefore, by definition, did not have AD before in vivo biomarkers became available.[39] Consequently, analyses of historical data should be limited to those where biomarker confirmation of AD was used. Second, older age leads to an exponential increase in all-cause mortality rates,[40,41] as well as less aggressive forms of dementia. Both of these factors could simultaneously lead to under- and

over-estimation of treatment effects on ST. Finally, there is evidence that age-standardized rates of dementia have been declining over the last few decades in higher-income countries,[42] perhaps due to a "compression" effect whereby people are increasingly developing dementia later in life and living with it for a shorter amount of time.[43]

Thus, when determining clinical meaningfulness by ST between treatment and non-treatment groups, there are several factors to take into count. These include age of symptom onset, age at the start of treatment, background demographics, and also longitudinal changes between cohorts. Nevertheless, if such factors can be controlled for and treatment groups can be shown to have increased ST, then we consider it both likely and meaningful that such treatments are modifying the disease process.

Conclusions

We consider that ST should be used to ground claims of disease modification in AD. We argue that, currently, the over-reliance on amyloid-lowering drugs has led to neglect of the long-term benefits of memantine and acetylcholinesterase inhibitors, which we do not consider to be merely symptomatic, since they demonstrate some long-term disease-modifying effects. We argue for increased use of acetylcholinesterase inhibitors in research (as a standard of care rather than placebo) and care settings (to reduce under-prescribing). Finally, we consider that if anti-amyloid treatments are truly disease-modifying as is claimed by the proponents and defenders of the amyloid hypothesis, then they should increase ST more than memantine or acetylcholinesterase inhibitors.

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References

1. Planche V, Villain N. US Food and Drug Administration approval of aducanumab: Is amyloid load a valid surrogate end point for Alzheimer disease clinical trials? *JAMA Neurol*. 2021;78(11):1307. doi:10.1001/jamaneurol.2021.3126
2. Biogen. Biogen to Realign Resources for Alzheimer's Disease Franchise. Cambridge, USA: Biogen; 2024. https://investors.biogen.com/node/27501/pdf. Accessed August 12, 2024.
3. Daly T, Kurkinen M. Measuring our language about anti-amyloid antibodies in Alzheimer's disease: Technical, theoretical, and lay language considerations. *Clin Neurol Neurosurg*. 2024;241:108314. doi:10.1016/j.clineuro.2024.108314
4. Kurkinen MT. Lecanemab (Leqembi) is not the right drug for patients with Alzheimer's disease. *Adv Clin Exp Med*. 2023;32(9):943–947. doi:10.17219/acem/171379
5. Eli Lilly and Company. Lilly's KisunlaTM (donanemab-azbt) Approved by the FDA for the Treatment of Early Symptomatic Alzheimer's Disease. Indianapolis, USA: Eli Lilly and Company; 2024. https://investor.lilly.com/node/51026/pdf. Accessed August 12, 2024.
6. Kurkinen MT. Donanemab: Not two without a third. *Adv Clin Exp Med*. 2023;32(10):1085–1087. doi:10.17219/acem/172673
7. Walsh F. EU regulator rejects Alzheimer's drug lecanemab. *BBC*. 24 July, 2024. https://www.bbc.com/news/articles/crgm0v1ne08o. Accessed August 12, 2024.
8. Planche V, Villain N. Advocating for demonstration of disease modification: Have we been approaching clinical trials in early Alzheimer disease incorrectly? *JAMA Neurol*. 2023;80(7):659. doi:10.1001/jamaneurol.2023.0815
9. Jack CR, Andrews JS, Beach TG, et al. Revised criteria for diagnosis and staging of Alzheimer's disease: Alzheimer's Association Workgroup. *Alzheimers Dement (N Y)*. 2024;20(8):5143–5169. doi:10.1002/alz.13859
10. Dubois B, Villain N, Frisoni GB, et al. Clinical diagnosis of Alzheimer's disease: Recommendations of the International Working Group. *Lancet Neurol*. 2021;20(6):484–496. doi:10.1016/S1474-4422(21)00066-1
11. Daly T, Keuck L. Alzheimer's disease: Engaging with an unstable category. In: Schramme T, Walker M, eds. *Handbook of the Philosophy of Medicine*. Dordrecht, the Netherlands: Springer Netherlands; 2024:1–24. doi:10.1007/978-94-017-8706-2_113-1
12. Høilund-Carlsen PF, Revheim ME, Alavi A, Satyamurthy N, Barrio JR. Amyloid PET: A questionable single primary surrogate efficacy measure on Alzheimer immunotherapy trials. *J Alzheimers Dis*. 2022;90(4):1395–1399. doi:10.3233/JAD-220841
13. Committee on Ethical and Scientific Issues in Studying the Safety of Approved Drugs, Board on Population Health and Public Health Practice, Institute of Medicine. *Ethical and Scientific Issues in Studying the Safety of Approved Drugs*. Washington, D.C., USA: National Academies Press; 2012:13219. doi:10.17226/13219
14. Ebell MH, Barry HC, Baduni K, Grasso G. Clinically important benefits and harms of monoclonal antibodies targeting amyloid for the treatment of Alzheimer disease: A systematic review and meta-analysis. *Ann Fam Med*. 2024;22(1):50–62. doi:10.1370/afm.3050
15. Walsh S, Merrick R, Milne R, Nurock S, Richard E, Brayne C. Considering challenges for the new Alzheimer's drugs: Clinical, population, and health system perspectives. *Alzheimers Dement (N Y)*. 2024;20(9):6639–6646. doi:10.1002/alz.14108
16. McLaughlin J, Scotton WJ, Hardy JA, Shoai M. Assessing clinical progression measures in Alzheimer's disease trials: A systematic review and meta-analysis. Preprint posted online August 29, 2023. medRxiv. doi:10.1101/2023.08.29.23294771
17. Panza F, Lozupone M, Bellomo A, Imbimbo BP. Do anti-amyloid-β drugs affect neuropsychiatric status in Alzheimer's disease patients? *Ageing Res Rev*. 2019;55:100948. doi:10.1016/j.arr.2019.100948
18. Rollo J, Crawford J, Hardy J. A dynamical systems approach for multiscale synthesis of Alzheimer's pathogenesis. *Neuron*. 2023;111(14):2126–2139. doi:10.1016/j.neuron.2023.04.018
19. Kurkinen M. Anti-amyloid therapies do not slow Alzheimer's disease progression. *Dement Neuropsychol*. 2023;17:e20230099. doi:10.1590/1980-5764-dn-2023-0099
20. Daly T. A philosophy of science approach to the amyloid hypothesis of Alzheimer's disease. *Eur J Neurosci*. 2024;60(5):4707–4722. doi:10.1111/ejn.16500
21. Ishibashi K, Miura Y, Wagatsuma K, Ishiwata K, Ishii K. Changes in brain amyloid-β accumulation after donepezil administration. *J Clin Neurosci*. 2017;45:328–329. doi:10.1016/j.jocn.2017.08.025
22. Daly T, Kepp KP, Imbimbo BP. Are lecanemab and donanemab disease-modifying therapies? *Alzheimers Dement (N Y)*. 2024;20(9):6659–6661. doi:10.1002/alz.14114
23. Howard R, McShane R, Lindesay J, et al. Donepezil and memantine for moderate-to-severe Alzheimer's disease. *N Engl J Med*. 2012;366(10):893–903. doi:10.1056/NEJMoa1106668
24. Xu H, Garcia-Ptacek S, Jönsson L, Wimo A, Nordström P, Eriksdotter M. Long-term effects of cholinesterase inhibitors on cognitive decline and mortality. *Neurology*. 2021;96(17):e2220–e2230. doi:10.1212/WNL.0000000000011832
25. Havreng-Théry C, Oquendo B, Zolnowski-Kolp V, et al. Cholinesterase inhibitors and memantine are associated with a reduced mortality in nursing home residents with dementia: A longitudinal observational study. *Alzheimers Res Ther*. 2024;16(1):117. doi:10.1186/s13195-024-01481-0
26. Yaghmaei E, Lu H, Ehwerhemuepha L, et al. Combined use of Donepezil and Memantine increases the probability of five-year survival of Alzheimer's disease patients. *Commun Med*. 2024;4(1):99. doi:10.1038/s43856-024-00527-6
27. Lazzeroni LC, Halbauer JD, Ashford JW, et al. Memantine is associated with longer survival than donepezil in a Veterans Affairs Prescription Database, 1997 to 2008. *J Alzheimers Dis*. 2013;36(4):791–798. doi:10.3233/JAD-130662

28. Barnes J, Bartlett JW, Wolk DA, Van Der Flier WM, Frost C. Disease course varies according to age and symptom length in Alzheimer's disease. *J Alzheimers Dis*. 2018;64(2):631–642. doi:10.3233/JAD-170841
29. Kukull WA, Brenner DE, Speck CE, et al. Causes of death associated with Alzheimer disease: Variation by level of cognitive impairment before death. *J Am Geriatr Soc*. 1994;42(7):723–726. doi:10.1111/j.1532-5415.1994.tb06531.x
30. Clark TG, Bradburn MJ, Love SB, Altman DG. Survival analysis part I: Basic concepts and first analyses. *Br J Cancer*. 2003;89(2):232–238. doi:10.1038/sj.bjc.6601118
31. National Institute on Aging. Alzheimer's Disease Fact Sheet. Bethesda, USA: National Institutes of Health (NIH); 2023. https://www.nia.nih.gov/health/alzheimers-and-dementia/alzheimers-disease-fact-sheet. Accessed August 12, 2024.
32. Espay AJ, Herrup K, Imbimbo BP, Kepp KP, Daly T. Recalibrating the risk-benefit profiles of lecanemab and donanemab: Scales, immunoreactivity, and changes in amyloid-β42. *J Alzheimers Dis*. 2024;99(3):877–881. doi:10.3233/JAD-240171
33. National Institute on Aging. How Is Alzheimer's Disease Treated? Bethesda, USA: National Institutes of Health (NIH); 2023. https://www.nia.nih.gov/health/alzheimers-treatment/how-alzheimers-disease-treated. Accessed August 12, 2024.
34. Millum J, Grady C. The ethics of placebo-controlled trials: Methodological justifications. *Contemp Clin Trials*. 2013;36(2):510–514. doi:10.1016/j.cct.2013.09.003
35. Levin J, Vöglein J, Quiroz YT, et al. Testing the amyloid cascade hypothesis: Prevention trials in autosomal dominant Alzheimer disease. *Alzheimers Dement (N Y)*. 2022;18(12):2687–2698. doi:10.1002/alz.12624
36. Pavisic IM, Nicholas JM, O'Connor A, et al. Disease duration in autosomal dominant familial Alzheimer disease: A survival analysis. *Neurol Genet*. 2020;6(5):e507. doi:10.1212/NXG.0000000000000507
37. Salloway S, Farlow M, McDade E, et al. A trial of gantenerumab or solanezumab in dominantly inherited Alzheimer's disease. *Nat Med*. 2021;27(7):1187–1196. doi:10.1038/s41591-021-01369-8
38. Holmes C, Boche D, Wilkinson D, et al. Long-term effects of Aβ42 immunisation in Alzheimer's disease: Follow-up of a randomised, placebo-controlled phase I trial. *Lancet*. 2008;372(9634):216–223. doi:10.1016/S0140-6736(08)61075-2
39. Doody RS, Thomas RG, Farlow M, et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014;370(4):311–321. doi:10.1056/NEJMoa1312889
40. Brookmeyer R, Evans DA, Hebert L, et al. National estimates of the prevalence of Alzheimer's disease in the United States. *Alzheimers Dement (N Y)*. 2011;7(1):61–73. doi:10.1016/j.jalz.2010.11.007
41. Jack CR, Therneau TM, Lundt ES, et al. Long-term associations between amyloid positron emission tomography, sex, apolipoprotein E and incident dementia and mortality among individuals without dementia: Hazard ratios and absolute risk. *Brain Commun*. 2022;4(2):fcac017. doi:10.1093/braincomms/fcac017
42. Knopman DS. The enigma of decreasing dementia incidence. *JAMA Netw Open*. 2020;3(7):e2011199. doi:10.1001/jamanetworkopen.2020.11199
43. Dufouil C, Beiser A, Chêne G, Seshadri S. Are trends in dementia incidence associated with compression in morbidity? Evidence from the Framingham Heart Study. *J Gerontol B Psychol Sci Soc Sci*. 2018;73(Suppl 1):S65–S72. doi:10.1093/geronb/gby001

Supporting open science: *Advances in Clinical and Experimental Medicine* and preprints

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Abstract

This editorial outlines the issue of preprints in scholarly communication. It presents the policy regarding them in *Advances in Clinical and Medical Problems* and a summary of papers released as preprints and subsequently published in this journal or rejected until July 10, 2024. The introduction discusses the definition of preprint, and leading preprint servers are listed. Policies of 2 such services – Research Square and medRxiv – most frequently chosen by *Adv Clin Exp Med* authors are then described, followed by a broad outline of the advantages of preprints and controversies surrounding them, based on selected literature on this topic. The next section discusses the policies of most renowned medical journals and publishers regarding preprints. The preprint policy of *Adv Clin Exp Med* is then thoroughly explained, as well as its reasons. All papers previously released as preprints and published in this journal in 2021–2024 are presented, focusing on meaningful differences between them. Rejected papers previously released as preprints, submitted to *Adv Clin Exp Med* in 2022–2024, are also listed and discussed. The conclusion is that the basis for endorsing preprints in this journal is not that they benefit this journal but that they serve the scientific community as a whole and science in general by facilitating rapid dissemination of results and fostering immediate assessment of those results by other investigators and debate around them. The most justified line of action is educating authors about the benefits and problems related to preprints.

Key words: policy, peer review, open science, scientific journal, preprints

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Introduction

One of the core rules of publication ethics in scientific journals is that a manuscript submitted to such periodical can neither be already published nor considered for publication elsewhere. In other words, it is strictly forbidden to submit the same paper to 2 or more journals concurrently (submitting an already published paper is a much more apparent case since it would mean blatant self-plagiarism). The above is called the Ingelfinger Rule after Franz J. Ingelfinger, editor-in-chief of the *New England Journal of Medicine* in 1967–1976.[1,2] However, a severe definitional controversy arises: Are publication and dissemination the same? Since it is understood that peer review (regardless of its specific form – double-blind, single-blind, open, etc.) is a prerequisite to consider a journal "scientific" or "scholarly", should texts made publicly available without peer review be considered "published"? One of the first researchers to ask themselves this question were investigators from the National Institutes of Health (NIH), who in 1961 began to circulate papers before their publication within an experimental network called Information Exchange Groups (IEGs) to ensure access to information about new discoveries as rapidly as it was possible back then, well before the advent of the Internet.[3] This experiment was shut down in 1967 after several journals refused to accept articles circulated as preprints.[4]

The term 'preprint' originates from the epoch when manuscripts were disseminated before they were published in a physical, printed form. Nowadays, many scientific journals do not have a printed version at all, and even if they do, it plays only a minor, if not marginal, role in their dissemination, but the term itself remains unchanged ("print" is a synonym for "formal publication" in this context).

What is a preprint? Definition attempt

Although many issues related to preprints are a subject of ongoing controversy, a clear definition of preprint may be attempted relatively easily. It is a manuscript (a paper/article means a published text in this editorial) that is:

1) scientific/scholarly in nature;
2) has not been published in a peer-reviewed scientific journal;
3) has not been peer-reviewed in any form;
4) has been released by the authors themselves, with them retaining all copyright;
5) has been deposited on the Internet in a freely accessible way (usually on a dedicated preprint server);
6) has (in most cases) a digital object identifier (DOI) assigned.

The Committee on Publication Ethics (COPE) defined a preprint as "a scholarly manuscript posted by the author(s) in an openly accessible platform, usually before or in parallel with the peer review process".[5] The adverb "usually" is crucial here – an article disseminated this way may be simultaneously submitted to a scientific journal; however, it is not an element of the definition, and some preprints are never submitted, or submitted but never accepted for publication.[6] Berg et al.[7] emphasized that preprint is a "complete scientific manuscript" and not a collection of raw data or work-in-progress (on the other hand, such "completeness" does not preclude improvements). They also pointed out that a preprint is commonly defined as being (or assumed to be) openly available online – all leading preprint servers are free of charge. Responsibility for the distribution of preprints is traditionally considered to be that of the author, a component of the definition that is often implicit in the verbs used to describe dissemination of preprints, such as, "sharing", "posting" and "self-archiving". The preprint definition was also discussed by Chiarelli et al.,[8] but without reaching any definitive conclusion.

In literature, definitions of preprints that somewhat restrict the above characteristics can be found. Blatch-Jones et al.[9] defined preprint as an open and accessible scientific manuscript or report shared publicly through a preprint server before being submitted to a journal (and not concurrently with submission). Chaleplioglou and Koulouris[10] add that a preprint is an entire scientific manuscript presenting a complete work or work-in-progress, but nevertheless must be an explicit and connotative presentation of the hypothesis, the rationale, the methodology, and the resulting research evidence that supports, rejects or revises the initial hypothesis. The authors retain the right to adapt their work in the future, enrich, modify, or reproduce any part of it in another version submitted for publication elsewhere. The main differences between scientific journals and preprint servers (and thus between peer-reviewed papers and preprinted manuscripts) were outlined in a table by Alfonso and Crea.[11] A definition employed by medRxiv also includes a negative component, stating what characteristics do not describe preprints: "Readers should be aware that articles on medRxiv have not been finalized by authors, might contain errors, and report information that has not yet been accepted or endorsed in any way by the scientific or medical community".[12]

Posting generally occurs on the day of submission or the next day (medRxiv declares 2–4 days).[12] There is typically no formal peer review of the article before it is posted online; however, it is checked for plagiarism and offensive/dangerous content; the only requirement is that the article be scientific (see section "Policy of preprint servers" below). Papers are neither typeset nor edited linguistically before being posted online. Preprint servers have no impact factor (IF), and authors retain the copyright of their articles.[13]

The role of preprints in the scientific landscape

In the last 10 years, preprints are perceived as more and more important in the circulation of scientific knowledge, and their number is steadily rising. Large number

of deposited preprints from various fields of science shown by Chaleplioglou and Koulouris[10] is one thing, but a survey recently conducted by Ni and Waltman[14] presents both the growing familiarity with preprinting and popularity of reading and posting preprints, as well as enhanced willingness to do so if a given scholar has not used this form of dissemination yet. Although preprints are more visible in physics and astronomy, followed by mathematics and computer science, and their position is much weaker in other research areas, the recent COVID-19 pandemic was met with a surge in a number of biomedical preprints – COVID-19 preprints were posted early in the pandemic, and in its early phases represented a significant proportion of the COVID-19 literature.[15] In more instances than before, manuscripts disseminated initially in this form or sometimes only in such a way contain important results (or at least data and hypotheses) also in the field of medicine. Therefore, it can be safely stated that preprints are becoming an important complement to published papers in the circulation of scientific knowledge, while being in no sense a dissemination mode that is to supplant or compete with established methods of scholarly communication. The usage of preprints can be seen as an attempt to (at least to some extent) alleviate the shortcomings of the peer-reviewed journal model of scientific publication – especially the long waiting time for publication and financial issues related to both high article-processing charges and paywall-mode access to published papers, employed by many journals.

Where are preprints deposited?

Dissemination of preprints in the current sense of this term has been possible since the emergence of the Internet at the turn of the 1980s and 1990s. The first dedicated preprint archive, arXiv (https://arxiv.org/), was launched in 1991, with Hyper Articles en Ligne (HAL; https://hal.archives-ouvertes.fr/) following in 2001. A visible rise in the popularity of preprints has been observed since 2010 – all the most popular services in medical, scientific publishing were started in the last 12 years: bioRxiv (http://biorxiv.org/) in 2013, Authorea (http://authorea.com/) also in 2013, Preprints.org (https://www.preprints.org/) in 2016, Open Science Framework (OSF) Preprints (https://osf.io/preprints/) in 2017, Research Square (https://www.researchsquare.com/) in 2018, and medRxiv (http://medrxiv.org/) in 2019. All the above preprint servers allow the deposited manuscript to be assigned a DOI.[16] Chaleplioglou and Koulouris[10] provided an exhaustive review of 22 preprint services and their policies, including those listed above. The most complete list of preprint repositories has been compiled (and is constantly updated) by the Directory of Open Access Preprint Repositories (DOAPR; https://doapr.coar-repositories.org/repositories/). This directory provides a list of preprint repositories that are available to the research community. It helps researchers find the most appropriate platform for a given manuscript, enabling them to browse through existing repositories by discipline, location, language, functionalities, and other facets. The directory is jointly managed by Centre pour la Communication Scientifique Directe (CCSD) and Confederation of Open Access Repositories (COAR).

Advice for researchers regarding preprints

In several popular science magazine articles, advice on disseminating one's manuscript as a preprint (and if yes – how to do it) may be found. Brock,[17] in an article in *Nature Index*, provided 10 tips for submitting a successful preprint, while Bourne et al.[18] listed and explained 10 simple rules to consider regarding preprint submission. Ettinger et al.[19] presented a more thorough guide to preprinting for early-career researchers, and Sarabipour et al.[20] described early-career researchers' perspectives on preprints. Guidelines regarding preprints were also released by, i.a., Columbia University,[21] University of Oxford,[22] UK Research and Innovation,[23] NIH,[24] University of Surrey,[25] University of Melbourne,[26] Eastern Michigan University,[27] Harvard Countway Library,[28] and University of Hong Kong Libraries.[29] Finally, a practical guide to the preprints has been released as a preprint in the Zenodo repository by Hettne et al.[30]

Since January 30, 2023, the National Library of Medicine (NLM) started to make preprints resulting from research funded by the NIH available via PubMed Central (PMC) and, by extension, PubMed.[31,32]

Objectives

This editorial in an attempt to:

1) define preprints as a form of dissemination in the scientific milieu;
2) outline the advantages of preprints and controversies around them (in a general context of scientific publications) as well as policies of 2 selected preprint servers;
3) provide some insight into attitudes of key stakeholders within the scientific community to preprints;
4) present policies of leading medical publishers and journals regarding preprints;
5) describe the approach to this form of dissemination in 1 scientific medical journal – *Advances in Clinical and Experimental Medicine* (*Adv Clin Exp Med*) – including the preprint policy of this periodical and reasons to this policy;
6) present how papers which have previously been released as preprints are present in this journal;
7) assess whether the aforementioned policy requires modification;
8) discuss future directions for preprint policies and their potential evolution in the context of open science (OS);
9) suggest future research on preprints, including areas that require further investigation.

Policies of preprint servers

It should be noted that although the abovementioned preprint servers do not offer peer-review services, it does not mean that everyone can post anything on such server, regardless of the quality of the disseminated material and/or its character. Preprints are approved for posting after moderation but not peer review. Policies of 2 selected preprint servers will be briefly discussed – Research Square and medRxiv. The latter has been chosen as the most respected preprint service dedicated to medical manuscripts; the former because of its popularity among *Adv Clin Exp Med* authors, though it accepts submissions from all research fields. The policies of both servers are similar, but there are some differences.

Research Square

Research Square does not conduct peer review on preprints before posting.[33] Submitted manuscripts are checked for appropriate ethics and consent statements, disclosure of competing interests, absence of patient identifiers, and inappropriate, alarming, highly controversial or pseudoscientific claims. Articles with firm conclusions, especially without fully accessible data, may also be screened out. The following manuscript types are accepted: research articles, systematic reviews, method articles, short reports, case reports, and data notes. Only research articles with complete methods and results sections will be considered for posting. Non-systematic reviews, theories and commentaries are not eligible for preprinting. Manuscripts reporting negative results are welcome. For medical research, it is considered critical that data be made fully accessible, and it is strongly encouraged. A submission already published in a journal will not be posted as a preprint. Submissions with missing figures, reference lists or other critical components may be rejected. Authors should include a competing interest statement and funding disclosure.[33]

Once a preprint posted on Research Square receives a DOI, it cannot be removed from the platform without cause – i.e., issues that cannot be addressed by submitting a revised version of the preprint. If a preprint is withdrawn from any leading preprint server, the content is removed, leaving behind basic metadata like title, authors, and the reason for withdrawal if the author chooses to provide it. In rare circumstances, a preprint is removed from the site altogether. In these cases, the preprint is replaced with text indicating that the manuscript has been removed. Also, in such a situation, wherever possible, the title and author list remain in place. Research Square may initiate a withdrawal on a preprint if its staff have reason to believe there are issues with research conduct or ethics associated with the work or if a later version of the article has been retracted from a journal.[33]

Research Square supports versions of preprints – the revision will be posted under the same DOI, and the original version of the manuscript will remain accessible. There is a limit of 5 revisions for 1 preprint (medRxiv does not limit the number of versions).[33]

Preprints at Research Square are indexed through Crossref, Europe PMC and Google Scholar. PubMed Central indexes a limited subset of preprints.

medRxiv

All manuscripts posted on medRxiv are screened for plagiarism, non-scientific content, inappropriate article types (i.e., not health-related, narrative reviews and case studies, among others), and material that could potentially endanger the health of individual patients or the public.[34] The latter may include, but is not limited to, studies describing dual-use research of concern and works that challenge or could compromise accepted public health measures and advice regarding infectious disease transmission, immunization and therapy. Submissions are also checked for ethical oversight, clinical trial registration and information that might identify a patient/participant.[12] Authors should declare at submission that all relevant ethical guidelines have been followed, all necessary institutional review board (IRB) and/or ethics committee approvals have been obtained, all necessary patient/participant consent has been obtained, and the appropriate institutional forms archived. Authors should include a competing interest statement and funding disclosure.[35]

The manuscript must not have been posted elsewhere (including other preprint server), nor should it have been accepted for publication in a journal (medRxiv is the only leading preprint server that addresses the issue of simultaneous submissions to 2 or more preprint servers). To allow screening, medRxiv is open only to manuscripts in English. Authors must submit the appropriate research reporting checklists defined by the EQUATOR network as supplementary files. Clinical trials must be registered with an internationally recognized trial registry with the trial ID included. A manuscript posted on medRxiv can be revised at any time until it is accepted for publication in a journal, provided that the journal to which the authors submitted the manuscript does not forbid posting versions that include changes made in response to the peer review process. If authors withdraw their manuscript from the server, a statement explaining the reason for the withdrawal is posted on the manuscript page to which the DOI defaults; the original manuscript is still accessible via the Info/History tab on medRxiv, and a "Withdrawn" watermark is added to the *pdf of all posted versions of the main text of the manuscript.[35] The authors may also revise a paper, but the fact that a revision (or multiple revisions) occurred is revealed to the reader – the revision will be posted under

the same DOI, and the original version of the manuscript will remain accessible.

Of note, similar policies regarding withdrawal and versioning are employed by the OSF.[36,37]

The medRxiv is indexed by Crossref, Google Scholar, Semantic Scholar, Europe PMC, and Web of Science's Preprint Citation Index. In addition, preprints reporting research funded by the NIH are indexed by PubMed.[12]

It is important to note here that neither Research Square nor medRxiv declare adherence to any COPE or International Committee of Medical Journal Editors (ICMJE) guidelines on their websites. The screening procedures employed by the preprint servers were analyzed by Kirkham et al.,[38] and also in a research letter by Malički et al.[39]

Advantages of preprints

The advantages of preprints are, at the same time, the main reasons for their rising popularity among researchers and the broadening acceptance of this practice among journal editors and research funders. As Brainard[40] noted in the context of the surge in published preprints during the COVID-19 pandemic, they did not cause a revolution in scientific publishing; however, because of the characteristics discussed below, preprints are becoming more and more popular.

Rapid dissemination

First, preprints provide an opportunity to release the results of one's research immediately, without delays caused by prolonged peer-review and editorial processes. Janda et al.[41] revealed in 2022 that among the preprints posted on medRxiv, 77.0% (1,077 out of 1,399) were published in peer-reviewed journals within a median of 6 months after posting, which shows that manuscripts released using this venue are indeed visible to the scientific community. The issue of the time from submission to publication has been investigated, among others, by Huisman and Smits,[42] Andersen et al.[43] (for biomedical journals), Zimmer et al.[44] (for genetic journals), Harlianto and Harlianto[45] (for urology journals), Sebo et al.[46] (for general medical journals), and Lee at al.[47] (for South Korean medical journals). All the above analyses emphasized that the waiting time for publication is considered too long by all stakeholders (according to Andersen et al.,[43] the mean timespan from submission to publication varies from 91 to 639 days, while the median timespan is 70–558 days). Because a preprint is only checked for its essential characteristics, not peer-reviewed, it becomes available on a preprint server after a few days. Consequently, research results may be communicated to the scientific community as soon as they are obtained, which may be crucial in rapidly developing fields of knowledge.

Access to new data

Members of the scholarly community can use the presented data, concepts and methods in their research; in this way, preprints contribute to more robust development of science in general. Other researchers may also offer the preprint's authors a chance for scientific collaboration, which stimulates the globalization of science and academic mobility. The above is particularly important in medicine because clinical research benefits from open and timely access to new data. Preprints can "accelerate" science, particularly useful, e.g., in combatting outbreaks of diseases.[48] Even if the presented results are not validated yet, the idea outlined in a preprinted manuscript may inspire other researchers when rapid development of new therapies, vaccines, etc. is paramount.

It is also worth noting here that in light of studies by Janda et al.[41] and Bero et al.,[49] most clinical studies posted as preprints and subsequently published in peer-reviewed journals have concordant study characteristics, results and final interpretations – the former analyzed the preprints posted on medRxiv while the latter a sample of COVID-19-related publications. Also, Brierley et al.[50] showed that although preprints and their published versions differed to a certain degree, the majority of these changes do not qualitatively change the conclusions of the paper. Carneiro et al.[51] showed that peer-reviewed articles had, on average, higher quality of reporting than preprints, although the difference was slight. This suggests that authors tend to release the versions of biomedical papers as preprints close to the final authors' version, which is subsequently submitted to a journal (provided, of course, that such submission occurs at all). Nelson et al.[52] assessed the robustness of evidence reported in preprints in 100 matched preprint–journal article pairs and rated it highly. Akbaritabar et al.[53] analyzed the differences only in reference list between preprints and their published versions, and noted that they were more pronounced in medical publications than in other fields of science.

Establishing priority

The most prominent advantage of preprints – rapid dissemination – is related to establishing priority (Elmore[13] calls it "documentation of the history of ideas"). Since preprint servers deposit a manuscript with a date stamp proving when exactly it has been submitted,[8] authors of the yet unpublished article can publicly declare when at least the draft/first version of their paper describing their original research was already in existence (which can also be important when applying for grant or employment). Moreover, Poremski et al.[54] argued that predatory journals would not be able to thrive like nowadays if authors were able to secure a legitimate place in the literature for their unreviewed work – provided that in their particular situation, DOI suffices. They are not in dire need of publishing, even in a journal of disputable quality.

Opening science

A significant advantage is that the most popular preprint servers are not-for-profit, open-access (OA) services, allowing for free access to recent scientific developments for all interested parties, with access to the Internet as the only prerequisite. Researchers and other professionals (e.g., doctors) from low-income countries can contribute to the global scientific community by releasing their own preprints and offering comments to other investigators' manuscripts even without any institutional support. While OA is still not a default mode of scientific publishing, dissemination of preprint is barrier-free by definition (Elmore[13] called it "democratization of the information flow"). Sever et al.,[55] among others, perceive preprints as the future of OS. In this vein, preprints can be seen as a model of self-publishing and self-archiving, in which the authors present their work publicly while retaining complete control over the content and full copyright.

Fostering debate

A preprinted manuscript can be further disseminated using social and lay media, professional Internet forums and other similar venues, allowing fellow scientists for commenting on it, pointing out its strong and weak aspects, and providing the authors with suggestions on how to improve it. Most leading preprint servers, including medRxiv and Research Square, also give the opportunity for commenting on each posted manuscript. Therefore, professional debate around its contents can be fostered before publication, enabling authors to enhance their work even before it is submitted to a scientific journal[11] or receive valuable feedback concerning their work in general. Authors can obtain input from a wider audience than a few peer reviewers.[13] Although Clemens[56] argues that negative comments in reaction to a preprint may discourage early-career researchers, it has to be emphasized here that critique and criticism (sometimes somewhat harsh) are a part of the scientific community, and scientists have to be ready from the beginning of their professional development to accept it.

Some researchers may also decide to release a preprint to test their peers reaction to a specific hypothesis, without the intention of submitting the manuscript to any journal in the form deposited on the preprint server. So far, no research is available showing whether such debate actually occurs and whether it has any meaningful influence on the contents of the released preprints. On the other hand, it should be noted that this does not necessarily mean that there are no researchers who benefit from such feedback; it means that this undoubtedly important issue awaits professional investigation.

Communicating controversial or negative outcomes

Preprints also provide a possibility to present adverse or controversial outcomes that could otherwise be deemed by peer reviewers unfit for publication in a regular journal.[13] Negative results can also be necessary for developments in a particular field since they may prove (or at least suggest) that a certain approach or method does not yield meaningful information; therefore, other researchers may refrain from considering it and save time and money for different ideas.

Boosting citations?

Finally, there is the open problem of whether preprints play a much more practical role from a researcher's or journal editor's point of view and boost the number of citations of the published paper (assuming that in this context, a preprint is only an intermediate stage). Results obtained by Fu and Hughey[57] suggest that releasing a preprint is associated with more attention and citations for the peer-reviewed article. Xu et al.[58] showed that the better the altmetrics and citations of preprints, the better performance when the preprints published in journals, while Fraser et al.[59] found that bioRxiv-deposited journal articles had sizably higher citation and altmetric counts compared to papers not deposited on this server before publication, as did Serghiou and Ioannidis.[60] Xie et al.[61] claimed that preprints correlate with more citations, but their study has been released only as a preprint and has not been peer-reviewed or otherwise validated. However, all these studies are still limited and do not allow for extrapolations or generalizations for all published papers that were previously released as preprints.

Controversy around preprints

Reliability of preprints

The most crucial concern raised is related to this exact feature of preprint which ushers their most prominent advantage: They are not peer reviewed. Thanks to this, they can be disseminated at once, but for exactly the same reason there is no guarantee of their scientific soundness. Some researchers expressed assurance that the academic community will self-regulate itself and detect faulty preprint publications; however, before this happens, inexperienced researchers or lay people may confuse a preprint with a legitimate, peer-reviewed scientific paper. Lack of peer reviews means that presented results and conclusions have to be closely scrutinized by readers (Bagdasarian et al.[62] discussed this issue in the context of the COVID-19 pandemic) because preprints may include a wide array of errors and/or dubious claims which will remain in circulation until a specialist

reads a given manuscript closely and alarms the public of its shortcomings. Moreover, lack of quality control may favor "salami-slicing" publishing – dividing one large paper into several shorter ones and releasing them as preprints independent of each other to obtain more publications.[11,63] Although the staff of preprint services verify manuscripts deposited on the most popular servers, this verification is brief and concerns mainly the scientific character of the paper; moreover, there are no studies in the literature examining the thoroughness and reliability of such verification.

Faulty screening

Additionally, no method is currently available to screen preprint submissions for conflicts of interest[13] – Research Square requires authors to disclose such conflict. Still, it is unclear whether any verification in this regard occurs.[33] If such a paper is cited in other research papers or reposted on social media, confusion may spread, as in the case of the preprint concerning hydroxychloroquine use to treat COVID-19, summarized by Kang and Oh.[3] Moreover, there are no standards regarding data sharing and open data concerning preprinted manuscripts – Research Square "strongly encourages" data sharing but does not require it.[33] This issue has been outlined by Strcic et al.[64] in the context of papers on COVID-19, while McGuinness and Sheppard[65] conducted a descriptive analysis of the data availability statements accompanying medRxiv preprints and a comparison with their published counterparts.

Preprints allow the scientific community to learn about discoveries before they are published; however, their dissemination may lead – at least potentially – to scientific misconduct since investigators with ulterior motives may attempt to scoop other research team's ideas and publish their results before the original team releases their paper in a peer-reviewed journal. On the one hand, the availability of predatory journals with very short turnaround time (because of only feigned peer review) theoretically makes such scoop possible; on the other hand, the probability of such occurrence seems very low in light of the current literature.[55] Nevertheless, such concern has been voiced[3] (mainly in the context of research representing a commercialization value), and they should be taken seriously. The risk of deliberate plagiarism is low because manuscripts deposited on most popular preprint servers are searchable for anti-plagiarism services (e.g., iThenticate) like published papers. The issue of scooping possibility in relation to preprints has been extensively discussed in a dedicated FAQ section on the ASAPbio server.[66]

Duplicate publication

Even though the similarity (sometimes reaching 100%) between the preprint and the manuscript submitted to a scientific journal is not seen as self-plagiarism (since a preprint is not a publication), the surge in the number of papers released as preprints prior to publication in a journal causes another significant problem: duplicate publications. The preprint servers do not monitor whether the deposited manuscripts end up published; therefore, it is the authors' duty to complement the preprint following publication of the paper with a note about such publication and a DOI of the published version, with the latter (and not the preprint) considered the version of record (i.e., final version).[67] However, many author neglect to fulfil this duty; they leave the preprint unchanged (this issue is discussed further on examples from our journal in the "Papers in *Adv Clin Exp Med* previously released as preprints in 2021–2024" section). Such neglect has a visible result: from our editors' experience, it can be observed that often other researchers cite a preprint, unaware that when they submitted their manuscript, a published, in some instances, significantly revised version of the cited article has already been published. In our opinion, it would be sufficient when, before citing the preprint, the authors would search on the Internet for papers with the same title and authors – in most cases, such a simple procedure would allow for finding the peer-reviewed version of the record (provided that such version exists at all). However, some authors seem unaware that it is so easy to avoid confusion. Checking in this way the preprints included in the reference lists is a standard procedure in editing a manuscript accepted for publication. When it is revealed that a preprint instead of a peer-reviewed version has been cited (which results in "citation dilution" because citations of one paper are "diluted" between 2 DOIs treated by the databases as 2 separate entities), our editors inform the authors about this fact since the version of the record can be substantially updated. The authors may wish to revise the corresponding passages of their paper in light of such updates. Finally, each journal should employ a citation format that ensures discerning between published papers and preprints, as pointed out by Kang and Oh.[3]

In this regard, the ICMJE recommends that the authors should: 1) inform a journal if the work submitted has been posted on a preprint server; 2) provide a link to the preprint to indicate in the text of the manuscript that a preprint is available and how reviewers can access that preprint; 3) ensure that preprints are amended to point readers to subsequent versions of the work, including the published article. In addition, authors should not post the published article in the preprint archive, nor should interim versions produced during the peer-review process incorporate revisions based on journal feedback. Finally, when a preprint article has been subsequently published in a peer-reviewed journal, authors should cite the subsequently published article rather than the preprint article whenever appropriate.[68]

Unequal status of preprints and their citability

It is also of utmost importance that all researchers in all fields of science and scholarly knowledge are fully aware

of the unequal status of preprints compared to peer-reviewed papers published in scientific journals. Although several funding entities – the Medical Research Council and Wellcome Trust – accept preprints in grant applications, such practice is far from uniform.[69] The same applies to citing preprints in other scientific publications – although most journals do not discourage it, a research paper with its reference list including many preprints may be treated as less reliable by peer reviewers.

Another concern also relates to citing preprints and their unequal status – and this is a two-edge sword. On the one hand, in a survey by Ni and Waltman[14] several respondents expressed doubts that their preprints would be cited at all because of low credibility of such dissemination form. Some researchers may be wary to release a manuscript as a preprint – since what is the point when one of the main expectations among all authors is to be cited? On the other hand, many scholars may also refrain from citing preprints – for 2 reasons: 1) disputable reliability of such sources may be frowned upon by peer reviewers if the reference list of a given manuscript comprises several such positions (even if certain topics are so far discussed only in preprints); and 2) not all citation standards include clear guidelines how to cite preprints in a way not would not be confused with papers already in press (from the experience of the authors of this editorial, it is clear that many less experienced authors confuse an in press paper – i.e., already accepted for publication but not yet published – with a manuscript only under review, which indeed can be cited only if it is concurrently available as a preprint).

Possibility of confusion among laypeople

Some scholars have voiced concerns that lay public, especially journalists, who need to become more familiar with the tenets and reality of scientific publishing, may confuse preprints with peer-reviewed articles or even be unaware of the status difference between them. Consequently, dubious or unverified claims may be presented to the public by journalists in mainstream media or private persons in social media as verified scientific facts (while actually they are yet to be scrutinized). This can have dire consequences in medical sciences – possible harm to health may be a considerable danger in some instances. There is also a possibility that some authors may attempt to publicize claims of questionable scientific merit, using the media to attract attention as they are unlikely to win the approval of the scientific community – such practice is sometimes called 'science by press conference'.[70] During the COVID-19 pandemic, Fraser et al.[15] noted that despite the warning messages provided by medRxiv and bioRxiv, COVID-19 preprints have received unprecedented coverage on online media platforms. Concerns were also raised that unverified claims in preprints can be used by politicians and physicians to advocate for specific treatments of disputable effectiveness or to support their political agenda.[71]

The most popular preprint servers place disclaimers on the 1st page of each deposited manuscript to minimize the danger of confusing it with a peer-reviewed journal paper – e.g., Research Square uses the formula "This is a preprint; it has not been peer-reviewed by a journal",[33] while medRxiv states that "this article is a preprint and has not been peer-reviewed. It reports new medical research that has yet to be evaluated and so should not be used to guide clinical practice",[12] as seen in a preprint by Vazquez-Rodriguez et al.[72] Peiperl[73] proposed placing a digital watermark on all pages ("preprint, not peer-reviewed") instead of a small disclaimer just on page 1 to deter the reporting of unverified data by the lay media.

Even the term 'preprint' is prone to mislead less experienced journalists and researchers because it says nothing about the peer review itself and can be confused with 'Epub ahead of print', which is a peer-reviewed paper published without pagination before it appears in a specific issue of a given journal, sometimes many months in advance (almost all papers published in *Adv Clin Exp Med* first appear as ahead of print versions). Therefore, Ravinetto et al.[74] proposed terms like 'unrefereed manuscript' or 'manuscript awaiting peer review' or 'non-reviewed manuscript' to avoid misunderstandings, while Mullins[75] pointed out that preprints that have not been subsequently published in a peer-reviewed journal and their authors do not intend to do so should be called "no-prints" to steer clear of suggesting that some form of publication occurred or is likely to happen.

Information overload

Already, so many papers are published in an ever-multiplying number of scientific journals that it becomes impossible to keep track of them in one's field of interest, which causes concerns about general information overload in the scientific community. It is said to be additionally amplified by the circulation of preprints, mainly due to them being an opportunity to disseminate low-quality research while circumventing the peer review, which serves as a gatekeeper in journals.[63] There are no studies aiming to cover all preprints released in a given field of science in a restricted period; therefore, it is so far impossible to assess whether this concern is legitimate.

Retractions of preprints

Preprints are a part of one of most prominent problems concerning scientific publishing: Although there is a mechanism of retraction, which enables to remove seriously flawed papers from circulation while at the same time appropriately informing the readers about the reasons for such action (in a retraction note) and retaining the basic information about the paper (title, authors and journal of publication, etc.), there is no universal mechanism of relaying the fact of retraction to databases and

other entities where the paper, often in full-text version, is available. The same applies to preprints – if authors cite a preprint, but does not access the manuscript as it is available on the preprint server, relying only on the version in the database, they may be unaware that it has been retracted. However, several major preprint servers – including arXiv, medRxiv and Preprints.org, but excluding Research Square – are included into the Retraction Watch Database (http://retractiondatabase.org/RetractionSearch.aspx). Should a preprint deposited in a repository present in this database be retracted, it would be visible after a proper search – not only the fact of retraction, but also the reason for it. The Retraction Watch Database address also the issue of preprint versioning: If a retracted preprint has multiple versions with different DOIs, all of them are entered into this database and the term "Revision" is added to the revised versions of the original article in the description of the article type.[76]

Lowering editorial standards

Last but not least, there is a controversy regarding the editorial aspect of preprints. As it has been pointed out above, such materials are neither typeset nor do they undergo any other editing, neither linguistic nor technical. They are deposited by authors as *doc/*docx files, and are available usually as *pdf files obtained by simple conversion of the source file. Such low technical standard of dissemination – from the experience of authors of the present editorial – may influence some researchers' expectations concerning the editing process, particularity its speed and thoroughness. Many authors appear genuinely surprised when they learn that their paper will not be published immediately following final acceptance and that it will be edited and typeset. When provided with the galley proof with many remarks and corrections, they sometimes expect that the *pdf file may be at this stage simply "reverted" to the *doc/*docx form, which is impossible – not only at this stage, but at any stage after typesetting (a manuscript after typesetting cannot be converted into text and *pdf form back and forth at will).

Examples of notable medical COVID-19 preprints

In this section, both examples of preprints that contained valuable scientific knowledge and of those which were debunked and removed from circulation will be briefly discussed. The presented cases do not prove anything in themselves, but they show that both praise and criticism of this dissemination form are warranted.

A preprinted manuscript by Pradhan et al.[77] titled "Uncanny similarity of unique inserts in the 2019-nCoV spike protein to HIV-1 gp120 and Gag" was published on bioRxiv on January 31, 2020, and quickly attracted attention of the scientific community, with a flurry of comments both directly on the preprint server and on Twitter, where the authors posted an URL to the preprint. Commenters contended that the author's methods seemed rushed, and the findings were at most a coincidence. Already on February 2, the manuscript was withdrawn and a following note appeared in its place: "This paper has been withdrawn by its authors. They intend to revise it in response to comments received from the research community on their technical approach and their interpretation of the results. If you have any questions, please contact the corresponding author."[77] The title and information about authors are still in place and the older (i.e., full) version of the paper is available, albeit with a large watermark "Withdrawn" on each page. However, in several databases, this preprint is marked not as withdrawn, but retracted, which is an important difference. Preprints may be withdrawn also for reasons not related to their content (e.g., conflict among authors), just like papers submitted to a journal but not yet published; however, a published paper can only be retracted (withdrawals or removals are rare) and such move is in most instanced connected to its scientific flaws or plagiarism.

Luckily, all of that happened before a single news outlet with any reach covered the paper. Already on February 8, 2020, Zhang et al.[78] released as a preprint (also on bioRxiv) a 1st version of a manuscript reanalyzing the data provided by Pradhan et al. and heavily criticizing the methodology employed in their paper. What is important, this manuscript was later published in a peer-reviewed version.[79] Later that year, claims of Pradhan et al. were invalidated in other peer-reviewed papers, among them in a study by Xiao et al.[80]

This example showcases that the scientific community is able to rapidly react to preprints of low quality. However, it should also be noted that such appropriate answer may be ascribed to the subject of the paper in light of the unfolding COVID-19 pandemic in its early stages, when all scientific materials concerning this issue were actively sought and received unprecedented attention. We cannot be sure that a faulty manuscript about a less timely topic would be debunked so swiftly and thoroughly.

An example of the positive role of preprints during the same time – early months of the COVID-19 pandemic – is a paper by Guan et al., released first as a preprint on medRxiv[81] on February 9, 2020, and just less than a month later (on February 28, 2020) in the *New England Journal of Medicine*.[82] Nineteen days seem not a long time; however, the crisis was spreading like a wildfire and such rapid dissemination truly made a substantial difference: The authors had shared the clinical symptoms, laboratory abnormalities and radiologic findings of over 1,000 patients with COVID-19 well before many American or European clinicians gained direct experience with SARS-CoV-2. The initial preprint provided an early window into one of the largest threat that COVID-19 would pose for patients

and the healthcare system – many experts became aware that the increasing number of patients with acute respiratory distress syndrome would shortly dwarf the number of available ventilators around the world. Awake prone positioning and shared ventilation, which were critical components of the global strategy to contend with the limited ventilator supply during the height of the pandemic, would not be known in many healthcare facilities around the world so quickly if the authors would wait for a peer-reviewed publication.[83] *New England Journal of Medicine* conducted the review process with a breathtaking speed, but the authors could not assume that when submitting the paper only a month before. Their decision about releasing the preprint and promoting it in social media was both warranted and expedient. Nevertheless, also in this case it should be borne in mind that in a less critical situation even a very important preprint may be overlooked – but this is a general consequence of a sheer number of scientific articles published every day; a peer-reviewed paper appearing is a journal other than top-ranking may also slip attention.

Perspective of various stakeholders

Several studies have analyzed the approach to preprint among members of the scientific community, focusing, however, primarily on researchers. Soderberg et al.[84] provided an interdisciplinary survey of researchers regarding the credibility of preprints, while Chiarelli et al.[8] conducted a broad study combining a literature review and survey among different members of the scientific community concerning perception of and attitude to preprints. In 2 very recent (2024) studies, Ni and Waltman[14] separately surveyed researchers in different parts of the globe (China, then USA and Europe, and finally the rest of the world), while Biesenbender et al.[85] surveyed life sciences researchers who posted COVID-19-related preprints. In order to provide at last a partial insight into this issue, these 4 studies are discussed below.

Soderberg et al.[84] in 2020 asked 3,759 researchers across a wide range of disciplines about the credibility of individual preprints and preprint services. Among their respondents, 69.73% of the sample felt slightly to strongly favorable towards preprints, while only 15.16% felt opposed to preprints and 14.95% felt neutral. The average percentage of favorable responses among medical authors was lower but still high (51%). Usage of preprints was associated with preprint views/downloads more than by preprint submissions: 70.63% of all surveyed researchers had viewed/downloaded preprints either a few or many times, while only 29.85% had submitted a preprint a few or many times. Medicine had the lowest levels of viewing/downloading of any discipline (48.11%). Information about OS content and independent verification of author claims were rated as highly important for judging preprint credibility, and peer views and author information were rated as less important.

Chiarelli et al.[8] conducted 38 semi-structured interviews of various stakeholders – mostly active researchers, but also research funders, administrators of research-performing organizations and preprint server providers – chiefly from the fields of biology, chemistry and psychology, between October 2018 and January 2019. The respondents expressed uncertainty and diverse views on both preprint definition and position in the scientific landscape. The main concerns were related to the lack of quality assurance and the Ingelfinger rule. Doubts and concerns were also recorded regarding the financial stability and business models of preprint servers, since some of them are owned and managed by non-governmental organizations (NGOs), some by universities and other research institutions, and others by private publishing companies. Although most of these institutions profess long-term preservation policy, respondents were not sure whether manuscripts disseminated only as preprints will be available indefinitely. The interviewees commented that if preprints were to play a more significant role in scholarly communication, major improvements to the preprints infrastructure would be needed – i.a., incorporation of preprints into scholarly and publisher workflows, provision for production of preprints in standards-based formats (e.g., *xml) and greater consideration of preservation services. The conclusions were that community norms are crucial and have not significantly changed in many cases, therefore constraining individuals' decisions. There was, nevertheless, some willingness to experiment, particularly amongst general OA supporters. There was some awareness of potential benefits becoming evident in practice but still at low levels; evidence of incomplete knowledge or misunderstandings amongst some researchers relating to preprints was also clearly visible.

Ni and Waltman[14] performed an online survey of corresponding authors of papers published in 2021 and early 2022 and indexed in the Web of Science database, asking them about familiarity with preprinting, ways of learning about it, experience with reading preprints, experience with posting them, willingness to post preprints in the future, as well as about attitudes toward preprinting (benefits of preprinting, concerns about it and ways to encourage it). Overall, 45% of the European survey participants reported to be "very familiar" or "extremely familiar" with preprinting, with 38% of participants from other continents answering in the same vein. Even more declared reading preprints (at least a few per year) – among medical researchers, approx. 70% regardless of the world part. Around 50% from the same group stated that they released at least 1 preprint during their career, and a similar percentage declared that they are willing to do so in the future (again or for the first time), while approx. 30% did not plan to engage in preprinting. The most emphasized benefits of preprints among medical investigators were: availability in OA (32–43% of respondents, depending on the continent, mentioned

this advantage), acceleration of scientific communication (31–38%), lack of publication charges (29–36%), establishing priority (24–34%), additional exposure (23–28%), early feedback (14–24%), and showing progress for grant/job applications (13–25%). More citations and sharing results that do not fit into journals were mentioned less often. Among the concerns raised most frequently were: low reliability and credibility (30–48%), sharing before peer review (25–41%), premature media coverage (23–53%), copyright/licensing uncertainty (16–27%), incompatibility with journal policies (17–19%), danger of getting scooped (13–35%), undermining peer-reviewed journals (9–24%), and lack of recognition for preprints (8–30%), with information overload and possibility of harmful comments on preprints appearing only seldom. Free-text responses revealed additional concerns: about quality of preprints, multiple versions of the same paper, citing preprints in peer-reviewed papers, and possible accusations of self-plagiarism. These authors also pointed out lower adoption of preprints in medical and health sciences when compared, e.g., to physics, astronomy, mathematics, and computer science. While reading preprints is more or less equally common in different countries, survey participants in the USA and Europe reported higher familiarity with preprinting and stronger commitment to posting preprints than those from other parts of the world.

Biesenbender et al.,[85] also very recently (2024), conducted a survey of life sciences researchers who have posted COVID-19-related preprints regarding their experiences and motivations. The most often cited motivations to release a preprint were: 1) to increase awareness of one's research; 2) to stake a claim on one's findings; 3) to receive early feedback; 4) to encourage increased citations of one's work; and 5) to promote possible research collaborations. The most often voiced concerns were that: 1) preprints lack quality assurance; 2) there is a risk of incorrect reporting by lay media; and 3) comments and feedback are generally not helpful. Many respondents also believed that in 5 years, preprinting will be a common practice in their research field. They then analyzed 8 popular preprint repositories regarding the number of posted preprints. Interestingly, survey and preprint server analysis have presented different, if not contradicting, results: While the majority of surveyed researchers were willing to continue posting preprints, the numbers of preprints published, especially on servers for the life sciences, have stagnated or declined. Also, while certain preprints garnered substantial citations during the COVID-19 pandemic, this has not resulted in a significant shift in researchers' publishing behavior, and the posting of preprints has not become a routine. The researchers concluded that the sustainability of preprint publishing practices is more strongly influenced by disciplinary norms and practices than by external shocks (as the COVID-19 pandemic).

The contents of the 4 above surveys can be summarized as follows: preprints are more and more accepted within the scientific community – at least among researchers, who are aware of several of both potential benefits and possible problems associated with this form of dissemination. However, the lack of trust emphasized by Chiarelli et al.[8] persists – the authors do not trust other researchers (possibility of scooping, releasing low-quality research, harmful comments), journal editors (incompatibility with journal policies, accusations of self-plagiarism) and journalists (premature, sensational coverage in media).

An international, cross-sectional survey of preprinting attitudes among biomedical researchers was also conducted by Ng et al.,[86] but their methodology makes a comparison with the studies presented above an undertaking outside the scope of this paper.

Policies of other journals

General tendencies

Smart[63] divided the relationship between journals and preprints into 6 steps to acceptance: uneasy relationship (nostalgia for strict Ingelfinger Rule), acceptance, encouragement, participation (loose collaboration with preprint services), and sub-merger (a close alliance of preprint servers, journals and peer review services). In 2020, Klebel et al.[87] described the policy of most of the 171 major academic journals across disciplines they examined in this regard as "unclear". During the last 4 years, the situation has probably improved, but the policies reviewed for the present study significantly differ in their specificity; however, they are clear enough to allow for comparisons. For example, in the same year, Massey et al.[88] published a cross-sectional study of preprint policies among the 100 clinical journals with the highest IF. They showed that 86% of journals allow for submitted articles to be previously posted as preprints. Policies of leading biomedical journals were also outlined in 2020 in an editorial by Flanagin et al.,[71] while Vlasschaert et al. presented the policies of scientific journals from the field of nephrology.[89]

Journals or publishers who do not accept papers previously released as preprint are only a fraction of all scientific medical periodicals.[90] Among them are all periodicals owned by the British Editorial Society of Bone & Joint Surgery, as well as *Journal of Orthopaedic Research* (owned by Wiley) and *Clinical Orthopaedics and Related Research* (owned by Wolters Kluwer).

Conversely, many journals do not stipulate anything particular apart from disclosing the existence of the preprint during submission and adding a note about the version of the record following publication; such policies will be called "unrestricted" in this section.

Examples of an unrestricted policy are the rules concerning preprints employed by Taylor & Francis,[91] Cambridge University Press[92] and the *Journal of Clinical Medicine Research*,[93] which do not include any further requirements.

Centers for Disease Control and Prevention (CDC) state that they will consider publication manuscripts posted on reputable, not-for-profit preprint servers on a case-by-case basis,[94] which is a slightly more cautious approach but still open to preprint practice.

Specific attitudes

Narrowing the rules covering preprint only to non-commercial preprint services is standard, though not uniform; however, this lack of uniformity may be caused by the fact that all the most popular preprint servers listed above are free of charge. Some journals accept only disseminating preprints through non-commercial servers (e.g., arXiv, Open Science Framework, Zenodo) – such policy was adopted by, e.g., the American Association for Physics in Medicine.[90] It should be noted here that authors' or institutional websites (e.g., repositories offered by universities) are also considered non-commercial. Therefore, e.g., BioMed Central (BMC) explicitly accepts sharing preprints also on such servers.[95]

Wiley company gives the authors a choice regarding the abovementioned note: They can use such disclaimer or post the final published version of the article immediately after publication on the non-commercial preprint server instead of the previous preprint version,[96] which prevents the circulation of different versions of the same paper. Conversely, Elsevier stipulates that preprints should not be added to or enhanced to appear more like, or to substitute for, the final versions of articles.[97] American Society of Clinical Oncology (ASCO) journals somewhat mix the 2 above approaches and stipulate that, on the one hand, no revisions should be posted to the preprint server during the manuscript's peer review process, while on the other hand, revisions following publication must not deviate from the final version of the manuscript published by ASCO.[98]

Some differences in preprint policies concern licensing. Springer journals stipulate that the authors must disclose the DOI of the preprint and the licensing terms offered by the preprint server[99] because these terms affect how the preprint may be shared and reused.

Some journals and publishers state that the manuscript versions that have been altered due to the peer review process may not deposited. This pertains, i.e., to journals published by the American Heart Association (AHA),[100] American Thoracic Society, European Respiratory Society, Cell Press journals (owned by Elsevier), and Japan Society for Cell Biology.[90] The American Association for Cancer Research (AACR) and *Science* journals use a slightly different formula: While a manuscript is considered, no versions revised in response to editorial input and peer review should be posted on a preprint server.[101,102] European Molecular Biology Organization Press states that no updated versions may be posted to preprint servers after initial submission to the journal – there is also a slight difference here because there is no mention that the update must be in any way related to the peer review process (the authors can revise the preprint also for other reasons, but in this case it is forbidden as well).[90]

The American Psychiatric Association Publishing and the Journal of the American Medical Association (JAMA) Network stipulate that the submitted manuscript must add meaningful new information above that is already in the preprint. This narrows the number of preprinted papers that can be considered for peer reviews in these journals because many submitted papers previously disseminated as preprints are identical or almost identical to versions available on preprint servers.[90]

Specific preprint policies of different journals and publishers differ in minor details. *BMJ* declares that it "fully supports and encourages archiving of preprints" provided that: 1) they are deposited on non-profit servers; 2) the authors inform about the preprint during submission and include its DOI; 3) a given paper is not a case report (due to patient confidentiality concerns); 4) the authors add the following text to the preprint following the publication of a given paper in *BMJ*: "This article has been published in [insert full citation] following peer review and can also be viewed on the journal's website at [insert DOI]." *BMJ* does not restrict the license chosen when posting a preprint version of work, but authors must retain the copyright of their work when posting to a preprint server.[103] Policies of *Nature* portfolio journals are identical – the only difference is the lack of specific provisions regarding case reports.[104]

Several entities further connected the preprint dissemination with publishing articles in their journals. Authors submitting papers to *PLoS One* journals can choose to deposit them concurrently on medRxiv or bioRxiv; the preprints are checked for suitability according to the rules of the respective preprint server. Otherwise, the preprint policy of these journals can be defined as unrestricted.[105] BioMed Central has partnered with Research Square to provide In Review, a journal-integrated solution for preprint sharing.[95] Sage Publishing also launched its preprint server – Advance.[106]

Summary

From the above, it can be inferred that the global trends are unequivocal: Preprints are more and more widely not only accepted but actively encouraged – some publishers and journals even themselves offer the authors the opportunity to release their manuscripts in this form as soon as a given article is submitted to their journal. Periodicals that consider preprints prior publication are only a tiny minority – since the advent of preprints, several journals changed their policies in favor of them, while in the literature, not a single case of a decision in the opposite direction is noted. As for now, the status of peer-reviewed papers compared to unrefereed preprints remains unchallenged. However, there are voices such as Neylon et al.,[107] which suggest that the role of preprints in specific fields of knowledge

becomes so paramount that the objective "state" of the article (work-in-progress, unpublished but completed, published, with other possibilities) can become divergent from the "standing" a given scientific community attaches to it.

Adv Clin Exp Med preprint policy

Our journal endorses and encourages the practice of disseminating the pre-peer-reviewed versions of scientific papers through established non-profit preprint servers such as BioRxiv, medRxiv Research Square, or Authorea, as well as on authors' or institutional websites, while not requiring it in any capacity. This policy does not interfere with the policies of funding institutions, employers or other entities, which may stipulate such release in their regulations concerning OA. Posting of preprints is not considered prior publication by the editorial office of *Adv Clin Exp Med* and a preprinted manuscript is treated as any other following submission.

Preprints are defined as an author's version of a research manuscript before formal peer review at a journal, deposited on a public server. Preprints may be posted before or during the peer review process, but not after acceptance in the journal and certainly not following publication (we also do not enourage authors to update the preprint following publication in order to let it mirror the version of record – it could cause further confusion because it is not a popular practice).. Versions of a manuscript that have been altered as a result of the peer review process may not be deposited on the preprint server – the above means that the authors can disseminate a preprint following submission (due to due to the possible length of the peer review process), but its text has to be either the version initially submitted or any earlier version, but no changes related to the peer reviewers' remarks can be implemented (changes unrelated to peer-review process are authors' choice). The reason for this prohibition is that by definition and as stated above, a preprint is a version of a research manuscript before formal peer review at a journal and, therefore, cannot include any content incorporated in consequence of such review. A preprint that has been peer-reviewed in a journal is no longer a preprint but a postprint – a research journal article after it has been peer-reviewed and accepted for publication but before it has been typeset and formatted by the journal.[8,108]

Adv Clin Exp Med does not formulate any stipulations regarding the licensing terms stated by the preprint servers since it is a golden OA journal – articles published on the journal's website (https://advances.umw.edu.pl/en/home/) are licensed under Creative Commons Licenses (CC), which means they can be freely distributed and shared so that other people can build their work based on them. Licenses offered by most popular preprint servers are either CC ones or at least do not create any legal conflict; therefore, we decided not to state any conditions regarding preprint licenses because it could cause unnecessary confusion among authors.[109]

If the article is already accessible online as a registered preprint on any website or in any database and has been already assigned with a DOI, such information, together with a URL of the registered preprint, has to be provided during submission of the paper as well as appear in the cover letter. Once the preprint is published in *Adv Clin Exp Med*, it is the author's responsibility to ensure that the preprint record is updated with a publication reference, including the DOI and a URL link to the published version of the article on the journal website. The editors of our journals check for the above annotation within the preprint periodically following publication to make sure that all authors comply with this policy.

Adv Clin Exp Med has not entered a partnership with any already established preprint server, owns no in-house preprint server, and has no intention to undertake any such initiatives.

Reasons for *Adv Clin Exp Med* preprint policy

In general, the advantages of preprints outweigh the controversy surrounding them and their potential dangers. Disseminating preprints is a practice widespread enough both among authors and publishers that rejecting it would mean that many high-quality papers would be desk-rejected even before the peer-review stage simply because of high Similarity Index (SI).

Supporting OS

We consider the opportunity to present the scientific developments to the global community of scholars as soon as they are available and foster discussion around them before the publication, as well as other most essential advantages of preprints the critical reasons for endorsing them in *Adv Clin Exp Med*. Our position is that rapid dissemination of research results in order to boost the development of knowledge does not necessarily contradict the need for peer review as the best-known method to secure professional assessment and verification of submitted manuscripts, provided that there is a clear distinction between refereed and unrefereed material. In our opinion, renowned preprint services like those that *Adv Clin Exp Med* authors choose most frequently (Research Square, bioRxiv, medRxiv, and Authorea) are a form of dissemination that assures such distinction thanks to their format and layout, which differ significantly from those of peer-reviewed journal. Nevertheless, the preprint servers should consider informing the readers even more clearly and unambiguously than they currently do that the manuscript they read has yet to be peer-reviewed and what it entails (the so-called caveat lector note).

Rapid dissemination

An ideal situation would be a peer review process much more rapid than nowadays. Still, in the absence of working solutions to this long-known problem, we can at least not deter authors from using available and widely accepted tools to at least to some extent circumvent this obstacle. Establishing priority is also an essential reason in this context – a preprint available in OA mode in its entirety is much more helpful in claiming priority than any document from the editorial office confirming that a given manuscript is under peer review or even has been accepted for publication because such documents contain only the paper's title and the authors' names, not its whole contents.

As a medical journal, we aim to support and expedite the development of knowledge in this field, not obstruct it – we endorse the practice of discussing preprints on dedicated servers and in social media and improving manuscripts as a result of such feedback. Like many other journals, we may also be wary of publishing controversial or negative results – but such content should also be able to find its way to the scientific community. Rapid dissemination of results as an advantage of preprints became paramount during the recent COVID-19 pandemic when a massive, unprecedented surge in released preprints was observed; we consider it as evidence supporting our stance.[110] Watson[111] even stated that rapid data sharing during COVID-19 has changed science forever. Preprints enabled both rapid dissemination of discoveries and quick disproving of only ostensibly promising avenues of research, which in turn allowed for rapid adjustment of health policies in different countries.[112] Premature or insufficiently proven claims were quickly detected and debunked by the scientific community, which has shown (at least to some degree) that the global science system is indeed self-regulatory and low-quality or fraudulent preprint manuscripts will not circulate unchecked indefinitely. The quality of COVID-19-related research has also been analyzed by Fraser et al.,[15] Singh and Ravinetto,[113] Kodvanj et al.,[114] Majumder and Mandl,[115] Vlasschaert et al.,[116] Wang et al.,[117] and Gianola et al.[118]

Authors in control

We also think that preprints empower authors with more control over the results of their work – they can (in a certain framework of preprint servers) inform others about their ideas and results on their own account. On the one hand, this reduces (to some degree) the dependence of researchers on the journals as the only channels of publishing science; on the other, it does not undermine the peer review verification system as a whole, since preprints are, in most fields of knowledge, only a complement, not a replacement of traditional peer-reviewed publishing.

The editors of *Adv Clin Exp Med* acknowledge that preprint dissemination may interfere with the anonymity of the authors because the reviewers may find the preprint on the Internet and thus learn about the authors' identities. Moreover, should a discussion occur in social media or on other websites where the authors promoted their preprint, a peer reviewer may also inadvertently read such remarks and be influenced by them. Despite these potential problems, we believe the advantages of the abovementioned preprints outweigh the potential interference with the peer-review process.

Papers in *Adv Clin Exp Med* previously released as preprints in 2021–2024

In 2022–2023, 9 papers were published in *Adv Clin Exp Med* that were previously disseminated as preprints; in 2024 (until July 10), 6 such papers were already published. Endorsement of preprints in our journal has been decided only in early 2021, so there are no data from the previous years that could allow for pointing out trends in the popularity of preprint release among our authors. In 2021, only 2 papers already available as preprints were published, but such a low number does not enable any meaningful comparisons – it is possible, e.g., that authors who wished to disseminate their work as preprint abstained from submitting their articles to *Adv Clin Exp Med* because they were not sure whether the editorial office followed the Ingelfinger Rule. The experience of editors shows that changes in editorial policy in this journal take a few months to be broadly recognized among prospective authors.

Outline of preprinted papers published in *Adv Clin Exp Med*

Papers published in *Adv Clin Exp Med* since the implementation of the preprint endorsement policy, which were identified as made available using preprint servers, are summarized in Table 1.[119–135]

Overall, 2 such papers were published in our journal in 2021, 6 in 2022, 3 in 2023, and 6 in 2024 (until July 10). However, 3 from the last group are ahead of print articles which will appear in regular issues in 2025 (1/2025, 2/2025 and 4/2025) and 1 is an ahead of print publication from the 12/2024 issue; this makes 17 papers disseminated as preprints in total. We need to find an explanation for the surge in preprinted papers in 2022. Among the 17 papers, 11 came from China, 3 from Poland, 1 from India, 1 from the Czech Republic, and 1 from Mexico. These numbers roughly reflect the composition of the country of origin of papers submitted to *Adv Clin Exp Med* – from January 1, 2021, to July 10, 2024, 63% of papers were from China and 14% from Poland. There is no evidence that funding institutions or other institutional policies

Table 1. Papers published in *Advances in Clinical and Experimental Medicine* (*Adv Clin Exp Med*), which were available as preprints at the time of submission

Article	Country	Title	Preprint server	SI	Additional information
Li et al., 2021[119]	China	miR-874 ameliorates retinopathy in diabetic rats by NF-κB signaling pathway	Research Square	97%	Information about version of record being the paper published in *Adv Clin Exp Med* only in the *pdf version available on the preprint server.
Liu et al., 2021[120]	China	Silencing of lncRNA SNHG12 inhibits proliferation and migration of vascular smooth muscle cells via targeting miR-766-5p/EIF5A axis	Research Square	82%	–
Guo et al., 2022[121]	China	Metformin protects against abdominal aortic aneurysm by Atg7-induced autophagy	Research Square	69%	–
Li et al., 2022[122]	China	Does a single dose of palonosetron have any role in preventing acute chemotherapy-induced nausea and vomiting in pediatric osteosarcoma patients without dexamethasone? A randomized clinical trial	Authorea	33%	iThenticate did not detect the preprint.
Putowski et al., 2022[123]	Poland	High intraoperative pulse pressure is a risk factor for postoperative acute kidney injury in a cohort of abdominal surgery patients: An exploratory study	Research Square	69%	–
Ji et al., 2022[124]	China	Hypoxia-inducible factor-2α promotes EMT in esophageal squamous cell carcinoma through the Notch pathway	Research Square	75%	Information about version of record being the paper published in *Adv Clin Exp Med* only in the *pdf version available on the preprint server.
Begum et al., 2022[125]	India	Efficacy of different intensity of aquatic exercise in enhancing remyelination and neuronal plasticity using cuprizone model in male Wistar rats	Research Square	97%	–
Sun et al., 2022[126]	China	Significance of detecting the levels of miR-29a, survivin and interferon gamma release assay in patients with lung cancer and tuberculosis	Research Square	77%	–
Sang et al., 2023[127]	China	Bone marrow mesenchymal stem cell-derived exosomes attenuate the maturation of dendritic cells and reduce the rejection of allogeneic transplantation	Research Square	95%	–
Grotowska et al., 2023[128]	Poland	Fluid resuscitation, but not inhaled nitric oxide, improves microcirculation in septic pigs	Research Square	67%	–
Ventruba et al., 2023[129]	Czech Republic	The contribution of donated human embryos suitable for the production of embryonic stem cells to increase the quality of life: Selection and preparation of embryos in the Czech Republic	Research Square	91%	–
Yang et al., 2024[130]	China	Correlation analysis of patients with diabetic foot ulcers treated with tibial cortex transverse transport surgery and platelet-to-lymphocyte ratio and monocyte-to-neutrophil ratio	Research Square	97%	Ahead of print (4/2025)
Li et al., 2024[131]	China	Small RNA sequencing highlights a potential regulatory network mediated by Gecko miRNA affecting the prognosis of hepatocellular carcinoma	Research Square	68%	Ahead of print (2/2025); Editorial Note: The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.
Szczepanowski et al., 2024[132]	Poland	Application of machine learning in predicting frailty syndrome in patients with heart failure	Research Square	88%	Information about version of record being the paper published in *Adv Clin Exp Med* only in the *pdf version available on the preprint server.
Zheng et al., 2024[133]	China	Integrated analysis of a competing endogenous RNA network reveals a ferroptosis-related 6-lncRNA prognostic signature in clear cell renal cell carcinoma	Research Square	99%	Ahead of print (12/2024)
Yong et al., 2024[134]	China	Differential expression of miRNA-769-5p and Smad2 in patients with or without oral cGVHD	Research Square	89%	Ahead of print (1/2025)
Vázquez-Rodríguez et al., 2024[135]	Mexico	Fc-gamma receptor expression and cytokine responses to intravenous human immunoglobulin in whole blood from non-pregnant and pregnant women and newborns	medRxiv	43%	–

SI – Similarity Index

in China or Poland encourage or stipulate disseminating papers as results of funded research more strongly than such institutions in other countries. Among the preprint servers chosen by the authors, Research Square was clearly the preferred one, with 15 papers stored there; 1 article was deposited in Authorea and 1 in medRxiv. The reason for this is most probably the snowball effect – for years, this server has been unchallenged as the most popular among medical researchers, and many of them might not consider any other service when choosing a preprint server, even though policies of Research Square and medRxiv are similar.[33,35] There are no visible patterns regarding the topics of the preprinted papers compared to all papers published in *Adv Clin Exp Med* – as shown in Table 1, the thematic scope of this whole group of articles is vast and covers diverse areas of medical sciences. This is in concert with the journal's scope in general – it published papers that deal with all clinical and experimental medicine.

Differences between preprints and published papers

Several studies discussed the scope of differences between preprint versions of medical papers and the final articles that appeared in peer-reviewed journals.[13,41,51] Although such an area of research is outside the scope of this paper, some insight might be provided by the results of the anti-plagiarism similarity check performed using the iThenticate service provided by Crossref (Lynnfield, USA). A study by Li et al.[122] was disseminated as a preprint using the Authorea service, which, for technical reasons, precluded it from being identified as a preprint during the verification above (the authors revealed the existence of the preprint in the cover letter). Among the remaining 16 papers, 8 (50%) remained very similar to the preprinted version, with the SI indicated by iThenticate varying between 88% and 99% (cf. Table 1). However, the reader should bear in mind that the percentage of changes does not necessarily reflect their significance; large parts of text can be rewritten for linguistic editing reasons, while changes small in terms of percentage may deeply modify the presented results (e.g., altered numerical values). Journals that accept preprinted papers do not require the most recent version deposited on the preprint server to be identical to the manuscript submitted to the editorial office – some only stipulate that it cannot be changed following submission, either as a result of peer-reviewers' remarks or at all; therefore, the SI in the range of 43–82% among the other 8 preprinted papers published in *Adv Clin Exp Med* is not a surprise. The authors may revise the paper substantially between posting it as a preprint and submitting it to a journal, and they do not have to post the exact submitted version on the preprint server – they may decide to leave the preprinted version unaltered. Such revisions may concern both the scientific content of the text and its structure and language.

Impact on citations

As has been mentioned before, several authors[57–61] discussed the question whether preprints boost the number of citations of their published versions. Unfortunately, it is impossible to analyze this issue on the example of articles previously disseminated as preprint and subsequently published in *Adv Clin Exp Med*, since none of those papers was cited more than 1–2 times and none of them was among the above-average cited papers published in this journal (the current IF for *Adv Clin Exp Med* is 2.1). Therefore, it cannot be tracked whether this single citation or 2 citations are in any way related to the preprint deposition.

Duplicate publication issues

An issue of particular importance is the persistence of preprints on dedicated servers even after the version of record (i.e., the published version) of the manuscript appears. This creates a paradoxical situation when both unrefereed and peer-reviewed versions of the same paper are in circulation. A paper can undergo many changes during the peer-review process, and the published version may differ substantially from the submitted one. Consequently, the preprint version may be misleading for the reader because it may include content that was put into question by the reviewers and altered as a result. Leading preprint services do not allow the removal of preprints (and published ones) from their servers entirely, considering them a part of the scientific record.[9,33] Therefore, the preprint reflecting the submitted version can still be available and citable, even though it presents only the imperfect, sometimes even flawed version of the manuscript.

An example of an unclear situation regarding a preprint is the paper by Li et al.[131] It has been withdrawn from Research Square with a withdrawal note placed (cf. Table 1), which provides the following reasons for withdrawal: "The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author."[136] The note is dated August 30, 2023, while the submission to *Adv Clin Exp Med* took place 5 days later – on September 4, 2023. Whether the withdrawal occurred due to publication in *Adv Clin Exp Med* or for other reasons is unclear. It is possible that the authors attempted to avoid duplicate publication by withdrawing the preprint rather than supplementing it with a note about the version of record; in our opinion, such action intensifies the confusion instead of minimizing it (the authors did not react to contact attemps after the editors learned about the withdrawal note).

However, a note may appear at the beginning of the preprinted paper indicating that the version of the record is not the preprint but the published version, with a DOI

or a URL of the published article. Such note about the version of the record being the paper published in *Adv Clin Exp Med* appears in 3 papers (conf. Table 1), but only in the *pdf versions available at the preprint server, not on the HTML subpages of the respective preprints.[119,124,132] This may lead to a situation when a researcher or a media outlet mistakenly cites the unrefereed preprint instead of the peer-reviewed article because when searched using the title in web browsers, preprint frequently appears directly or almost directly below the published version in PubMed and on the journal's website. There is also a possibility that the preprint is erroneously taken for a paper entirely different from the version of the record listed above in the browser (this can happen when the reader neglects to compare the title and author list of both releases). Therefore, the editorial office of *Adv Clin Exp Med* stipulated as follows in the instructions for authors[109]: "Once the preprint is published in *Adv Clin Exp Med*, it is the author's responsibility to ensure that the preprint record is updated with a publication reference, including the DOI and a URL of the published version of the article on the journal website."

Rejected papers previously released as preprints in 2022–2024

The rejection rate in *Adv Clin Exp Med* is very stable and, since 2021, has oscillated around 80% each year. Therefore, many more manuscripts were submitted to our journal following a preprint release that did not make it to publication. Since the SI, which allows for (apart from unmasking plagiarism cases) the detection of a preprint (should the authors neglect to reveal its existence in the submission form), was consistently measured for all submitted manuscripts only from December 2021, we decided to sift the rejected papers for preprints releases only beginning from those submitted later than January 1, 2022.

Overall, from January 1, 2022, to July 15, 2024, 72 manuscripts previously released as preprints were submitted to *Adv Clin Exp Med*, of which 15 were published and 57 were rejected (there were no preprinted manuscripts among the withdrawn ones; manuscripts still under peer review were not considered since their fate is yet undecided). The total rejection rate for the given period is 19.62%. In comparison, for the previously preprinted papers, it is 20.83%, so the difference is negligible. It does not prove any difference in the quality between manuscripts released as a preprint and all papers published in our journal.

Nevertheless, it is interesting to investigate at which stages of the article assessment process the abovementioned 57 papers were rejected. Nineteen were rejected during the peer review stage because of negative reviews, while 15 were recommended for rejection by section editors resulting from a preliminary assessment before peer review. The rest did not pass even the initial verification stage. Authors of 13 manuscripts disclosed that they released them beforehand as preprints in the submission form or after being asked by the editors when the SI check revealed the preprint existence in the SI report; however, they failed to implement during the initial verification the editor's remarks concerning other aspects of the paper (e.g., references formatting or the overall structure of the paper). Authors of 10 papers did not inform the editors in advance about the preprints. When the SI report uncovered this negligence, they did not address the (repeated and accompanied by a URL to the preprint) question about this issue. Our editors always assume the goodwill of the authors, so failing to disclose a preprint in the submission form is never a reason for rejection – provided that the authors confirm that they made a preprint release when asked about it; should they remain silent, the paper is rejected after 60 days. We cannot explain this lack of response (even though the authors were informed that *Adv Clin Exp Med* endorses preprint dissemination) nor make any informed supposition regarding the authors' motivation for breaking contact with the editors at so an early stage of manuscript assessment.

Conclusions

Should our preprint policy be changed?

The analysis of all preprinted papers submitted to *Adv Clin Exp Med* showed that their rejection rate is similar to the general rejection rate for all submitted manuscripts; consequently, it can be stated that the papers previously released as preprints do not differ in quality compared to all papers published in our journal. Therefore, the justification for endorsing preprints in *Adv Clin Exp Med* is not that they benefit this journal but that they serve the scientific community as a whole and science in general by facilitating rapid dissemination of results, fostering immediate assessment of those results by other investigators and debate around them, as well as by allowing to establish priority for researchers for whom this is important for any reasons. Though legitimate, concerns and controversies surrounding preprints are primarily rooted in potentialities and not established facts; thus, the policy of our journal will remain favorable to preprints unless their disadvantages are scientifically proven or at least many cases of harm caused by preprints emerge.

As stated at the end of the "Policies of other journals" section, the status of preprints in the scientific community is dynamic and the only things we can be sure of are more changes. We are unable to predict these changes and, in consequence, to act in advance. Arguments presented in the sections "Advantages of preprints" and "*Adv Clin Exp Med* preprint policy" clearly show that there is no way back and our stipulations regarding preprints may

become more detailed, encompassing more aspects of this issue, but we would contradict our dedication to advancing medical knowledge and promoting OS if we decided to stop considering preprinted papers for publication.

Therefore, we believe the most justified line of action in light of the knowledge amassed to date is educating authors – specifically, regarding 3 aspects of preprints. First, only a tiny fraction of papers submitted to *Adv Clin Exp Med* are released as preprints; the authors should be more aware of the advantages of preprinting for them and the scientific world. Second, some authors still seem to be unsure and wary whether disseminating the paper this way before publication could cause rejection by a journal; they should know that in most periodicals, it is not a problem nowadays, but at the same time, they should be aware that a preprint is not equal to a peer-reviewed publication, which can be crucial in the context of, e.g., scientific output assessment or grant application. Third, several authors neglected to amend their preprinted manuscripts with information about the published version of the record; however, we have no means to enforce our policy following publication. The authors should be educated on why it is essential from both ethical points of view and for practical reasons because preprint citations are not monitored. If 2 or more versions of a given paper (preprint(s) and published version of record) are circulating simultaneously, some researchers can erroneously cite the preprint because they are unaware that a published version exists, and the authors do not benefit as much as when their published paper is cited. Authors who neglect to link the preprint to the version of the record are presumable oblivious to this danger.

Future directions for preprint policies and their potential evolution in the context of OS

During the last 15 years, the preprints have been rapidly evolving, concerning both their role in the scientific landscape and the growing number of services dedicated to their dissemination. It is not probable that preprints will be treated on par with papers – peer review remains the most reliable gatekeeping mechanism. However, based on the information and hypotheses presented in the cited literature on the subject, the following tendencies in this evolution can be pointed out.

The most important area of preprint development is the issue most often raised in their context – the fact that they are unrefereed manuscripts. Some initiatives have already been launched to provide at least some of the preprints with reviewing – at least those whose authors aim at ultimately publishing them in peer-reviewed journals. This issue was exhaustively discussed on a theoretical level in an important preprint by Avissar-Whiting et al.[137] They offered a definition of preprint review and outlined what sets it apart from other types of more informal feedback on preprints, presented the benefits of preprint review, and called to action for stakeholders in the scholarly communication ecosystem to further promote preprint review.

Such action can take 3 direction. One is launching peer-review services independent of specific journals or publishers. Review Commons is an initiative recently launched by European Molecular Biology Organization (EMBO) and ASAPbio. It functions as a journal-independent peer review service to which authors submit preprints and obtain peer reviews that they can then present to journals to consider.[138–140] Another idea was recently implemented by the journal *eLife*, which since 2021 exclusively reviews papers already published as preprints.[140] The 3rd direction are overlay journals – OA academic journals that does not produce their own content, but select from texts that are already freely available online and contact the authors. Many overlay journals derive their content from preprint servers; therefore, in these periodicals, preprinting is also a prerequisite for submission.[141,142]

These initiatives show that the future of preprints is in closer ties with peer-reviewed journals – integrating preprinting in journal submission workflows and general closer cooperation between journals and preprint servers – rather than in becoming and alternative mode for dissemination. If such mechanisms become norm, it can also result in more preprints reaching the publication stage. Given the rising popularity of preprints, it also possible that in the coming years, last journals who regard preprints as previous publication would abandon this interpretation of the Ingelfinger Rule and at least concede to reviewing such cases on an individual basis. A survey conducted by Fraser et al.[143] among authors of preprints deposited on bioRxiv showed that the core of the problem is shifting from journal policies to authors' perceptions – disproportionately many authors as recent as 2022 were convinced that still many scientific journals (much more than in reality) consider releasing a preprint a breach of the above rule; however, Fraser et al. pointed out that it may be due to still unclear policies of many journals.[143] This means that endorsement of preprints by editors must go hand in hand with policies' clarification.

The COVID-19 pandemic clearly showed that more thorough screening of manuscripts deposited on preprint servers in both possible (at least to some extent) and important. While peer-reviewing of all preprinted materials would diminish their primary advantage over peer-reviewed papers – the speed of dissemination – the leading preprint services seem to tighten both the requirements and the verification mechanisms, especially regarding scientific content, dubious claims, conflict of interest, and data sharing. This should include also mandatory cross-linking of preprints to their published peer-reviewed versions, stipulated by both preprint servers and journal editors, which should curb the issue of citation dilution. Licensing in regard to preprints should also become more uniform since now it is not always clear from authors' perspective

whether the terms offered by preprint servers will not cause problems later, when the paper is published.

An evolution of preprints policies among funders and not only journals can be observed. Some grant funders, such as the Bill and Melinda Gates Foundation,[144] NIH[31,32] and the European Research Council (ERC), oblige beneficiaries to make their papers available under green OA status, in which self-archiving by authors (inckuding preprinting) is permitted. If all research funders required their grantees to post their manuscripts first on preprint servers, the widespread desire to provide immediate free access to the world's scientific output would be achieved with minimal effort and expense.[55]

During the COVID-19 pandemic, a marked change was observed in the use of preprints in policy documents; it is another proof that the status of preprints is improving in the view of organizational (public and private alike) and state bodies. It is not clear yet whether this tendency was specific only to the global health emergency situation or will persist – the latter is more probable in light of funders' moves towards OS. More accepting attitude to citing preprints in medical publications will be probably also visible in the near future.

Some members of the scientific community – like Ni and Waltman[14] – emphasize that research institutions and research funders should recognize and reward researchers for preprinting their work because in doing it they promote OS. This is especially important for early-career researchers. It can also be assumed that preprints will be more welcome in resumes and evaluation sheets of researchers, albeit not as a sole or primary mode of disseminating one's achievements. For example, the University of California Davis (UC Davis) and the New York University Grossman School of Medicine both allow preprints to be included as evidence for promotion. While this is encouraging, we suspect that such policies are rare, leaving it unclear for job applicants and faculty whether to list preprints on their curricula vitae.[145]

Policies of databases are also changing. Preprints are indexed in many of them, but searching, retrieval and accessibility of preprints is another issue, since currently there are no cross-platform searchable aggregators specific for preprints and the end-user is relied upon external content indexing by Google Scholar, SHARE, Microsoft Academic, Unpaywall, Crossref, Europe PMC, PubMed, Prepubmed, or SciLit search engines.[10] In the near future, preprints may become more integrated into the searching and aggregating tools available for researchers, which would in turn boost their citability.

In the long term, double-blind review may be impossible to maintain if authors have posted their article in a preprint server,[63] since the reviewers will be able to learn the authors' names by simply checking the title of the manuscript or a passage from it using an ordinary web browser. It may lead to abandonment of double-blind peer review in favor of open peer-review, like in the model adopted since 2021 by the journal *eLife*.[140] The scale of this process would depend on the ratio of manuscripts submitted to journals being released as preprint before submission or during the peer-review process.

Suggestions for future research on preprints

Although the literature on preprints is abundant, several issues were only slightly touched or not referred to at all in scientific papers. Therefore, we want to offer our suggestions for future research concerning this topic.

The most important issue in the context of scientific publishing seems to be the long-term impact of preprints on citation rates. This area of research forks into 3 subissues: 1) What impact releasing preprints has on the citation rates of the same papers after they are published in a peer-reviewed journal? 2) Is citation dilution between preprint and peer-reviewed version of the same articles a real or only a perceived problem? 3) Are preprints themselves frequently cited? Attempts were made to answer the 1st question – this issue has been discussed, among others, by Fu and Hughey,[57] Xu et al.,[58] Fraser et al.,[59] Serghiou and Ioannidis,[60] and Xie et al.,[61] but this area of research is still in its infancy and no definite conclusions have been reached. Añazco et al.[146] showed that COVID-19-related preprints which were eventually published had a higher citation count as preprints when compared to unpublished preprints, and that published preprints had a significantly higher citation count after publication in a scholarly journal compared to as a preprint; however, their study had a limited scope and is a proof that broader studies would probably yield more reliable results.

Another key and yet only cursorily investigated problem is the perception of preprints among the general public. In our opinion, the most important specific question in this regard can be formulated as follows: Is the general public able to discern between preprinted and peer-reviewed papers? Experiences from the COVID-19 pandemic have shown that laypeople, lay media and politicians alike are in some cases unable to distinguish between a scientific publication, a preprint and a press release presenting some research results. This not a new problem: Penfold and Polka[147] described a case of bioRxiv preprint linking cell phone radiation to cancer in rats, which in 2016 was reported on without mentioning its preprint status; however, so far this issue has not been systematically analyzed. The results of such investigations would (and should) have considerable influence on appropriate marking of preprints as such on all most popular preprint servers (as for now, the policies are not uniform). Moreover, it would be an unprecedented opportunity to bridge the scientific and media communities to create a consensus on the reporting of preprints in order to at least partially avoid mistakes made during the pandemic in the future.[15] However, it should be borne in mind that laypeople (including many

journalists) may be simply unaware what does it mean that a manuscript has not been peer-reviewed since knowledge about tenets of scientific publishing is not widespread even among educated people (it is not part of the high school or even university curriculum in many countries if a given person does not attempt to launch a career in the scholarly milieu). What is obvious to researchers is certainly not self-evident among the general public. Therefore, educating broader audience should also take into account the abovementioned basic rules of disseminating knowledge in the scientific community.

A related issue is the true scope of reaction to preprints both on preprint severs and in social and lay media, also outside the context of the pandemic. An often raised (and discussed in this paper) advantage of the preprints is that they are to foster professional debate about their contents even before a paper is published (or even if it is not published); however, there are only single studies showing whether such debate actually occurs and how many people participate in it – both on preprint servers and in social media (X (formerly Twitter), Facebook, etc.), where the authors post links to the released but unrefereed manuscripts. For example, Rzayeva et al.[148] conducted a survey among authors of pandemic-related preprints deposited on arXiv, bioRxiv, medRxiv, and ChemRxiv in 2020, of whom 53% reported that they had received feedback on their preprints. However, only a quarter of the feedback received by respondents consisted of detailed comments. Respondents also reported that, compared to preprint feedback, journal peer review was more likely to lead to major changes to their work, suggesting that journal peer review provides significant added value compared to feedback received on preprints. This suggests that the possibility of valuable debate about a given preprint may be overrated so far; this matter must be investigated further.

Another issue requiring thorough investigation is at least an estimation of the real (not only perceived) possibility of scooping. Approximately 2% of respondents in bioRxiv's survey reported having suffered a loss in ability to claim priority or to publish in the journal of their choice as a result of having published a preprint.[147] It does not mean that these researchers were directly scooped (journal policies rejecting preprint could also be at play), but such voices show that such possibility is not only an unfounded, unjustified fear. If such act of scientific misconduct can actually happen, concerns voiced by authors in surveys discussed in this editorial are warranted and should be widely known – in order to let authors considering a preprint release make an informed decision.

Papers presenting surveys mentioned above focused on the perspective of researchers as authors – but not as peer reviewers. It would be very informative to have some insight how scientists serving as peer reviewers perceive the value of preprints and whether they are willing to engage in reviewing also preprints if such mechanism was to be wider established.

Sustainability of preprint services and servers is also an important but only scarcely discussed issue. While the preprint services most frequently chosen by the authors are owned and managed by respectable institutions, their model of financing and sustaining their functioning is not as stable as, e.g., dark archives like CLOCKSS or large databases like Scopus, Web of Science Core Collection, PubMed, PMC, Medline, or Ovid. A broad study describing, comparing and assessing the stability of these models would be paramount to the whole scientific community and could stimulate discussion about creating more sustainable mechanisms for preprint dissemination, especially if they are viewed as a form of OS.

Related to this field of research is the impact of preprints on changing hierarchies in global science, specifically concerning the country of origin of both authors and manuscripts. The question here is: Do preprint infrastructures and social mechanisms develop with issues of diversity, equity and de-colonialization of science taken into account?[147] Fry and MacGarvie[149] showed that COVID-19-related preprints from China received significantly less attention than those covering the same topic and originating from the USA, so certainly this an issue worth attention. It is also an open problem why researchers outside Europe and USA are more cautious about preprints – this issue was clearly observed by Ni and Waltman,[14] who offered various hypotheses concerning the causes of this disparity; however, no clear explanation was yet offered. Biesenbender et al.[150] conducted a survey about experiences and attitudes towards posting and using preprints in the Global South as opposed to the Global North. It could be observed that a greater percentage of participants from the Global South expressed agreement with the proposed benefits of posting preprints than those from the Global North. This shows that there is an unexplored potential among researchers from developing countries regarding participation in OS, also in the form of preprints. Barriers blocking these scientists from broader participation in the preprint initiatives must be clearly identified and – if possible – overcome.

Last but not least, policies regarding preprint citation employed by different publishers and journals warrant a comparison in one source. The American Medical Association (AMA) reference standard required by *Adv Clin Exp Med* includes a specific preprint notation, but other reference models may allow for distinguishing preprints and papers accepted but not yet published (so-called in press articles) not clearly enough. Since we as editors sometimes observe authors themselves assuming that a paper still under review in a journal can be cited as paper "in press", it is crucial to both show the authors how to cite preprints properly and to make them aware that a paper not accepted yet can be released as a preprint and cited as such while a paper already accepted but not yet published can be cited either as a preprint or as an in press article (having in mind that only the former mode allows

the reader for checking its contents immediately, without waiting for publication).

More interesting open questions regarding the future of preprints were proposed in a preprint manuscript by Puebla et al.[151]

The editorial staff of *Adv Clin Exp Med* remains vigilant regarding the evolving role of preprints in scientific publishing – our policy must never lag behind the development of OS.

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References

1. Relman AS. The Ingelfinger Rule. *N Engl J Med*. 1981;305(14):824–826. doi:10.1056/NEJM198110013051408
2. Angell M, Kassirer JP. The Ingelfinger Rule revisited. *N Engl J Med*. 1991;325(19):1371–1373. doi:10.1056/NEJM199111073251910
3. Kang H, Oh HC. Current concerns on journal article with preprint: *Anesthesia and Pain Medicine* perspectives. *Anesth Pain Med*. 2023;18(2):97–103. doi:10.17085/apm.23036
4. Cobb M. The prehistory of biology preprints: A forgotten experiment from the 1960s. *PLoS Biol*. 2017;15(11):e2003995. doi:10.1371/journal.pbio.2003995
5. Committe on Publication Ethics (COPE). *COPE Discussion Document: Preprints*. Eastleigh, UK: Committe on Publication Ethics (COPE); 2018. doi:10.24318/R4WByao2
6. Kaiser J. The preprint dilemma. *Science*. 2017;357(6358):1344–1349. doi:10.1126/science.357.6358.1344
7. Berg JM, Bhalla N, Bourne PE, et al. Preprints for the life sciences. *Science*. 2016;352(6288):899–901. doi:10.1126/science.aaf9133
8. Chiarelli A, Johnson R, Pinfield S, Richens E. Preprints and scholarly communication: An exploratory qualitative study of adoption, practices, drivers and barriers. *F1000Res*. 2019;8:971. doi:10.12688/f1000research.19619.2
9. Blatch-Jones AJ, Recio Saucedo A, Giddins B. The use and acceptability of preprints in health and social care settings: A scoping review. *PLoS One*. 2023;18(9):e0291627. doi:10.1371/journal.pone.0291627
10. Chaleplioglou A, Koulouris A. Preprint paper platforms in the academic scholarly communication environment. *Journal of Librarianship and Information Science*. 2023;55(1):43–56. doi:10.1177/09610006211058908
11. Alfonso F, Crea F. Preprints: A game changer in scientific publications? [published correction appeared in: *Eur Heart J*. 2023;44(10):870. doi:10.1093/eurheartj/ehad024]. *Eur Heart J*. 2023;44(3):171–173. doi:10.1093/eurheartj/ehac665
12. medRxiv. Frequently asked questions (FAQ). Laurel Hollow, USA: Cold Spring Harbor Laboratory; 2024. https://www.medrxiv.org/about/FAQ#unrefereed. Accessed August 21, 2024.
13. Elmore SA. Preprints: What role do these have in communicating scientific results? *Toxicol Pathol*. 2018;46(4):364–365. doi:10.1177/0192623318767322
14. Ni R, Waltman L. To preprint or not to preprint: A global researcher survey. *J Assoc Inf Sci Technol*. 2024;75(6):749–766. doi:10.1002/asi.24880
15. Fraser N, Brierley L, Dey G, et al. The evolving role of preprints in the dissemination of COVID-19 research and their impact on the science communication landscape. *PLoS Biol*. 2021;19(4):e3000959. doi:10.1371/journal.pbio.3000959
16. Wikipedia. List of preprint repositories. In: *Wikipedia*. San Francisco, USA: Wikimedia Foundation; 2024. https://en.wikipedia.org/wiki/List_of_preprint_repositories. Accessed August 21, 2024.
17. Brock J. 10 tips for submitting a successful preprint. *Nature Index*. https://www.nature.com/nature-index/news/tips-how-to-most-successful-preprint-research-science-submission-study. Published May 26, 2020. Accessed August 21, 2024.
18. Bourne PE, Polka JK, Vale RD, Kiley R. Ten simple rules to consider regarding preprint submission. *PLoS Comput Biol*. 2017;13(5):e1005473. doi:10.1371/journal.pcbi.1005473
19. Ettinger CL, Sadanandappa MK, Görgülü K, Coghlan KL, Hallenbeck KK, Puebla I. A guide to preprinting for early-career researchers. *Biol Open*. 2022;11(7):bio059310. doi:10.1242/bio.059310
20. Sarabipour S, Debat HJ, Emmott E, Burgess SJ, Schwessinger B, Hensel Z. On the value of preprints: An early career researcher perspective. *PLoS Biol*. 2019;17(2):e3000151. doi:10.1371/journal.pbio.3000151
21. Columbia University Irving Medical Center. What is a preprint? Is it the right publishing choice for you? New York, USA: Columbia University; 2021. https://library.cumc.columbia.edu/kb/what_is_preprint. Accessed August 21, 2024.
22. University of Oxford. Preprints. Oxford, UK: University of Oxford; 2024. https://openaccess.ox.ac.uk/preprints. Accessed August 21, 2024.
23. UK Research and Innovation. Preprints. Swindon, UK: UK Research and Innovation; 2024. https://www.ukri.org/who-we-are/mrc/our-policies-and-standards/research/preprints/. Accessed August 21, 2024.
24. National Institutes of Health (NIH). Reporting preprints and other interim research products. Bethesda, USA: National Institutes of Health (NIH); 2017. https://grants.nih.gov/grants/guide/notice-files/NOT-OD-17-050.html. Accessed August 21, 2024.
25. University of Surrey. Preprints. Guildford, UK: University of Surrey; 2024. https://www.surrey.ac.uk/library/open-research/preprints. Accessed August 21, 2024.
26. University of Melbourne. Preprints. Melbourne, Australia: University of Melbourne; 2024. https://unimelb.libguides.com/openresearch/preprints. Accessed August 21, 2024.
27. Eastern Michigan University. Preprints. Ypsilanti, USA: Eastern Michigan University; 2023. https://guides.emich.edu/preprints. Accessed August 21, 2024.
28. Harvard Countway Library. Open access publishing. Boston, USA: Harvard University; 2024. https://guides.library.harvard.edu/c.php?g=1097539&p=8004417. Accessed August 21, 2024.
29. University of Hong Kong Libraries. Preprint. Hong Kong, China: University of Hong Kong; 2023. https://libguides.lib.hku.hk/preprint. Accessed August 21, 2024.
30. Hettne K, Aardening R, Van Gorp D, et al. A practical guide to preprints: Accelerating scholarly communication. Preprint posted online October 10, 2021. *Zenodo*. doi:10.5281/ZENODO.5600535
31. National Institutes of Health (NIH). NIH Preprint Pilot. Bethesda, USA: National Institutes of Health (NIH); 2024. https://www.ncbi.nlm.nih.gov/pmc/about/nihpreprints/. Accessed August 21, 2024.
32. National Institutes of Health (NIH). NIH Preprint Pilot accelerates and expands discovery of research results. Bethesda, USA: National Institutes of Health (NIH); 2022. https://www.nlm.nih.gov/news/NIH_Preprint_Pilot_Accelerates_Expands_Discovery_Research_Results.html. Accessed August 21, 2024.
33. Research Square. Editorial policies. London UK: Springer Nature; 2024. https://www.researchsquare.com/legal/editorial. Accessed August 21, 2024.
34. medRxiv. Screening process. Laurel Hollow, USA: Cold Spring Harbor Laboratory; 2024. https://connect.medrxiv.org/news/2022/06/13/screening_procedures. Accessed August 21, 2024.
35. medRxiv. Submission guide. Laurel Hollow, USA: Cold Spring Harbor Laboratory; 2024. https://www.medrxiv.org/submit-a-manuscript. Accessed August 21, 2024.
36. Open Science Framework (OSF). Preprint: FAQs. Charlottesville, USA: Center for Open Science; 2023. https://help.osf.io/article/230-preprint-faqs. Accessed August 21, 2024.
37. Open Science Framework (OSF). Withdrawing a preprint. Charlottesville, USA: Center for Open Science; 2024. https://help.osf.io/article/186-withdrawing-a-preprint. Accessed August 21, 2024.
38. Kirkham JJ, Penfold NC, Murphy F, et al. Systematic examination of preprint platforms for use in the medical and biomedical sciences setting. *BMJ Open*. 2020;10(12):e041849. doi:10.1136/bmjopen-2020-041849
39. Malički M, Jeroncic A, Ter Riet G, et al. Preprint servers' policies, submission requirements, and transparency in reporting and research integrity recommendations. *JAMA*. 2020;324(18):1901. doi:10.1001/jama.2020.17195
40. Brainard J. No revolution: COVID-19 boosted open access, but preprints are only a fraction of pandemic papers. *Science*. 2021;373(6560):9058. doi:10.1126/science.acx9058

41. Janda G, Khetpal V, Shi X, Ross JS, Wallach JD. Comparison of clinical study results reported in medRxiv preprints vs peer-reviewed journal articles. *JAMA Netw Open*. 2022;5(12):e2245847. doi:10.1001/jamanetworkopen.2022.45847
42. Huisman J, Smits J. Duration and quality of the peer review process: The author's perspective. *Scientometrics*. 2017;113(1):633–650. doi:10.1007/s11192-017-2310-5
43. Andersen MZ, Fonnes S, Rosenberg J. Time from submission to publication varied widely for biomedical journals: A systematic review. *Curr Med Res Opin*. 2021;37(6):985–993. doi:10.1080/03007995.2021.1905622
44. Zimmer RL, Mancuso ACB, Matte U, Ashton-Prolla P. Analysis of the interval between submission and publication in genetics journals. *PLoS One*. 2023;18(5):e0284866. doi:10.1371/journal.pone.0284866
45. Harlianto NI, Harlianto ZN. Time from submission to publication in urology journals: A look at publication times before and during Covid-19. *Heliyon*. 2023;9(3):e14233. doi:10.1016/j.heliyon.2023.e14233
46. Sebo P, Fournier JP, Ragot C, Gorioux PH, Herrmann FR, Maisonneuve H. Factors associated with publication speed in general medical journals: A retrospective study of bibliometric data. *Scientometrics*. 2019;119(2):1037–1058. doi:10.1007/s11192-019-03061-8
47. Lee Y, Kim K, Lee Y. Publication delay of Korean medical journals. *J Korean Med Sci*. 2017;32(8):1235. doi:10.3346/jkms.2017.32.8.1235
48. Johansson MA, Reich NG, Meyers LA, Lipsitch M. Preprints: An underutilized mechanism to accelerate outbreak science. *PLoS Med*. 2018;15(4):e1002549. doi:10.1371/journal.pmed.1002549
49. Bero L, Lawrence R, Leslie L, et al. Cross-sectional study of preprints and final journal publications from COVID-19 studies: Discrepancies in results reporting and spin in interpretation. *BMJ Open*. 2021;11(7):e051821. doi:10.1136/bmjopen-2021-051821
50. Brierley L, Nanni F, Polka JK, et al. Tracking changes between preprint posting and journal publication during a pandemic. *PLoS Biol*. 2022;20(2):e3001285. doi:10.1371/journal.pbio.3001285
51. Carneiro CFD, Queiroz VGS, Moulin TC, et al. Comparing quality of reporting between preprints and peer-reviewed articles in the biomedical literature. *Res Integr Peer Rev*. 2020;5(1):16. doi:10.1186/s41073-020-00101-3
52. Nelson L, Ye H, Schwenn A, Lee S, Arabi S, Hutchins BI. Robustness of evidence reported in preprints during peer review. *Lancet Glob Health*. 2022;10(11):e1684–e1687. doi:10.1016/S2214-109X(22)00368-0
53. Akbaritabar A, Stephen D, Squazzoni F. A study of referencing changes in preprint-publication pairs across multiple fields. *J Infometrics*. 2022;16(2):101258. doi:10.1016/j.joi.2022.101258
54. Poremski D, Falissard B, Fegert J, et al. Moving from 'personal communication' to 'available online at': Preprint servers enhance the timeliness of scientific exchange. *Child Adolesc Psychiatry Ment Health*. 2019;13(1):42. doi:10.1186/s13034-019-0301-4
55. Sever R, Eisen M, Inglis J. Plan U: Universal access to scientific and medical research via funder preprint mandates. *PLoS Biol*. 2019;17(6):e3000273. doi:10.1371/journal.pbio.3000273
56. Clemens A. 5 disadvantages of preprints. Prague, Czech Republic: Researchers' Writing Academy; 2022. https://annaclemens.com/blog/downsides-publishing-preprint/. Accessed August 21, 2024.
57. Fu DY, Hughey JJ. Releasing a preprint is associated with more attention and citations for the peer-reviewed article. *eLife*. 2019;8:e52646. doi:10.7554/eLife.52646
58. Xu F, Ou G, Ma T, Wang X. The consistency of impact of preprints and their journal publications. *J Infometrics*. 2021;15(2):101153. doi:10.1016/j.joi.2021.101153
59. Fraser N, Momeni F, Mayr P, Peters I. The relationship between bioRxiv preprints, citations and altmetrics. *Quantitative Science Studies*. 2020;1(2):618–638. doi:10.1162/qss_a_00043
60. Serghiou S, Ioannidis JPA. Altmetric scores, citations, and publication of studies posted as preprints. *JAMA*. 2018;319(4):402. doi:10.1001/jama.2017.21168
61. Xie B, Shen Z, Wang K. Is preprint the future of science? A thirty year journey of online preprint services. Preprint posted online February 17, 2021. *arXiv*. doi:10.48550/ARXIV.2102.09066
62. Bagdasarian N, Cross GB, Fisher D. Rapid publications risk the integrity of science in the era of COVID-19. *BMC Med*. 2020;18(1):192. doi:10.1186/s12916-020-01650-6
63. Smart P. The evolution, benefits, and challenges of preprints and their interaction with journals. *Sci Ed*. 2022;9(1):79–84. doi:10.6087/kcse.269
64. Strcic J, Civljak A, Glozinic T, Pacheco RL, Brkovic T, Puljak L. Open data and data sharing in articles about COVID-19 published in preprint servers medRxiv and bioRxiv. *Scientometrics*. 2022;127(5):2791–2802. doi:10.1007/s11192-022-04346-1
65. McGuinness LA, Sheppard AL. A descriptive analysis of the data availability statements accompanying medRxiv preprints and a comparison with their published counterparts. *PLoS One*. 2021;16(5):e0250887. doi:10.1371/journal.pone.0250887
66. ASAPbio. Scooping FAQ. San Francisco, USA: ASAPbio; 2020. https://asapbio.org/preprint-info/preprint-faq#qaef-923. Accessed August 21, 2024.
67. Moustafa K. Postprints-to-preprints linkage to enhance access to scientific literature. *Account Res*. 2023;30(7):542–546. doi:10.1080/08989621.2021.2019024
68. International Committee of Medical Journal Editors (ICMJE). International Committee of Medical Journal Editors Recommendations. Vancouver, Canada: International Committee of Medical Journal Editors (ICMJE); 2024. https://www.icmje.org/recommendations/. Accessed August 21, 2024.
69. ASAPbio. Funder policies. San Francisco, USA: ASAPbio; 2021. https://asapbio.org/funder-policies. Accessed August 21, 2024.
70. Moore A. Bad science in the headlines: Who takes responsibility when science is distorted in the mass media? *EMBO Rep*. 2006;7(12):1193–1196. doi:10.1038/sj.embor.7400862
71. Flanagin A, Fontanarosa PB, Bauchner H. Preprints involving medical research: Do the benefits outweigh the challenges? *JAMA*. 2020;324(18):1840. doi:10.1001/jama.2020.20674
72. Vazquez-Rodriguez S, Arriaga-Pizano LA, Mancilla-Herrera I, et al. Fc-gamma receptor expression and cytokine response to intravenous human immunoglobulin in mothers and neonates. Preprint posted online September 30, 2021. *medRxiv*. doi:10.1101/2021.09.28.21264275
73. Peiperl L; on behalf of the PLoS Medicine Editors. Preprints in medical research: Progress and principles. *PLoS Med*. 2018;15(4):e1002563. doi:10.1371/journal.pmed.1002563
74. Ravinetto R, Caillet C, Zaman MH, et al. Preprints in times of COVID19: The time is ripe for agreeing on terminology and good practices. *BMC Med Ethics*. 2021;22(1):106. doi:10.1186/s12910-021-00667-7
75. Mullins M. Opinion: The problem with preprints. *The Scientist*. https://www.the-scientist.com/opinion-the-problem-with-preprints-69309. Published November 1, 2021. Accessed August 15, 2024.
76. Retraction Watch. Retraction Watch Database User Guide. New York, USA: Center for Scientific Integrity (CFS); 2024. https://retractionwatch.com/retraction-watch-database-user-guide/. Accessed September 3, 2024.
77. Pradhan P, Pandey AK, Mishra A, et al. RETRACTED: Uncanny similarity of unique inserts in the 2019-nCoV spike protein to HIV-1 gp120 and Gag. Preprint posted online February 2, 2020. *bioRxiv*. doi:10.1101/2020.01.30.927871
78. Zhang C, Zheng W, Huang X, Bell EW, Zhou X, Zhang Y. Protein structure and sequence re-analysis of 2019-nCoV genome does not indicate snakes as its intermediate host or the unique similarity between its spike protein insertions and HIV-1. Preprint posted online February 8, 2020. *bioRxiv*. doi:10.1101/2020.02.04.933135
79. Zhang C, Zheng W, Huang X, Bell EW, Zhou X, Zhang Y. Protein structure and sequence reanalysis of 2019-nCoV genome refutes snakes as its intermediate host and the unique similarity between its spike protein insertions and HIV-1. *J Proteome Res*. 2020;19(4):1351–1360. doi:10.1021/acs.jproteome.0c00129
80. Xiao C, Li X, Liu S, Sang Y, Gao SJ, Gao F. HIV-1 did not contribute to the 2019-nCoV genome. *Emerg Microbes Infect*. 2020;9(1):378–381. doi:10.1080/22221751.2020.1727299
81. Guan WJ, Ni ZY, Hu Y, et al. Clinical characteristics of 2019 novel coronavirus infection in China. Preprint posted online February 9, 2020. *medRxiv*. doi:10.1101/2020.02.06.20020974
82. Guan WJ, Ni ZY, Hu Y, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. 2020;382(18):1708–1720. doi:10.1056/NEJMoa2002032

83. Guterman EL, Braunstein LZ. Preprints during the COVID-19 pandemic: Public health emergencies and medical literature. *J Hosp Med*. 2020;15(10):634–636. doi:10.12788/jhm.3491
84. Soderberg CK, Errington TM, Nosek BA. Credibility of preprints: An interdisciplinary survey of researchers. *R Soc Open Sci*. 2020; 7(10):201520. doi:10.1098/rsos.201520
85. Biesenbender K, Toepfer R, Peters I. Life scientists' experience with posting preprints during the COVID-19 pandemic [published online as ahead of print on April 5, 2024]. *Scientometrics*. 2024. doi:10.1007/s11192-024-04982-9
86. Ng JY, Chow V, Santoro LJ, et al. An international, cross-sectional survey of preprint attitudes among biomedical researchers. *F1000Res*. 2024;13:6. doi:10.12688/f1000research.143013.1
87. Klebel T, Reichmann S, Polka J, et al. Peer review and preprint policies are unclear at most major journals. *PLoS One*. 2020;15(10):e0239518. doi:10.1371/journal.pone.0239518
88. Massey DS, Opare MA, Wallach JD, Ross JS, Krumholz HM. Assessment of preprint policies of top-ranked clinical journals. *JAMA Netw Open*. 2020;3(7):e2011127. doi:10.1001/jamanetworkopen.2020.11127
89. Vlasschaert C, Giles C, Hiremath S, Lanktree MB. Preprint servers in kidney disease research: A rapid review. *Clin J Am Soc Nephrol*. 2021;16(3):479–486. doi:10.2215/CJN.03800320
90. Wikipedia. List of academic publishers by preprint policy. In: *Wikipedia*. San Francisco, USA: Wikimedia Foundation; 2024. https://en.wikipedia.org/wiki/List_of_academic_publishers_by_preprint_policy. Accessed August 21, 2024.
91. Taylor & Francis. What are preprints and preprint servers? Abingdon-on-Thames, UK: Taylor & Francis; 2023. https://authorservices.taylorandfrancis.com/publishing-your-research/making-your-submission/posting-to-preprint-server/. Accessed August 21, 2024.
92. Cambridge University Press. Preprint policy. Cambridge, UK: Cambridge University Press; 2024. https://www.cambridge.org/core/services/open-access-policies/open-access-journals/preprint-policy#. Accessed August 21, 2024.
93. Journal of Clinical Medicine Research. Preprints policy. Richmond Hill, Canada: Journal of Clinical Medicine Research; 2023. https://www.jocmr.org/index.php/JOCMR/about/editorialPolicies#custom-16. Accessed August 21, 2024.
94. Emerging Infectious Diseases. Policy on preprint publication. Atlanta, USA: Centers for Disease Control and Prevention (CDC); 2024. https://wwwnc.cdc.gov/eid/page/preprint-policy. Accessed August 21, 2024.
95. BioMed Central. Preprint sharing and citation. London, UK: BioMed Central; 2023. https://www.biomedcentral.com/getpublished/editorial-policies#preprint+sharing+and+citation. Accessed August 21, 2024.
96. Wiley. Wiley's preprint policy. Hoboken, USA: Wiley; 2024. https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/preprints-policy.html. Accessed August 21, 2024.
97. Elsevier. Article sharing. Amsterdam, the Netherlands: Elsevier; 2023. https://www.elsevier.com/about/policies-and-standards/sharing. Accessed August 21, 2024.
98. American Society of Clinical Oncology (ASCO) Publications. Preprint policy. Alexandria, USA: American Society of Clinical Oncology (ASCO); 2023. https://ascopubs.org/authors/journal-policies#preprint-policy. Accessed August 21, 2024.
99. Springer Nature. Preprint sharing. London UK: Springer Nature; 2023. https://www.springer.com/gp/open-access/preprint-sharing/16718886. Accessed August 21, 2024.
100. American Heart Association (AHA). Prior publication policy. Dallas, USA: American Heart Association (AHA); 2023. https://www.ahajournals.org/prior-publication-policy. Accessed August 21, 2024.
101. American Association for Cancer Research (AACR). Editorial policies. Philadelphia, USA: American Association for Cancer Research (AACR); 2024. https://aacrjournals.org/pages/editorial-policies. Accessed August 21, 2024.
102. Science. Prior publication and presentation at meetings. Washington D.C., USA: American Association for the Advancement of Science (AAAS); 2024. https://www.science.org/content/page/science-journals-editorial-policies#prior-publication. Accessed August 21, 2024.
103. BMJ Author Hub. Preprints. London, UK: BMJ Group; 2024. https://authors.bmj.com/policies/preprints/. Accessed August 21, 2024.
104. Nature. Preprints & conference proceedings. London, UK: Springer Nature; 2024. https://www.nature.com/nature-portfolio/editorial-policies/preprints-and-conference-proceedings. Accessed August 21, 2024.
105. PLoS One. Preprints. San Francisco, USA: Public Library of Science (PLoS); 2024. https://journals.plos.org/plosone/s/preprints. Accessed August 21, 2024.
106. Sage Publishing. Prior publication. Thousand Oaks, USA: Sage Publishing; 2024. https://us.sagepub.com/en-us/nam/prior-publication. Accessed August 21, 2024.
107. Neylon C, Pattinson D, Bilder G, Lin J. On the origin of nonequivalent states: How we can talk about preprints. *F1000Res*. 2017;6:608. doi:10.12688/f1000research.11408.1
108. Wikipedia. Postprint. In: *Wikipedia*. San Francisco, USA: Wikimedia Foundation; 2024. https://en.wikipedia.org/wiki/Postprint. Accessed August 21, 2024.
109. Advances in Clinical and Experimental Medicine. Instructions for authors. Wrocław, Poland: Wroclaw Medical University Press; 2024. https://advances.umw.edu.pl/en/instructions-for-authors/. Accessed August 21, 2024.
110. Brierley L. Lessons from the influx of preprints during the early COVID-19 pandemic. *Lancet Planet Health*. 2021;5(3):e115–e117. doi:10.1016/S2542-5196(21)00011-5
111. Watson C. Rise of the preprint: How rapid data sharing during COVID-19 has changed science forever. *Nat Med*. 2022;28(1):2–5. doi:10.1038/s41591-021-01654-6
112. Besançon L, Peiffer-Smadja N, Segalas C, et al. Open science saves lives: Lessons from the COVID-19 pandemic. *BMC Med Res Methodol*. 2021;21(1):117. doi:10.1186/s12874-021-01304-y
113. Singh JA, Ravinetto R. COVID-19 therapeutics: How to sow confusion and break public trust during international public health emergencies. *J Pharm Policy Pract*. 2020;13(1):47. doi:10.1186/s40545-020-00244-0
114. Kodvanj I, Homolak J, Virag D, Trkulja V. Publishing of COVID-19 preprints in peer-reviewed journals, preprinting trends, public discussion and quality issues. *Scientometrics*. 2022;127(3):1339–1352. doi:10.1007/s11192-021-04249-7
115. Majumder MS, Mandl KD. Early in the epidemic: Impact of preprints on global discourse about COVID-19 transmissibility. *Lancet Glob Health*. 2020;8(5):e627–e630. doi:10.1016/S2214-109X(20)30113-3
116. Vlasschaert C, Topf JM, Hiremath S. Proliferation of papers and preprints during the coronavirus disease 2019 pandemic: Progress or problems with peer review? *Adv Chronic Kidney Dis*. 2020; 27(5):418–426. doi:10.1053/j.ackd.2020.08.003
117. Wang Y, Cao Z, Zeng DD, Zhang Q, Luo T. The collective wisdom in the COVID-19 research: Comparison and synthesis of epidemiological parameter estimates in preprints and peer-reviewed articles. *Int J Infect Dis*. 2021;104:1–6. doi:10.1016/j.ijid.2020.12.040
118. Gianola S, Jesus TS, Bargeri S, Castellini G. Characteristics of academic publications, preprints, and registered clinical trials on the COVID-19 pandemic. *PLoS One*. 2020;15(10):e0240123. doi:10.1371/journal.pone.0240123
119. Li R, Yuan H, Zhao T, et al. miR-874 ameliorates retinopathy in diabetic rats by NF-κB signaling pathway. *Adv Clin Exp Med*. 2021;30(4): 421–430. doi:10.17219/acem/130602
120. Liu W, Che J, Gu Y, Song L, Jiao Y, Yu S. Silencing of lncRNA SNHG12 inhibits proliferation and migration of vascular smooth muscle cells via targeting miR-766-5p/EIF5A axis. *Adv Clin Exp Med*. 2021; 30(6):591–598. doi:10.17219/acem/133496
121. Guo J, Wang Z, Xue M, et al. Metformin protects against abdominal aortic aneurysm by Atg7-induced autophagy. *Adv Clin Exp Med*. 2021;31(1):59–69. doi:10.17219/acem/142026
122. Li Z, Li C, Li P, Li Y, Lai J, Rastogi S. Does a single dose of palonosetron have any role in preventing acute chemotherapy-induced nausea and vomiting in pediatric osteosarcoma patients without dexamethasone? A randomized clinical trial. *Adv Clin Exp Med*. 2022; 31(3):223–230. doi:10.17219/acem/142332
123. Putowski Z, Krzych Ł, Czajka S. High intraoperative pulse pressure is a risk factor for postoperative acute kidney injury in a cohort of abdominal surgery patients: An exploratory study. *Adv Clin Exp Med*. 2022;31(5):511–517. doi:10.17219/acem/145946

124. Ji Z, Bao S, Li L, Wang D, Shi M, Liu X. Hypoxia-inducible factor-2α promotes EMT in esophageal squamous cell carcinoma through the Notch pathway. *Adv Clin Exp Med*. 2022;31(7):795–805. doi:10.17219/acem/147270
125. Begum Z, Subramanian V, Raghunath G, Gurusamy K, Vijayaraghavan R, Sivanesan S. Efficacy of different intensity of aquatic exercise in enhancing remyelination and neuronal plasticity using cuprizone model in male Wistar rats. *Adv Clin Exp Med*. 2022;31(9):999–1009. doi:10.17219/acem/148112
126. Sun L, Li H, Fu Q, Hu S, Zhao W. Significance of detecting the levels of miR-29a, survivin and interferon gamma release assay in patients with lung cancer and tuberculosis. *Adv Clin Exp Med*. 2022;31(10):1073–1080. doi:10.17219/acem/150306
127. Sang H, Zhao R, Lai G, et al. Bone marrow mesenchymal stem cell-derived exosomes attenuate the maturation of dendritic cells and reduce the rejection of allogeneic transplantation. *Adv Clin Exp Med*. 2023;32(5):551–561. doi:10.17219/acem/156643
128. Grotowska M, Harbut P, Frostell C, Gozdzik W. Fluid resuscitation, but not inhaled nitric oxide, improves microcirculation in septic pigs. *Adv Clin Exp Med*. 2022;32(6):667–676. doi:10.17219/acem/156700
129. Ventruba T, Ventruba P, Ješeta M, et al. The contribution of donated human embryos suitable for the production of embryonic stem cells to increase the quality of life: Selection and preparation of embryos in the Czech Republic. *Adv Clin Exp Med*. 2023;32(8):901–907. doi:10.17219/acem/158777
130. Yang S, Pan K, Hua Q, et al. Correlation analysis of patients with diabetic foot ulcers treated with tibial cortex transverse transport surgery and platelet-to-lymphocyte ratio and monocyte-to-neutrophil ratio [published online as ahead of print on June 27, 2024]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/187765
131. Li Z, Zhao J, Lu L, et al. Small RNA sequencing highlights a potential regulatory network mediated by Gecko miRNA affecting the prognosis of hepatocellular carcinoma [published online as ahead of print on April 29, 2024]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/185253
132. Szczepanowski R, Uchmanowicz I, Pasieczna AH, et al. Application of machine learning in predicting frailty syndrome in patients with heart failure. *Adv Clin Exp Med*. 2024;33(3):309–315. doi:10.17219/acem/184040
133. Zheng Q, Gong Z, Lin S, Ou D, Lin W, Shen P. Integrated analysis of a competing endogenous RNA network reveals a ferroptosis-related 6-lncRNA prognostic signature in clear cell renal cell carcinoma [published online as ahead of print on March 12, 2024]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/176050
134. Yong XZ, Zhou YX, Wu TT, et al. Differential expression of miRNA-769-5p and Smad2 in patients with or without oral cGVHD [published online as ahead of print on February 14, 2024]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/181147
135. Vázquez-Rodríguez S, Arriaga-Pizano LA, Mancilla-Herrera I, et al. Fc-gamma receptor expression and cytokine responses to intravenous human immunoglobulin in whole blood from non-pregnant and pregnant women and newborns [published online as ahead of print on January 5, 2024]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/174566
136. Li Z, Zhao J, Tong D, et al. WITHDRAWN: Investigating the miRNA of the Chinese herb Gecko on the cross-species regulation network of hepatocellular carcinoma. Preprint posted online August 30, 2023. *Research Square*. doi:10.21203/rs.3.rs-2204354/v2
137. Avissar-Whiting M, Belliard F, Bertozzi SM, et al. Advancing the culture of peer review with preprints. Preprint posted online April 3, 2023. *Research Square*. doi:10.31219/osf.io/cht8p
138. Lemberger T, Pulverer B. Review Commons: Pre-journal peer review. *EMBO Rep*. 2019;20(12):e49663. doi:10.15252/embr.201949663
139. Sever R, Carvalho T. What is the future of preprint peer review? *Acta Med Port*. 2023;36(4):225–226. doi:10.20344/amp.19675
140. Eisen MB, Akhmanova A, Behrens TE, et al. Peer review without gatekeeping. *eLife*. 2022;11:e83889. doi:10.7554/eLife.83889
141. Rousi AM, Laakso M. Overlay journals: A study of the current landscape. *Journal of Librarianship and Information Science*. 2024;56(1):15–28. doi:10.1177/09610006221125208
142. Ursić L, Gudelj D, Tomić V, Marušić M, Marušić A. Analysing overlay journals: The state-of-the-art in 2021 and possible perspectives. *Learned Publishing*. 2022;35(4):640–649. doi:10.1002/leap.1491
143. Fraser N, Mayr P, Peters I. Motivations, concerns and selection biases when posting preprints: A survey of bioRxiv authors. *PLoS One*. 2022;17(11):e0274441. doi:10.1371/journal.pone.0274441
144. Brainard J. In a bold bid to avoid open-access fees, Gates foundation says grantees must post preprints. *Science*. April 1, 2024. doi:10.1126/science.zqvd4bu
145. Maggio LA, Costello JA, Artino AR. Describing the landscape of medical education preprints on medRxiv: Current trends and future recommendations. *Acad Med*. 2024;99(9):981–986. doi:10.1097/ACM.0000000000005742
146. Añazco D, Nicolalde B, Espinosa I, et al. Publication rate and citation counts for preprints released during the COVID-19 pandemic: The good, the bad and the ugly. *PeerJ*. 2021;9:e10927. doi:10.7717/peerj.10927
147. Penfold NC, Polka JK. Technical and social issues influencing the adoption of preprints in the life sciences. *PLoS Genet*. 2020;16(4):e1008565. doi:10.1371/journal.pgen.1008565
148. Rzayeva N, Henriques SO, Pinfield S, Waltman L. The experiences of COVID-19 preprint authors: A survey of researchers about publishing and receiving feedback on their work during the pandemic. *PeerJ*. 2023;11:e15864. doi:10.7717/peerj.15864
149. Fry C, MacGarvie M. Author country of origin and attention on open science platforms: Evidence from COVID-19 preprints. *Management Science*. 2024;70(8):5426–5444. doi:10.1287/mnsc.2023.4936
150. Biesenbender K, Smirnova N, Mayr P, Peters I. The emergence of preprints: Comparing publishing behaviour in the Global South and the Global North [published online as ahead of print on January 15, 2024]. *Online Information Review*. 2024. doi:10.1108/OIR-04-2023-0181
151. Puebla I, Polka J, Rieger O. Preprints: Their evolving role in science communication. Preprint posted online February 18, 2021. *Open Science Framework (OSF)*. doi:10.31222/osf.io/ezfsk

The clinical impact of plasma estrogen receptor-1 mutation in patients with metastatic breast cancer: A meta-analysis

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Abstract

Background. The relevance of the discovered plasma *ESR1* mutations in positive metastatic breast cancer (BC) patients who had progressing disease after aromatase inhibitor (AI)-based therapy is still being debated.

Objectives. We conducted this meta-analysis to explore the prognostic and predictive role of plasma *ESR1* mutations in patients with progressive BC who have previously received AI therapy.

Materials and methods. We searched for relevant studies in the PubMed, Embase and Cochrane Library databases to be included in the meta-analysis. This study was performed to compute combined hazard ratios (HRs) with 95% confidence intervals (95% CIs) for the progression-free survival (PFS) rate and overall survival (OS) rate. Subgroup and sensitivity analyses were also performed. The heterogeneity between studies was evaluated using the I^2 statistic.

Results. In this meta-analysis, a total of 1,844 patients with metastatic BC and positive for estrogen receptors (ERs) were enrolled from 8 articles. The analysis revealed that patients with circulating *ESR1* mutations had significantly worse PFS (HR: 1.34; 95% CI: 1.17–1.55; $p < 0.001$) and OS (HR: 1.59; 95% CI: 1.31–1.92; $p < 0.001$) compared to wild-type *ESR1* patients. Subgroup analysis showed that the types of plasma *ESR1* mutations were associated with differences in the prognosis of metastatic BC. The D538G mutation showed a statistically significant lower PFS ($p = 0.03$), while the Y537S mutation was not significantly correlated with PFS ($p = 0.354$).

Conclusions. According to the findings of this meta-analysis, the assessment for plasma *ESR1* mutations may provide prognostic and clinical guidance regarding subsequent endocrine therapy decisions for ER-positive, metastatic BC patients who had received prior therapy with AIs.

Key words: meta-analysis, metastatic breast cancer, aromatase inhibitors, *ESR1* mutations, prognostic role

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Background

Breast cancer (BC) is among the most common tumors, with about 75% of BC cases being positive for estrogen receptors (ERs).[1,2] The cornerstone of treatment are endocrine therapies such as ER modulators or aromatase inhibitors (AIs).[3] The preferred initial treatment for metastatic breast cancer (MBC) is endocrine therapy; however, most patients are at risk for endocrine resistance during treatment.[4,5]

Several mechanisms and biomarkers have been associated with endocrine resistance, but the clinical application of these mechanisms has not yet been reached.[6] One crucial factor in their resistance is a mutation occurring in the binding domain of estrogen receptor 1 (ESR1) encoding ER-α.[7] In vitro investigations have provided evidence that the *ESR1* mutation gives rise to an ER that is constantly active, regardless of ligand binding. This constitutive activation of the ER leads to increased cell proliferation and reduced responsiveness to endocrine therapies.[8] This particular mutation has been the subject of extensive research in the past 10 years, with a focus on understanding its biochemical and molecular impacts, as well as its significance in determining suitable treatment options and identifying potential weaknesses that can be targeted therapeutically.[9]

A set of *ESR1* gene mutations has been identified in previous studies from patients with metastatic BCs. The prevalence of *ESR1* mutations is much higher in metastatic BC compared to primary cancers, especially in patients previously treated with AIs.[10,11]

The incidence of *ESR1* mutations among patients with MBC who have undergone AI therapy ranges from around 20% to 40%, with variations observed depending on the specific areas of metastatic disease.[12] Estrogen receptor 1 mutations were detected in about 55% of biopsies from ER-positive metastatic BC patients.[13] About 40% of MBCs acquire the *ESR1* mutations, which confers resistance to AI therapy in MBC.[14]

The most common mutations found in several investigations are the D538G and Y537S variants. The E380Q mutation is the 3rd most prevalent identified mutation.[15] Recently, most studies have focused on detecting *ESR1* gene mutations in circulating tumor DNA (ctDNA) for easier sampling as an alternative to tumor tissue biopsies.[16] Several clinical studies have provided evidence that *ESR1* mutations in the plasma are associated with a decreased progression-free survival (PFS) rate following AI treatment.[17–19] However, the utility and reliability of *ESR1* mutations in the plasma as a predictive tool for BC prognosis is still controversial due to the limitations in the current evidence. These limitations include a wide discrepancy in mutations assessed, drugs used, plasma processing methods, and techniques. Moreover, most studies included a limited number of patients, resulting in inconsistent findings. The aforementioned limitations render them insufficient in their capacity to effectively assess the varying impacts of various *ESR1* mutations and their utility in the prediction of efficacy of a specific treatment, such as AIs or fulvestrant.

Objectives

Therefore, the present meta-analysis aimed to assess the clinical utility of *ESR1* mutations in ctDNA and its impact on PFS and overall survival (OS) among MBC patients with ER-positive BC. We also carried out subgroup analysis to evaluate the relevance of the most frequent types of *ESR1* mutations, including D538G and Y537S, and their predictive significance in therapies based on AI regimens and fulvestrant.

Materials and methods

The current meta-analysis study was conducted based on the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines.[20]

Search strategy and study selection

Observational, retrospective or prospective studies reporting the effect of *ESR1* mutations on the therapeutic outcomes of MBC patients with positive ER BC were included. Only clinical trials conducted on patients, regardless of the language used, were eligible for the analysis. The size or follow-up duration of the study did not limit the inclusion. At least 1 of the outcomes, including PFS and OS rates, had to be measured in the eligible studies. Commentaries, review articles and irrelevant studies were excluded.

Data sources and identification

First, we systematically searched the different electronic databases, including Embase, Cochrane Library and PubMed, until August 2022. The terms adapted and applied for searching each electronic database included BC, breast neoplasm, tumor, ctDNA, cell-free DNA (cfDNA), plasma, ESR1, ER, and therapy, as summarized in Table 1. No restrictions were applied regarding the location, design or language of the study. After the initial examination of the titles and abstracts, we gathered all eligible publications using EndNote software (Clarivate Plc, London, UK) into a single file to omit duplications. Studies that did not report the impact of *ESR1* mutations on PFS or OS among MBC patients were excluded. The retrieved publications were investigated for relevant outcome data. Publications with missing data were excluded.

Screening

A pre-designed form was utilized to summarize main features and properties of the studies. The main

Table 1. Search strategy for each database

Database	Search strategy
PubMed	#1 "breast cancer"[MeSH Terms] OR "breast neoplasm"[All Fields] OR "ESR1 mutation"[All Fields] OR "breast metastasis"[All Fields] #2 "aromatase inhibitor"[MeSH Terms] OR "PFS"[All Fields] OR "OS"[All Fields] OR "progression-free survival"[All Fields] OR "overall survival"[All Fields] OR "endocrine therapy"[All Fields] (word variations have been searched) #3 #1 AND #2
Embase	#1 'breast cancer'/exp OR 'breast neoplasm'/exp OR 'ESR1 mutation'/exp OR 'breast metastasis'/exp #2 'aromatase inhibitor'/exp OR 'PFS'/exp OR 'OS'/exp OR 'progression-free survival'/exp OR 'overall survival'/exp OR 'endocrine therapy'/exp (word variations have been searched) #3 #1 AND #2
Cochrane Library	#1 (breast cancer):ti,ab,kw OR (breast neoplasm):ti,ab,kw OR (ESR1 mutation):ti,ab,kw OR (breast metastasis):ti,ab,kw #2 (aromatase inhibitors):ti,ab,kw OR (PFS):ti,ab,kw OR (OS):ti,ab,kw OR (progression-free survival):ti,ab,kw OR (overall survival):ti,ab,kw OR (endocrine therapy):ti,ab,kw (word variations have been searched) #3 #1 AND #2

parameters included the surname of the first author, study timeframe, region, year of publication, age, the assessed *ESR1* mutation types, study techniques, subject number, therapeutic regimens, and outcome data such as hazard ratios (HRs) of PFS and OS. In cases of missing survival data, a Kaplan–Meier curve was used instead to extract data using the method described by Tierney et al.[21] A unique identification number was used for each citation. In case of a study's suitability for analysis, according to the applied inclusion criteria, data were retrieved by 2 of the authors individually. In cases of discrepancy, the corresponding author was consulted for the final decision. Each study was appraised by 2 authors who examined the procedural quality of the relevant trials individually.

The risk of bias

For the study's procedural quality evaluation and risk of bias assessment, the Cochrane risk-of-bias tool was implemented for randomized trials v. 2 (RoB 2).[22] The quality of selected publications was evaluated in duplicates, and discrepancies were cleared by consulting the corresponding author. The bias evaluation criteria included the assignment of the study to one of the following categories: the complete fulfillment of quality standards warranted the study classification to the low risk of bias category, partial fulfillment of the quality requirements (1 or more missing or not adequately clarified) warranted the publication categorization as having a moderate risk of bias, and the high risk of bias category applied to publications which failed to meet the quality standards. Reassessment of the original publication was applied in cases of any inconsistencies.

Eligibility

The main findings focused on the relation of *ESR1* gene mutations in BC patients with metastasis. A summary was created based on the presence of outcome data regarding PFS or OS. The identified records were screened initially based on their titles and abstract, and then the full text was reviewed. We conducted a comprehensive search to identify all available and relevant studies to identify the latest developments and limitations in the existing literature. Well-designed randomized controlled or comparative research studies, either prospective or retrospective, which enrolled patients with positive ER MBC in whom procedure of intervention included the detection of *ESR1* gene mutations by ctDNA or cfDNA at baseline of endocrine therapy, and which had adequately described data to estimate the overall pooled effect size of survival status association with *ESR1* mutations were included in this meta-analysis. Published case reports, abstracts, editorials, review articles, animal experiments, commentaries, studies with missing duplicate reports or incomplete data, studies lacking therapy information at baseline analysis of *ESR1* mutations, and research studies with irrelevant objectives were excluded from this meta-analysis.

Sensitivity analysis and subgroup assessment

Sensitivity analyses were applied only to studies that showed a high risk of bias or high heterogeneity to evaluate the impact on research findings.

Statistical analyses

Pooled HRs, 95% confidence interval range (95% CI), and p-values were estimated. We used Review Manager (RevMan v. 5.3; Nordic Cochrane Centre, Copenhagen, Denmark) and STATA v. 10.0 (StataCorp LLC, College Station, USA) for data analysis and visualization. The data were presented in forest plots. For computing statistical heterogeneity, we used the I^2 index, which was valued up to 100%.[17] The I^2 value of about 0% denoted no heterogeneity, while I^2 values of 25%, 50% and 75% denoted low, moderate and high heterogeneity, respectively.[23,24] The discrepancy between the included studies regarding eligibility criteria, population characteristics, potential bias, chemotherapeutic regimens, sample type applied, and study interventional arms was evaluated to determine the appropriate model

to employ in our study, whether it be a fixed-effect model or a random-effects model. The random-effects model was employed for all analyses based on the evaluation of disparities. For subgroup analyses, we stratified the outcomes per result category. We quantitatively assessed publication bias by Egger's regression,[25] Begg's rank correlation tests[26] and visual inspection of Begg's funnel plots. The entire estimated p-values were two-tailed. A p-value for differences amongst comparisons of < 0.05 denoted a statistically significant difference.

Results

The main characteristics of the included studies

The search of electronic databases resulted in 214 publications. After the search was restricted to clinical trials, 142 of them were retrieved. The removal of irrelevant studies and duplicates yielded 8 relevant articles for inclusion and analysis. Two randomized clinical trials were presented in a report by Fribbens et al.[27] (SoFEA and PALOMA3) and were independently analyzed. Figure 1 summarizes the search strategy for the databases and the selection for meta-analysis.

Table 2 illustrates 8 randomized trials[27–34] published between 2016 and 2021. All trials were retrospective, including prospective-retrospective studies (n = 5) using the baseline archived plasma from randomized trials. The sample size in the eligible studies ranged from 16 to 541 patients, with a total of 1,844 patients enrolled. All participants received prior therapy with AIs with different subsequent treatment regimens, as listed in Table 2. The risk of bias in the selected studies was assessed in accordance with the Cochrane Collaboration tool. Study selection was based on 95% agreement between the 2 authors, and they both agreed 100% on the quality rate of the selected studies.

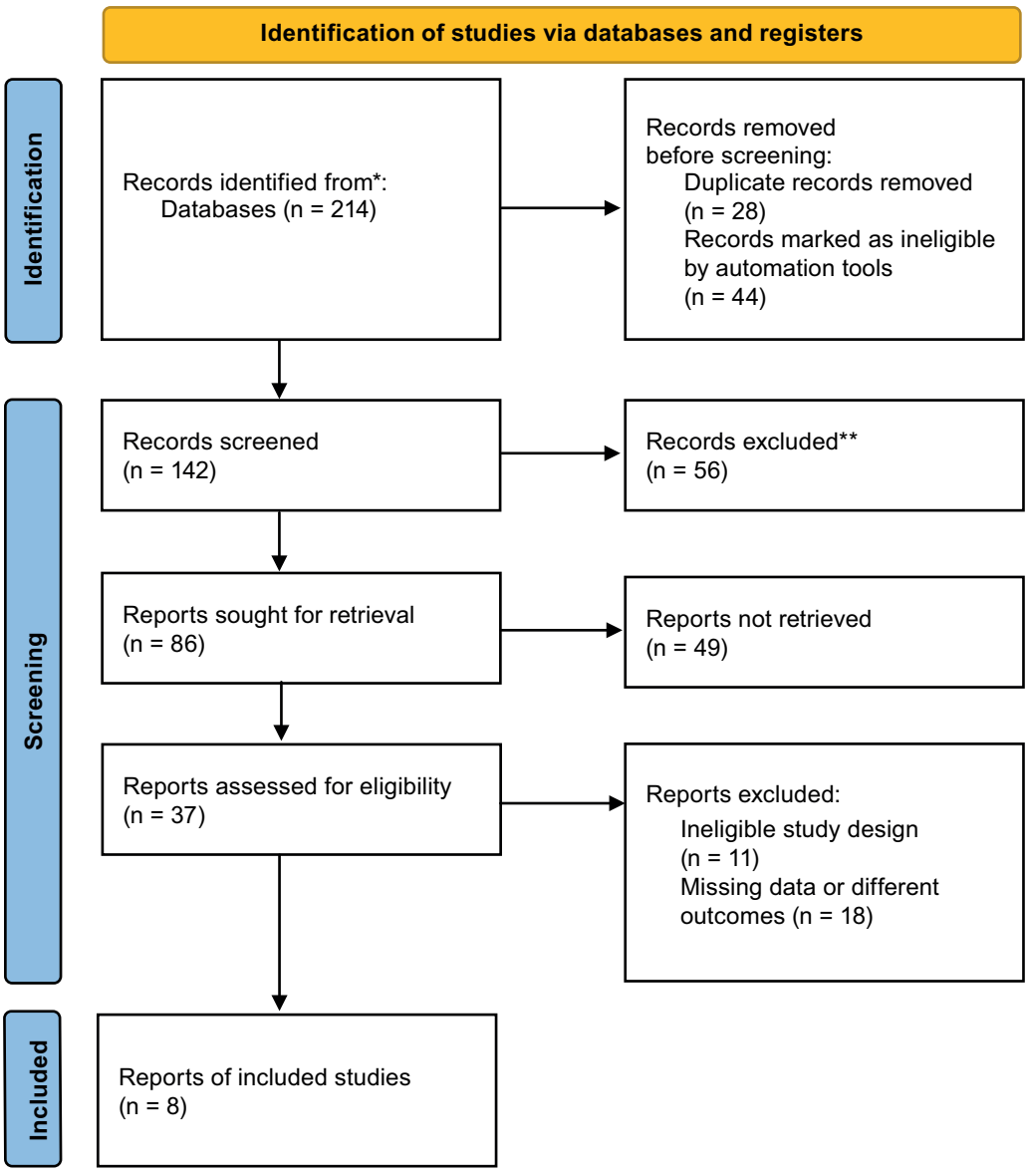

Fig. 1. Flowchart of the study search strategy

Table 2. Characteristics of the selected studies

Outcome(s) of interest measured	Subsequent therapy	Sample type	Sample size	Study ID, year
PFS, OS PFS	fulvestrant and anastrozole, exemestane fulvestrant and palbociclib/placebo	ctDNA	161 360	Fribbens et al., 2016[27] SoFEA PALOMA
PFS, OS	AI-based therapy	cfDNA	144	Clatot et al., 2016[28]
PFS	AI-based therapy	ctDNA	171	Schiavon et al., 2016[29]
PFS	fulvestrant and pictilisib	ctDNA	153	Spoerke et al., 2016[30]
PFS, OS	exemestane and placebo/everolimus	cfDNA	541	Chandarlapaty et al., 2016[31]
PFS, OS	AI-based therapy, fulvestrant, and exemestane	ctDNA	383	Turner et al., 2020[32]
PFS	AI-based therapy	cfDNA	103	Zundelevich et al., 2020[33]
PFS, OS	chemotherapy, cyclin-dependent kinase inhibitor and endocrine therapy	ctDNA	59	Muendelin et al., 2021[34]

AI – aromatase inhibitor; cfDNA – cell free DNA; ctDNA – circulating tumor DNA; PFS –progression-free survival; OS – overall survival.

ESR1 gene mutations and progression-free survival rate

The association of plasma *ESR1* mutations in MBC patients with PFS was investigated in 8 studies with 11 outcomes for PFS data. The pooled estimated effect size showed a statistically significant worsened PFS with *ESR1* mutations using the unchanged wild-type (WT) *ESR1* as a comparison group (HR: 1.34; 95% CI: 1.17–1.55; $p < 0.001$, $z = 4.12$) among MBC patients with positive ER, as illustrated in Fig. 2. The heterogeneity level was moderate in the HRs of the individual trials for PFS ($I^2 = 52\%$).

ESR1 gene mutations and overall survival rate

The pooled estimated HR for OS from 6 studies with 7 outcomes using a random effects model was 1.59 (95% CI: 1.31–1.92; $p < 0.001$, $z = 4.71$), suggesting a prognostic link of *ESR1* gene mutations in study participants with ER-positive MBC. The forest plot of the OS analysis is shown in Fig. 3. The heterogeneity level was moderate in the HRs of the individual trials for OS ($I^2 = 71\%$).

Subgroup analysis according to the type of *ESR1* mutations was described in only 3 studies, which reported the association of individual types of mutations with PFS. The estimated pooled HRs with the D538G mutation showed shorter PFS (HR: 1.42; 95% CI: 1.06–2.11; $p = 0.031$) using the WT *ESR1* mutation as a comparator in BC patients assigned to endocrine therapy and the heterogeneity level was low ($I^2 = 2\%$). Unlike Y537S mutation, which showed high heterogeneity ($I^2 = 78\%$) and its association with PFS was statistically nonsignificant (HR: 1.55, 95% CI: 0.61–2.33; $p = 0.354$).

Based on subsequent therapy for MBC patients, the subgroup stratification for AI-based subsequent therapy showed a statistically significant pooled HR of 1.78 (95% CI: 1.37–2.32; $p < 0.001$, $I^2 = 67\%$) for PFS and 1.37 for OS (95% CI: 1.03–1.80; $p = 0.033$, $I^2 = 20\%$). These findings suggest that circulating *ESR1* mutations predict a lower PFS for patients

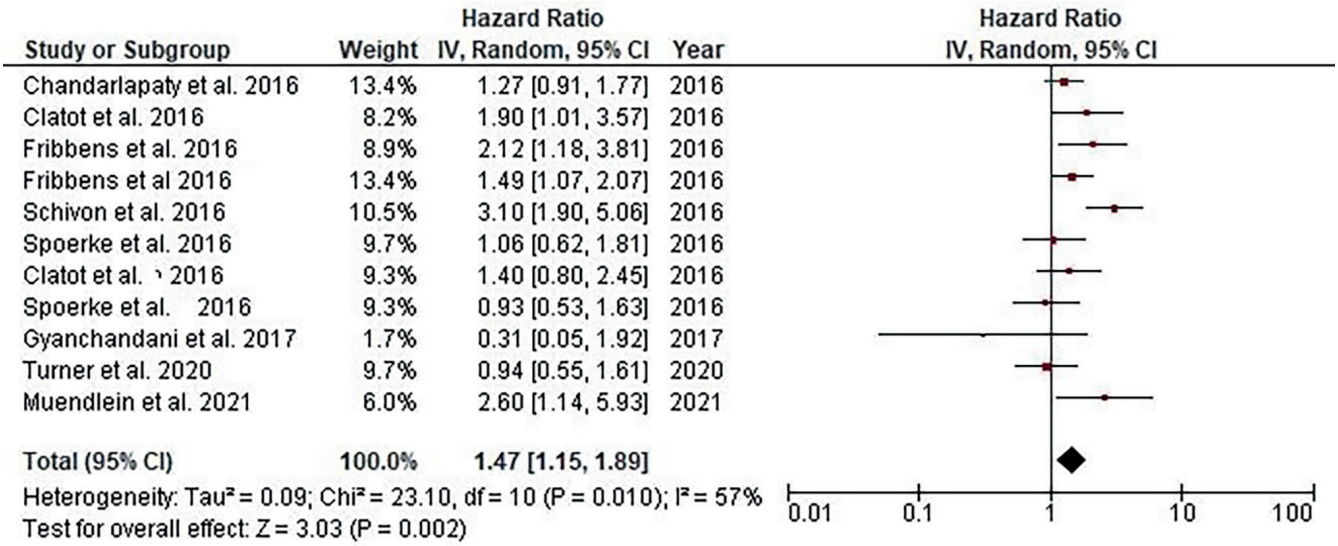

Fig. 2. Forest plot of the *ESR1* gene mutations' effect on the progression-free survival (PFS) rate

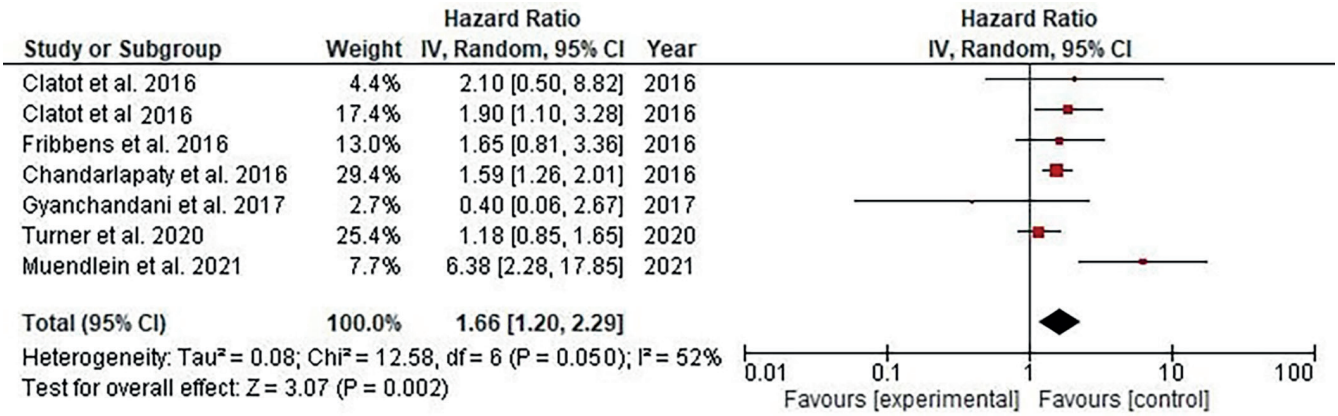

Fig. 3. Forest plot of *ESR1* gene mutations' effect on the overall survival (OS) rate

on subsequent AI therapy. The analysis of fulvestrant subsequent treatment showed a nonsignificant correlation with PFS (HR: 1.56, 95% CI: 0.96–2.16; p = 0.081).

Publication bias

The Egger's regression analysis and Begg's test did not detect any evidence of publication bias among studies investigating the association between *ESR1* mutations (Egger's test: p = 0.592 and 0.741; Begg's test: p = 0.931 and 0.548) and PFS and OS, respectively (Supplementary Table 1). The same was observed during the visual evaluation of Begg's funnel plots, which showed a symmetrical pattern of distribution of the studies (Fig. 4).

Discussion

The present meta-analysis investigated the association between survival status and *ESR1* mutations in plasma among patients with MBC. The pooled HRs for PFS and OS between *ESR1* mutations and WT *ESR1* were 1.48 (95% CI: 1.28–1.73; p < 0.001) and 1.55 (95% CI: 1.30–1.83; p < 0.001), respectively. The results of this study revealed a notable differentiation between BC cases with and without mutations in the *ESR1* gene. Specifically, cases with ESR1 mutations had inferior outcomes in terms of PFS and OS.

Several preclinical trials reported tumor growth suppression with fulvestrant in cell lines with *ESR1* mutations.[35,36] These findings are consistent with our findings of subgroup analysis based on subsequent therapy, where PFS was significantly reduced in patients assigned to subsequent AI therapy with *ESR1* mutations – unlike fulvestrant therapy, which did not significantly favor patients with WT *ESR1*, implying that *ESR1* gene mutations may not be associated with intrinsic or acquired resistance to fulvestrant. Therefore, our findings strengthen the evidence which supports fulvestrant over AI in the treatment of MBC patients.[37,38] Moreover, these outcomes highlight the implication of plasma *ESR1* testing to better guide the selection of endocrine-based treatment.

The advantages of using cfDNA/ctDNA over tissue biopsies for *ESR1* mutation detection have been addressed in several studies. The potential of detecting several additional mutations with the use of cfDNA or ctDNA highlighted the advantage of liquid biopsy.[39,40] In addition,

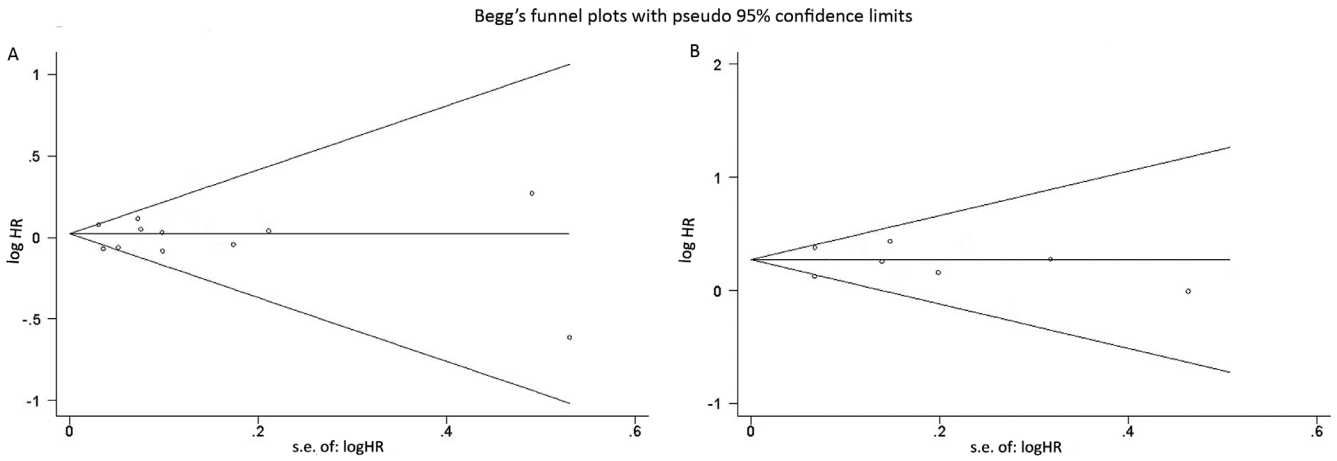

Fig. 4. Begg's funnel plots for publication bias. A. Studies on progression-free survival rate; B. Studies assessing overall survival (OS) rate

the feasibility of serum sample collection enables the sequential assessment of mutations at baseline and during treatment.[39] The increase in *ESR1* mutations during therapy suggests disease progression as a consequence of AI treatment. Upon fulvestrant therapy, if *ESR1* mutated clones decay, this would allow an AI re-challenge in those patients.

We also assessed the effect of *ESR1* mutation types on PFS and OS. The existing analysis showed how distinct *ESR1* mutations (Y537S and D538G) differentially impact patterns of disease progression and patient's survival. Our findings showed that the D538G mutation was associated with shorter PFS compared to WT *ESR1*, but the same was not observed with the Y537S mutation. However, these findings are inconsistent with many basic studies that reported the highest ER activity with Y537S mutations.[41,42] This discrepancy in our findings could be attributed to the limited number of studies included and the high statistical heterogeneity detected (I^2 = 78%). Other mutations, such as E380Q, L536R, Y537N, and Y537C in circulating DNA, have not been investigated in the available literature. Therefore, further studies evaluating all *ESR1* domain mutations and the distinction between them based on their clinical effect are needed.[41,43,44] This meta-analysis highlights the association of *ESR1* mutations with an unfavorable prognosis, as reflected by reduced PFS and OS. The clinical implications of *ESR1* analysis are significant, but issues remain concerning how frequently testing should be performed, taking the patient's age into consideration, and how effectively each type of mutation reacts to treatment. High-quality prospective studies to optimize therapeutic options for regulating ER signaling prior to the onset of extensive disease metastasis would thus be a sensible next step for future trials. Moreover, the literature lacks studies aimed at investigating the potential of *ESR1* mutations as a means of real-time and dynamic monitoring of tumor progression and therapeutic effectiveness in patients with MBC who exhibit resistance to endocrine therapy.

This meta-analysis has several strengths. First, all selected trials for analysis were relevant to our study, which assessed the clinical implications of *ESR1* mutations on disease progression and survival. Thus, the risk of bias is limited. Second, this study provided updated and comprehensive evidence from the literature about an emerging topic in the management of BC and provided guidance for clinicians with patients who have different *ESR1* mutations.

Although our findings are promising, several limitations can also be discussed.

Limitations

The main limitation is the wide diversity between study protocols and the use of potentially biased evidence, with considerable heterogeneity between the trials. Second, the relatively small sample size might affect the certainty of the estimates. Finally, some outcome data were extracted from curves, which might affect the accuracy of the results.

Conclusions

The identification of *ESR1* mutations in circulating DNA analyses has been found to be indicative of a poorer OS in patients who have received prior treatment with an AI. It may seem prudent to consider the analysis of circulating ctDNA for prognosis prediction and directing the choice of endocrine therapy in MBC patients positive for ERs, especially those who did not adequately respond to AI therapy. Moreover, *ESR1* mutations and WT *ESR1* can be considered different subtypes of advanced BC with positive ERs. Future research may help better establish the use of plasma DNA sampling in clinical practice, improve our understanding of the clinical impact of the different types of *ESR1* mutations, and potentially guide therapeutic selection.

Supplementary data

The Supplementary materials are available at: https://doi.org/10.5281/zenodo.10212351. The package includes the following file:

Supplementary Table 1. Publication bias and heterogeneity test results among studies in overall and subgroup analyses.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

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References

1. Watkins EJ. Overview of breast cancer. *JAAPA*. 2019;32(10):13–17. doi:10.1097/01.JAA.0000580524.95733.3d
2. Cardoso F, Senkus E, Costa A, et al. 4th ESO–ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC 4). *Ann Oncol*. 2018;29(8):1634–1657. doi:10.1093/annonc/mdy192
3. Gombos A. Selective oestrogen receptor degraders in breast cancer: A review and perspectives. *Curr Opin Oncol*. 2019;31(5):424–429. doi:10.1097/CCO.0000000000000567
4. Lei JT, Anurag M, Haricharan S, Gou X, Ellis MJ. Endocrine therapy resistance: New insights. *Breast*. 2019;48(Suppl 1):S26–S30. doi:10.1016/S0960-9776(19)31118-X
5. Isik A, Eken H, Soyturk M, Firat D, Yilmaz I. A rare presentation of accessory breast in axilla. *Galician Med J*. 2016;23(4):E201645. doi:10.21802/gmj.2016.4.5

6. Lloyd MR, Wander SA, Hamilton E, Razavi P, Bardia A. Next-generation selective estrogen receptor degraders and other novel endocrine therapies for management of metastatic hormone receptor-positive breast cancer: Current and emerging role. *Ther Adv Med Oncol*. 2022;14:175883592211136. doi:10.1177/17588359221113694
7. Hermida-Prado F, Jeselsohn R. The *ESR1* mutations: From bedside to bench to bedside. *Cancer Res*. 2021;81(3):537–538. doi:10.1158/0008-5472.CAN-20-4037
8. Wang P, Bahreini A, Gyanchandani R, et al. Sensitive detection of mono- and polyclonal *ESR1* mutations in primary tumors, metastatic lesions, and cell-free DNA of breast cancer patients. *Clin Cancer Res*. 2016; 22(5):1130–1137. doi:10.1158/1078-0432.CCR-15-1534
9. O'Leary B, Cutts RJ, Liu Y, et al. The genetic landscape and clonal evolution of breast cancer resistance to palbociclib plus fulvestrant in the PALOMA-3 trial. *Cancer Discov*. 2018;8(11):1390–1403. doi:10.1158/2159-8290.CD-18-0264
10. Hong R, Xu B. Breast cancer: An up-to-date review and future perspectives. *Cancer Commun (Lond)*. 2022;42(10):913–936. doi:10.1002/cac2.12358
11. Xia S, Lin Q. Estrogen receptor bio-activities determine clinical endocrine treatment options in estrogen receptor-positive breast cancer. *Technol Cancer Res Treat*. 2022;21:153303382210903. doi:10.1177/15330338221090351
12. Brett JO, Spring LM, Bardia A, Wander SA. *ESR1* mutation as an emerging clinical biomarker in metastatic hormone receptor-positive breast cancer. *Breast Cancer Res*. 2021;23(1):85. doi:10.1186/s13058-021-01462-3
13. Tay TKY, Tan PH. Liquid biopsy in breast cancer: A focused review. *Arch Pathol Lab Med*. 2021;145(6):678–686. doi:10.5858/arpa.2019-0559-RA
14. Williams MM, Spoelstra NS, Arnesen S, et al. Steroid hormone receptor and infiltrating immune cell status reveals therapeutic vulnerabilities of *ESR1*-mutant breast cancer. *Cancer Res*. 2021;81(3):732–746. doi:10.1158/0008-5472.CAN-20-1200
15. Toy W, Weir H, Razavi P, et al. Activating *ESR1* mutations differentially affects the efficacy of ER antagonists. *Cancer Discov*. 2017;7(3): 277–287. doi:10.1158/2159-8290.CD-15-1523
16. Dustin D, Gu G, Fuqua SAW. *ESR1* mutations in breast cancer. *Cancer*. 2019;125(21):3714–3728. doi:10.1002/cncr.32345
17. Sundaresan TK, Sequist LV, Heymach JV, et al. Detection of T790M, the acquired resistance *EGFR* mutation, by tumor biopsy versus noninvasive blood-based analyses. *Clin Cancer Res*. 2016;22(5):1103–1110. doi:10.1158/1078-0432.CCR-15-1031
18. Forshew T, Murtaza M, Parkinson C, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012;4(136):136ra68. doi:10.1126/scitranslmed.3003726
19. Sundaresan TK, Dubash TD, Zheng Z, et al. Evaluation of endocrine resistance using *ESR1* genotyping of circulating tumor cells and plasma DNA. *Breast Cancer Res Treat*. 2021;188(1):43–52. doi:10.1007/s10549-021-06270-z
20. Stroup DF. Meta-analysis of observational studies in epidemiology: A proposal for reporting. *JAMA*. 2000;283(15):2008. doi:10.1001/jama.283.15.2008
21. Tierney JF, Stewart LA, Ghersi D, Burdett S, Sydes MR. Practical methods for incorporating summary time-to-event data into meta-analysis. *Trials*. 2007;8(1):16. doi:10.1186/1745-6215-8-16
22. Cochrane Collaboration. RoB 2: A revised Cochrane risk-of-bias tool for randomized trials. 2019. https://methods.cochrane.org/bias/resources/rob-2-revised-cochrane-risk-bias-tool-randomized-trials. Accessed December 6, 2019.
23. Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, Welch VA, eds. Cochrane Handbook for Systematic Reviews of Interventions. 2nd ed. Chichester, UK: Wiley & Sons; 2019. doi:10.1002/9781119536604
24. Higgins JPT. Measuring inconsistency in meta-analyses. *BMJ*. 2003; 327(7414):557–560. doi:10.1136/bmj.327.7414.557
25. Egger M, Smith GD, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ*. 1997;315(7109):629–634. doi:10.1136/bmj.315.7109.629
26. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics*. 1994;50(4):1088. doi:10.2307/2533446
27. Fribbens C, O'Leary B, Kilburn L, et al. Plasma *ESR1* mutations and the treatment of estrogen receptor–positive advanced breast cancer. *J Clin Oncol*. 2016;34(25):2961–2968. doi:10.1200/JCO.2016.67.3061
28. Clatot F, Perdrix A, Augusto L, et al. Kinetics, prognostic and predictive values of *ESR1* circulating mutations in metastatic breast cancer patients progressing on aromatase inhibitor. *Oncotarget*. 2016;7(46): 74448–74459. doi:10.18632/oncotarget.12950
29. Schiavon G, Hrebien S, Garcia-Murillas I, et al. Analysis of *ESR1* mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Sci Transl Med*. 2015;7(313):313ra182. doi:10.1126/scitranslmed.aac7551
30. Spoerke JM, Gendreau S, Walter K, et al. Heterogeneity and clinical significance of *ESR1* mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. *Nat Commun*. 2016;7(1):11579. doi:10.1038/ncomms11579
31. Chandarlapaty S, Chen D, He W, et al. Prevalence of *ESR1* mutations in cell-free DNA and outcomes in metastatic breast cancer: A secondary analysis of the BOLERO-2 clinical trial. *JAMA Oncol*. 2016;2(10):1310. doi:10.1001/jamaoncol.2016.1279
32. Turner NC, Swift C, Kilburn L, et al. *ESR1* mutations and overall survival on fulvestrant versus exemestane in advanced hormone receptor-positive breast cancer: A combined analysis of the phase III SoFEA and EFECT trials. *Clin Cancer Res*. 2020;26(19):5172–5177. doi:10.1158/1078-0432.CCR-20-0224
33. Zundelevich A, Dadiani M, Kahana-Edwin S, et al. *ESR1* mutations are frequent in newly diagnosed metastatic and loco-regional recurrence of endocrine-treated breast cancer and carry worse prognosis. *Breast Cancer Res*. 2020;22(1):16. doi:10.1186/s13058-020-1246-5
34. Muendlein A, Geiger K, Gaenger S, et al. Significant impact of circulating tumour DNA mutations on survival in metastatic breast cancer patients. *Sci Rep*. 2021;11(1):6761. doi:10.1038/s41598-021-86238-7
35. Yates LR, Gerstung M, Knappskog S, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. *Nat Med*. 2015;21(7):751–759. doi:10.1038/nm.3886
36. De Santo I, McCartney A, Migliaccio I, Di Leo A, Malorni L. The emerging role of *ESR1* mutations in luminal breast cancer as a prognostic and predictive biomarker of response to endocrine therapy. *Cancers*. 2019;11(12):1894. doi:10.3390/cancers11121894
37. Gu G, Dustin D, Fuqua SA. Targeted therapy for breast cancer and molecular mechanisms of resistance to treatment. *Curr Opin Pharmacol*. 2016;31:97–103. doi:10.1016/j.coph.2016.11.005
38. Pascual J, Attard G, Bidard FC, et al. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: A report from the ESMO Precision Medicine Working Group. *Ann Oncol*. 2022;33(8):750–768. doi:10.1016/j.annonc.2022.05.520
39. Buono G, Gerratana L, Bulfoni M, et al. Circulating tumor DNA analysis in breast cancer: Is it ready for prime-time? *Cancer Treat Rev*. 2019;73:73–83. doi:10.1016/j.ctrv.2019.01.004
40. Openshaw MR, Page K, Fernandez-Garcia D, Guttery D, Shaw JA. The role of ctDNA detection and the potential of the liquid biopsy for breast cancer monitoring. *Expert Rev Mol Diagn*. 2016;16(7): 751–755. doi:10.1080/14737159.2016.1184974
41. Elgendy M, Saeed H, Abou-Taleb H. Assessment of educated people awareness level and sources about COVID-19. *Int J Clin Med Res*. 2023;1(1):37–48. doi:10.61466/ijcmr1010004
42. Shaaban M, Mohamed A. Determining the efficacy of N-acetyl cysteine in treatment of pneumonia in COVID-19 hospitalized patients: A meta-analysis. *Int J Clin Med Res*. 2023;1(2):37–48. doi:10.61466/ijcmr1020006
43. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R. *ESR1* mutations: A mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol*. 2015;12(10):573–583. doi:10.1038/nrclinonc.2015.117
44. Palacín-Aliana I, García-Romero N, Asensi-Puig A, Carrión-Navarro J, González-Rumayor V, Ayuso-Sacido Á. Clinical utility of liquid biopsy-based actionable mutations detected via ddPCR. *Biomedicines*. 2021;9(8):906. doi:10.3390/biomedicines9080906

Effect of post-extubation inspiratory muscle training on diaphragmatic function in mechanically ventilated patients: A randomized controlled trial

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Abstract

Background. Diaphragmatic dysfunction is a common problem in patients who have been mechanically ventilated.

Objectives. The study aimed to evaluate the effectiveness of inspiratory muscle training (IMT) on diaphragm muscle thickness and function in mechanically ventilated patients.

Materials and methods. A single-blind trial was conducted. Twenty patients were randomly assigned to either the conventional physiotherapy (CP) group or to the IMT group for 5 days following extubation. The CP group received only CP, while the IMT group received CP in addition to IMT. Ten healthy controls (HCs) underwent IMT. Maximum inspiratory pressure (MIP) and physical function were recorded. Diaphragm excursion (DE), diaphragm thickness at the end of inspiration (Td_i), diaphragm thickness at the end of expiration (Td_e), peak contraction velocity (PCV), and peak relaxation velocity (PRV) were evaluated with ultrasonography before and after the intervention.

Results. The IMT group and HCs showed significant improvements in DE ($p = 0.005$; $p = 0.005$, respectively), PCV ($p = 0.028$; $p = 0.015$, respectively) and PRV ($p = 0.029$; $p = 0.020$, respectively) after 5 days of IMT. A significant increase in MIP was recorded in all groups after the intervention (CP: $p = 0.044$; IMT: $p = 0.005$; HC: $p < 0.001$). There was a significant improvement in the Medical Research Council (MRC) and the Physical Function in Intensive Care Test (PFIT) scores in both the CP and IMT groups ($p < 0.001$ and $p < 0.001$, respectively).

Conclusions. Inspiratory muscle training improves diaphragmatic functions, including MIP, diaphragm excursion, PCV, and PRV. We think that IMT applied after extubation may serve as a tool to prevent and facilitate the recovery of diaphragmatic function.

Key words: weaning, inspiratory muscle training, diaphragm dysfunction, diaphragmatic ultrasonography, tissue Doppler imaging

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Background

Mechanical ventilation (MV) is commonly used to alleviate the work of breathing and reduce diaphragm activity in patients with acute respiratory failure.[1] Even though MV is a crucial intervention, studies have shown that within 18–69 h after MV, diaphragm muscle fibers are more susceptible to proteolysis, and the respiratory muscles begin to deteriorate more rapidly. This leads to respiratory muscle weakness that is about twice as prevalent as extremity muscle weakness (63% compared to 34%) in patients who have been on MV for at least 24 h.[2,3] The progressive development of diaphragmatic atrophy during the early periods of MV indicates that there has been prolonged ventilation and an increased risk of complications associated with acute respiratory failure. In patients with respiratory failure, clinical outcomes may improve if diaphragmatic atrophy and function are preserved in the early stages of critical illness.[4] It has been demonstrated that MV-related dysfunction of the diaphragm and respiratory muscles contributes to prolonged MV, weaning failure, persistent dyspnea, and prolonged stay in the intensive care unit (ICU).[5,6]

Diaphragmatic ultrasound (US), which is a bedside, noninvasive and reproducible method, may be a good option for monitoring diaphragmatic structure abnormalities and function in the ICU. It provides reliable measures of diaphragm excursion (DE), diaphragm thickness (DT) and diaphragm thickening fraction (DTF). Tissue Doppler imaging (TDI) is a commonly used method that measures the velocity of moving tissues; however, there is limited research on diaphragmatic TDI. Soilemezi et al. found that the contraction and relaxation velocities of diaphragmatic tissue were significantly lower in the ICU patients compared to the healthy volunteers, which is correlated with a failure to wean. The use of diaphragmatic ultrasound and TDI may early detect diaphragmatic dysfunction and prompt intervention in the ICU.[7]

Inspiratory muscle training (IMT) provides resistance to the respiratory muscles independent of inspiratory flow, based on threshold pressure load and maximum inspiratory pressure.[8,9] It facilitates weaning in mechanically ventilated patients, including those with weaning difficulties such as chronic obstructive pulmonary disease (COPD), by increasing their respiratory muscles' strength and endurance.[10] Inspiratory muscle training can be used as a feasible, well-tolerated clinical strategy to prevent diaphragmatic dysfunction and improve short- and the long-term clinical outcomes.

Objectives

The main objective of our study was to evaluate the effect of IMT on diaphragmatic structure and function in respiratory ICU patients after extubation. This study is the first to investigate the ultrasonographic effects of IMT on diaphragm structure and function following extubation. It is anticipated that this research can make significant contributions to the relevant literature and can potentially serve as a pioneering reference for future studies in this context.

Materials and methods

Study design

A prospective randomized controlled trial (RCT) was conducted as a single-center, single-blind study at a university hospital in the Respiratory ICU. The study was approved by the Marmara University Faculty of Medicine Clinical Research Ethics Committee (protocol No. 09.2020.976) and was performed in accordance with the Declaration of Helsinki. The study was registered as a prospective RCT at clinicaltrails.gov (registration No. NCT05303623).

The study involved 3 groups: IMT group, conventional physiotherapy (CP) group and healthy controls (HCs). Randomizer.org was used to assign patients to either the IMT or the CP group after confirmation of eligibility and baseline assessments.

Participants

Healthy controls aged 18–80 years with a body mass index (BMI) less than 40 kg/m^2 and without any chronic disease or ongoing treatment were included in the study. For the patient group, the inclusion criteria were as follows: age 18–80 years, mechanically ventilated for more than 2 days, Sedation Agitation Score = 4, and hemodynamic stable (heart rate <140 beats/min and stable blood pressure). Patients with severe arrhythmia, congestive heart failure, unstable ischemic heart disease, lack of alertness and cooperation, chest wall trauma and/or deformity, progressive neuromuscular diseases, excessive secretion (requiring aspiration every hour), continuous use of sedative drugs, or home MV before ICU admission were excluded.

Intervention

The CP group received CP, including breathing exercises, thoracic expansion exercises, coughing and gradual mobilization, once a day for 5 days after extubation. In addition to CP, the IMT group underwent IMT while in a high sitting position under the supervision of a physiotherapist. Inspiratory muscle training was performed at 30% of maximum inspiratory pressure (MIP) for 5 days, twice a day, consisting of 30 breaths, 4 sets, 6–8 breaths per set, and 2-min rest between sets using a threshold-loaded PowerBreathe Medic Plus® device (PowerBreathe International Ltd. Southam, UK). The intervention was

terminated if hemodynamic instability occurred before, during or after treatment. Inspiratory muscle training was performed on HCs using the same protocol as that used for the IMT group.

Measurements

Characteristics of patients

Demographic details such as age, gender, body weight, height, and BMI (Table 1), as well as clinical features of patients such as the primary diagnosis upon admission to ICU, duration of MV, length of stay in the ICU, total hospitalization time, medications, comorbidities, and medical history were included (Table 2). The severity of the illness was assessed using the APACHE II within 24 h of ICU admission.

Ultrasonographic assessments

Ultrasonographic assessments were performed on each patient's right hemidiaphragm while lying on the bed at 20–30°. Examinations were performed using a Mindray ultrasound device (Mindray. Shenzhen, China). Diaphragm thickness, excursion and diaphragm tissue Doppler imaging are presented in Fig. 1. The Supplementary files provide further information regarding the ultrasonographic measurements.

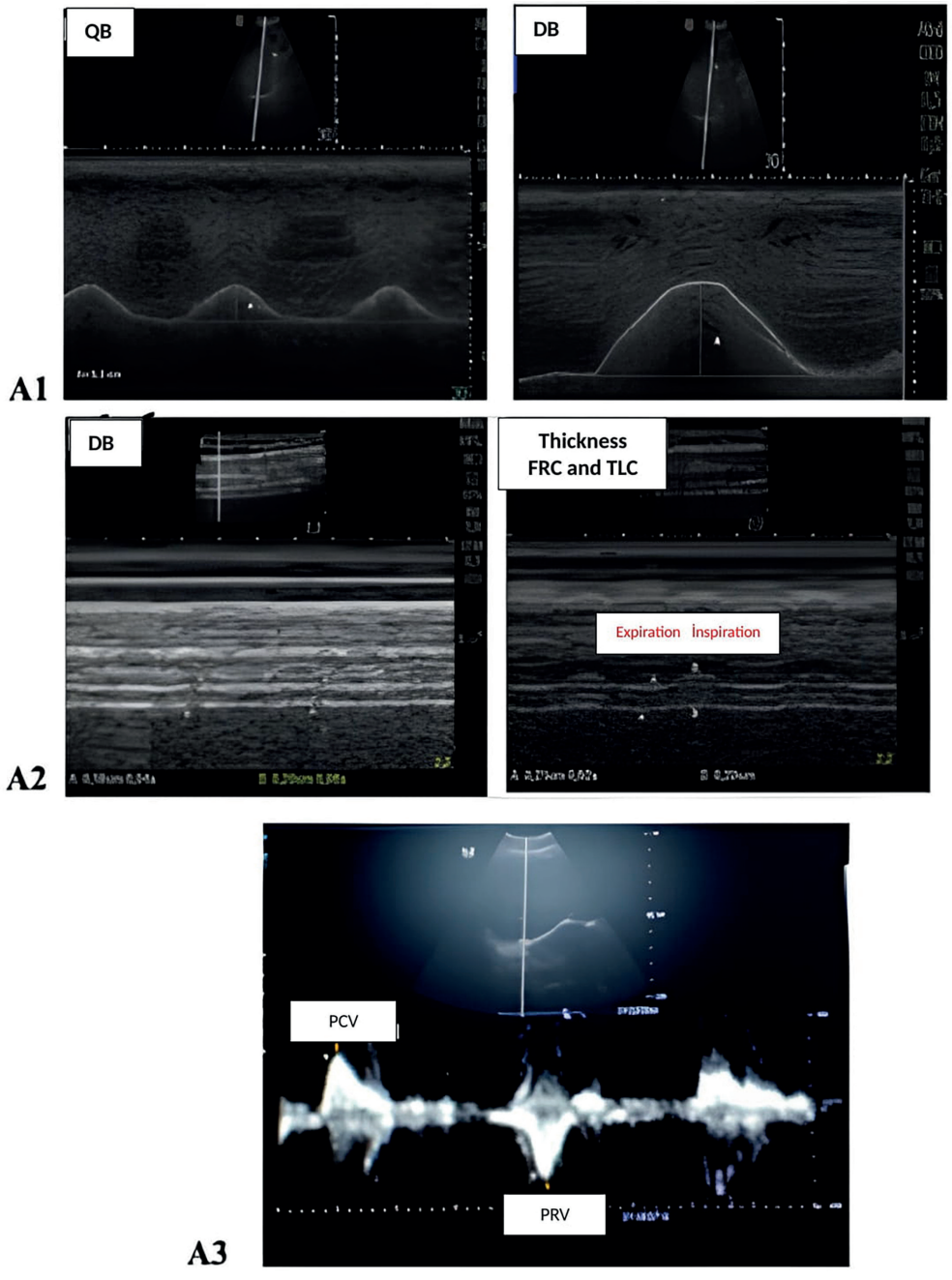

Fig. 1. Panel of diaphragm ultrasound images

A1. M-mode ultrasonography of the right hemidiaphragm showing mobility during quiet breathing (QB) and deep breathing (DB); A2. M-mode ultrasonography of the right hemidiaphragm showing normal end-inspiratory thickness (FRC), normal thickening (TF) and maximal thickness (TLC); A3. Tissue Doppler imaging of right diaphragm showing peak contraction velocity (PCV) and peak relaxation velocity (PRV)

Table 1. Demographics of study participants

Characteristics	CP (n = 10)	IMT (n = 10)	Healthy controls (n = 10)	p-value[a]	Post hoc test p-value[b] G1–G2 G1–G3 G2–G3
Age [year]; M ±SD	62.8 ±16.37	64.10 ±8.21	51.8 ±11.4	0.081[a] F = 2.762	–
Gender [M/F]	8/2	8/2	5/5	0.240[a] X^2 = 2.857	–
Weight [kg], M ±SD	66 ±18.5	79.2 ±8.5	82.5 ±15.5	0.045[a] F = 3.493	0.168 0.057 0.999
Height [cm], M ±SD	165.4 ±6.2	166.1 ±6.7	168.2 ±9.06	0.685[a] F = 0.383	–
BMI [kg/cm^2], M ±SD	23.5 ±5.31	28.93 ±4.4	28.8 ±3.6	0.017[a] F = 4.744	0.035 0.042 0.999

CP – conventional physiotherapy; IMT – inspiratory muscle training; M – mean; SD – standard deviation; BMI – body mass index; [a] one way analysis of variance (ANOVA) test; [b] Tukey's honest significant difference and Games–Howell post hoc tests were performed following ANOVA; G1 – CP group; G2 – IMT group; G3 – healthy control.

Table 2. Baseline characteristics of patients

Characteristics		CP (n = 10)	IMT (n = 10)	p-value
Diagnosis, n (%)	COPD yes/no	9 (90%)/1 (10%)	6 (60%)/4 (40%)	0.051[a] X^2 = 1.053
	Toxic gas inhalation yes/no	1 (10%)/9 (90%)	0 (0%)/10 (100%)	0.305[a] X^2 = 1.574
	Postoperative pneumonia yes/no	0 (0%)/10 (100%)	1 (10%)/9 (90%)	0.531[a] X^2 = 2.102
	Cardiogenic pulmonary edema yes/no	0 (0%)/10 (100%)	1 (10%)/9 (90%)	0.531[a] X^2 = 2.102
	Interstitial lung disease yes/no	0 (0%)/10 (100%)	1 (10%)/9 (90%)	0.763[a] X^2 = 0.001
	Metabolic acidosis yes/no	1 (10%)/9 (90%)	0 (0%)/10 (100)%	0.305[a] X^2 = 1.574
Comorbidities, n (%)	Hypertension yes/no	4 (40%)/6 (60%)	3 (30%)/7 (70%)	0.639[a] X^2 = 0.220
	Diabetus mellitus yes/no	8 (80%)/2 (20%)	4 (40%)/6 (60%)	0.003[a] X^2 = 9.899
	Atrial fibrillation yes/no	1 (10%)/9 (90%)	1 (10%)/9 (90%)	0.763[a] X^2 = 0.001
	Obstructive sleep apnea yes/no	0 (%0)/10 (100%)	1 (10%)/9 (90%)	0.305[a] X^2 = 1.606
	Sequela of tuberculosis yes/no	0 (%0)/10 (100%)	1 (10%)/9 (90%)	0.305[a] X^2 = 1.606
	Anxiety disorder yes/no	0 (%0)/10 (100%)	1 (10%)/9 (90%)	0.305[a] X^2 = 1.606
	Chronic heart failure yes/no	0 (%0)/10 (100%)	1 (10%)/9 (90%)	0.136[a] X^2 = 2.224
Lenght of stay in ICU [days], M ±SD		7.6 ±2.80	7.3 ±2.00	0.910[b] t = 0.278
Lenght of stay in hospital [days], M ±SD		11.7 ±1.80	12.5 ±5.00	0.999[b] t = –0.481
Intubation time [days], M ±SD		4.5 ±1.60	3.9 ±1.20	0.390[b] t = 0.931
APACHE II scores, M ±SD		26.8 ±4.46	27.0 ±4.4	0.922[b] t = –0.099

CP – conventional physiotherapy; IMT – inspiratory muscle training; COPD – chronic obstructive pulmonary disease; M – mean; SD – standard deviation; APACHE II – acute physiology and chronic health evaluation score; ICU – intensive care unit; [a] – Pearson's X^2 test; [b] – independent sample t-test.

B and M mode ultrasonographic evaluation of the diaphragm

B-Mode US at the zone of apposition was used to demonstrate diaphragm muscle between 2 parallel echogenic lines, the diaphragmatic pleura and peritoneal fascia, as previously described.[11] A 10 MHz linear array transducer was positioned perpendicular to the chest wall near the mid-axillary line between the 8th and 11th intercostal spaces. Diaphragm thickness (DT) was measured in the M mode at the end of inspiration (Td_i) and at the end of expiration (Td_e). The diaphragm thickening fraction (DTF) was calculated according to the formula (Eq. 1)

$$DTF = \frac{(\text{end-inspiratory thickness} - \text{end-expiratory thickness})}{\text{end-expiratory thickness}} \times 100\% \quad (1)$$

The subcostal approach was used to evaluate diaphragm excursion (DE). A 5 MHz convex probe was positioned below and parallel to the costal margin between the anterior axillary line (AAL) and the midclavicular line. The diaphragm was visualized with B-Mode US and the DE was calculated with M-Mode US; a single investigator analyzed all US recordings blinded to the clinical outcomes and the patient's research group. All ultrasound measurements were repeated on at least 3 separate breaths and the highest value was recorded.

Tissue Doppler imaging

Tissue Doppler imaging (TDI) views were obtained using a convex probe with a frequency of 2.7 MHz, placed in the subcostal area between the midclavicular and anterior axillary lines. The probe was angled to ensure that the ultrasound waves reached the diaphragm as perpendicular as possible. A sample volume of 5.0 mm and velocity scale of 20.6 cm/s were selected. Two parameters were measured on each TDI waveform: peak contraction velocity (PCV), defined as the maximal diaphragmatic velocity during contraction, measured in cm/s; and peak relaxation velocity (PRV), defined as the maximal diaphragmatic velocity during relaxation, measured in cm/s. Participants were instructed to perform deep breathing during TDI measurements after 8–10 tidal breaths. The highest value for the 3 deep breaths was recorded.

Maximum inspiratory pressure

Maximum inspiratory pressure was measured using the method described using Marini et al. with the MicroRPM® device (Care Fusion, Wokingham, UK), which uses a one-way expiratory valve to selectively allow exhalation while blocking inspiration.[12] Patients were positioned in a high sitting position and performed 3 inspiratory maneuvers. The highest value was recorded. There should be less than 10% or 10 cm H_2O difference between the best measured MIP values.

Physical assessment

Limb muscle strength was assessed using the Medical Research Council (MRC) score which involves evaluating the manual muscle strength of 6 muscle groups bilaterally. Each muscle group was scored on a scale of 0 to 5, with 0 indicating no contraction and 5 indicating full-force contraction. The muscle groups evaluated were shoulder abductors, elbow flexors, wrist extensors, hip flexors, knee extensors, and ankle dorsiflexors. The maximum possible MRC score was 60, and an MRC score of less than 48 was characteristic of ICU-acquired weakness. The Physical Function in Intensive Care Test (PFIT) was performed according to Skinner et al.[13] The PFIT included sit-to-stand with assistance, marching in place, and evaluation of shoulder flexion and knee extension strength. The number of steps taken while marching in place and the time taken to complete each component were recorded. The PFIT score ranges from 0 to 12.

Statistical analyses

IBM SPSS for Windows 22.0 (IBM Corp., Armonk, USA) was used to analyze the data. Numbers, percentages, means (M), and standard deviations (SD) were used for descriptive statistics. Shapiro–Wilk test was used to assess the distribution pattern of the variables (Supplementary data). Results are expressed as mean ± standard deviation (M ±SD) for normal distribution or median (Q1–Q3) for non-normal distribution. Differences between the ratios of categorical variables in the independent groups were analyzed using Pearson χ^2 and Fisher's exact tests. Within group analyses, paired sample t-tests were used for data that followed a normal distribution before and after intervention, while the Wilcoxon signed rank test was used for data that did not follow a normal distribution. Between group analyses, the one-way analysis of variance (ANOVA) test was applied to normally distributed data. Homogeneity of variances in the one-way ANOVA test was assessed using the Levene's test (online supplement). For results where the Levene's test yielded $p < 0.05$, the Welch's test significance values were assessed. The Tukey's honest significant difference post hoc tests were performed following analysis of variance. The sample size with the G*Power 3.1.9.7 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower) program (the number of participants that should be included in each group for 80% power) was calculated as at least 10 people (effect size: 0.3, α:0.05).[14]

Results

A total of 122 patients and 11 HCs were recruited between September 2021 and December 2022. Twenty patients and 10 HCs were included in the analysis. A flowchart of the patient and HC subject selection process is presented in Fig. 2. Inspiratory muscle training group completed 95%

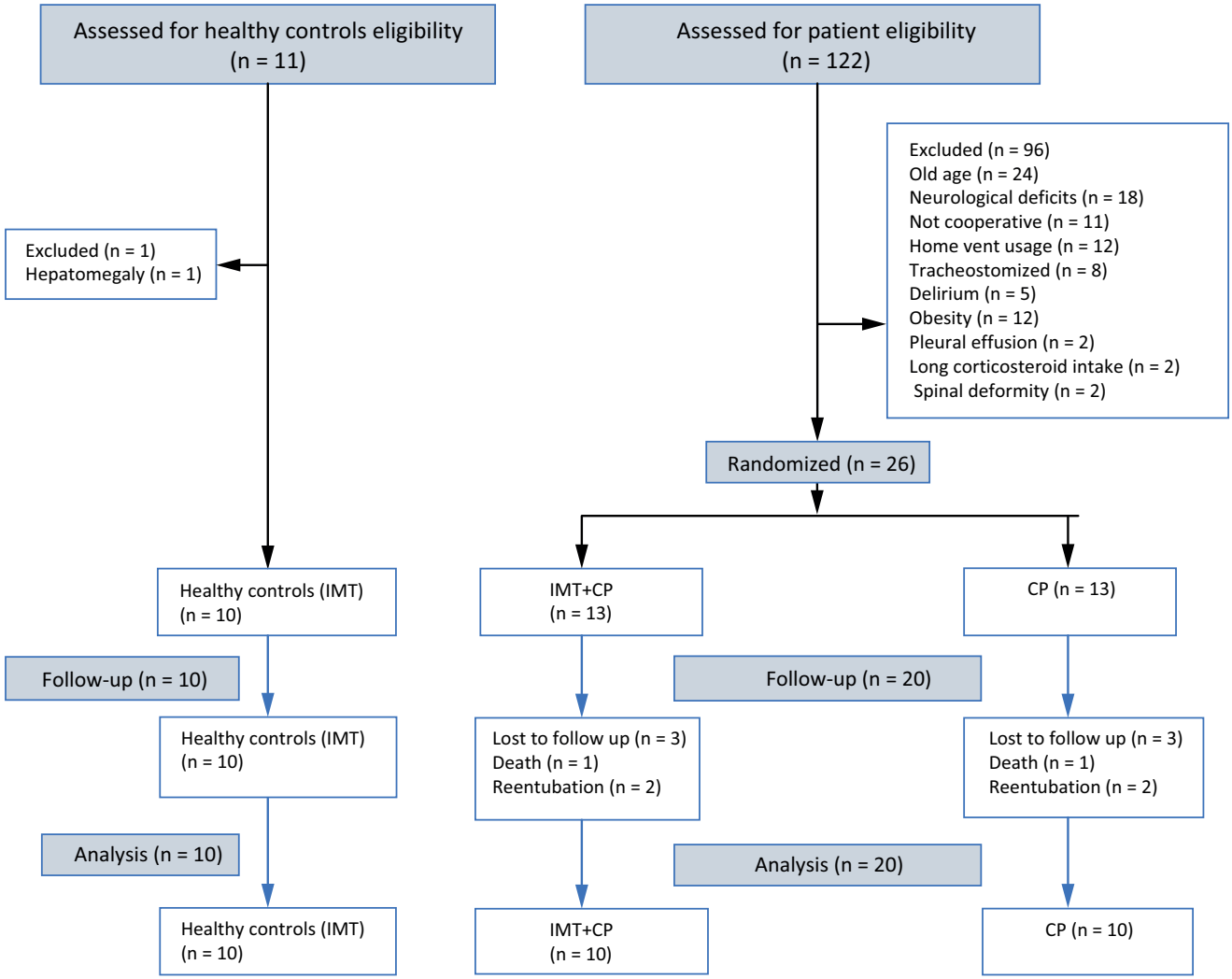

Fig. 2. Flowchart of the study

of planned IMT and physiotherapy sessions, CP group completed 80% of planned physiotherapy sessions, and HC group completed 95% of planned IMT sessions. The participants' characteristics are presented in Table 1. The weight and BMI of the HC group were significantly higher than of the CP group ($p = 0.045$). The IMT group had a significantly higher BMI than the CP group ($p = 0.017$). The baseline characteristics and features of patients were similar for most of the parameters, ICU stay duration ($p = 0.910$) and length of hospital stay ($p = 0.999$) as shown in Table 2. The effects of the intervention on diaphragm function in patients and HCs are presented in Table 3. Diaphragm excursion measurements did not significantly change pre- and post-intervention in the CP group ($p = 0.285$), but significantly increased in the HCs and IMT groups post-intervention compared to pre-intervention ($p = 0.005$ and $p = 0.005$, respectively). The increase in PCV measurements was significantly higher in the IMT and HCs than in the CP group ($p = 0.028$ and $p = 0.015$, respectively). There was a statistically significant difference in the change in PRV pre- and post-intervention between the IMT and HC groups ($p = 0.029$ and $p = 0.020$, respectively). The effects of intervention on MIP in patients and HCs are presented in Table 3. A statistically significant increase was found between pre- and post-intervention MIP in all groups (CP, $p = 0.044$; IMT, $p = 0.005$; and HCs, $p < 0.001$). Only the increase in predicted MIP% in the healthy group was statistically significant ($p < 0.001$). There was no significant difference in pre-intervention MRC and PFIT scores among CP and IMT groups. In the CP group, post-intervention MRC (51.2 ±6.61) and PFIT score (9.00 ±1.41) improved statistically compared to pre-intervention MRC score (47.8 ±7.33) and PFIT score (4.0 ±0.47; $p < 0.001$; $t = -5.667$, $p < 0.001$; $t = -10.607$). In the IMT group, post-intervention MRC (55.6 ±4.79) and PFIT score (10.30 ±2.45) improved statistically compared to pre-intervention MRC score (51.0 ±6.4) and PFIT score (4.80 ±1.38) ($p < 0.001$; $t = -5.438$, $p < 0.001$; $t = -11.524$).

Discussion

This study is the first to demonstrate that IMT can improve diaphragmatic function and MIP in mechanically

Table 3. Comparison of outcomes within groups

Outcomes	Outcomes and groups	Pre-mean	Post-mean	Estimated mean difference (post–pre) mean (95% CI)	Within group p-value
DE [cm]; median (Q1–Q3)	CP group	4.35 (4.97–3.53)	4.81 (3.70–6.08)	0.46 (–1.68–2.95)	0.285[a]; Z = –1.070
	IMT group	3.55 (3.06–4.60)	4.31 (3.77–5.16)	0.76 (0.06–1.98)	0.005[a]; Z = –2.805
	HC group	5.53 (5.38–5.99)	6.24 (5.48–6.57)	0.71 (0.05–1.21)	0.005[a]; Z = –2.803
Td_i [cm]; median (Q1–Q3)	CP group	0.24 (0.21–0.32)	0.25 (0.21–0.30)	0.01 (–0.18–0.07)	0.553[a]; Z = –593
	IMT group	0.28 (0.24–0.32)	0.28 (0.25–0.42)	0.00 (–0.03–0.47)	0.315[a]; Z = –323
	HC group	0.26 (0.24–0.31)	0.28 (0.26–0.32)	0.02 (0.00–0.06)	0.011[a]; Z = –2.555
Td_e [cm]; median (Q1–Q3)	CP group	0.18 (0.16–0.28)	0.20 (0.16–0.23)	0.02 (–0.10–0.02)	0.810[a]; Z = –240
	IMT group	0.22 (0.19–0.25)	0.24 (0.21–0.31)	0.02 (0.00–0.38)	0.200[a] Z = –679
	HC group	0.21 (0.20–0.22)	0.22 (0.21–0.24)	0.01 (–0.01–0.02)	0.058[a] Z = –1.897
DTF (%); M ±SD	CP group	27.63 ±10.6	28.56 ±14.41	–0,93 (–8.94–7.07)	0.798[b];t = –0.263
	IMT group	28.31 ±11.91	28.22 ±9.03	0.093 (–7.33–7.51)	0.978[b];t = 0.028
	HC group	23.78 ±8.99	28.37 ±8.03	–4.59 (–11.7–2.54)	0.179[b];t = –1.456
PCV [cm/s]; M ±SD	CP group	11.05 ±2.30	11.12 ±2.21	–0.07 (–1.29–1.14)	0.893[b]; t = –0.139
	IMT group	9.09 ±2.21	10.02 ±2.60	–0.927 (–1.72––0.12)	0.028[b]; t = –2.625
	HC group	11.02 ±2.39	14.45 ±2.70	–0.738 (–1.29––0.18)	0.015[b]; t = –3.015
PRV [cm/s]; M ±SD	CP group	11.5 ±2.63	10.68 ±1.81	0.81 (–0.68–2.32)	0.251[b]; t = 1.227
	IMT group	11.30 ±3.67	12.9 ±3.43	–1.59 (–2.97––0.19)	0.029[b]; t = –2.587
	HC group	12.90 ±2.98	14.45 ±2.72	–1.55 (–2.79––0.30)	0.020[b]; t = –2.825
MIP; M ±SD	CP group	33.6 ±10.50	40.5 ±14.00	6.9 (–13.81–0.015)	0.044[b]; t = –2.257
	IMT group	36.4 ±13.11	52.5 ±19.0	–16.1 (–21.83––10.36)	0.005[b]; t = –6.348
	HC group	73.1 ±16.0	86.7 ±20.06	–13.6 (19.25––7.945)	<0.001[b]; t = 5.440
MIP% predicted; M ±SD	CP group	34.30 ±10.3	35.55 ±20.9	–1.24 (–15–13)	0.510[b]; t = –0.200
	IMT group	38.51 ±12.84	52.7 ±27.7	–14.18 (–30.7–2.35)	0.084[b]; t = –1.940
	HC group	76.58 ±14.7	91.48 ±20.6	–14.90 (–20.72–9.07)	<0.001[b]; t = –5.791

95% CI – 95% confidence interval; HC – healthy control; CP – conventional physiotherapy; IMT – inspiratory muscle training; DE – diaphragm excursion; Td_i – diaphragm thickness at the end of inspiration; Td_e – diaphragm thickness at end of expiration; DTF – diaphragm thickness fraction; PCV – peak contraction velocity; PRV – peak relaxation velocity; MIP – maximum inspiratory pressure; M – mean; SD – standard deviation; [a] Wilcoxon signed rank test; [b] paired sample t-test.

ventilated patients during the post-extubation period. A study reported that IMT did not reduce hospitalization time or ICU duration, despite being used in conjunction with CP.[5] The diaphragm excursion of HCs was higher than that of the IMT and CP groups at baseline, suggesting that MV negatively affected diaphragm contractility. Our findings also showed that both the IMT group and HCs had greater improvements in diaphragm excursion than those who did not receive IMT. A normative study conducted on healthy volunteers found that the average diaphragmatic excursion was 5.96 cm, with variation according to age and gender.[15] Furthermore, previous studies have shown that diaphragmatic excursion is associated with disease severity, functional capacity and respiratory function in COPD patients.[16,17] These findings suggest that improving diaphragmatic excursion through IMT may restore respiratory function and facilitate weaning in mechanically ventilated patients.

Research has shown that the loss of diaphragm muscle thickness is particularly significant in the first 3 days after MV, even after just 24 h.[18] While numerous studies have examined diaphragm atrophy and function using ultrasonography during MV, there is still a lack of understanding regarding the specific changes in the structure and function of the diaphragm after extubation. Further research is required to fully elucidate the effects of MV on the diaphragm and its recovery after extubation. Our study revealed that the diaphragmatic thickness in the CP group decreased after 5 days of extubation, whereas the diaphragmatic thickness in the group that received IMT increased. Although only the HCs showed a statistically significant increase, the slight increase in the IMT group compared to the decreased diaphragmatic thickness in the control group is an encouraging finding that suggests that IMT may help prevent ongoing diaphragmatic atrophy and maintain respiratory function after extubation. Further research is needed to determine the clinical significance of these findings, including any potential effects on patient outcomes or respiratory function.

A previous research found that there was no significant correlation between diaphragmatic thickness and extubation success in mechanically ventilated patients.

However, they found that the DTF was a positive predictor of extubation success and was strongly associated with the duration of MV and length of stay in the ICU.[19] Some studies have reported no significant differences in DTF between COPD patients and HCs due to increased airway resistance and air trapping in patients with COPD.[20,21] In this study, there were no significant changes in DTF in any group after the intervention, possibly due to the majority of patients in the Respiratory ICU had increased airway resistance and air trapping.

A study assessed real-time diaphragm tissue velocity in healthy individuals and ICU patients using TDI. Pioneer research reported that the PCV and PRV measurements were similar in healthy individuals. However, the TDI patterns of successfully weaned patients differed from those who failed to wean.[8] In obstructive pulmonary diseases, decreased diaphragm displacement due to air trapping and increased respiratory muscle load can lead to loss of diaphragmatic relaxation velocity. This may result in a mechanical disadvantage and delay in expiration by the functional residual capacity. Diaphragm perfusion occurs primarily during relaxation, and it may be inversely related to diaphragm fatigue as the diaphragm rapidly reaches the optimal length when perfused.[22] This study is the only RCT that evaluated the effect of physiotherapy intervention on the velocity of the diaphragm using TDI. We found that IMT increased the PCV, and the PRV decreased in the CP group, whereas it increased in the IMT groups. Supplementary Fig. 1 presents the change in diaphragm peak contraction and peak relaxation velocities of the groups pre- and post-intervention. Our study suggests that IMT may be a feasible alternative to prevent the possible loss of diaphragmatic contraction and relaxation velocities during the weaning process.

Several studies have previously reported an increase in MIP in mechanically ventilated patients who received IMT.[23–25] Papadopoulos et al. reported that MIP improved post-extubation in patients who received chest physiotherapy, which included breathing exercises and bronchial hygiene techniques, compared with those who did not receive chest physiotherapy.[26] Our findings are consistent with prior research in this regard. Similarity-based learning may have contributed to these improvements. A study that applied 50% MIP intensity to patients during weaning, along with T-tube trials, reported a decrease in the MIP value in the intervention group.[27] In contrast, our study observed an increase in MIP without any complications during the IMT sessions, indicating the appropriateness of our training load. In healthy individuals, IMT provides an 8–45% improvement in working MIP, whereas shorter training provides lower improvement in MIP. This phenomenon may be attributed to the dose–response relationship, as in skeletal muscles.[28,29] It is hypothesized that the comparable progress in physical function and muscle strength in both the CP and IMT groups can be attributed to the early mobilization and physiotherapy approaches. In a previous RCT, 2 weeks of IMT application during the post-extubation period did not result in reduced hospitalization and reintubation rates.[4] These findings appear to be in agreement with our study. Future multicenter studies with larger patient populations are warranted to further investigate this issue. Another important point that needs to be highlighted is the increasing significance of point-of-care ultrasound (POCUS) in physiotherapeutic decision-making and management.[30] In this context, our research may provide valuable clinical insights for future studies in this field.

Limitations

One of the limitations of this study was the exclusion of 6 patients due to 4 reintubations and 2 deaths, which resulted in a loss of data. However, these events were not related to the intervention. Furthermore, no complications were observed during either IMT or CP sessions. Although our study was conducted under changing conditions during the pandemic period, it focused on the effects of IMT on the diaphragm in the early post-extubation period. In our study, the intervention was applied to intensive care patients and the HC group, and the changes in diaphragmatic functions were observed pre- and post-intervention. However, future studies with different patient groups or longer follow-up periods may provide different perspectives by providing more information about the effectiveness of the IMT on diaphragm function. Another limitation of our study is that we did not follow-up on the primary outcomes beyond 5 days after extubation. It is possible that some patients may require continued IMT beyond the initial 5-day training period, especially since most did not achieve close to full recovery in diaphragm function and predicted MIP% values during the study.

Conclusions

This study demonstrates that IMT initiated on the day of extubation and continued 5 days significantly improves the functional parameters of the diaphragm. We have concluded that IMT applied after extubation may serve as a tool to prevent and facilitate the recovery of diaphragmatic function. Our study results highlight the need for more effective post-extubation care protocols, and suggest that IMT should be considered as a respiratory ICU clinical routine intervention to support the post-extubation process in mechanically ventilated patients.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10066482. The package includes the following files:

Supplementary Fig. 1. Changes in diaphragm excursion, peak contraction and relaxation velocity within groups.

Supplementary File 1. Radiological assessments.

Supplementary Table 1. Normality test and Levene's assumptions.

Supplementary Table 2. Pre- and post-intervention outcomes (95% CI).

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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References

1. Moodie L, Reeve J, Elkins M. Inspiratory muscle training increases inspiratory muscle strength in patients weaning from mechanical ventilation: A systematic review. *J Physiother*. 2011;57(4):213–221. doi:10.1016/S1836-9553(11)70051-0.
2. Levine S, Nguyen T, Taylor N, et al. Rapid disuse atrophy of diaphragm fibers in mechanically ventilated humans. *N Engl J Med*. 2008; 358(13):1327–1335. doi:10.1056/NEJMoa070447
3. Bissett BM, Leditschke IA, Neeman T, et al. Inspiratory muscle training to enhance recovery from mechanical ventilation: A randomised trial. *Thorax*. 2016;71(9):812–819. doi:10.1136/thoraxjnl-2016-208279
4. Goligher EC, Dres M, Fan E, et al. Mechanical ventilation-induced diaphragm atrophy strongly impacts clinical outcomes. *Am J Respir Crit Care Med*. 2018;197(2):204–213. doi:10.1164/rccm.201703-0536OC
5. Bissett B, Gosselink R, van Haren FMP. Respiratory muscle rehabilitation in patients with prolonged mechanical ventilation: A targeted approach. *Crit Care*. 2020;24(1):103. doi:10.1186/s13054-020-2783-0
6. Kim WY, Suh HJ, Hong SB, Koh Y, Lim CM. Diaphragm dysfunction assessed by ultrasonography: Influence on weaning from mechanical ventilation. *Crit Care Med*. 2011;39(12):2627–2630. doi:10.1097/CCM.0b013e3182266408
7. Soilemezi E, Savvidou S, Sotiriou P, Smyrniotis D, Tsagourias M, Matamis D. Tissue Doppler imaging of the diaphragm in healthy subjects and critically ill patients. *Am J Respir Crit Care Med*. 2020;202(7):1005–1012. doi:10.1164/rccm.201912-2341OC
8. Moodie LH, Reeve JC, Vermeulen N, Elkins MR. Inspiratory muscle training to facilitate weaning from mechanical ventilation: Protocol for a systematic review. *BMC Res Notes*. 2011;4:283. doi:10.1186/1756-0500-4-283
9. Aldrich TK, Karpel JP. Inspiratory muscle resistive training in respiratory failure. *Am Rev Respir Dis*. 1985;131(3):461–462. doi:10.1164/arrd.1985.131.3.461
10. Elkins M, Dentice R. Inspiratory muscle training facilitates weaning from mechanical ventilation among patients in the intensive care unit: A systematic review. *J Physiother*. 2015;61(3):125–134. doi:10.1016/j.jphys.2015.05.016
11. Matamis D, Soilemezi E, Tsagourias M, et al. Sonographic evaluation of the diaphragm in critically ill patients: Technique and clinical applications. *Intensive Care Med*. 2013;39(5):801–810. doi:10.1007/s00134-013-2823-1
12. Marini JJ, Smith TC, Lamb V. Estimation of inspiratory muscle strength in mechanically ventilated patients: The measurement of maximal inspiratory pressure. *J Crit Care*. 1986;1(1):32–38. doi:10.1016/S0883-9441(86)80114-9
13. Skinner EH, Berney S, Warrillow S, Denehy L. Development of a physical function outcome measure (PFIT) and a pilot exercise training protocol for use in intensive care. *Crit Care Resusc*. 2009;11(2):110–115. PMID:19485874
14. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007;39(2):175–191. doi:10.3758/bf03193146
15. Scarlata S, Mancini D, Laudisio A, Raffaele AI. Reproducibility of diaphragmatic thickness measured by M-mode ultrasonography in healthy volunteers. *Respir Physiol Neurobiol*. 2019;260:58–62. doi:10.1016/j.resp.2018.12.004
16. Shiraishi M, Higashimoto Y, Sugiya R, et al. Diaphragmatic excursion is correlated with the improvement in exercise tolerance after pulmonary rehabilitation in patients with chronic obstructive pulmonary disease. *Respir Res*. 2021;22(1):271. doi:10.1186/s12931-021-01870-1
17. Güneş S, Genç A, Aytür YK, et al. Effects of pulmonary rehabilitation on diaphragm thickness and contractility in patients with chronic obstructive pulmonary disease. *Turk J Med Sci*. 2022;52(1):89–96. doi:10.3906/sag-2105-345
18. Schepens T, Verbrugghe W, Dams K, et al. The course of diaphragm atrophy in ventilated patients assessed with ultrasound: A longitudinal cohort study. *Crit Care*. 2015;19:422. doi:10.1186/s13054-015-1141-0
19. Dubé BP, Dres M, Mayaux J, et al. Ultrasound evaluation of diaphragm function in mechanically ventilated patients: Comparison to phrenic stimulation and prognostic implications. *Thorax*. 2017;72(9):811–818. doi:10.1136/thoraxjnl-2016-209459
20. Baria MR, Shahgholi L, Sorenson EJ, et al. B-mode ultrasound assessment of diaphragm structure and function in patients with COPD. *Chest*. 2014;146(3):680–685. doi:10.1378/chest.13-2306
21. Eryüksel E, Cimşit C, Bekir M, et al. Diaphragmatic thickness fraction in subjects at high-risk for COPD exacerbations. *Respir Care*. 2017;62(12):1565–1570. doi:10.4187/respcare.05646
22. Hussain SN, Roussos C, Magder S. Effects of tension, duty cycle, and arterial pressure on diaphragmatic blood flow in dogs. *J Appl Physiol (1985)*. 1989;66(2):968–976. doi:10.1152/jappl.1989.66.2.968
23. Martin AD, Smith BK, Davenport PD, et al. Inspiratory muscle strength training improves weaning outcome in failure to wean patients: A randomized trial. *Crit Care*. 2011;15(2):R84. doi:10.1186/cc10081
24. Dixit A, Prakash S. Effects of threshold inspiratory muscle training versus conventional physiotherapy on the weaning period of mechanically ventilated patients: A comparative study. *Int J Physiother Res*. 2014;2:424–428. https://www.ijmhr.org/ijpr_articles_vol2_2/IJPR-2014-609.pdf. Accessed December 13, 2023.
25. Condessa RL, Brauner JS, Saul AL, Baptista M, Silva AC, Vieira SR. Inspiratory muscle training did not accelerate weaning from mechanical ventilation but did improve tidal volume and maximal respiratory pressures: A randomised trial. *J Physiother*. 2013;59(2):101–107. doi:10.1016/S1836-9553(13)70162-0
26. Papadopoulos ES, Kyprianou TH, Nanas S. Chest physiotherapy is effective in the management of intensive care unit patients immediately after extubation. *Crit Care*. 2005;9(Suppl 1):P128. doi:10.1186/cc3191
27. Shimizu JM, Manzano RM, Quitério RJ, et al. Determinant factors for mortality of patients receiving mechanical ventilation and effects of a protocol muscle training in weaning. *Man Ther Posturology Rehabil J*. 2014;12:180. doi:10.17784/mtprehabjournal.2014.12.180
28. Sonetti DA, Wetter TJ, Pegelow DF, Dempsey JA. Effects of respiratory muscle training versus placebo on endurance exercise performance. *Respir Physiol*. 2001;127(2–3):185–199. doi:10.1016/s0034-5687(01)00250-x
29. Sheel AW. Respiratory muscle training in healthy individuals: Physiological rationale and implications for exercise performance. *Sports Med*. 2002;32(9):567–581. doi:10.2165/00007256-200232090-00003
30. Smith M, Hayward S, Innes S. A proposed framework for point of care lung ultrasound by respiratory physiotherapists: Scope of practice, education and governance. *Ultrasound J*. 2022;14(1):24. doi:10.1186/s13089-022-00266-6

Analysis of aerosol generation during Er:YAG laser-assisted caries treatment: A randomized clinical trial

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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. Maintaining biosafety in dental practice involves the effective elimination of aerosols produced during dental treatment.

Objectives. To assess the quantity of aerosols and aerobic bacteria in the air during the treatment of caries.

Materials and methods. The study involved 60 patients with a total of 60 molar teeth (n = 60) in the mandible who were divided into 2 groups based on caries treatment method. Group 1 (G1, n = 30) received treatment with a conventional dental turbine (W&H Synea TA-98LC; W&H, Bürmoos, Austria), while group 2 (G2, n = 30) underwent treatment with an Er:YAG (erbium-doped yttrium aluminium garnet) laser (LightWalker, Fotona, Slovenia). Measurements of aerosol particles between 0.3 µm and 10.0 µm near the operator's mouth were taken using the PC200 laser particle counter (Trotec GmbH, Schwerin, Germany). The number of aerobic bacteria in the air was determined using 60 Petri plates with a microbiological medium (Columbia agar with 5% sheep blood) and the sedimentation method. A control group (G3) was established to measure initial aerosol levels and initial total number of bacteria colony-forming units (CFUs) before each treatment.

Results. In G1 (dental turbine), the median value of aerosol particles was 57,021 (42,564–67,568), while in G2 (Er:YAG laser), it was significantly lower at 33,318 (28,463–35,484) ($p < 0.001$). The median total bacteria count per cubic meter of air in G1 (conventional dental turbine + high volume evacuator (HVE)), G2 (Er:YAG laser + HVE) and G3 (control group before caries treatment) were 734 (420–988), 158 (96–288) and 48 (32–74), respectively, with a statistically significant difference between the groups ($p < 0.001$).

Conclusions. The use of Er:YAG laser during caries treatment resulted in a 41.6% reduction in aerosol amounts and a 78.5% decrease in the total bacterial count (TBC) compared to treatment with a dental turbine.

Key words: bacteria, dentistry, aerosols, biohazards

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Background

Aerosols, defined as minute suspended particles with prolonged air suspension characteristics, raise concerns in dental procedures due to their capacity to serve as potential vectors for transmitting infectious agents, including bacteria, viruses and fungi.[1–4] Dental aerosols exhibit a spectrum of particle sizes, typically spanning from submicron dimensions (<1 μm) to well over 100 μm in diameter, with the majority falling within the 10–30 μm range.[1,5] The utilization of a dental turbine in conservative dentistry procedures generates elevated concentrations of aerosols, which can be classified into 4 categories: respiratory aerosols, bioaerosols, water spray originating from rotary instruments, and a composite of bioaerosols and water spray characterized by significant dispersion potential.[6,7] It is important to emphasize that aerosol particles of smaller dimensions, particularly those with a diameter less than 5 μm, pose an increased risk as they have the potential to be inhaled into the finer recesses of the pulmonary system.[8] Conversely, particles exceeding 50 μm in diameter typically lead to the formation of splatter patterns and are not inhalable, rendering them less hazardous.[6,9] Therefore, the reduction of water spray quantities in various dental procedures holds paramount importance in mitigating the risk of viruses and bacteria transmission within the field of dentistry.[1,2,10]

The utilization of water–air spray cooling in dental rotary tools results in the emission of aerosols that contain various microorganisms, such as viruses, bacteria and fungi.[8,11,12] The imperative lies in the reduction of aerosol production during dental procedures, as it directly impacts the biological safety of the dental environment.[13] Our prior investigations, conducted both in vitro and in vivo, have demonstrated the generation of substantial quantities of potentially perilous aerosols during dental treatments, thereby elevating the risk of pathogen transmission.[1,2,14] The use of rotary dental devices, including both high-speed and low-speed ones, in addition to ultrasonic scalers, is associated with the most elevated levels of aerosol concentration and the potential for transmitting bacteria, viruses and fungi within the dental clinical setting.[5,8,15] Transmission pathways for pathogens during dental procedures involve aerosols containing saliva and blood, along with dental instruments and handpieces that may become contaminated.[11,16,17] Aerosols possess significant potential for carrying and disseminating viral and bacterial infections. Consequently, it is imperative for dental practitioners to acknowledge and mitigate the risk of microbial transmission, particularly when dealing with patients in the incubation phase of illness, those who are unaware of their condition, or those choosing to conceal their disease.[18–20]

Scientific literature has demonstrated the positive impact of lasers on various dental treatments as they eradicate viruses, bacteria and fungi.[21–23] Certain lasers create aerosols or induce cavitation effects in fluids.[24–28] Erbium lasers find extensive application in the dental field, including in procedures like caries removal, endodontic irrigation, periodontal and implant treatments, as well as orthodontic bracket and prosthetic crown removal.[28–36] There is a growing recognition of the potential health hazards linked to aerosols generated by laser procedures. These aerosols are often comprised of smaller particles in contrast to respiratory droplets, which differ significantly in size from viruses even though viruses are typically much smaller than the cells they target.[2,24] Existing literature confirms the use of lasers for virus inactivation in dentistry.[25,26] However, it is crucial to note that heat generated by lasers and vaporization of soft tissue may lead to smoke production, which may contain infectious particles.[24,25]

The composition of aerosols resulting from cavity preparation with high-speed turbines and erbium lasers is influenced by a complex interplay of factors related to the tools, tissues, settings, patient factors, operator techniques, environmental conditions, and the size and airborne characteristics of the particles generated.[1,2,37] Minimizing the overall volume of water spray and aerosols within dental facilities plays a crucial role in reducing the potential for airborne transmission of pathogens.[18,38] Dental procedures frequently employ suction devices, such as salivary ejectors and high-pressure evacuators, to eliminate aerosols. An in vitro study demonstrated that an enhanced suction device with an extended suction tip significantly reduced aerosol levels during in vitro caries removal. This study further established that broader suction systems outperformed conventional suction tips in aerosol removal. Furthermore, when compared to other instruments used for caries removal, an Er:YAG (erbium-doped yttrium aluminium garnet) laser coupled with a traditional evacuator generated fewer aerosols.[1,2] The current study aimed to determine whether these in vitro findings translate to in vivo benefits for patients treated in dental offices.

Objectives

The principal aim of this study was to determine whether there is a discernible difference in the volume of aerosols and bacterial count generated during caries treatment in a dental office when utilizing either a conventional dental turbine or an Er:YAG laser in a human model.

Materials and methods

Study design and setting

The study was a randomized controlled clinical trial. Prior to commencing, permission was granted by the Local Ethics Committee at the Faculty of Dentistry's (Wroclaw Medical University; approval No. KB-737/2021), and all participants provided informed consent in adherence to the Declaration of Helsinki. The clinical trial was registered with ClinicalTrials.gov (identifier: NCT05988359).

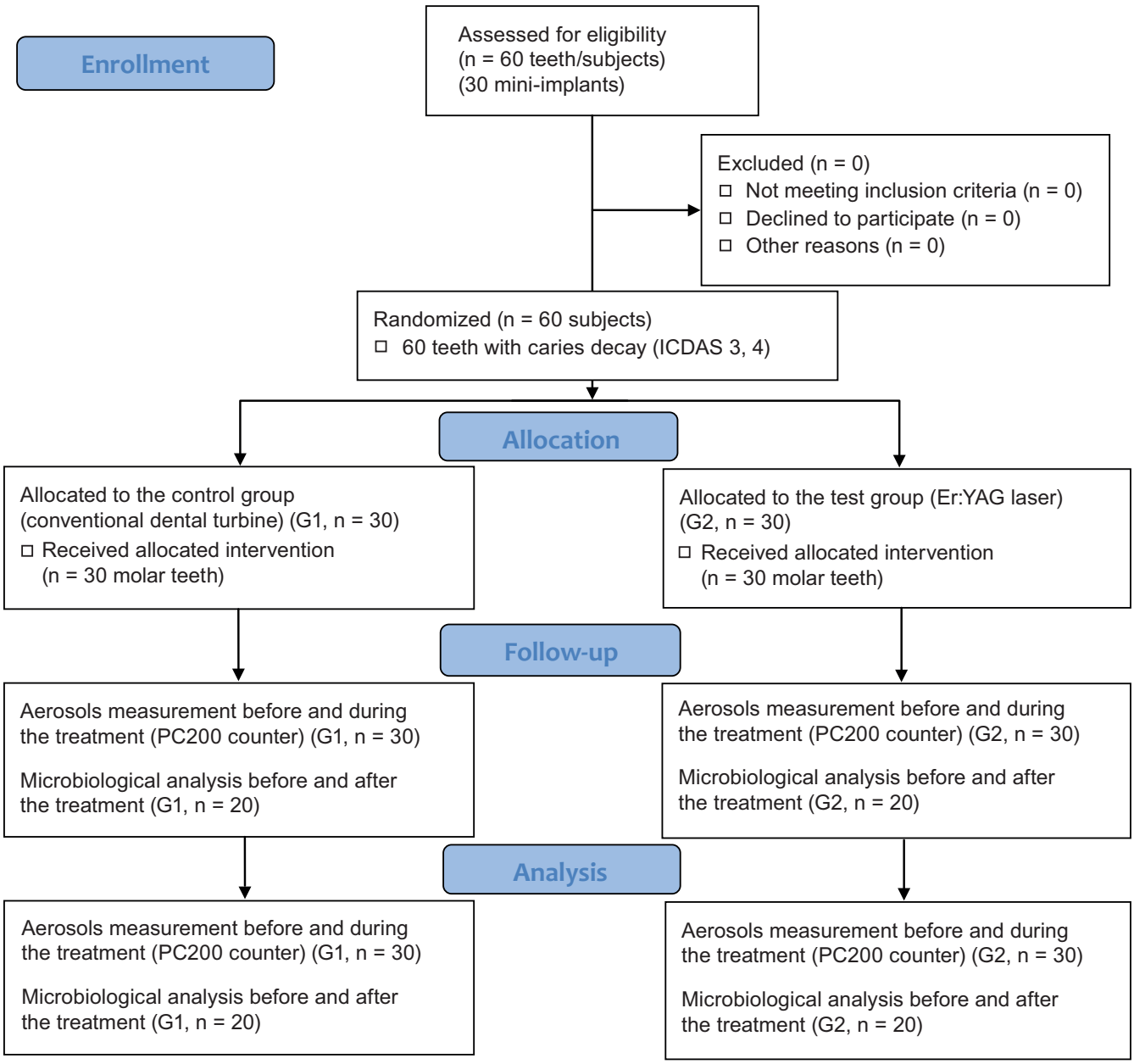

Fig. 1. Participants who were treated in accordance with the Consolidated Standards for Reporting Trials (CONSORT) 2010 guidelines

Participants

The study included a cohort of 60 participants comprised of 39 women and 21 men, all presenting with moderate caries decay as per the International Caries Detection and Assessment System (ICDAS 3 and 4) in a total of 60 molar teeth located in the mandibular region. The mean age of the participants was 29.4 ±5.8 years. The sample size for each group, consisting of 30 study participants, was determined using G*Power software (Kiel University, Kiel, Germany). This calculation was based on our prior research,[1,2] considering a significance level of 0.05, effect size (d) of 0.71, a 95% confidence interval (95% CI), and 85% statistical power. All participants were selected to meet specific inclusion criteria, which included having moderate caries decay (ICDAS 3 and 4), non-use of anti-inflammatory medications, non-smoking status, absence of systemic illnesses, no antibiotic usage within the last 2 months, no uncontrolled diabetes or untreated periodontal disease, and having received hygienist treatment beforehand (Fig. 1).

Data sources and measurement

Participant groups and procedure for treating caries

The study involved treating 60 patients with caries decay for a total of 60 molar teeth located in the mandible. The study participants were separated randomly

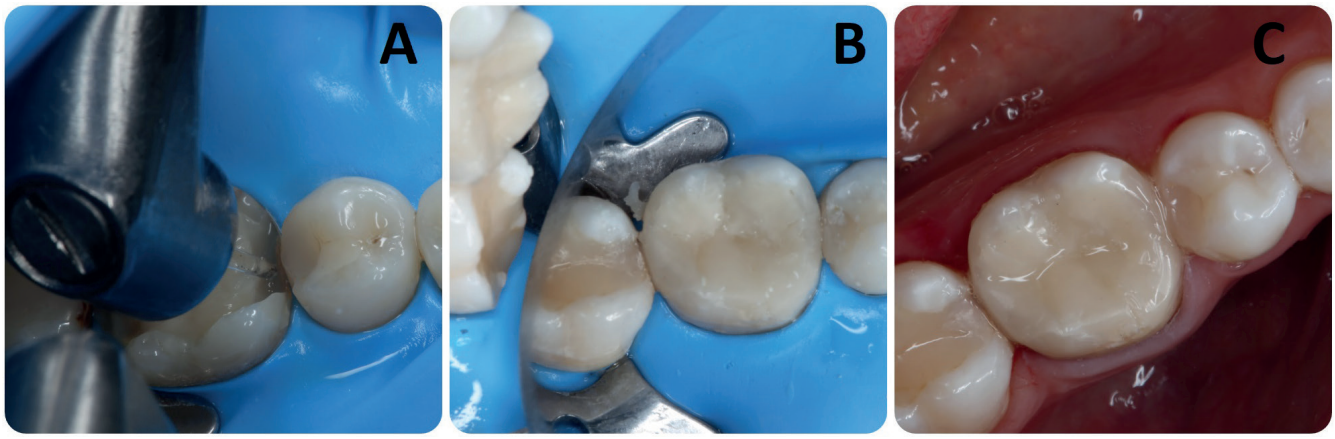

Fig. 2. Clinical pictures of caries treatment with Er:YAG laser. A. Er:YAG laser contra-angle handpiece (LightWalker, Fotona, Slovenia); B. Immediately after cavity restoration with composite material; C. Final tooth cavity restoration

into 2 groups utilizing the www.randomizer.org website. The 1st group, denoted as G1 (n = 30), underwent caries treatment utilizing a round diamond bur (#014) on a W&H Synea TA-98LC high-speed handpiece (W&H, Bürmoos, Austria). The handpiece operated at a speed of 200,000 revolutions per minute (rpm) with water cooling maintained at a flow rate of 30 mL/min. The mean time for caries preparation was 60 s.

The 2nd group, referred to as G2 (test, n = 30), underwent caries treatment using the Er:YAG laser contra-angle handpiece (LightWalker, Fotona, Slovenia) (Fig. 2). Laser parameters consisted of an energy setting of 300 mJ, frequency set at 20 Hz, power set at 6 W, energy density 38.2 J/cm^2, power density 764 W/cm^2, medium short pulse (MSP) mode (100 µs), and a tip diameter of 1 mm. Additionally, a water/air coolant at a flow rate of 30 mL/min was used. The mean time for caries preparation was 200 s. In both experimental groups, a standard evacuator EM19 EVO (Monoart® Euronda, Vicenza, Italy) was deployed for the removal of aerosols generated during caries treatment. Prior to each treatment session, baseline aerosol levels and the initial total bacterial count (TBC) expressed in colony-forming units (CFUs) were assessed, establishing the G3 control group.

Protocol for measuring aerosols

The assessment of aerosol particle quantities (primary outcome) at the examination sites was conducted using the PC200 counter (Trotec GmbH, Schwerin, Germany). The counter's nozzle was positioned 2 cm away from the operator's mouth. Utilizing the aerosols detector, measurements were taken for 6 distinct aerosol fractions, spanning diameters from 0.3 µm to 10.0 µm. The counter was initiated immediately prior to each treatment session and deactivated upon completion of caries treatment. The cumulative count of particles encompassing all fractions was tabulated, and subsequent mean values were compared between the groups.

Quantifying the concentration of airborne particulate matter using sedimentation analysis

The Koch sedimentation technique was employed to ascertain the total count of aerobic bacteria (secondary outcome) present within the air of a dental facility. Sixty Petri plates containing Columbia agar with 5% sheep blood medium were used for the quantification of aerobic bacteria. Twenty plates were exposed for a duration of 60 min before the commencement of treatment (designated as the control, G3, n = 20). Subsequently, they were closed promptly prior to the initiation of caries treatment. Forty additional plates were opened at the outset of the treatment, employing either a conventional turbine (G2, n = 20) or the Er:YAG laser (G1, n = 20), and were sealed after a 60-min interval. Each caries treatment procedure lasted less than 30 min. Measurements were conducted at a height of 1 m above the floor and situated 2 m from the patient's mouth, positioned centrally within the office. The bacterial specimens were incubated for a 48-h period at a temperature of 37°C, and the extent of microbiological contamination was computed as the overall number of CFUs per m^3 of air using the formula:

$$L = a \times 1000/\pi r^2 \times k,$$

where:
L – microbial contamination level in [CFU/m^3];
a – the quantity of bacterial colonies cultivated on the plate;
r – radius of the Petri dish [cm];
k – the exposure time coefficient, denoted as 'k,' is determined by multiplying the exposure time 't' (measured in min) by 1/5.

Dental office surface and air standardization

Between each patient, surfaces were cleaned by wiping down all areas, including dental chairs, lasers and handles, with a disinfectant cleaner to remove visible debris. The aspiration system was then cleaned with a cleaning solution

Table 1. The quantity of aerosol particles measured at the mouth of the operator before (control/initial measurement) and after caries treatment using either an Er:YAG laser or a dental turbine (ANOVA Kruskal–Wallis test)

Groups	ANOVA Kruskal–Wallis; H (2; n = 90) = 78,3711; p < 0.001			
	n	median	lower-top quartiles	p-value
Turbine + HVE (G1)	30	57,021	(42,564–67,568)	G1 vs G2; <0.001 G1 vs G3; <0.001 G2 vs G3; <0.001
Er:YAG + HVE (G2)	30	33,318	(28,463–35,484)	
Control (G3)	30	29,129	(29,178–29,784)	

ANOVA – analysis of variance; HVE – high volume evacuator; n – number of measurements; G1 – group 1; G2 – group 2; G3 – group 3 (control).

run through it to clear debris and disinfect internal components. Suction lines were also disinfected to maintain proper function. External surfaces were treated with disinfectant, which was left for 10 min. The dental office environment underwent a series of standardization procedures. The office space encompassed an area of 20 m^2 with all windows and doors securely closed and the air conditioning system deactivated. An air purifier, specifically the NV1050 model manufactured by Novaerus (Dublin, Ireland), boasting an air exchange rate of 800 m^3/h, was employed to maintain aerosol levels within the range of 28,000–30,000 particles/m^3 for each procedure. Continuous monitoring was conducted at 1-min intervals while the air purifier was in operation. Each dental treatment was conducted after completion of air standardization within the designated range. Control measurements were taken by positioning a sensor at the central point of the office, and it took on average approx. 5 min to cleanse the air to the specified levels.

Statistical analyses

Normal distribution of the data was tested using the Shapiro–Wilk test (Supplementary Fig. 1–6). Normal distribution was not observed for most of the data. The quantity of aerosols, as measured using the particle's detector, and TBC (expressed as CFU/m^3) throughout the caries treatments were analyzed using Kruskal–Wallis analysis of variance (ANOVA) and a post hoc Dunn's test with Bonferroni correction. For statistical analysis, Statistica software v. 13.3 (StatSoft Inc., Tulsa, USA) was used. Significance was attributed to any values falling below the threshold of p = 0.05.

Results

Quantity of aerosol particles generated during caries treatment (primary outcome)

The Er:YAG laser produced a significantly lower level of aerosol particles at the operator's level during caries treatment in contrast to the standard dental turbine (p < 0.001). The 1st group (conventional dental turbine + HVE) had a median aerosol value of 57,021 (42,564–67,568), while G2 (Er:YAG laser + HVE) had a lower value of 33,318 (28,463–35,484). When comparing the results of both groups, it was found that use of the Er:YAG laser reduced aerosol amounts during caries removal by 41.6% compared to the high-speed turbine. However, it is important to note that for both methods, the initial level of aerosols in the office 29,129 (29,178–29,784) significantly increased at the end of the treatment (p < 0.001) (Table 1).

Concentration of aerobic bacteria after caries treatment (secondary outcome)

The median count of total bacteria per m^3 of air in G1 (conventional dental turbine + HVE), G2 (Er:YAG laser + HVE) and G3 (control group before caries treatment) were 734 (420–988), 158 (96–288) and 48 (32–74), respectively. During caries treatment, employing a dental turbine (G1, p < 0.001) and Er:YAG laser (G2, p < 0.001) led to a notable increase in bacteria CFU levels in the dental office air compared to the initial levels in the control group (G3). Use of the Er:YAG laser resulted in a significantly lower total bacteria count compared to the standard dental turbine (p < 0.001). Additionally, utilizing the Er:YAG laser during caries removal resulted in a 78.5% decrease in TBC compared to the standard dental turbine (Table 2).

Discussion

Ensuring air purity in dental facilities is vital in mitigating the risk of microbial transmission.[6,39] Various measures, including air decontamination, protective masks and surface disinfection, have been adopted to enhance safety and promote good air quality within treatment rooms.[6,20] The primary approach to mitigating bioaerosol transmission during dental procedures centers on eradicating the accumulation of bioaerosols and cooling sprays within the oral cavity.[1,2] The purpose of this study was to examine aerosol levels in the air during caries treatment by removing generated aerosols before they enter the dental office environment. This strategy resulted in a substantial decrease in microbial risks present in the dental office air, with a 41.6% decrease in aerosol levels achieved using the Er:YAG laser

Table 2. The median value of the total bacterial count (measured in CFU/m^3) assessed at the central location within the dental office (ANOVA Kruskal–Wallis test)

Groups	ANOVA Kruskal–Wallis; H (2, n = 60) = 52,5042; p < 0.001			
	n	median	lower-top quartiles	p-value
Turbine + HVE (G1)	20	734	(420–988)	G1 vs G2; <0.001 G1 vs G3; <0.001 G2 vs G3; <0.001
Er:YAG + HVE (G2)	20	158	(96–288)	
Control (G3)	20	48	(32–74)	

ANOVA – analysis of variance; HVE – high volume evacuator; TBC – total bacteria count; CFU – colony-forming unit; n – number of microbiological plates; G1 – group 1; G2 – group 2; G3 – group 3 (control).

compared to the high-speed dental handpiece. Moreover, using the Er:YAG laser, there was a 78.5% decline in total bacteria count when treating caries compared to standard dental turbines. These in vivo findings corroborated previously published in vitro studies.[1,2]

The principal aim of this study was to assess the null hypothesis that there is no notable disparity in aerosol generation between dental caries decay removal using a burr on a turbine and an Er:YAG laser. For both methods, an additional dental standard high-volume evacuator (HVE) was applied. The study findings revealed that the use of Er:YAG laser for dental caries treatment considerably reduced the level of aerosol in the dental office compared to standard dental turbine, which is consistent with our previous in vitro study.[2] Moreover, in the present trial, use of the Er:YAG laser reduced aerosol particles in dental office air by 41.6% compared to the high-speed turbine. These results align with our previous published in vivo research, in which we observed a 40% decrease in the quantity of aerosols when treating dental decay utilizing a broader evacuator in conjunction with a dental turbine.[14] Other researchers have also recently demonstrated the effectiveness of using HVE to reduce the concentration of aerosols in dental facilities.[10,24,40,41] Results from Harrel et al.[40] and Jacks[10] showed significantly better efficiency of HVE than salivary ejector for aerosol removal, which is consistent with our previous in vitro studies. Findings from in vivo research by Nulty et al.[41] were congruent with our results, providing further evidence of aerosol reduction during dental procedures. Our present study is the first randomized clinical trial conducted in vivo that examined the use of an Er:YAG laser and not only different suction systems or rotary instruments.

It should also be highlighted that the findings of comparative studies conducted with various mouth rinses, including chlorhexidine, reinforce the efficacy of this method.[42] These studies have consistently demonstrated a significant reduction in the number of microorganisms that may escape from a patient's mouth through aerosols during dental treatments.[42] However, in the air exhaled by the patient and within the tissues affected by caries, there are still bacteria which, in order to maintain the safety of medical staff, should be eliminated during the dentist's work in the patient's oral cavity. The hydration level during the removal of carious tissues (dentin) plays a significant role in shaping the characteristics of the aerosol produced during dental procedures. Research by Timbrell and Eccles[43] highlighted that grinding enamel and dentine in surgical procedures without water cooling results in a fine aerosol with a substantial respirable fraction, posing potential health risks to dentists. Additionally, the use of water spray in high-speed grinding demands attention due to its high respirability, raising concerns about droplets potentially acting as efficient carriers of microorganisms into the dentist's lungs. To mitigate these risks, it is essential to utilize effective high-volume suction apparatus. The function of erbium lasers on tissue is dependent on the interaction with water, which results in the elevation of hydrogen molecule vibrations and thermal energy.[27,44] This creates movement within the liquid, resulting in water evaporation and the potential production of aerosols during dental procedures such as caries removal, endodontics and periodontal pocket treatment.[30,31,34,45–47] However, our previous in vitro and present in vivo investigations found that the Er:YAG laser application resulted in a significantly lower increase in aerosols compared to other procedures in a dental office.[1,2] In contrast to initial measurements, the dental turbine and Er:YAG laser increased aerosols by 96% and 11.4%, respectively. In our previous research, we implemented 3 distinct Er:YAG lasers.[2] These lasers operate based on comparable physical phenomena for operation on tissues, but discrepancies in cooling fluid delivery methods may lead to dissimilar aerosol particle generation during dental procedures.[1,2,24] We discovered that the type of cooling system utilized in the laser has a notable effect on the generation of aerosols.[2] The erbium lasers used in the present study use coolant supply lines with 3 endpoints in the handpiece's head. When using this kind of laser, the tissue is cooled with 3 water streams.[2] The outcomes from our prior in vitro experimentation showed that the aerosol increase in the air was greater for the laser with its cooling system built in the handpiece than for the laser where the water and air supply lines were integrated into the tip (and not just into the handpiece). However, we did not utilize the laser with a cooling system in the tip during our research due to its inefficacy in vaporizing tooth enamel, primarily because of its lower maximal power.[1,2]

The global spread of bacterial infections has raised serious public health concerns.[48–50] The 2nd aim of the study was to evaluate the level of aerobic bacteria in the air when treating dental caries decay. Our findings indicate

an increase in bacterial count in CFU/m^3 in the air for both treatment methods (dental turbine and laser) compared to initial measurements before treatment. Other studies have also reported an increase in airborne bacterial, fungal load during dental treatment and significant room contamination when high-speed instruments were used.[18,51] In a study by Manarte-Monteiro et al.,[51] it was revealed that the level of airborne bacteria increased at both 0.5 m and 2 m distances during endodontic treatment. Correspondingly, Rautemaa et al.[18] found substantial room contamination within the 0–2 m range when high-speed dental instruments were employed, resulting in an average of 970 CFU/m^3/h. Additionally, Szymańska[4] demonstrated that fungi concentration in the air ranged from 4 × 101 CFU/m^3 to 34 × 101 CFU/m^3 during caries dental decay removal. Our current study found that the highest median bacterial count was observed for conventional turbines with 734 (420–988) CFU/m^3 after 1 h. In contrast, the Er:YAG laser led to a 78.5% reduction in TBC compared to the conventional handpiece used at the patient's mouth during caries treatment. These findings coincide with our recently published research, in which we found an 84.5% reduction of TBC during caries treatment with a high-speed turbine when compared to standard HVE for the wider intraoral suction system.[14]

Limitations

The study contains several limitations. The primary reason why there are restrictions on the use of erbium lasers instead of conventional rotary instruments in dentistry is their high cost. Furthermore, operating these devices safely in vivo requires expertise and knowledge. In this study, the conventional Koch sedimentation method, which has limited accuracy, was used to measure the TBC in the office air. Other measurement methods for microbial air analysis, such as air sampling with impactors (e.g., Andersen samplers), need to be evaluated in a dental office. Another limitation is the scarcity of existing literature addressing the impact of different water systems in dental handpieces on aerosol levels. Furthermore, our clinical research compared the amount of aerosol particles and TBC generated by dental turbine vs Er:YAG laser without identification of specific colonies. Considering the influence of oral microflora on aerosol composition is important for ensuring accurate and comparable results in any study or research involving dental procedures. However, for studies that identify bacterial colonies, it is recommended to have patients with similar oral microflora.

Further research is necessary to assess the efficacy of Er:YAG lasers for decontamination during dental treatment, as well as randomized clinical trials to evaluate their impact on reducing aerosols. Additionally, investigations should be conducted to determine how other methods for caries treatment, such as dental sandblasting, affect aerosol levels and microbial air concentration.

Conclusions

The use of a traditional high-speed handpiece for treating dental caries produces a significant number of aerosols that can linger in the air of a dental clinic for a prolonged time, which can increase the risk of transmission of infection between medical staff and patients. This study demonstrated a significant reduction in the amount of aerosols generated when using an Er:YAG laser compared to a standard dental turbine. Additionally, the Er:YAG laser significantly reduced bacterial amounts in the office air compared to the dental turbine. The incorporation of erbium laser technology during dental caries therapy not only contributed to the enhancement of air quality within the treatment area, but also played a pivotal role in elevating the broader biological safety standards within the dental office setting.

Supplementary data

The Supplementary materials are available at https://zenodo.org/doi/10.5281/zenodo.10043159. The package contains the following files:

Supplementary Fig. 1. Results of the Shapiro–Wilk test for TBC in group 1 (G1).

Supplementary Fig. 2. Results of the Shapiro–Wilk test for TBC in group 2 (G2).

Supplementary Fig. 3. Results of the Shapiro–Wilk test for TBC in group 3 (G3).

Supplementary Fig. 4. Results of the Shapiro–Wilk test for aerosol amount in group 1 (G1).

Supplementary Fig. 5. Results of the Shapiro–Wilk test for aerosol amount in group 2 (G2).

Supplementary Fig. 6. Results of the Shapiro–Wilk test for aerosol amount in group 3 (G3).

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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References

1. Matys J, Grzech-Leśniak K. Dental aerosol as a hazard risk for dental workers. *Materials (Basel)*. 2020;13(22):5109. doi:10.3390/ma13225109
2. Grzech-Leśniak K, Matys J. The effect of Er:YAG lasers on the reduction of aerosol formation for dental workers. *Materials (Basel)*. 2021; 14(11):2857. doi:10.3390/ma14112857

3. Böke ES, Keleş A, Keskin C, Tanrıverdi Çaycı Y, Turk T. Are aerosol control devices effective in preventing the spread of dental aerosol? *PeerJ*. 2022;10:e13714. doi:10.7717/peerj.13714
4. Szymańska J. Exposure to airborne fungi during conservative dental treatment. *Ann Agric Environ Med*. 2006;13(1):177–179. PMID:16841889.
5. Liu MH, Chen CT, Chuang LC, Lin WM, Wan GH. Removal efficiency of central vacuum system and protective masks to suspended particles from dental treatment. *PLoS One*. 2019;14(11):e0225644. doi:10.1371/journal.pone.0225644
6. Dominiak M, Różyło-Kalinowska I, Gedrange T, et al. COVID-19 and professional dental practice: The Polish Dental Association Working Group recommendations for procedures in dental office during an increased epidemiological risk. *J Stomatol*. 2020;73(1):1–10. doi:10.5114/jos.2020.94168
7. Pitak-Arnnop P, Auychai P, Neff A. Dental aerosols should not be ignored during the COVID-19 pandemic until proven otherwise. *J Evid Based Dent Pract*. 2022;22(3):101745. doi:10.1016/j.jebdp.2022.101745
8. Rafiee A, Carvalho R, Lunardon D, et al. Particle size, mass concentration, and microbiota in dental aerosols. *J Dent Res*. 2022;101(7):785–792. doi:10.1177/00220345221087880
9. He Z, Gao Q, Henley A, et al. Efficacy of aerosol reduction measures for dental aerosol generating procedures. *Aerosol Sci Technol*. 2022;56(5):413–424. doi:10.1080/02786826.2022.2040729
10. Jacks ME. A laboratory comparison of evacuation devices on aerosol reduction. *J Dent Hyg*. 2002;76(3):202–206. PMID:12271865.
11. Graetz C, Plaumann A, Tillner A, Salzer S, Bielfeldt J, Dorfer C. Efficacy versus health risks: An in vitro evaluation of power-driven scalers. *J Indian Soc Periodontol*. 2015;19(1):18–24. doi:10.4103/0972-124X.145796
12. Harrel SK, Molinari J. Aerosols and splatter in dentistry: A brief review of the literature and infection control implications. *J Am Dent Assoc*. 2004;135(4):429–437. doi:10.14219/jada.archive.2004.0207
13. Remington WD, Ott BC, Hartka TR. Effectiveness of barrier devices, high-volume evacuators, and extraoral suction devices on reducing dental aerosols for the dental operator: A pilot study. *J Am Dent Assoc*. 2022;153(4):309–318.e1. doi:10.1016/j.adaj.2021.08.011
14. Matys J, Gedrange T, Dominiak M, Grzech-Leśniak K. Quantitative evaluation of aerosols produced in the dental office during caries treatment: A randomized clinical trial. *J Clin Med*. 2023;12(14):4597. doi:10.3390/jcm12144597
15. Bentley CD, Burkhart NW, Crawford JJ. Evaluating spatter and aerosol contamination during dental procedures. *J Am Dent Assoc*. 1994;125(5):579–584. doi:10.14219/jada.archive.1994.0093
16. Guderian DB, Loth AG, Weiß R, Diensthuber M, Stöver T, Leinung M. In vitro comparison of surgical techniques in times of the SARS-CoV-2 pandemic: Electrocautery generates more droplets and aerosol than laser surgery or drilling. *Eur Arch Otorhinolaryngol*. 2021;278(4):1237–1245. doi:10.1007/s00405-020-06330-y
17. Allison JR, Dowson C, Pickering K, et al. Local exhaust ventilation to control dental aerosols and droplets. *J Dent Res*. 2022;101(4):384–391. doi:10.1177/00220345211056287
18. Rautemaa R, Nordberg A, Wuolijoki-Saaristo K, Meurman JH. Bacterial aerosols in dental practice: A potential hospital infection problem? *J Hosp Infect*. 2006;64(1):76–81. doi:10.1016/j.jhin.2006.04.011
19. Miller RL. Characteristics of blood-containing aerosols generated by common powered dental instruments. *Am Ind Hyg Assoc J*. 1995;56(7):670–676. doi:10.1080/15428119591016683
20. Sotiriou M, Ferguson SF, Davey M, et al. Measurement of particle concentrations in a dental office. *Environ Monit Assess*. 2008;137(1–3):351–361. doi:10.1007/s10661-007-9770-7
21. Romero SS, Do Vale KL, Remolina VG, et al. Oral hygiene associated with antimicrobial photodynamic therapy or lingual scraper in the reduction of halitosis after 90 days follow up: A randomized, controlled, single-blinded trial. *Photodiagnosis Photodyn Ther*. 2021;33:102057. doi:10.1016/j.pdpdt.2020.102057
22. Stona P, Silva Viana ED, Santos Pires LD, Blessmann Weber JB, Floriani Kramer P. Recurrent labial herpes simplex in pediatric dentistry: Low-level laser therapy as a treatment option. *Int J Clin Pediatr Dent*. 2014;7(2):140–143. doi:10.5005/jp-journals-10005-1252
23. Golob Deeb J, Smith J, Belvin BR, Lewis J, Grzech-Leśniak K. Er:YAG laser irradiation reduces microbial viability when used in combination with irrigation with sodium hypochlorite, chlorhexidine, and hydrogen peroxide. *Microorganisms*. 2019;7(12):612. doi:10.3390/microorganisms7120612
24. Garden JM. Viral disease transmitted by laser-generated plume (aerosol). *Arch Dermatol*. 2002;138(10):1303–1307. doi:10.1001/archderm.138.10.1303
25. Garden JM, O'Banion MK, Shelnitz LS, et al. Papillomavirus in the vapor of carbon dioxide laser-treated verrucae. *J Urol*. 1989;141(1):223–224. doi:10.1016/S0022-5347(17)40716-6
26. Hughes PSH, Hughes AP. Absence of human papillomavirus DNA in the plume of erbium:YAG laser-treated warts. *J Am Acad Dermatol*. 1998;38(3):426–428. doi:10.1016/S0190-9622(98)70500-6
27. Grzech-Leśniak K, Nowicka J, Pajączkowska M, et al. Effects of Nd:YAG laser irradiation on the growth of *Candida albicans* and *Streptococcus mutans*: In vitro study. *Lasers Med Sci*. 2019;34(1):129–137. doi:10.1007/s10103-018-2622-6
28. Matys J, Jaszczak E, Flieger R, Kostrzewska-Kaminiarz K, Grzech-Leśniak K, Dominiak M. Effect of ozone and diode laser (635 nm) in reducing orthodontic pain in the maxillary arch: A randomized clinical controlled trial. *Lasers Med Sci*. 2020;35(2):487–496. doi:10.1007/s10103-019-02896-0
29. Matys J, Grzech-Leśniak K, Flieger R, Dominiak M. Assessment of an impact of a diode laser mode with wavelength of 980 nm on a temperature rise measured by means of k-02 thermocouple: Preliminary results. *Dent Med Probl*. 2016;53(3):345–351. doi:10.17219/dmp/62575
30. Dominiak M, Matys J. Assessment of pain when uncovering implants with Er:YAG laser or scalpel for second stage surgery. *Adv Clin Exp Med*. 2016;25(6):1179–1184. doi:10.17219/acem/62456
31. Matys J, Flieger R, Dominiak M. Assessment of temperature rise and time of alveolar ridge splitting by means of Er:YAG laser, piezosurgery, and surgical saw: An ex vivo study. *BioMed Res Int*. 2016;2016:9654975. doi:10.1155/2016/9654975
32. Matys J, Świder K, Flieger R, Dominiak M. Assessment of the primary stability of root analog zirconia implants designed using cone beam computed tomography software by means of the Periotest® device: An ex vivo study. A preliminary report. *Adv Clin Exp Med*. 2017;26(5):803–809. doi:10.17219/acem/65069
33. Matys J, Hadzik J, Dominiak M. Schneiderian membrane perforation rate and increase in bone temperature during maxillary sinus floor elevation by means of Er:YAG laser: An animal study in pigs. *Implant Dent*. 2017;26(2):238–244. doi:10.1097/ID.0000000000000520
34. Grzech-Leśniak K, Matys J, Żmuda-Stawowiak D, et al. Er:YAG laser for metal and ceramic bracket debonding: An in vitro study on intrapulpal temperature, SEM, and EDS analysis. *Photomed Laser Surg*. 2018;36(11):595–600. doi:10.1089/pho.2017.4412
35. Grzech-Leśniak K, Bencharit S, Skrjanc L, Kanduti D, Matys J, Deeb JG. Utilization of Er:YAG laser in retrieving and reusing of lithium disilicate and zirconia monolithic crowns in natural teeth: An in vitro study. *Appl Sci*. 2020;10(12):4357. doi:10.3390/app10124357
36. Matys J, Botzenhart U, Gedrange T, Dominiak M. Thermodynamic effects after diode and Er:YAG laser irradiation of grade IV and V titanium implants placed in bone: An ex vivo study. Preliminary report. *Biomed Tech (Berl)*. 2016;61(5):499–507. doi:10.1515/bmt-2015-0135
37. Szymańska J. Dental bioaerosol as an occupational hazard in a dentist's workplace. *Ann Agric Environ Med*. 2007;14(2):203–207. PMID:18247451.
38. Mirhoseini SH, Koolivand A, Bayani M, et al. Quantitative and qualitative assessment of microbial aerosols in different indoor environments of a dental school clinic. *Aerobiologia*. 2021;37(2):217–224. doi:10.1007/s10453-020-09679-z
39. Matys J, Grzech-Leśniak K, Dominiak M. Disinfectants and devices for surface and air disinfection in dental offices. *J Stomatol*. 2020;73(4):200–205. doi:10.5114/jos.2020.98267
40. Harrel SK, Barnes JB, Rivera-Hidalgo F. Aerosol and splatter contamination from the operative site during ultrasonic scaling. *J Am Dent Assoc*. 1998;129(9):1241–1249. doi:10.14219/jada.archive.1998.0421
41. Nulty A, Lefkaditis C, Zachrisson P, Van Tonder Q, Yar R. A clinical study measuring dental aerosols with and without a high-volume extraction device. *Br Dent J*. November 2020. doi:10.1038/s41415-020-2274-3

42. Agarwal N, Daigavane P, Kamble R. Comparative evaluation of Chlorhexidine and Triphala with Rajat Bhasma jelly as against chlorhexidine mouth rinse in prevention of bacterial accumulation in fixed orthodontic assembly: A randomized interventional study. *F1000Res*. 2023;12:548. doi:10.12688/f1000research.133532.1
43. Timbrell V, Eccles JD. The respirability of aerosols produced in dentistry. *J Dent*. 1973;2(1):21–31. doi:10.1016/S0300-5712(73)80006-5
44. Grzech-Leśniak K, Matys J, Jurczyszyn K, et al. Histological and thermometric examination of soft tissue de-epithelialization using digitally controlled Er:YAG laser handpiece: An ex vivo study. *Photomed Laser Surg*. 2018;36(6):313–319. doi:10.1089/pho.2017.4413
45. Matys J, Świder K, Flieger R. Laser instant implant impression method: A case presentation. *Dent Med Probl*. 2017;54(1):101–106. doi:10.17219/dmp/66363
46. Zakrzewski W, Dobrzynski M, Kuropka P, et al. Removal of composite restoration from the root surface in the cervical region using Er:YAG laser and drill: In vitro study. *Materials (Basel)*. 2020;13(13):3027. doi:10.3390/ma13133027
47. Kiryk J, Matys J, Nikodem A, et al. The effect of Er:YAG laser on a shear bond strength value of orthodontic brackets to enamel: A preliminary study. *Materials (Basel)*. 2021;14(9):2093. doi:10.3390/ma14092093
48. Jori G, Fabris C, Soncin M, et al. Photodynamic therapy in the treatment of microbial infections: Basic principles and perspective applications. *Lasers Surg Med*. 2006;38(5):468–481. doi:10.1002/lsm.20361
49. Liu W, Wang R, Vedarethinam V, Huang L, Qian K. Advanced materials for precise detection and antibiotic-free inhibition of bacteria. *Mater Today Adv*. 2022;13:100204. doi:10.1016/j.mtadv.2021.100204
50. Fanghänel J, Gedrange T, Proff P. The face-physiognomic expressiveness and human identity. *Anat Anz*. 2006;188(3):261–266. doi:10.1016/j.aanat.2005.11.013
51. Manarte-Monteiro P, Carvalho A, Pina C, Oliveira H, Manso MC. Air quality assessment during dental practice: Aerosols bacterial counts in an universitary clinic. *Rev Port Estomatol Med Dent Cir*. 2013;54(1):2–7. doi:10.1016/j.rpemd.2012.10.002

Autoimmune cytopenias in patients with malignant lymphoma: A multicenter report by the Polish Lymphoma Research Group

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Abstract

Background. Autoimmune cytopenias (ACs), including immune thrombocytopenia (ITP), autoimmune hemolytic anemia (AIHA) and autoimmune granulocytopenia, are rare complications observed in lymphoma patients. They may appear before, during or after lymphoma diagnosis, whether the patients had disease progression or not.

Objectives. This study aims to correlate ACs with lymphoma type, disease course and prognosis. We performed a multicenter retrospective analysis of adult patients with malignant lymphoma and ACs coexistence diagnosed and treated in centers aligned with the Polish Lymphoma Research Group (PLRG).

Materials and methods. The analysis covers the years 2016–2022 and included 51 patients comprised of 23 women and 28 men. Of these, 35 patients were diagnosed with AIHA, 15 patients with ITP and 1 patient with both AIHA and ITP.

Results. The most common type of lymphoma was Hodgkin lymphoma (HL) (12 patients) and diffuse large B-cell lymphoma (DLBCL) (14 patients). At the time of diagnosis, 31 (61%) of patients had stage 4 of HL or DLBCL, according to Ann Arbor classification. In total, the response to treatment was evaluated in 50 patients, with 25 being in complete remission and 6 in partial remission. We observed that B cell symptoms ($p = 0.036$), bone marrow involvement ($p = 0.073$), splenomegaly ($p = 0.025$), and more than 2 lines of treatment were more common in AIHA compared to ITP patients. Conversely, eucopenia ($p = 0.056$) and ACs without lymphoma progression ($p = 0.002$) were more often diagnosed in ITP patients.

Conclusions. In the study group, relapsed and refractory disease was observed more often, and shorter overall survival (OS) was noted in patients with DLBCL. We found that AC is associated with a worse prognosis in comparison to the general population of lymphoma patients. There were no differences in response to AC therapy. To have more accurate data, a larger group, as part of a multicenter study, should be evaluated.

Key words: lymphoma, ITP, AIHA, immune complications, autoimmune cytopenia

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Background

Autoimmune cytopenias (ACs) are a group of heterogeneous but closely related conditions defined by immune-mediated destruction of hematologic cell lineages, including white blood cells (neutrophils), red blood cells and platelets.[1] This destruction can be primary or secondary to other illnesses. Autoimmunity results from a complex interplay of genetic and environmental factors, including infections and drugs. Autoimmune cytopenias can be formally classified as idiopathic, consisting of single lineage destruction, including autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP) and, more rarely, autoimmune granulocytopenia (AG), as well as multi-lineage destruction, known as Evans syndrome.[2] The relative frequency of each of the clinical forms of ACs remains uncertain, with conflicting results from different studies. Older literature reported the highest rates of AIHA with lower rates of ITP and multi-lineage autoimmune cytopenia. The diagnosis of AC is usually made according to the criteria of the respective disease, and in some cases, antibodies are detected without symptomatic disease.

Secondary AC results from another cause, including medications, rheumatologic disorders, immunodeficiencies, lymphoproliferative disorders, malignancies, or as a complication of organ or hematopoietic stem cell transplant (HSCT).[3] The association between lymphoma and AC has long been noted. While definitive evidence linking the causality of these diseases is rare, there is compelling evidence of their co-occurrence. Autoimmune cytopenia may be diagnosed before, at presentation, or at any point during the course of lymphoma, and may also be observed in both untreated and treated patients. Autoimmune cytopenia occurs in approx. 5–10% of chronic lymphocytic leukemia (CLL) patients,[4] although so far, the exact prevalence of AC in lymphoma patients remains unclear. The frequency of AC varies among entities, with a high prevalence in certain types of lymphomas. Most AC are associated with B cell lymphomas and are much less frequent with T cell non-Hodgkin lymphomas (NSLs).

Objectives

Because of the rarity of lymphoma-associated AC, the data on the clinical characteristics, treatment of the disease, as well as prognosis are extremely poor and based mostly on case reports, with some larger groups described. Therefore, taking into consideration the scarcity of data and simultaneously the need to increase physician awareness of the diagnosis of ACs in lymphoma patients, we conducted a retrospective study at the Polish Lymphoma Research Group (PLRG) between 2016 and 2022.

Materials and methods

Data source

The study was performed on behalf of the Extranodal Lymphomas Working Group of the PLRG, which is a voluntary organization comprising hematological and oncological centers in Poland that provide care for lymphoma patients. All member centers were invited to participate in this study and provide additional study-specific data about eligible patients.

Study population and outcome

This study was a retrospective analysis of all patients who were diagnosed with ACs either simultaneously or sequentially with lymphoma. The analysis includes patients diagnosed during the period 2016–2022.

The primary objective of the study was to analyze the outcome of lymphoma treatment, i.e., overall survival (OS) of the patients with ACs and mortality. The secondary objectives were to examine the clinical presentation of lymphoma, the efficacy of lymphoma treatment, progression-free survival (PFS), and factors associated with OS and PFS.

Diagnosis of autoimmune cytopenia

The presence of AC, namely AIHA, ITP and AG, was respectively recorded based on patients' medical records. The diagnosis of AIHA was based on the presence of anemia (a hemoglobin level below 12 g/dL), and laboratory evidence of hemolysis, namely strong direct antiglobulin test (DAT) positivity, elevated absolute reticulocyte count, an elevated lactate dehydrogenase (LDH), an elevated indirect bilirubin level, and a low serum haptoglobin level. Immune thrombocytopenia was diagnosed in patients with a platelet count below 100,000/μL in whom other causes of thrombocytopenia have been ruled out.

All cases had biopsy-confirmed lymphoma and were diagnosed with AC at any time before, concurrently or after. The histological lymphoma diagnosis was established based on the 2008 World Health Organization (WHO) classification.[5]

We assessed the incidence, clinical characteristics, treatment strategies, and outcome of lymphoma patients experiencing AC, and compared their data with concurrent patients with Hodgkin lymphoma (HL) and diffuse large B-cell lymphoma (DLBCL) population who had no evidence of AC.

Response to treatment

Response to treatment was assessed as proposed by the most recent system, known as the Lugano classification, which applies to both HL and NHL.[6] Overall survival was calculated from the lymphoma diagnosis

to the last follow-up visit or death. Progression-free survival was estimated as the interval between the date of diagnosis and the estimated date of progression or death or end of follow-up.

Statistical analyses

Kaplan–Meier plots and log-rank tests were performed to visualize and compare survival curves. The median follow-up was calculated using the Schemper and Smith method. Pearson's χ^2 test was used to analyze the independence of categorical variables. Appropriate corrections were used where needed: the Yates's correction for continuity or Fisher's exact test. In general, if any of the expected frequencies of the 2×2 table was below 15, Yates's correction was used, but when it was below 5, the Fisher's exact test was used.

P-values <0.05 were considered significant. The Shapiro–Wilk test was used to confirm where the continuous variables had a normal distribution. Depending on the variable distribution, they were presented as mean ± standard deviation (M ±SD). A normal probability plot of continuous variables (age at diagnosis) is provided in Fig. 1. All statistical analyses were performed in MedCalc (MedCalc Software Ltd, Ostend, Belgium).

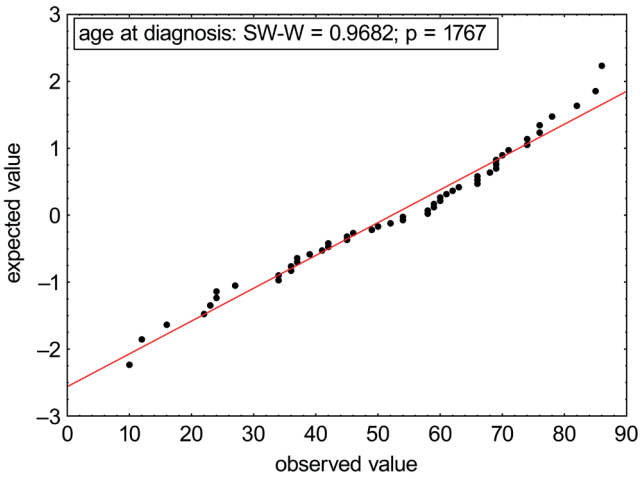

Fig. 1. Normal probability plot of age at diagnosis

Results

Patients

We identified 51 patients diagnosed with AC and lymphoma in 5 PLRG centers. Of these, 23 (45%) were women, and the mean age at lymphoma diagnosis was 52 years (95% confidence interval (95% CI): 46.7–57.7) ±19.7. Thirty-five patients were diagnosed with AIHA, 15 with ITP and 1 patient with both AIHA and ITP. The most common diagnosis of lymphoma was DLBCL (15 patients) and HL (12 patients), followed by indolent lymphoma, including marginal zone lymphoma, follicular lymphoma, mantle cell lymphoma (19 patients) and T cell lymphoma (5 patients). Thirty-one patients (61%) were in stage IV according to the Ann Arbor classification at the time of diagnosis (23 AIHA and 8 ITP, 66% and 53%, respectively), although most patients were in a good clinical stage (Eastern Cooperative Oncology Group (ECOG)-0 in 16 patients, ECOG-1 in 26 patients, 31% and 51%, respectively). Thirty-four patients (68%) had B cell symptoms (27 AIHA and 7 ITP, 77% and 47%, respectively), and 36 patients (71%) had extranodal lymphoma at the time of diagnosis (26 AIHA and 10 ITP, 74% and 67%, respectively). Interestingly, in the AIHA group, 14/34 patients (41%) had bone marrow involvement. A high prognostic index was found in 26 out of 49 patients (53%), which was adequate for their specific type of lymphoma (20 AIHA and 6 ITP, 57% and 40%, respectively). The detailed patient characteristics are provided in Table 1.

We then made the comparison between the AIHA and ITP group with all parameters according to patient characteristics (age, sex, ECOG, B symptoms, nodal and extranodal lymphoma), comorbidities, risk factors (Ann Arbor classification, prognostic index, type of lymphoma), morphology, biochemistry and response to the treatment, observing some major differences. It was seen that in the AIHA group (27/34, 79%), significantly more patients had B symptoms than in the ITP group (7/15, 47%) ($\chi^2 = 3.90$, $p = 0.049$, χ^2 test with Yates's correction). In the AIHA group, LDH during the lymphoma diagnosis was significantly higher than in the ITP group ($p = 0.002$). Interestingly, patients with AIHA had disease localized in the spleen less often than in the ITP group (2.9% compared to 25.0%, $p = 0.029$, Fisher's exact test). Moreover, the AIHA group had AC diagnosed without disease progression more frequently than in ITP ($p = 0.039$). While not a significant result, when comparing the AIHA group to ITP, we observed less type 2 diabetes ($p = 0.186$, Fisher's exact test), while asthma ($p = 0.295$, Fisher's exact test) was more frequently observed. The data also suggested that in the AIHA group, bone marrow tended to be more frequently involved in the lymphoma ($p = 0.203$, Fisher's exact test). The treatment analysis showed that patients with AIHA had radiotherapy less often in relapsed and refractory disease ($p = 0.263$, Fisher's exact test). Moreover, intravenous immunoglobulin (IVIG) and splenectomy were used more frequently in the ITP group as a salvage treatment ($p = 0.235$ and $p = 0.294$ respectively; Fisher's exact test). All data are shown in Table 2.

Treatment

The median observation time of our cohort was 78.9 months (95% CI: 61.4–143.2 months), and 49 out of 51 patients received treatment for lymphoma. A variety of different protocols was used for first-line therapy, including 47 patients (96%) treated with chemotherapy and 27 patients (55%) with immunotherapy, while 8 patients (16%) required radiotherapy. Of these, 20 patients (39%),

Table 1. Patient characteristics

AC		AIHA	ITP	Total
Number of patients		35	16	51
Sex	female male	15 (43%) 20 (57%)	8 (50%) 8 (50%)	23 (45%) 28 (55%)
At lymphoma diagnosis				
Age of diagnosis [years], median (range)		59 (18–89)		
Lymphoma type	DLBCL HL indolent B-cell T-cell	10 (26%) 6 (17%) 16 (46%) 3 (11%)	5 (31%) 6 (39%) 3 (18%) 2 (12%)	15 (31%) 12 (24%) 19 (27%) 4 (8%)
Ann Arbor	I II III IV	3 (9%) 2 (6%) 6 (18%) 23 (67%)	3 (20%) 1 (7%) 3 (20%) 8 (53%)	6 (12%) 3 (6%) 9 (18%) 31 (64%)
Performance status according to ECOG	0 1 2 3	13 (37%) 18 (51%) 3 (9%) 1 (3%)	3 (19%) 8 (54%) 4 (27%) 0 (0%)	17 (32%) 26 (52%) 7 (14%) 1 (2%)
Comorbidities	yes no	24 (66%) 12 (34%)	10 (67%) 5 (33%)	34 (68%) 17 (32%)
B symptoms	yes no	27 (77%) 8 (23%)	7 (47%) 8 (53%)	34 (68%) 17 (32%)
Extranodal disease	yes no	26 (74%) 9 (26%)	10 (63%) 6 (37%)	36 (71%) 15 (29%)
Bone marrow involvement	yes no	14 (40%) 21 (60%)	3 (19%) 13 (81%)	17 (33%) 34 (67%)
Prognostic index	low intermediate high	3 (9%) 11 (32%) 20 (59%)	3 (20%) 6 (40%) 6 (40%)	6 (12%) 17 (35%) 26 (53%)

AIHA – autoimmune hemolytic anemia; ITP – immune thrombocytopenia; AC – autoimmune cytopenia; DLBCL – diffuse large B-cell lymphoma; ECOG – Eastern Cooperative Oncology Group; HL – Hodgkin lymphoma.

Table 2. Comparison between the AIHA and ITP groups (symptoms in bold were more frequent)

AC	AIHA	ITP	p-value
B symptoms	**27/35 (77%)**	7/15 (47%)	0.049
Spleen involvement with lymphoma	1/35 (3%)	**4/16 (25%)**	0.029
Elevated LDH	**30/35 (86%)**	4/16 (25%)	0.002
Immunotherapy at relapse	4/13 (31%)	**4/5 (80%)**	0.088
AC diagnosis with lymphoma progression	**3/13 (23%)**	0/5 (0%)	0.039
Diabetes	3/35 (9%)	**4/16 (25%)**	0.186
Asthma	**4/35 (11%)**	0/16 (0%)	0.295
Bone marrow involvement with lymphoma	**14/35 (40%)**	3/16 (19%)	0.203
Radiotherapy at relapse	0/35 (0%)	**1/5 (20%)**	0.263
AC treatment with IVIG	1/35 (3%)	**4/16 (25%)**	0.235
AC treatment with splenectomy	1/35 (3%)	**3/16 (19%)**	0.294

AIHA – autoimmune hemolytic anemia; ITP – immune thrombocytopenia; LDH – lactate dehydrogenase; IVIG – intravenous immunoglobulin; AC – autoimmune cytopenia.

i.e., 15 in AIHA group (44%), and 5 in ITP group (33%), were refractory or relapsed and needed salvage therapy. Finally, 5 patients (10%) had an autologous HSCT, and 2 patients (4%) underwent allogeneic HSCT (Table 3).

Among 49 patients eligible for response assessment, 25 (15 AIHA; 10 ITP) (51%) had complete response (CR) and 6 (3 AIHA; 3 ITP) (12%) had partial response (PR). Moreover, 19 patients (39%) had no response or had disease progression after first-line treatment (Table 3). We also observed 20 deaths (38%), with 15 related to lymphoma progression, 2 to hemophagocytic syndrome, 2 to AIHA progression, and 1 to ITP progression.

The diagnosis of AC in a majority of patients was made together with with lymphoma diagnosis or progression

Table 3. Response to treatment

AC		AIHA	ITP	Total
Lymphoma treatment chemotherapy immunotherapy radiotherapy		33/35 (94%) 32/35 (91%) 20/35 (57%) 5/35 (14%)	16/16 (100%) 15/16 (94%) 7/16 (44%) 3/16 (19%)	49/51 (96%) 47/51 (92%) 27/51 (53%) 8/51 (16%)
Response to treatment	CR PR PD	15/35 (43%) 3/35 (9%) 17/35 (49%)	10/16 (62%) 3/16 (19%) 3/16 (19%)	25/51 (49%) 6/51 (12%) 20/51 (39%)
Treatment in relapsed disease chemotherapy immunotherapy radiotherapy		14/35 (40%) 13/14 (93%) 4/14 (29%) 0/14 (0%)	5/16 (31%) 5/5 (100%) 4/5 (80%) 1/5 (20%)	19/51 (37%) 18/19 (95%) 8/19 (42%) 1/19 (5%)
autoHSCT alloHSCT		4/35 (11%) 1/35 (3%)	1/16 (6%) 1/16 (6%)	5/51 (10%) 2/51 (4%)
AC treatment 1 line 2 lines >3 lines		35/35 (100%) 0/35 (0%) 15/35 (43%) 20/35 (57%)	12/16 (75%) 2/16 (13%) 5/16 (31%) 5/16 (31%)	47/51 (92%) 2/51 (4%) 20/51 (39%) 27/51 (53%)
Deaths		15/35 (43%)	5/16 (31%)	20/51 (39%)

AIHA – autoimmune hemolytic anemia; ITP – immune thrombocytopenia; AC – autoimmune cytopenia; CR – complete response; PR – partial response; PD – progressive disease; autoHSCT – autologous hematopoietic stem cell transplantation; alloHSCT – allogeneic hematopoietic stem cell transplantation.

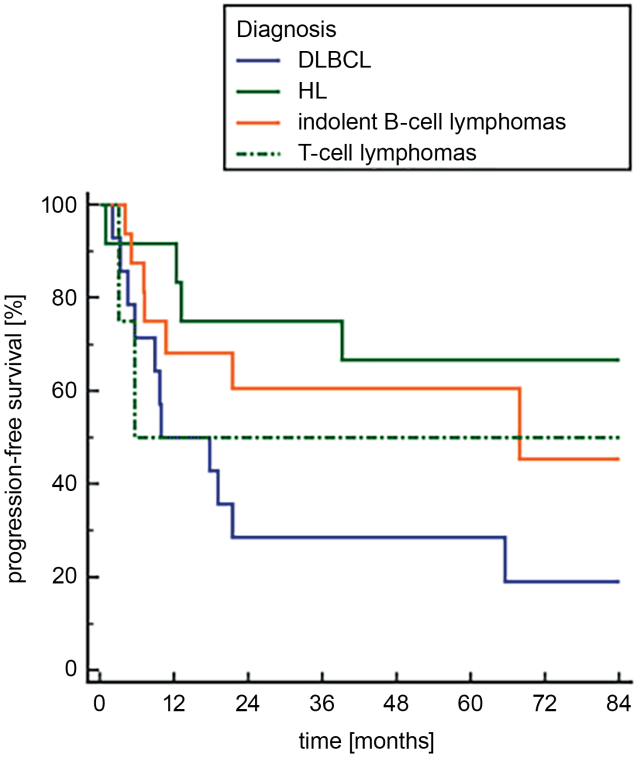

Fig. 2. Progression-free survival (PFS) in the studied groups of patients. Patients with DLBCL had a trend into poorer PFS compared to the other diagnosis groups, but the difference did not yield statistical significance (log-rank p-value = 0.063)

DLBCL – diffuse large B-cell lymphoma; HL – Hodgkin lymphoma.

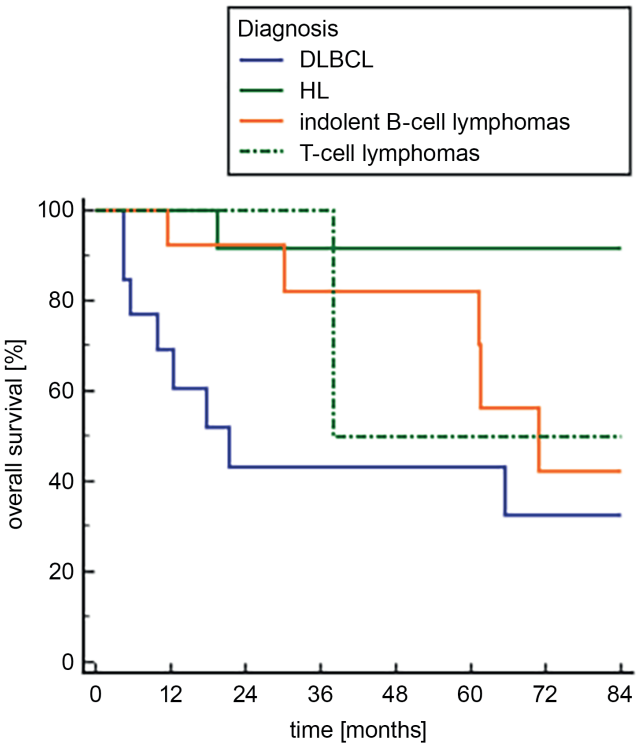

Fig. 3. Overall survival (OS) in the studied groups of patients. Patients with Hodgkin lymphoma (HL) had a significantly better outcome in terms of OS (median not reached) compared to other groups: DLBCL (median OS: 1.8 years), indolent B-cell lymphoma (median OS: 5.9 years) and T-cell lymphoma (median OS: 3.2 years). Log-rank p-value = 0.008

DLBCL – diffuse large B-cell lymphoma.

(35 out of 51 patients, 68%). A further 8 patients (16%) had their AC diagnosis before lymphoma, and 8 patients (16%) had AC after lymphoma diagnosis without disease progression. Out of 35 patients with a diagnosis of AIHA, all received treatment, with 8 patients (23%) receiving 1 line of treatment, 6 (17%) had 2 lines of treatment and 21 (60%) had 3 or more lines of treatment. Out of 16 patients with ITP diagnosis, 2 were not treated at all (12.5%), 5 (31%) had 1 line of treatment, 7 (44%) had 2 lines of therapy and 2 (12.5%) had 3 or more lines.

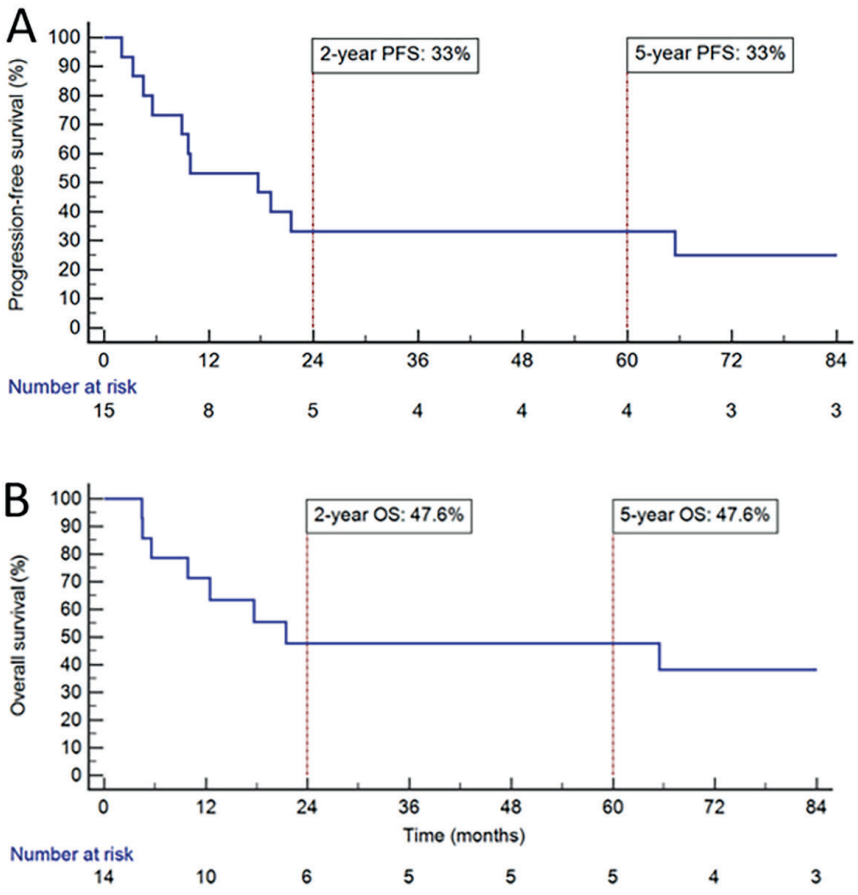

Fig. 4. Progression-free survival (PFS) (A) and overall survival (OS) (B) in the diffuse large B-cell lymphoma (DLBCL) group. The median PFS was 17.7 months (95% confidence interval (95% CI): 8.9–65.5), with a 2-year PFS rate of 33% and a 5-year PFS rate of 33%. The median OS for this group was 21.4 months (95% CI: 9.9–65.5) with a 2-year OS rate of 47.6% and a 5-year OS rate of 47.6%

Survival analysis

The data on survival was available for 50 patients, and the median observation time in our cohort was 78.9 months (95% CI: 61.4–143.2 months). The median PFS in the whole cohort was 65.5 months (95% CI: 13.2–288.4), although there was a trend towards a lower PFS in DLBCL patients compared to the other groups (log-rank p-value = 0.063) (Fig. 2). The median OS of the whole study group was 27.2 years (95% CI: 65.5–325.9 months), and patients with HL had a significantly better outcome in terms of OS (median not reached) compared to other groups: DLBCL (median OS: 1.8 years), indolent B-cell lymphoma (median OS: 5.9 years) and T cell lymphoma (median OS: 3.2 years). Log-rank p-value was 0.008 (Fig. 3).

For the DLBCL group (survival data available for 15 patients), median PFS was 17.7 months (95% CI: 8.9–65.5), with a 2-year PFS rate of 33% and 5-year PFS rate of 33% (Fig. 4A). Median OS for this group was 21.4 months (95% CI: 9.9–65.5) with a 2-year OS rate of 47.6% and a 5-year OS rate of 47.6% (Fig. 4B). For the HL group, the median PFS was not reached, with a 2-year PFS rate of 75.0% and a 5-year PFS rate of 66.7% (Fig. 5A). Moreover, the median OS was not reached, with a 2-year OS rate of 91.7% and a 5-year OS rate of 91.7% (Fig. 5B). Moreover, patients with DLBCL had both shorter PFS (log-rank p = 0.0115) and OS (p = 0.003) when compared to patients with HL.

Discussion

Despite the long recognition of the coexistence of lymphoma and AC, the exact characteristics of patients with AC and lymphoma remain poorly defined. In our multicenter study, we investigated a large cohort of Polish patients, and we analyzed their clinical and prognostic profiles. To the best of our knowledge, this study is the largest to report AC-associated lymphoma. Previously, the largest cohort reported so far was Hu et al., with 28 patients with B cell and T cell lymphoma.[7] In their study group, 24 patients were diagnosed with AIHA and 6 with ITP. Similar to our study with 51 patients, the majority were DLBCL (10 out of 28; 36%), followed by HL and B cell indolent lymphoma (less than 30%).

Our study revealed a distinct pattern of a worse prognostic profile, with 61% of investigated patients in an advanced stage of the disease (66% AIHA; 53% ITP), i.e., at stage IV according to the Ann Arbor classification for lymphoma diagnosis. The IPI adequate for different types of lymphoma diagnosis was high in 57% of AIHA patients and 40% of those with ITP. Moreover, 68% of patients had B symptoms (77% AIHA; 47% ITP) and 71% had extranodal disease (74% AIHA; 67% ITP), both known to be poor prognostic factors for DLBCL patients. It was interesting that 41% of AIHA patients had bone marrow involvement. The extranodal involvement of lymphoma observed

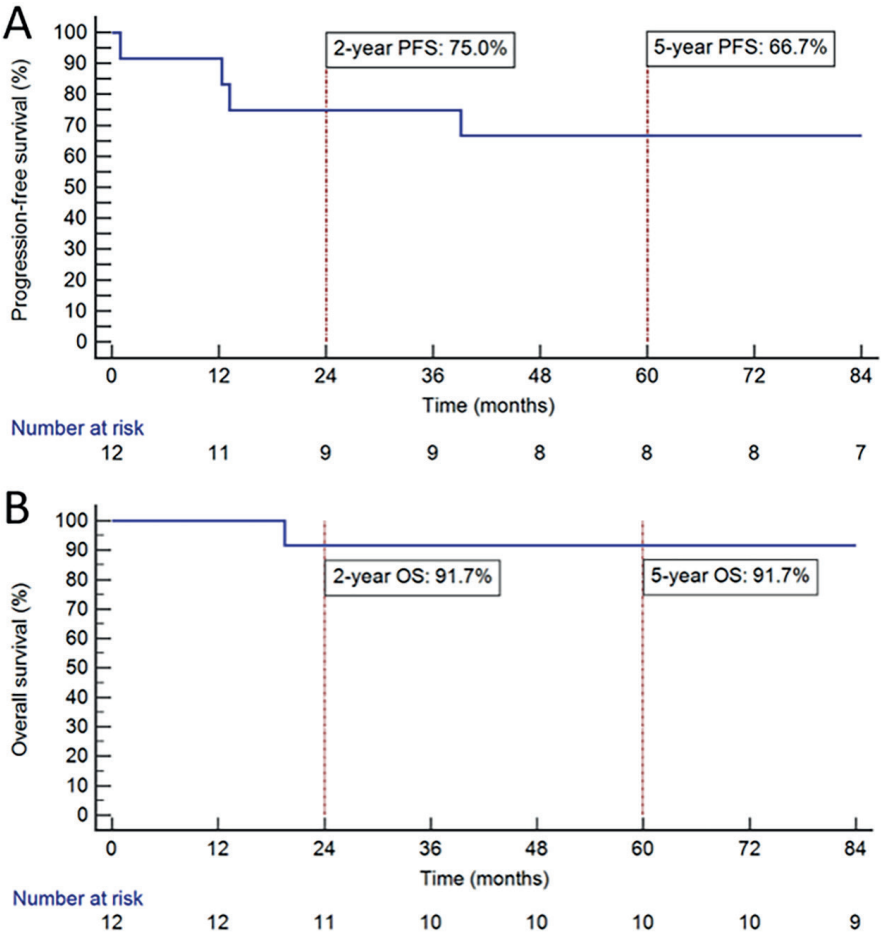

Fig. 5. Progression-free survival (PFS) (A) and overall survival (OS) (B) in the Hodgkin lymphoma (HL) group. The median PFS was not reached with a 2-year PFS rate of 75.0% and a 5-year PFS rate of 66.7%. Median OS was not reached with a 2-year OS rate of 91.7% and a 5-year OS rate of 91.7%

in patients who also had an AC diagnosis was consistent with findings published by Hu et al.[7] Similar to our results, in work by Pinczes et al. examining 16 patients with HL and AC, the authors observed advanced disease features such as stage III/IV, bone marrow involvement and B symptoms.[8] The same observation was published by Dimou et al., who reported a remarkable predominance of advanced-stage lymphoma.[9] All these results are consistent with our data in a larger group.

Although the number of AC cases was small, we found statistically significant differences between the AIHA and ITP groups. We observed that B symptoms, elevated LDH and AC diagnosis connected with lymphoma progression were statistically more likely in AIHA compared to ITP patients and may be associated with worse clinical outcome in the AIHA group. Conversely, spleen involvement with lymphoma was more often found in ITP patients. We have also seen a trend that AIHA patients were diagnosed with asthma more often and had bone marrow involvement with lymphoma during the diagnosis. Moreover, the ITP group seemed to be more often diabetic and was treated with radiotherapy following relapse. However, to conduct a more accurate analysis between these 2 groups, a larger number of patients is necessary.

Lechner and Chen observed that out of 39 lymphoma patients with AC, 13 relapsed or were refractory to first-line treatment.[2] This was similar to our PLRG database, in which we were able to identify 39% of relapsed and refractory disease (44% in AIHA and 33% in the ITP group). In the relapsed and refractory group, 5 patients undergo autologous HSCT (10%) and 2 allogeneic HSCT (4%). Historically, most patients are cured with anthracycline-containing immunochemotherapy, and approx. 1 in 4 experience primary refractory disease or relapse (25–30%).[10] Similar data were published by the Hemato-Oncology Latin America Observational Registry Study (HOLA), in which out of 578 DLBCL patients, 29% had relapsed or refractory AC.[11] Furthermore, response to first-line treatment in our group was poor, with CR in 51% and PR in 12%. Moreover, 20 patients died (38%), mainly due to disease progression. In other studies with higher subject numbers, 20–30% of patients with NHL and 15% with HL will not be able to achieve a CR with standard induction.[12,13] Our experience suggests that the coexistence of AC and lymphoma is associated with more aggressive clinical behavior, inferior outcomes and increased risk of death. However, our study numbers were too low to draw a definitive conclusion.

In the present study, we found that the median PFS was 17.7 months in the DLBCL group, a much lower result

compared to data from other studies. In the HOLA study, the median PFS was 7.7 years.[11] Moreover, our 5-year PFS was much worse, with 33% in our study compared to 56.2% in the HOLA study. Moreover, 2- and 5-year OS for our DLBCL group were both 47.6%; again, a much lower result compared to other large cohort studies. In data from an article published by Mauer et al. in the SEAL database, 2-year OS was 87% and 5-year OS was 80%.[14] We observed that in our patients with the concurrence of AC and lymphoma, not only efficacy but also OS was lower with AC than without. The possible explanation is that the development of AC affects the survival of lymphoma patients, especially in the DLBCL group.

Finally, we observed the trend of decreased PFS in DLBCL patients ($p = 0.063$) compared to other lymphoma groups. Moreover, patients with HL had significantly better outcomes in OS compared to all the other lymphoma groups. These results are consistent with the prognosis of HL and DLBCL in patients without AC. It may demonstrate that AC worsens the prognosis of all lymphoma subtypes, not only DLBCL. Still, the most important prognostic factor for OS and PFS is the histopathological type of lymphoma.

Limitations

The main limitation of this study is its retrospective nature, but evaluation of characteristics and outcomes of lymphoma-associated AC is not possible prospectively. Another limitation may be a relatively small sample size. However, the rarity of the diagnosis precludes the possibility of gathering a large group of patients, even if performed mutinationally. According to our knowledge, we pooled one of the biggest study groups so far. Nevertheless, we believe that the inclusion of all patients from all age groups and different lymphoma subtypes represents a real group and provides clinically useful information. Thus, it may be the main strength of this analysis.

Conclusions

Lymphoma patients with AC are more likely to present advanced stages of the disease and seem to have worse outcomes and responses to treatment. This association can be a significant prognostic factor, especially for the DLBCL group, both for PFS and OS. Moreover, AIHA patients have a stronger trend towards increased mortality. Patients who present with any kind of AC should always be assessed for relapse of refractory disease. Large prospective studies are needed to confirm our preliminary findings.

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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References

1. Váróczy L, Páyer E, Kádár Z, et al. Malignant lymphomas and autoimmunity: A single center experience from Hungary. *Clin Rheumatol*. 2012;31(2):219–224. doi:10.1007/s10067-011-1807-1
2. Lechner K, Chen YA. Paraneoplastic autoimmune cytopenias in Hodgkin lymphoma. *Leuk Lymphoma*. 2010;51(3):469–474. doi:10.3109/10428190903556394
3. Crickx E, Poullot E, Moulis G, et al. Clinical spectrum, evolution, and management of autoimmune cytopenias associated with angioimmunoblastic T-cell lymphoma. *Eur J Haematol*. 2019;103(1):35–42. doi:10.1111/ejh.13239
4. Hodgson K, Ferrer G, Montserrat E, Moreno C. Chronic lymphocytic leukemia and autoimmunity: A systematic review. *Haematologica*. 2011;96(5):752–761. doi:10.3324/haematol.2010.036152
5. Jaffe ES. The 2008 WHO classification of lymphomas: Implications for clinical practice and translational research. *Hematology Am Soc Hematol Educ Program*. 2009;2009(1):523–531. doi:10.1182/asheducation-2009.1.523
6. Johnson SA, Kumar A, Matasar MJ, Schöder H, Rademaker J. Imaging for staging and response assessment in lymphoma. *Radiology*. 2015;276(2):323–338. doi:10.1148/radiol.2015142088
7. Hu S, Zhou D, Wu Y, et al. Autoimmune disease-associated non-Hodgkin's lymphoma: A large retrospective study from China. *Ann Hematol*. 2019;98(2):445. doi:10.1007/s00277-018-3515-2
8. Pinczés LI, Szabó R, Miltényi Z, Illés Á. The impact of autoimmune cytopenias on the clinical course and survival of Hodgkin lymphoma. *Int J Hematol*. 2021;113(2):175–182. doi:10.1007/s12185-020-03021-6
9. Dimou M, Angelopoulou MK, Pangalis GA, et al. Autoimmune hemolytic anemia and autoimmune thrombocytopenia at diagnosis and during follow-up of Hodgkin lymphoma. *Leuk Lymphoma*. 2012;53(8):1481–1487. doi:10.3109/10428194.2012.660628
10. Sehn LH, Donaldson J, Chhanabhai M, et al. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. *J Clin Oncol*. 2005;23(22):5027–5033. doi:10.1200/JCO.2005.09.137
11. Pavlovsky M, Cubero D, Agreda-Vásquez GP, et al. Clinical outcomes of patients with B-cell non-Hodgkin lymphoma in real-world settings: Findings from the hemato-oncology Latin America observational registry study. *JCO Glob Oncol*. 2022;(8):e2100265. doi:10.1200/GO.21.00265
12. Coiffier B, Lepage E, Brière J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346(4):235–242. doi:10.1056/NEJMoa011795
13. Zahid U, Akbar F, Amaraneni A, et al. A review of autologous stem cell transplantation in lymphoma. *Curr Hematol Malig Rep*. 2017;12(3):217–226. doi:10.1007/s11899-017-0382-1
14. Maurer MJ, Habermann TM, Shi Q, et al. Progression-free survival at 24 months (PFS24) and subsequent outcome for patients with diffuse large B-cell lymphoma (DLBCL) enrolled on randomized clinical trials. *Ann Oncol*. 2018;29(8):1822–1827. doi:10.1093/annonc/mdy203

Unraveling the therapeutic potential of ginsenoside compound Mc1 in Alzheimer's disease: Exploring the role of AMPK/SIRT1/NF-κB signaling pathway and mitochondrial function

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Abstract

Background. Alzheimer's disease (AD) is a disabling neurodegenerating disorder characterized by chronic neuroinflammation, cognitive impairment and memory loss. Current treatment options for AD offer limited benefits, underscoring the urgent need for alternative therapeutics. Despite the promising effects of ginsenosides in neurodegenerative diseases, the therapeutic potential of ginsenoside compound Mc1 (GCMc1) in AD remains to be thoroughly investigated.

Objectives. This study aimed to investigate the therapeutic potential of GCMc1 in rats with AD and to elucidate the molecular mechanisms responsible for its effects.

Materials and methods. Alzheimer's disease was induced in Sprague Dawley rats through a single intracerebro-ventricular injection of amyloid-beta (Aβ)1-42 peptide. The animals were divided into 5 groups: a control group and 4 AD subgroups, with or without receiving 10 mg/kg of GCMc1 and/or 100 μg/kg of compound C intraperitoneally (ip.). Behavioral tests, mitochondrial function, inflammatory cytokines, and proteins expression were evaluated using the Morris water maze (MWM) test, fluorometry, enzyme-linked immunosorbent assay (ELISA), and immunoblotting techniques, respectively.

Results. Treatment with GCMc1 improved cognitive function, reduced hippocampal Aβ accumulation, and suppressed interleukin (IL)-1β, IL-10 and tumor necrosis factor alpha (TNF-α) levels. Ginsenoside compound Mc1 reduced mitochondrial reactive oxygen species (ROS) levels and membrane depolarization, increased adenosine triphosphate (ATP) levels, upregulated the expression of AMPK, PGC-1α and SIRT1 proteins, and downregulated the nuclear factor-kappa-B (NF-κB) expression. Importantly, co-administration of compound C, an AMPK inhibitor, attenuated the beneficial effects of GCMc1, suggesting the involvement of AMPK pathway in mediating GCMc1's neuroprotective effects.

Conclusions. We showed that GCMc1 confers substantial neuroprotection in rats with AD by modulating the AMPK/SIRT1/NF-κB signaling pathway. These findings highlight the potential of GCMc1 as a promising therapeutic agent for AD treatment.

Key words: neuroprotection, neuroinflammation, Alzheimer's disease, cognitive dysfunction, ginsenosides

Background

Alzheimer's disease (AD) is a neurological degenerating disorder that negatively affects the lives of a large population in the world.[1] It is characterized by an impairment in cognitive tasks, memory loss and behavioral abnormalities.[1] Amyloid beta (Aβ) plaques accumulation, neurofibrillary tangles formation and chronic neuroinflammation are considered as the main pathophysiological features of AD.[1,2] Several brain regions play pivotal roles in AD, with the hippocampus and neocortex being particularly affected.[3] The hippocampus, a vital structure for memory consolidation and spatial navigation, is among the first brain regions to exhibit significant damage in AD.[4] Neuronal loss and synaptic dysfunction within the hippocampus contribute to memory impairments commonly observed in AD patients.[4] Additionally, the neocortex, encompassing regions responsible for higher-order cognitive functions such as reasoning and language, experiences progressive degeneration in AD.[5] This degeneration disrupts the intricate neural networks that underlie complex cognitive processes, leading to deficits in reasoning, language and executive function.[6] Neurotransmitters also play a critical role in AD-related cognitive impairments.[7] Acetylcholine, a neurotransmitter involved in memory and learning processes, experiences substantial depletion in different regions of brain in AD. The loss of cholinergic neurons and the subsequent reduction in acetylcholine levels contribute to cognitive decline. Furthermore, excitotoxicity resulting from excessive glutamate release and imbalances in GABAergic neurotransmission can disrupt neural network stability and lead to neuronal damage and cognitive deficits in AD.[7,8] With an aging population, the frequency of AD is increasing, making it a major community health concern.[9] Despite significant efforts to develop effective treatments for AD, there are still no reliable treatments for this debilitating condition, and available therapies only provide modest benefits. Therefore, there is a need to explore alternative approaches for AD treatment.

It has been recently reported that the AMP-activated protein kinase (AMPK) signaling pathway is substantially involved in the regulation of energy metabolism, cellular stress response and neuroinflammation in AD.[10,11] AMP-activated protein kinase is a master regulator of energy homeostasis in cells. In the context of AD, energy metabolism is disrupted, and brain cells struggle to produce and utilize energy efficiently. Activation of AMPK helps restore the energy balance by enhancing processes like glucose uptake and mitochondrial biogenesis, preventing neuronal dysfunction and cell death.[12] In addition, chronic neuroinflammation is a hallmark of AD, and it plays a complex role in disease progression.[13] By inhibiting the activation of proinflammatory pathways, such as nuclear factor-kappa B (NF-κB), AMPK can help reduce the production of inflammatory molecules and cytokines and mitigate the damaging effects of neuroinflammation in AD.[14]

It has been shown that natural compounds, such as ginsenosides, have potential therapeutic benefits in AD.[5,16] Ginsenosides are a class of bioactive compounds found in the roots of ginseng, which have been shown to have neuroprotective effects in several types of neurodegenerative disorders.[6] Among these ginsenosides, ginsenoside compound Mc1 (GCMc1) has shown promising results in improving oxidative, apoptotic and inflammatory responses in the pathophysiology of different diseases in animal models.[17,18] Ginsenoside compound Mc1 is a deglycosylated ginsenoside that has higher pharmacological absorption, bioavailability and activity than deglycosylated ginsenosides.[17] In a recent study, the potential mechanisms underlying the cardioprotective effects of GCMc1 have been studied in an experimental cardiac injury model.[17] The study found that treatment with this compound improved cardiac function and reduced oxidative stress in the hearts and H9C2 cardiomyocytes under hypoxia/reoxygenation injury. The study also showed that GCMc1 increased the activity of AMPK and inhibited the activation of oxidative stress in vivo and in vitro.[17] Activation of AMPK-related signaling pathway and subsequent surviving proteins like situin-1 (SIRT1) can improve cognitive function, reduce Aβ and tau accumulation, and enhance mitochondrial function in animal model of AD.[11,18]

In addition to its effects on AMPK and downstream targets, GCMc1 may also exert its neuroprotective effects by inhibiting the activity of NF-κB-dependent proinflammatory mediators, which show a critical involvement in the pathogenicity of AD.[19] Proinflammatory cytokines and interleukins (ILs) including IL-1β and IL-6, as well as tumor necrosis factor alpha (TNF-α), have been shown to induce neuronal damage and promote the buildup of Aβ and tau proteins within different regions of the brain.[20] Here, mitochondrial dysfunction is closely linked to the production of neuroinflammation in the pathophysiology of AD.[21] Impaired mitochondrial function leads to energy deficits, oxidative stress, mitochondrial DNA damage, inflammasome activation, and neuronal vulnerability, all of which contribute to the chronic neuroinflammatory state observed in AD. Importantly, beneficial impact of GCMc1 on mitochondrial improvement has been reported in cerebral ischemia/reperfusion injury in hyperlipidemic rats.[22] Therefore, targeting mitochondrial activity and neuroinflammation through the safe and effective therapeutic compounds may be a promising strategy for treatment of AD.

Objectives

Given the promising effects of GCMc1 on the activity of signaling pathways regulating mitochondrial biogenesis and function, and on reducing NF-κB-dependent prinflammatory cytokines,[17,18,23] we investigated the impact of GCMc1 on AD outcomes and underlying mechanisms in a rat model.

Specifically, we evaluated the cognitive performance, Aβ accumulation, mitochondrial function, and neuroinflammation in rats with AD following treatment with GCMc1. We hypothesized that treatment with this agent will improve AD outcomes in rats by activating AMPK-dependent mitochondrial biogenesis, improving mitochondrial function and inhibiting NF-κB-dependent inflammatory cytokines. This study will provide valuable insights into the underlying therapeutic benefits of GCMc1 in AD and the mechanisms underlying its neuroprotective effects.

Materials and methods

Animal care and ethics statement

Male Sprague Dawley rats weighing 250–300 g were used in this study. The animals were kept under a controlled temperature of 22–24°C in a housing room with a 12 h light and 12 h dark cycle and given water and standard rat chow ad libitum. All experimental protocols and animal care were conducted in accordance with the guidelines and regulations of the National Institutes of Health (NIH) for the care and use of laboratory animals (2011, 8th ed., National Academy of Sciences, USA) and approved by the local Institutional Animal Care and Use Committee of the Wuhan University of Science and Technology, China (approval No. 20230501001).

Experimental design and sample size calculation

The animals were randomly assigned into 5 groups of 6 rats (total number of rats – 30). The 1st group was considered as the control group and received a vehicle (saline) solution. The 2nd group received 5 μg/μL Aβ peptide to induce AD-like symptoms. The 3rd group received Aβ peptide and 10 mg/kg of GCMc1.[17,18] The 4th group received Aβ peptide and then 100 μg/kg compound C, as AMPK inhibitor. The 5th group received Aβ peptide and then compound C plus GCMc1. The drugs were administered daily by intraperitoneal (ip.) injection for 10 days, starting 1 day after Aβ administration. Every day before each GCMc1 injection, AMPK inhibitor-receiving rats were given ip. injection of compound C (dissolved in dimethyl sulfoxide (DMSO) with the dosage of 100 μg/kg/day). The control and AD model groups were injected with the same volume of DMSO and vehicle. We performed a sample size assessment using PS calculator software, v. 3.1.2 (Informer Technologies, Inc, Los Angeles, USA), with assumptions of the 5% alpha level, 95% power and the standard deviation (SD) of 35% of the means, which indicated a sample size requirement of 5 rats for each experimental group. To ensure robustness, we included 1 additional rat in each group, resulting in a final sample size of 6 rats per experimental group.

Induction of AD model

The AD modeling was induced by a single intra-cerebro-ventricular injection of Aβ (1–42) peptide (5 μg/μL) (Sigma-Aldrich, St. Louis, USA), as previously described.[24] The stereotaxic surgery was performed under anesthesia using ip. injection of a combination of ketamine (65 mg/kg) and xylazine (15 mg/kg). The coordinates for creating holes in the skull were set as follows: 0.8 mm posterior to bregma, 1.5 mm lateral to the sagittal suture and 3.2 mm ventral to the surface of the skull. After fixation and recovery time, an injection of 5 μg/μL of the Aβ peptide solution was administered in the lateral ventricles of both hemispheres using Hamilton 26-gauge syringe. In the control group, a comparable procedure was followed with the injection of normal saline.

Behavioral tests

Behavioral (cognitive) tests were conducted in a Morris water maze (MWM) after drug administration and before surgery.[25] The MWM was a round pool with a diameter of 150 cm and a depth of 50 cm filled with water (24 ±1°C) made opaque with nontoxic white paint. The pool was virtually divided into 4 quadrants and a 10 × 10 cm hidden platform was located 1 cm below the water superficial level in the center of one quadrant. The rats underwent training for 5 days (4 trials for each day) to locate the hidden platform using distal cues. On the 6th day, the hidden platform was disconnected from the pool and the rats swam for 60 s in the pool to search the platform (probe trial). The following parameters were recorded: escape latency (time from start of search to finding the platform), time spent in the target quadrant (in which the platform was located at the time of training) and discrimination index (DI).

Tissue collection and preparation

After behavioral tests, the animals were anesthetized with ketamine (65 mg/kg) and xylazine (15 mg/kg), and the brains of animals were rapidly and carefully removed from their skulls immediately after dislocation of the neck. After locating the hippocampi within the brain (they are usually recognizable due to their distinct shape and location), the hippocampi were carefully removed from the brain tissue using scalpels. Once isolated, the hippocampi were immediately placed in a storage container and then stored at –80°C for further analysis. During tissue processing, the tissues were placed in the radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors and homogenized. The homogenates were centrifuged at 10,000 × g for 10 min at 4°C. Then the supernatants were collected for biochemical analysis.

Mitochondrial function indices

The following mitochondrial function indices were estimated in fresh hippocampal samples after extracting mitochondrial pellets using a commercial extraction kit (Thermo Fisher Scientific, Waltham, USA).

Measurement of mitochondrial membrane potential

The membrane potential of freshly obtained mitochondrial samples was assessed using a fluorescent dye (JC-1; Thermo Fisher Scientific).[26] Hippocampal tissues were homogenized in mitochondrial isolation buffer (10 mM Tris-HCl, 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.4) on ice, and centrifuged at 1,000 × g for 10 min at 4°C. The mitochondrial fraction was obtained from the supernatant by centrifugation at 10,000 × g for 10 min at 4°C. The isolated mitochondria were suspended in 1 mL of JC-1 buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM NaCl, 5 mM glucose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) containing 10 µM JC-1 dye and incubated at 37°C for 30 min. The fluorescent intensity of JC-1 was detected using a fluorescence spectrophotometric reader (PerkinElmer LS55; PerkinElmer, Waltham, USA) with excitation/emission wavelength of 490/530 nm for the green monomers and 540/590 nm for the red aggregates.

Measurement of mitochondrial ROS levels

Mitochondrial reactive oxygen species (ROS) levels were estimated by means of dichloro fluorescein diacetate (DCFDA) assay (Thermo Fisher Scientific).[27] Briefly, the hippocampal tissues were homogenized in isolation buffer and centrifuged at 600 g for 10 min at 4°C. The mitochondrial pellet was re-suspended in DCFDA containing buffer and incubated at 37°C for 30 min. Then, their fluorescent intensities were detected using the fluorescence spectrophotometric reader at excitation/emission wavelength of 485/535 nm.

Measurement of ATP levels

An ATP Bioluminescent Assay Kit (Sigma-Aldrich) was employed to measure the levels of adenosine triphosphate (ATP) in prepared samples.[28] The supernatant of hippocampal tissues was gathered and diluted to 10 µg/µL protein concentration. The diluted supernatants were mixed with the ATP assay reagent and incubated for 5 min at room temperature. The luminescence signal of the resulting solution was detected by means of a luminometer Victor3 (PerkinElmer).

Hippocampal Aβ accumulation and cytokines measurement

The levels of Aβ accumulation and cytokines IL-1β, IL-10 and TNF-α were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource Inc., San Diego, USA), based on the manufacturer's instructions.[29] Briefly, the previously prepared supernatants were incubated with the cytokine-specific antibody for 2 h, followed by the addition of a biotinylated secondary antibody for 1 h. The solution was then incubated with streptavidin-horseradish peroxidase (HRP) for 30 min, and the reaction was visualized using a substrate solution. The absorbances of solutions were detected at 450 nm using an ELISA reader (BioTek Instruments, Winooski, USA). The concentration of protein in the supernatant was measured using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific).

Western blotting

After preparing supernatants, an amount of about 30 µg of protein from each sample was separated with sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by an electrophoretic transfer to a nitrocellulose membrane (Bio-Rad, Hercules, USA).[30] The membrane was then placed in a dish containing 5% non-fat dry milk blocking buffer in Tris-buffered saline and 0.1% Tween 20 (TBST) for 1 h. This stage was followed by the incubation of the membranes with primary antibodies against total AMPK, phosphorylated AMPK, SIRT1, PGC-1α, phosphorylated p65-NFκB (1:1500; Cell Signaling Technology (CST), Danvers, USA), and GAPDH (1:1000; CST) in TBST at 4°C. After washing the membranes 5 times with TBST, they were soaked in HRP-conjugated secondary antibody (1:1500; SCT). One hour later, TBST was used again to rinse the membranes, and then the membranes were placed in the vicinity of the enhanced chemiluminescence (ECL) reagents so that the protein bands appeared. The protein bands were visualized by means of a visualizing device (Bio-Rad) and their intensities were quantified through ImageJ software (National Institutes of Health (NIH), Bethesda, USA). After selecting the specific lanes containing the protein bands of interest within the ImageJ, the pixel values of the bands were calculated and the intensities of the protein bands were normalized to the corresponding intensity of the GAPDH band.

Statistical analyses

Data analysis was performed with GraphPad Prism 7 software (GraphPad Software, San Diego, USA). Data were presented as median (interquartile range (IQR)). The differences between groups were analyzed using one-way

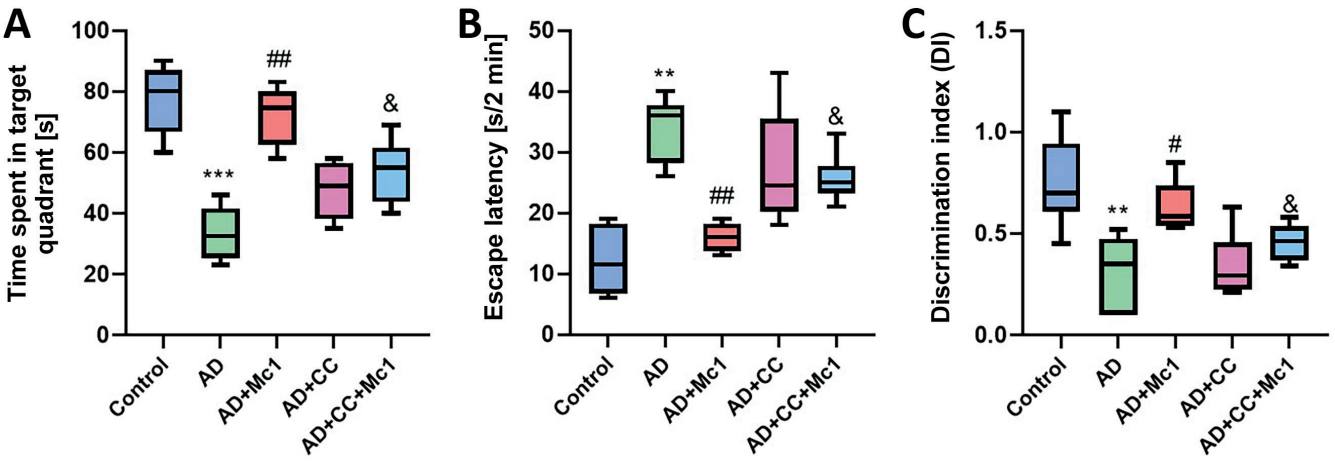

Fig. 1. Effect of ginsenoside compound Mc1 on behavioral cognitive functions in rats with Alzheimer's disease (AD). A. Time spent in target quadrant (in seconds); B. escape latencies (in seconds per 2 min); C. Discrimination index (DI). These cognitive functions were evaluated with the Morris water maze test. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The box plots represent the median and distribution of the data, where the box edges indicate the interquartile range (IQR; Q1–Q3); n = 6. ** < 0.01, and *** < 0.001 compared to control group; # < 0.05, and ## < 0.01 compared to AD group; & < 0.05 compared to AD+Mc1 group. GCMc1 – ginsenoside compound Mc1; CC – compound C

analysis of variance (ANOVA) and subsequent post hoc with Tukey's test. To ascertain the normality of the dataset, the Shapiro–Wilk test was conducted. Also, Bartlett's test was performed to validate the assumption of homogeneity of variances across groups. A p-value <0.05 was set to determine the statistical significance.

Results

Effect of GCMc1 on AD outcomes

We first evaluated the effect of GCMc1 on cognitive function in rats using the MWM trial. One-way ANOVA analysis revealed that in comparison to the AD group, rats treated with GCMc1 showed significantly shorter escape latencies ($p < 0.01$) and more times spending in the target quadrant ($p < 0.01$), indicating boosted spatial memory and learning. In addition, discrimination index was higher following GCMc1 administration compared with AD group ($p = 0.03$) (Fig. 1A–C). Also, we performed an ELISA assay for Aβ measurement and found that treatment with GCMc1 significantly decreased Aβ accumulation in the hippocampus ($p < 0.01$) compared to the AD group (Fig. 2). However, co-administration of compound C to inhibit AMPK pathway significantly reduced the beneficial impacts of GCMc1 on hippocampal Aβ accumulation as well as spatial learning and memory indices, as evidenced by a significant increase in escape latency ($p = 0.05$), decreased spending times in the target quadrant ($p = 0.03$) and lower discrimination index ($p = 0.04$) in comparison to the AD+Mc1 group (Fig. 1,2). These results suggest that GCMc1 has beneficial effects on AD outcomes and improves learning and memory features in rats, and that these effects are AMPK-dependent.

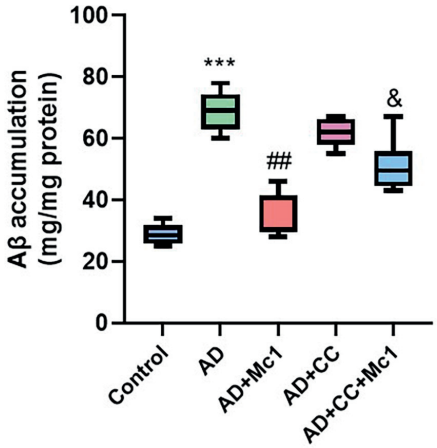

Fig. 2. Effect of ginsenoside compound Mc1 on hippocampal Aβ accumulation in rats with Alzheimer's disease (AD). Aβ accumulation was assessed with enzyme-linked immunosorbent assay (ELISA) method. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The box plots represent the median and distribution of the data, where the box edges indicate the interquartile range (IQR; Q1–Q3); n = 6. *** < 0.001 compared to control group; ## < 0.01 compared to AD group; & < 0.05 compared to AD+Mc1 group; GCMc1 – ginsenoside compound Mc1; CC – compound C

Effect of GCMc1 on mitochondrial function

We next investigated the effect of GCMc1 on mitochondrial function; the data were analyzed using one-way ANOVA and subsequent Tukey's post hoc tests. Mitochondrial function indices, including mitochondrial ROS levels, mitochondrial membrane potential changes and ATP levels, were measured in the hippocampal tissue samples (Fig. 3A–C). Induction of AD led to a significant rising of mitochondrial ROS level ($p < 0.001$) and declining of mitochondrial membrane potential and ATP levels ($p < 0.01$) in comparison to the control group.

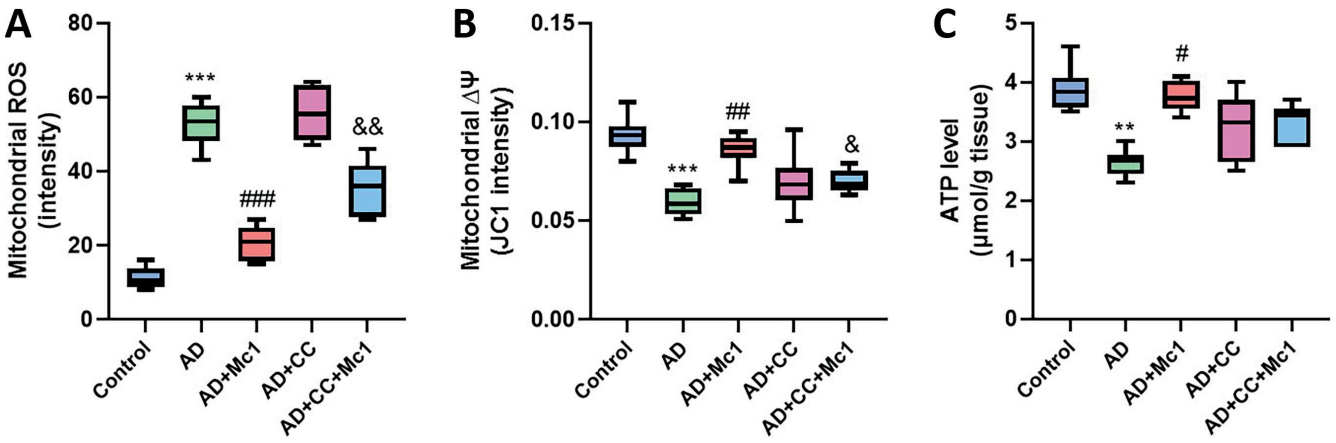

Fig. 3. Effect of ginsenoside compound Mc1 on mitochondrial function indices in rats with Alzheimer's disease (AD). A. Mitochondrial reactive oxygen species (ROS) levels; B. Mitochondrial membrane potential (ΔΨ); C. Adenosine triphosphate (ATP) levels. Mitochondrial ROS and ΔΨ were detected flourometrically and ATP levels were measured with a bioluminescent assay kit. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The box plots represent the median and distribution of the data, where the box edges indicate the interquartile range (IQR; Q1–Q3); n = 6; ** < 0.01, and *** < 0.001 compared to control group; # < 0.05, ## < 0.01, and ### < 0.001 compared to AD group; & < 0.05 and && < 0.01 compared to AD+Mc1 group. GCMc1 – ginsenoside compound Mc1; CC – compound C

Treatment with GCMc1 in the AD+Mc1 group showed significant improvement in all mitochondrial function parameters compared to the AD group ($p < 0.001$). However, in the AD+CC+Mc1 group, the protective effect of GCMc1 was abolished, as evidenced by a significant overproduction of mitochondrial ROS ($p < 0.01$) and mitochondrial membrane depolarization ($p = 0.05$) compared to the AD+Mc1 group (Fig. 3A, 3B). These results suggest that the activation of AMPK plays a role in the protective effects of GCMc1 on mitochondria in AD.

Effect of GCMc1 on cytokines levels

The levels of IL-1β, IL-10 and TNF-α cytokines were measured in the hippocampal tissue samples to estimate the extent of neuroinflammation. One-way ANOVA demonstrated that the levels of IL-1β and TNF-α were increased and the level of IL-10 was reduced in AD group when compared to the control group ($p < 0.001$) (Fig. 4). Treatment with GCMc1 showed significant reduction of proinflammatory cytokines IL-1β and TNF-α levels and elevation of anti-inflammatory cytokine IL-10 level compared to the AD group ($p < 0.001$ and $p < 0.001$). Conversely, administration of compound-C significantly eliminated the anti-inflammatory effects of GCMc1, so that it amplified the production of IL-1β ($p = 0.04$) and TNF-α ($p = 0.002$) levels and suppressed the production of IL-10 ($p = 0.003$) compared to the AD+Mc1 group ($p < 0.05$). The findings highlight the anti-neuroinflammatory potential of GCMc1 in AD, and the role of AMPK inhibition in partially reversing this effect.

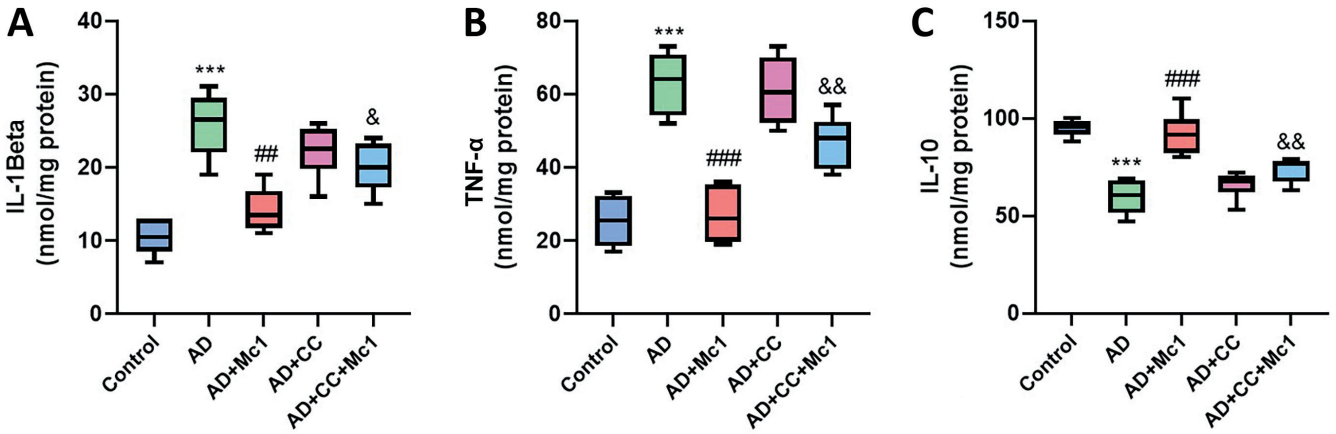

Fig. 4. Effect of ginsenoside compound Mc1 on hippocampal inflammatory cytokines levels in rats with Alzheimer's disease (AD). A. Interleukin-1 beta (IL-1β); B. Tumor necrotic factor alpha (TNF-α); C. Interleukin-10 (IL-10). The cytokines levels were assessed with enzyme-linked immunosorbent assay (ELISA) method. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey's post hoc. The box plots represent the median and distribution of the data, where the box edges indicate the interquartile range (IQR; Q1–Q3); n = 6; *** < 0.001 compared to control group; ## < 0.01, and ### < 0.001 compared to AD group; & < 0.05 and && < 0.01 compared to AD+Mc1 group. GCMc1 – ginsenoside compound Mc1; CC: compound C

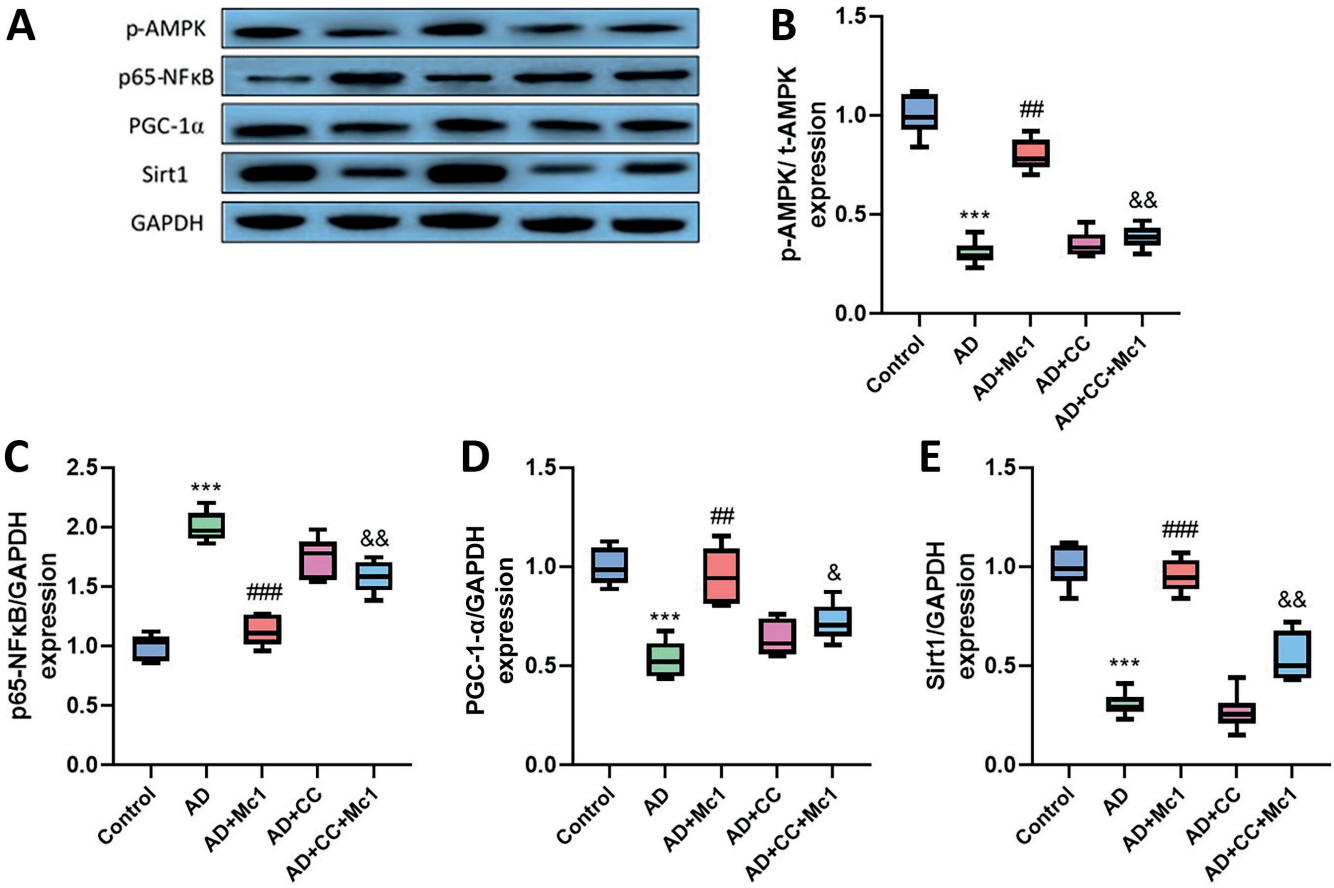

Fig. 5. Effect of ginsenoside compound Mc1 on signaling proteins expression in rats with Alzheimer's disease (AD). A. Western blotting images; B. Phosphorylated AMPK; C. p65-NFkB; D. PGC-1α; E. Sirt1. The expression of proteins was quantified using western blotting technique and subsequent visualization with enhanced chemiluminescence (ECL) reactions. Statistical differences were tested using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The box plots represent the median and distribution of the data, where the box edges indicate the interquartile range (IQR; Q1–Q3); n = 4; *** < 0.001 compared to control group; # < 0.01, and ### < 0.001 compared to AD group; & < 0.05, and && < 0.01 compared to AD+Mc1 group; GCMc1 – ginsenoside compound Mc1, CC – compound C.

Effect of GCMc1 on signaling proteins expression

Analysis of western blot findings with one-way ANOVA between groups showed that the AD group had a significant decrease in phosphorylated AMPK, PGC-1α, Sirt-1, and p65-NFκB compared to the control group ($p < 0.001$) (Fig. 5A–E). Treatment of rats with AD with GCMc1 showed significant upregulation of phosphorylated AMPK ($p < 0.01$) accompanied with higher expression of PGC-1α ($p < 0.01$), and Sirt-1 ($p < 0.001$), and lower expression of p65-NF-κB ($p < 0.001$) compared to the AD group. To explore the role of AMPK pathway activation in the effect of GCMc1 on signaling proteins expression, we measured the expression of proteins after AMPK blockade by compound C. We found that administration of compound C completely suppressed the effect of GCMc1 on AMPK phosphorylation ($p < 0.01$) and partially blocked PGC-1α ($p = 0.05$), SIRT1 and p65-NFκB expression ($p < 0.01$). The findings propose that AMPK pathway activation may be involved in the beneficial effect of GCMc1 on the relevant signaling proteins in rats with AD.

Discussion

The current study revealed the beneficial impact of GCMc1 on AD outcomes via an AMPK-dependent manner in a rat model. The results indicated that the administration of this compound ameliorated cognitive impairment, improved mitochondrial function and reduced inflammatory responses in the hippocampus of AD rats. These findings endorse that GCMc1 has potential therapeutic effects on AD by stimulating the AMPK/PGC-1α/SIRT1 signaling pathway and hindering NF-κB-dependent inflammatory cytokines production. In addition, the activation of AMPK by GCMc1 was required for the observed beneficial effects.

Ginsenosides are natural compounds derived from *Panax ginseng*, which has been traditionally used as a medicinal plant for various diseases.[31,32] Emerging reports have demonstrated that GCMc1 has anti-inflammatory, antioxidative and neuroprotective effects.[17,19,23] Our results suggested that that GCMc1 significantly improved cognitive performance in the AD rats as indicated by lower escape latency, higher time spending in target quadrant

and higher discrimination index. These findings are in line with previous reports in which ginsenoside compounds can recover cognitive function in AD model animals.[33] The mitochondrial dysfunction is a hallmark of AD pathology,[34] and our study demonstrated that GCMc1 improved mitochondrial function by reducing ROS production, increasing mitochondrial membrane potential and enhancing ATP production.

There is growing evidence indicating a connection between mitochondrial dysfunction and inflammatory responses in AD.[35] These dysfunctional mitochondria trigger a series of events that result in inflammation within the brain. Inflammatory responses, characterized by the release of proinflammatory mediators, exacerbate neuronal damage and contribute to the progression of AD pathophysiology.[20] Our study showed that GCMc1 repressed the production of IL-1β and TNF-α as proinflammatory cytokines, and amplified the production of IL-10 as anti-inflammatory cytokine in the hippocampus of AD rats. These findings confirm the previous reports regarding that ginsenosides can exert anti-inflammatory effects in AD models.[15,36] The interplay between mitochondrial dysfunction and inflammatory responses forms a vicious cycle, where inflammation further disrupts mitochondrial function, creating a detrimental feedback loop.[37] Intervening in this destructive cycle with GCMc1 could potentially decrease the AD-induced functional and structural changes in brain regions and enhance learning, memory and cognitive functions. Understanding and targeting this interaction may offer potential therapeutic strategies for combating AD.

Neuroplasticity deficits, characterized by synaptic loss and neuronal shrinkage in the prefrontal cortex and hippocampus, significantly contribute to the impairment of learning and memory.[38,39] The accumulation of Aβ within hippocampal neurons has detrimental effects on synaptic plasticity.[40] However, there is currently no available information on the potential impact of GCMc1 in this context. Nonetheless, previous research has suggested that other types of ginsenosides can positively influence these endpoints, resulting in cognitive improvement. For example, ginsenoside Rg1 treatment in experimental models not only increased the sensitivity of triggering synaptic responses and restored long-term potentiation (LTP), but also elevated the expression of proteins associated with synaptic plasticity, including glutamate receptor-1, synaptophysin and postsynaptic density 95 (PSD95).[41] Similarly, ginsenoside Rb1 treatment enhanced LTP and the transmission of glutamatergic and GABAergic signals in the hippocampal CA3 region.[42] This effect was linked to the sequential enhancement of PSD95 and α-synuclein expression in this region. Notably, ginsenoside Rb1 administration restored cholinergic dysfunction and promoted cell survival in the dentate gyrus and hippocampal CA3, indicating that ginsenosides had a positive impact not only on synaptic plasticity but also on the process of neurogenesis.[42,43] It is essential to highlight, however, that there is still a gap in research focusing on the specific mechanisms through which GCMc1 may target synaptic plasticity and neurogenesis to enhance cognitive function in AD.

AMP-activated protein kinase is a key regulator of cellular metabolic activity, and its normal function can improve mitochondrial function and reduce inflammation.[44] Our study demonstrated that GCMc1 activated AMPK and this activation was required for the observed beneficial effects on cognitive function, mitochondrial function and inflammation. Inhibition of AMPK activity by compound C abolished the protective effects of the drug in AD rats. Our results agree with the previous findings demonstrating that AMPK activation by ginsenosides is involved in their beneficial effects on mitochondrial function and inflammation.[45] PGC-1α and Sirt1 are downstream targets of AMPK, and play crucial roles in regulating mitochondrial biogenesis and function.[46] Our study showed that GCMc1 increased the expression of PGC-1α and Sirt1, indicating that improving mitochondrial function is likely due to the activation of the PGC-1α/Sirt1 pathway. In addition, GCMc1 reduced the activity of p65-NF-κB, suggesting that the observed anti-inflammatory property of this compound are intermediated through the inhibition of NF-κB signaling. NF-κB is a transcription molecule that controls various inflammatory cytokines expression, and its activation has been associated with neuroinflammation, neurodegeneration and cognitive impairment in patients with AD.[4] Interestingly, there is emerging evidence suggesting a cross-link among AMPK and NF-κB signalings.[48] The activation of AMPK has been shown to inhibit NF-κB activity, thereby attenuating the inflammatory response. Conversely, NF-κB activation can inhibit AMPK signaling, leading to further disruption of energy metabolism and mitochondrial dysfunction.[48] These findings support the hypothesis that the neuroprotective effects of GCMc1 on AD outcomes may be partly attributed to its ability to activate cerebral AMPK/PGC-1α/Sirt1/NF-κB signaling pathway, leading to the improvement of mitochondrial function and limitation of inflammatory responses in the hippocampus of rats. It is important to note that the exact mechanisms underlying the neuroprotective effects of GCMc1 in AD are still an active area of research. To fully understand its potential therapeutic benefits, further studies are needed to elucidate the contribution of other important mechanisms and mediators responsible for these effects, including the involvement of neurotrophic factors, mitochondrial homeostasis and biogenesis, apoptosis, autophagy, and neurotransmitter regulation.[49–51]

Limitations

There were some limitations to our research. First, we only used male rats in our study and future reseach should explore the effect of ginsenoside on female rats.

Second, we only used 1 AD model here, and similar studies are necessary to investigate the effects of GCMc1 in other AD models that are more similar to human patient's conditions. It is crucial to replicate the study in multiple AD models that better mimic the heterogeneity seen in human patients. Third, the mechanisms responsible for the activation of PGC-1α/Sirt1 pathway independent of AMPK by this compound in AD rats require further investigation.[51,52]

Conclusions

Our study demonstrated that the GCMc1 improved cognitive features and mitochondrial function, and reduced neuroinflammation in AD rats through the activation of AMPK and modulation of the downstream PGC-1α/Sirt1/NF-κB signaling pathway. The findings suggest that GCMc1 has the potential to be a valuable addition to the therapeutic strategy for AD. However, further research, including clinical trials, is needed to confirm GCMc1 translational application in human patients. Clinical studies can provide valuable insights into the GCMc1 safety, dosage and efficacy in a real-world clinical setting. Investigating the long-term effects of this compound on AD is essential to assess its potential as a sustainable treatment option for its clinical use. Finally, given the intricate pathophysiology of AD, the combination of this compound with other pharmacological or non-pharmacological interventions may lead to additive or synergistic effects in enhancing outcomes for individuals with AD.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10038799. The package includes the following file:

Supplementary File 1. Results of the statistical analysis of project data. The information regarding the results of the statistical analysis for each project's data is presented in this file, organized in accordance with the data order presented in the article.

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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References

1. Breijyeh Z, Karaman R. Comprehensive review on Alzheimer's disease: Causes and treatment. *Molecules*. 2020;25(24):5789. doi:10.3390/molecules25245789
2. DeTure MA, Dickson DW. The neuropathological diagnosis of Alzheimer's disease. *Mol Neurodegener*. 2019;14(1):32. doi:10.1186/s13024-019-0333-5
3. Wang X, Michaelis LM, Michaelis EK. Functional genomics of brain aging and Alzheimers disease: Focus on selective neuronal vulnerability. *Curr Genomics*. 2010;11(8):618–633. doi:10.2174/138920210793360943
4. Rao YL, Ganaraja B, Murlimanju BV, Joy T, Krishnamurthy A, Agrawal A. Hippocampus and its involvement in Alzheimer's disease: A review. *3 Biotech*. 2022;12(2):55. doi:10.1007/s13205-022-03123-4
5. Jobson DD, Hase Y, Clarkson AN, Kalaria RN. The role of the medial prefrontal cortex in cognition, ageing and dementia. *Brain Commun*. 2021;3(3):fcab125. doi:10.1093/braincomms/fcab125
6. Menon V, D'Esposito M. The role of PFC networks in cognitive control and executive function. *Neuropsychopharmacology*. 2022;47(1):90–103. doi:10.1038/s41386-021-01152-w
7. Yang Z, Zou Y, Wang L. Neurotransmitters in prevention and treatment of Alzheimer's disease. *Int J Mol Sci*. 2023;24(4):3841. doi:10.3390/ijms24043841
8. Kandimalla R, Reddy PH. Therapeutics of neurotransmitters in Alzheimer's disease. *J Alzheimers Dis*. 2017;57(4):1049–1069. doi:10.3233/JAD-161118
9. Xia X, Jiang Q, McDermott J, Han JJ. Aging and Alzheimer's disease: Comparison and associations from molecular to system level. *Aging Cell*. 2018;17(5):e12802. doi:10.1111/acel.12802
10. Wang X, Zimmermann HR, Ma T. Therapeutic potential of AMP-activated protein kinase in Alzheimer's disease. *J Alzheimers Dis*. 2019;68(1):33–38. doi:10.3233/JAD-181043
11. Assefa BT, Tafere GG, Wondafrash DZ, Gidey MT. The bewildering effect of AMPK activators in Alzheimer's disease: Review of the current evidence. *Biomed Res Int*. 2020;2020:9895121. doi:10.1155/2020/9895121
12. Curry DW, Stutz B, Andrews ZB, Elsworth JD. Targeting AMPK signaling as a neuroprotective strategy in Parkinson's disease. *J Parkinsons Dis*. 2018;8(2):161–181. doi:10.3233/JPD-171296
13. Onyango IG, Jauregui GV, Čarná M, Bennett JP, Stokin GB. Neuroinflammation in Alzheimer's disease. *Biomedicines*. 2021;9(5):524. doi:10.3390/biomedicines9050524
14. Li YQ, Chen Y, Jiang SQ, et al. An inhibitor of NF-κB and an agonist of AMPK: Network prediction and multi-omics integration to derive signaling pathways for acteoside against Alzheimer's disease. *Front Cell Dev Biol*. 2021;9:652310. doi:10.3389/fcell.2021.652310
15. Shi Z, Chen H, Zhou X, Yang W, Lin Y. Pharmacological effects of natural medicine ginsenosides against Alzheimer's disease. *Front Pharmacol*. 2022;13:952332. doi:10.3389/fphar.2022.952332
16. Zheng M, Xin Y, Li Y, et al. Ginsenosides: A potential neuroprotective agent. *Biomed Res Int*. 2018;2018:8174345. doi:10.1155/2018/8174345
17. Hong SH, Hwang HJ, Kim JW, et al. Ginsenoside compound-Mc1 attenuates oxidative stress and apoptosis in cardiomyocytes through an AMP-activated protein kinase-dependent mechanism. *J Ginseng Res*. 2020;44(4):664–671. doi:10.1016/j.jgr.2019.08.006
18. Liu L, Liu C, Fang L. AMPK-SIRT1 pathway dysfunction contributes to neuron apoptosis and cognitive impairment induced by sevoflurane. *Mol Med Rep*. 2020;23(1):56. doi:10.3892/mmr.2020.11694
19. Sun Y, Geng J, Wang D. Cardioprotective effects of ginsenoside compound-Mc1 and *Dendrobium nobile* Lindl against myocardial infarction in an aged rat model: Involvement of TLR4/NF-κB signaling pathway. *Eur J Inflamm*. 2021;19:205873922110005. doi:10.1177/20587392211000577
20. Rani V, Verma R, Kumar K, Chawla R. Role of pro-inflammatory cytokines in Alzheimer's disease and neuroprotective effects of pegylated self-assembled nanoscaffolds. *Curr Res Pharmacol Drug Discov*. 2023;4:100149. doi:10.1016/j.crphar.2022.100149
21. Yoo SM, Park J, Kim SH, Jung YK. Emerging perspectives on mitochondrial dysfunction and inflammation in Alzheimer's disease. *BMB Rep*. 2020;53(1):35–46. doi:10.5483/BMBRep.2020.53.1.274
22. Wang M, Li D. Ginsenoside-Mc1 reduces cerebral ischemia–reperfusion injury in hyperlipidemia through mitochondrial improvement and attenuation of oxidative/endoplasmic reticulum stress. *Arch Biol Sci (Beogr)*. 2022;74(2):159–168. doi:10.2298/ABS220212015W

23. Zhang Y, Xu K, Zhang Y. Ginsenoside-Mc1 reduces ischemia/reperfusion-induced cardiac arrhythmias through activating JAK2/STAT3 pathway and attenuating oxidative/endoplasmic reticulum stress in hyperlipidemic rats. *Turk J Biochem*. 2022;47(4):491–500. doi:10.1515/tjb-2021-0171
24. Cetin F, Yazihan N, Dincer S, Akbulut G. The effect of intracerebroventricular injection of beta amyloid peptide (1–42) on caspase-3 activity, lipid peroxidation, nitric oxide and nos expression in young adult and aged rat brain. *Turk Neurosurg*. 2012;23(2):144–150. doi:10.5137/1019-5149.JTN.5855-12.1
25. Othman MZ, Hassan Z, Che Has AT. Morris water maze: A versatile and pertinent tool for assessing spatial learning and memory. *Exp Anim*. 2022;71(3):264–280. doi:10.1538/expanim.21-0120
26. Sivandzade F, Bhalerao A, Cucullo L. Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe. *Bio Protoc*. 2019;9(1):e3128. doi:10.21769/BioProtoc.3128
27. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. *Antioxid Redox Signal*. 2014;20(2):372–382. doi:10.1089/ars.2012.4886
28. Chu Y, Park J, Kim E, Lee S. Fluorescent materials for monitoring mitochondrial biology. *Materials (Basel)*. 2021;14(15):4180. doi:10.3390/ma14154180
29. Alaaeldin R, Bakkar SM, Mohyeldin RH, Ali FEM, Abdel-Maqsoud NMR, Fathy M. Azilsartan modulates HMGB1/NF-κB/p38/ERK1/2/JNK and apoptosis pathways during renal ischemia reperfusion injury. *Cells*. 2023;12(1):185. doi:10.3390/cells12010185
30. Wang N, Jiang D, Zhou C, Han X. Alpha-solanine inhibits endothelial inflammation via nuclear factor kappa B signaling pathway. *Adv Clin Exp Med*. 2023;32(8):909–920. doi:10.17219/acem/158781
31. Liu CY, Zhou RX, Sun CK, et al. Preparation of minor ginsenosides C-Mc, C-Y, F2, and C-K from American ginseng PPD-ginsenoside using special ginsenosidase type-I from *Aspergillus niger* g.848. *J Ginseng Res*. 2015;39(3):221–229. doi:10.1016/j.jgr.2014.12.003
32. Lu JM, Yao Q, Chen C. Ginseng compounds: An update on their molecular mechanisms and medical applications. *Curr Vasc Pharmacol*. 2009;7(3):293–302. doi:10.2174/157016109788340767
33. Feng H, Xue M, Deng H, Cheng S, Hu Y, Zhou C. Ginsenoside and its therapeutic potential for cognitive impairment. *Biomolecules*. 2022;12(9):1310. doi:10.3390/biom12091310
34. Perez Ortiz JM, Swerdlow RH. Mitochondrial dysfunction in Alzheimer's disease: Role in pathogenesis and novel therapeutic opportunities. *Br J Pharmacol*. 2019;176(18):3489–3507. doi:10.1111/bph.14585
35. Galizzi G, Di Carlo M. Mitochondrial DNA and inflammation in Alzheimer's disease. *Curr Issues Mol Biol*. 2023;45(11):8586–8606. doi:10.3390/cimb45110540
36. Li J, Huang Q, Chen J, et al. Neuroprotective potentials of Panax Ginseng against Alzheimer's disease: A review of preclinical and clinical evidences. *Front Pharmacol*. 2021;12:688490. doi:10.3389/fphar.2021.688490
37. López-Armada MJ, Riveiro-Naveira RR, Vaamonde-García C, Valcárcel-Ares MN. Mitochondrial dysfunction and the inflammatory response. *Mitochondrion*. 2013;13(2):106–118. doi:10.1016/j.mito.2013.01.003
38. Price RB, Duman R. Neuroplasticity in cognitive and psychological mechanisms of depression: An integrative model. *Mol Psychiatry*. 2020;25(3):530–543. doi:10.1038/s41380-019-0615-x
39. Liu Y, Zhou G, Song L, et al. DEAD-Box helicase 17 promotes amyloidogenesis by regulating BACE1 translation. *Brain Sci*. 2023;13(5):745. doi:10.3390/brainsci13050745
40. Ma T, Klann E. Amyloid β: Linking synaptic plasticity failure to memory disruption in Alzheimer's disease. *J Neurochem*. 2012;120(s1):140–148. doi:10.1111/j.1471-4159.2011.07506.x
41. Nie L, Xia J, Li H, et al. Ginsenoside Rg1 ameliorates behavioral abnormalities and modulates the hippocampal proteomic change in triple transgenic mice of Alzheimer's disease. *Oxid Med Cell Longev*. 2017;2017:6473506. doi:10.1155/2017/6473506
42. Qu S, Meng X, Liu Y, Zhang X, Zhang Y. Ginsenoside Rb1 prevents MPTP-induced changes in hippocampal memory via regulation of the α-synuclein/PSD-95 pathway. *Aging*. 2019;11(7):1934–1964. doi:10.18632/aging.101884
43. O'Day DH. Calmodulin binding proteins and Alzheimer's disease: Biomarkers, regulatory enzymes and receptors that are regulated by calmodulin. *Int J Mol Sci*. 2020;21(19):7344. doi:10.3390/ijms21197344
44. Zhang J, Wang Y, Liu X, Dagda RK, Zhang Y. How AMPK and PKA interplay to regulate mitochondrial function and survival in models of ischemia and diabetes. *Oxid Med Cell Longev*. 2017;2017:4353510. doi:10.1155/2017/4353510
45. Huang Q, Gao S, Zhao D, Li X. Review of ginsenosides targeting mitochondrial function to treat multiple disorders: Current status and perspectives. *J Ginseng Res*. 2021;45(3):371–379. doi:10.1016/j.jgr.2020.12.004
46. Cantó C, Auwerx J. PGC-1α, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol*. 2009;20(2):98–105. doi:10.1097/MOL.0b013e328328d0a4
47. Sun E, Motolani A, Campos L, Lu T. The pivotal role of NF-kB in the pathogenesis and therapeutics of Alzheimer's disease. *Int J Mol Sci*. 2022;23(16):8972. doi:10.3390/ijms23168972
48. Abd El-Fattah EE, Saber S, Mourad AAE, et al. The dynamic interplay between AMPK/NFκB signaling and NLRP3 is a new therapeutic target in inflammation: Emerging role of dapagliflozin in overcoming lipopolysaccharide-mediated lung injury. *Biomed Pharmacother*. 2022;147:112628. doi:10.1016/j.biopha.2022.112628
49. Han Y, Liu D, Cheng Y, et al. Maintenance of mitochondrial homeostasis for Alzheimer's disease: Strategies and challenges. *Redox Biol*. 2023;63:102734. doi:10.1016/j.redox.2023.102734
50. Qi Y, Chen L, Shan S, Nie Y, Wang Y. Vitexin improves neuron apoptosis and memory impairment induced by isoflurane via regulation of miR-409 expression. *Adv Clin Exp Med*. 2020;29(1):135–145. doi:10.17219/acem/104556
51. Abubakar MB, Sanusi KO, Ugusman A, et al. Alzheimer's disease: An update and insights into pathophysiology. *Front Aging Neurosci*. 2022;14:742408. doi:10.3389/fnagi.2022.742408
52. Li Y, Hu K, Liang M, et al. Stilbene glycoside upregulates SIRT3/AMPK to promotes neuronal mitochondrial autophagy and inhibit apoptosis in ischemic stroke. *Adv Clin Exp Med*. 2021;30(2):139–146. doi:10.17219/acem/130608

Fc-gamma receptor expression and cytokine responses to intravenous human immunoglobulin in whole blood from non-pregnant and pregnant women and newborns

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Abstract

Background. Intravenous immunoglobulin (IVIG) can suppress the inflammatory response in adults, but its role in pregnant women and newborns is poorly studied. While the adult immune system is considered mature, it is immature in neonates and suppressed in pregnancy. Since the immune response differs in these 3 groups, the use of IVIG could differentially modulate the immune response.

Objectives. We aimed to explore the effect of IVIG on myeloid blood cells from non-pregnant women, pregnant women and newborns.

Materials and methods. Whole blood from healthy donors was incubated with lipopolysaccharide (LPS) and/or IVIG. After 0 h, 24 h and 48 h of culture, Fc-gamma receptor (CD16, CD32 and CD64) expression, monocyte and neutrophil bacterial phagocytosis, and cytokine and chemokine concentrations were determined in the supernatant.

Results. The baseline expression of monocyte CD16 was higher in newborns than in adult women, but the expression of CD32 and CD64 was similar between groups. Furthermore, LPS and IVIG stimulation, together or separately, did not change Fc-gamma receptor expression in monocytes or neutrophils and did not modify their phagocytosis capacity. On the other hand, IVIG did not downregulate the proinflammatory cytokine response induced by LPS in any group. Interestingly, IVIG induced a strong interleukin 8 (IL-8) response in neonates but not in non-pregnant or pregnant women.

Conclusions. Our results show that IVIG did not induce changes in Fc-gamma receptor expression, phagocytic ability, or the cytokine response to LPS in blood cells from neonates, non-pregnant or pregnant women. However, IVIG induced a strong IL-8 response in neonates that could improve immunity.

Key words: IVIG, Fc-gamma receptors, maternal-newborn response

Background

Intravenous human immunoglobulin (IVIG) is a serum-based polyclonal pharmaceutical preparation used as a medical treatment for immunodeficiencies and an immunomodulator treatment in patients with autoimmunity or infectious diseases.[1] Furthermore, IVIG has been used in pregnant women to treat fetal-neonatal alloimmune thrombocytopenia, antiphospholipid syndrome and recurrent pregnancy loss.[2,3] However, the ex vivo effects of IVIG on healthy pregnant women and newborns have been poorly analyzed.

Non-pregnant women, pregnant women and newborns have different immune responses. Adult female immunity is mature, pregnant women express tolerogenicity that suppresses the immune response to the fetus, while newborn immunity is immature. In addition, pregnant women and the fetus share multiple mechanisms that regulate the immune system and develop immunological tolerance during pregnancy.[4,5]

Usually, a lower immune response is detected in newborns when comparing adult and newborn immune mechanisms. However, the newborn is exposed to a different hormonal environment than non-pregnant women. Pregnancy appears to be useful for contrasting the immune response in non-pregnant women and newborns because pregnant women express unique tolerogenic and hormonal characteristics that they share with newborns.[6–9] Furthermore, non-pregnant women have a level of immune maturity shown by pregnant women when facing immunoregulatory challenges such as those invoked by IVIG.

The immunoregulatory effects of IVIG involve several mechanisms, such as inhibiting lymphocyte proliferative responses, limiting the inflammatory cytokine response, and apoptosis induction, among others.[10–12] Most of the mechanisms invoked by IVIG depend on binding to Fc-gamma receptors (FcγRs), expressed on most leukocytes.[12] Three FcγR have been described: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16).[13] CD64 is a high-affinity receptor for monomeric IgG, while CD32 and CD16 are low-affinity receptors for IgG. Monocytes constitutively express CD64, macrophages, neutrophils express CD32, and neutrophils and natural killer (NK) cells express CD16.[13] Upon binding of IgG to the FcγR, leukocytes mediate cytokine synthesis and phagocytosis.[12] However, FcγR expression is different in adults and newborns, with monocyte CD16 and CD32 expression lower in adults than in newborns, while CD64 expression is higher in granulocytes in newborns than in adults.[14] In mice, the cellular expression of FcγR changes after IVIG treatment.[15,16]

Objectives

We used an ex vivo model to explore the effects of IVIG on neonatal monocytes and granulocytes, and compare it with the non-pregnant and pregnant response. Whole blood (WB) was stimulated with lipopolysaccharide (LPS), and the effect of IVIG on the expression of FcγRs, monocyte and granulocyte phagocytic capacity as well as the proinflammatory response were analyzed.

Materials and methods

Patients and sample collection

The study included 3 groups: non-pregnant women (NP, n = 18), prepartum pregnant women (P, n = 15) and newborns (N, n = 18). All adult donors were healthy women (25 ±5 years old) at the time of blood collection, and patients with comorbidities such as overweight, obesity, diabetes, hypertension, autoimmunity, and immunosuppression were excluded. All babies were born at term (37–40.3 weeks of gestation) and had healthy anthropomorphic parameters. The Research Committee reviewed and approved our study (Project: HM: INV/2015:2020), which was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Peripheral blood (6 mL) was collected by venipuncture from NP and P participants, while umbilical cord blood (UCB) was collected by arterial umbilical venipuncture (n = 18) after placenta delivery. Blood samples were collected using Vacutainer® plastic sodium heparin tubes (Cat. No. 367876, BD Biosciences, Franklin Lakes, USA). Both UCB and peripheral blood were processed immediately after collection.

Cell culture

Whole blood (1 mL) was incubated in 24-well culture plates (cat. No. 13485; Costar, New York, USA), either alone or with 10 ng/mL of *Escherichia coli* O55: B5 LPS (cat. No. L2880; Sigma Aldrich, St. Louis, USA), 10 mg/mL of IVIG (cat. No. 5240IgG6; Kedrigamma Kedrion Group, Barga, Italy), or LPS/IVIG (both 10 ng/mL) for 0 h, 24 h and 48 h at 37°C with 5% CO_2.

Evaluation of Fcγ receptor expression in monocytes and neutrophils

After cell culture, 50 µL of WB was mixed with antibodies, including CD45-PacificOrange (cat. No. MHCD4530; Invitrogen, Waltham, USA), CD14-PE/Cy7 (cat. No. 301804; BioLegend, Santa Clara, USA), CD16-APC/Cy7 (cat. No. 302018; BioLegend), CD32-PE (cat. No. 303206; BioLegend), and CD64-APC (cat. No. 305014; BioLegend). Appropriate compensation was performed, and isotype controls were used for each antibody. After 15 min of incubation, erythrolysis was performed using FACS™ Lysing Solution (cat. No. 349202; BD Biosciences). The samples were washed twice with ×1 phosphate-buffered saline (PBS) (1,500 rpm, 5 min, 4°C) and resuspended

in 100 µL of PBS. Thirty thousand single leukocytes were acquired on a FACS Aria II flow cytometer (BD Biosciences), and FCS files were analyzed using Infinicyt v. 1.8 software (Cytognos, Salamanca, Spain). The leukocytes were single cells (FSC-A compared to FSC-H plot), CD45-positive, and with typical size and complexity (side scatter (SSC) compared to CD45 plot). Monocytes were SSC^{mid} forward scatter (FSC)$^{mid}CD45^{+}CD14^{+}$, and neutrophils were $SSC^{mid}FSC^{mid}CD45^{+}CD16^{+}$ cells. The percentage of CD64-, CD32- and CD16-positive cells and the mean fluorescence intensity (MFI) were calculated.

Phagocytosis assay

After cell culture, the samples were tested for phagocytosis according to the manufacturer's instructions (pHrodoTM Green *Escherichia coli* BioParticle Kit, cat. No. P35381; Invitrogen). Briefly, WB was precultured alone or with IVIG, LPS or both, and then incubated with pHrodo-conjugated opsonized *E. coli* bioparticles at 37°C or on ice for 15 min. Leukocytes were immunophenotyped using anti-CD45-PacificOrange (cat. No. MHCD4530; Invitrogen), CD14-PE/Cy7 (cat. No. 301804; BioLegend) and CD16-APC/Cy7 (cat. No. 302018; BioLegend). The cells were lysed, washed and counted, their viability was assessed, and they were resuspended in a wash buffer for acquisition on an Aria II BD cytometer. Phagocytosis was evaluated as the percentage of bacteria-positive monocytes or neutrophils, while the MFI value represented the presence of bacteria within a cellular acidic compartment. The nucleated phagocytes were discriminated using the SSC and FSC parameters, with neutrophils defined by the $SSC\text{-}A^{med}FSC\text{-}H^{med}CD45^{+}CD14^{+}$ phenotype.

Cytokine quantification

After culture, plasma cytokine quantification (interleukin (IL)-8, tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, IL-10, and IL-12) was performed according to the manufacturer's instructions (LEGENDplex human inflammation kit, cat. No. 740808; BioLegend). Data were acquired with a FACS Aria III BD cytometer. Logarithmic transformed data were used to construct standard curves fitted to 10 discrete points using a 4-parameter logistic model. Concentrations were calculated using interpolations of the corresponding reference curves.

Statistical analyses

Data analysis employed IBM SPSS Statistics for Windows v. 25.0 (IBM Corp., Armonk, USA) or GraphPad Prism 7.0 (GraphPad Software, San Diego, USA). Results are expressed as mean ± standard error (M ±SE) or mean ± standard deviation (M ±SD). Fc-gamma receptors and phagocytosis data were analyzed using a bootstrap repeated measures analysis of variance (RM-ANOVA), while cytokine kinetics analysis used two-way RM-ANOVA with Tukey´s multiple comparisons. The 95% confidence interval (95% CI) was calculated for each test, and $p < 0.05$ was considered statistically significant. Supplementary data contain the normality tests, bootstrap and Tukey's multiple comparisons analysis for conditions analyzed.

Results

Fc-gamma receptor expression in monocytes and granulocytes

The P blood samples contained the lowest percentage of monocytes (2.2 ±1.2%), which was lower than in N patients (8.9 ±4.4%, p = 0.001). Also, pregnant women had the highest percentage of granulocytes (69.2 ±6.1%), significantly more than N patients (40.9 ±10.4%, p = 0.001). Since the monocyte and granulocyte percentages differed between P and N, we calculated if the expression of FcγR per cell was different between the groups and calculated MFI/% for CD16, CD32 and CD64. Table 1 shows the index for each Fc-gamma receptor (FcγR) on monocytes and neutrophils.

At the beginning of the kinetics assessment, N showed the highest CD16 index in monocytes, with a statistical difference between the NP and N CD16 index (282 ±199, 95% CI: –109.4–674.5 compared to 2003.7 ±199; 95% CI: 1,611–2,395). After 24 h and 48 h of culture, the CD16 index decreased for each group, though only the N CD16 index decreased from 0 h to 48 h (2004 ±199; 95% CI: 1611–2,395 compared to 221.1 ±141.1; 95% CI: –56–498.3).

Treatment with IVIG, LPS or IVIG+LPS did not induce significant changes in the CD16 index during kinetics. Generally, the neutrophil CD16 index was similar in NP and P, and remained almost unchanged with or without IVIG or LPS. Meanwhile, the CD16 index in N neutrophils remained unchanged when WB was cultured alone. In contrast, IVIG, LPS and IVIG+LPS decreased the CD16 index in N after 48 h of culture (525 ±199; 95% CI: 133.2–917.3 compared to 273.8 ±141.1; 95% CI: –3.3–551; 241.6 ±141.1; 95% CI: –35.5–518.8; and 280.8 ±141.1, 95% CI: 3.6–558, respectively).

The monocyte CD32 index was similar between times and groups throughout, with only IVIG causing a significant change in the CD32 index between 24 h and 48 h in NP women (77.8 ±261; 95% CI: –434.8–590.6 compared to 345.6 ±261.1; 95% CI: –167–858.4). Also, the neutrophil CD32 index was changed by IVIG between 24 h and 48 h (111.3 ±261.1; 95% CI: –401.4–624 compared to 346.4 ±261.1; 95% CI: –166.2–859.2). Furthermore, N had the lowest CD32 index after 48 h of culture with IVIG, with the response lower than P and NP (194.6 ±182, 95% CI: –163.1–552.5 or 43.7 ±257.7; 95% CI: –462.3–549.8 compared to 346.4 ±261.1; 95% CI: –166.2–859.2, respectively).

Table 1. FcγR index on monocytes or neutrophils of non-pregnant women, pregnant women and newborns

Index	Hours								
	0			24			48		
	NP	P	N	NP	P	N	NP	P	N
CD16 MFI/% monocytes									
WB alone	282 ±275	549 ±350	1920 ±2427	47 ±28	342 ±248	319 ±298	80 ±35	125 ±35	526 ±437
+IVIG	282 ±275	549 ±350	1920 ±2427	862	296 ±122	167 ±112	134 ±96	132 ±70	358 ±195
+LPS	282 ±275	549 ±350	1920 ±2427	124	416 ±434	312 ±222	141 ±107	126 ±109	270 ±202
++IVIG+LPS	282 ±275	549 ±350	1920 ±2427	124	416 ±434	312 ±222	96 ±52	99 ±51	383 ±447
CD32 MFI/% monocytes									
WB alone	230 ±137	122 ±55	139 ±46	310 ±32	239 ±55	227 ±159	349 ±282	200 ±30	344 ±297
+IVIG	230 ±137	122 ±55	139 ±46	77 ±22	182 ±42	127 ±62	345 ±221	202 ±102	201 ±118
+LPS	230 ±137	122 ±55	139 ±46	198 ±161	192 ±106	211 ±137	216 ±148	144 ±45	183 ±147
+IVIG+LPS	230 ±137	122 ±55	139 ±46	198 ±161	192 ±32	211 ±137	197 ±102	160 ±9	172 ±106
CD64 MFI/% monocytes									
WB alone	232 ±60	208 ±110	135 ±26	225 ±49	349 ±91	164 ±103	300 ±103	191 ±53	155 ±76
+IVIG	232 ±60	208 ±110	135 ±26	276 ±164	270 ±68	150 ±34.99	281 ±187	204 ±21	110 ±13
+LPS	232 ±60	208 ±110	135 ±26	432 ±254	317 ±123	248 ±150	399 ±218	197 ±43	208 ±123
+IVIG+LPS	232 ±60	208 ±110	135 ±26	432 ±60	317 ±123	155 ±76	399 ±102	250 ±26	133 ±66
CD16 MFI/% neutrophils									
WB alone	386 ±447	432 ±276	351 ±337	784 ±739	444 ±235	369 ±302	273 ±276	328 ±211	324 ±253
+IVIG	386 ±447	432 ±276	351 ±337	409 ±109	392 ±267	431 ±313	312 ±269	341 ±211	324 ±238
+LPS	386 ±447	432 ±276	351 ±337	405 ±459	355 ±235	403 ±274	248 ±266	307 ±213	306 ±243
+IVIG+LPS	386 ±447	432 ±276	351 ±337	405 ±459	355 ±235	403 ±274	332 ±210	374 ±240	229 ±180
CD32 MFI/% neutrophils									
WB alone	83 ±24	145 ±81	89 ±10	129 ±90	88 ±41	80 ±36	216 ±173	63 ±26	120 ±88
+IVIG	83 ±24	145 ±81	89 ±10	111 ±90	37 ±6	45 ±7	346 ±263	43 ±7	42 ±15
+LPS	83 ±24	145 ±81	89 ±10	104 ±67	264 ±314	101 ±53	143 ±91	75 ±18	90 ±35
+IVIG+LPS	83 ±24	145 ±81	89 ±10	104 ±67	264 ±314	101 ±53	132 ±70	119 ±97	60 ±33
CD64 MFI/% neutrophils									
WB alone	100 ±71	159 ±49	93 ±27	133 ±89	148 ±24	190 ±128	177 ±227	346 ±320	164 ±106
+IVIG	100 ±71	159 ±49	93 ±27	141 ±111	184 ±84	141 ±67	151 ±182	169 ±51	187 ±119
+LPS	100 ±71	159 ±49	93 ±27	134 ±84	167 ±71	165 ±77	123 ±103	246 ±123	177 ±127
+IVIG+LPS	100 ±71	159 ±49	93 ±27	134 ±84	167 ±71	165 ±77	165 ±88	283 ±182	127 ±89

Data show the mean ± standard deviation (M ±SD) for each FcγR index on monocytes or neutrophils. Non-pregnant women (NP, n = 5), pregnant (P, n = 3) and newborns (N, n = 5). Differences among groups were calculated using a bootstrap repeated measures analysis of variance (RM ANOVA). The whole bootstrap RM ANOVA is shown in Supplementary Table 1. WB – whole blood; IVIG – intravenous immunoglobulin; LPS – lipopolysaccharide.

The monocyte CD64 index showed some differences between NP and P, with P monocytes from WB cultured alone having a lower CD64 index after 48 h than at 24 h of culture (191.3 ±257.7; 95% CI: –314.7–697.4 compared to 208.5 ±257; 95% CI: –297.5–714.6). Meanwhile, LPS led to a higher CD64 index after 24 h compared to 0 h of culture in the NP group (432.9 ±202.2; 95% CI: 35.7–830 compared to 232.9 ±199.6; 95% CI: –159–624.9). In contrast, N monocytes did not show statistical differences in the CD64 index. However, a lower CD64 index was detected in N monocytes than NP monocytes after 48 h of culturing WB alone (221.3 141.1; 95% CI: –55.8–498.5 compared to 300.8 ±202.2; 95% CI: –96.3–697.9), and with IVIG (146.8 ±141.1; 95% CI: –130.3–424 compared to 281.1 ±202.2; –95% CI: 116–678.3), LPS (130.3 ±141.1; 95% CI: –146.8–407.5 compared to 399.4 ±202; 95% CI: 2.2–796.6) or IVIG+LPS (146.8 ±141.1; 95% CI: –130.3–424 compared to 399.6 ±202.2; 95% CI: 2.4–796). These differences were not observed in the CD64 index of neutrophils, with only minor changes detected.

Effect of intravenous immunoglobulin on phagocytosis

Since IVIG can opsonize antigens or interact with FcγRs to enhance or reduce the phagocytic capacity of blood cells, we performed an ex vivo phagocytosis assay. We observed that monocytes from the NP and P groups had a similar percentage of cells that phagocytosed bacteria. On the contrary,

Table 2. Comparison of pHrodo *Escherichia coli* phagocytosis in monocytes and neutrophils of non-pregnant women, pregnant women and newborns in a 24-h culture

Phagocytosis	Hours					
	0			24		
	NP	P	N	NP	P	N
	% monocytes bacteria-positive					
IVIG	76.1 ±2.5	87.3 ±2.8	76 ±11.3	82.9 ±8.0	78.2 ±2.6	37.4 ±3.2
LPS	76.1 ±2.5	87.3 ±2.8	76 ±11.3	68.7 ±10.0	84.1 ±4.5	34.5 ±9
IVIG+LPS	76.1 ±2.5	87.3 ±2.8	76 ±11.3	74.3 ±1.5	88 ±1.0	38.5 ±4.5
	% neutrophils bacteria-positive					
WB alone	82.8 ±7.9	93.3 ±3.2	93.7 ±4.0	78.5 ±5.7	93.2 ±1.9	49.1 ±29.4
IVIG	82.8 ±7.9	93.3 ±3.2	93.7 ±4.0	51.2 ±15.9	92.5 ±3.5	54.8 ±9.6
LPS	82.8 ±7.9	93.3 ±3.2	93.7 ±4.0	52.8 ±14.1	94.9 ±6.2	51.4 ±4.3
IVIG+LPS	82.8 ±7.9	93.3 ±3.2	93.7 ±4.0	74.7 ±0.6	93.9 ±6.9	53.8 ±3.2

Data show the mean ± standard deviation (M ±SD) of percentage in monocytes and neutrophils. Non-pregnant (NP, n = 3), pregnant (P, n = 3) and newborns (N, n = 3). Differences between times and groups were calculated using a bootstrap repeated measures analysis of variance (RM ANOVA). The whole bootstrap RM ANOVA is shown in Supplementary Table 2. WB – whole blood ; IVIG – intravenous immunoglobulin; LPS – lipopolysaccharide.

the percentage of phagocytic monocytes in N was lower than in NP or P (74.5 ±1,076.6; 95% CI: –2,049.9–2,198.9 compared to 90.8 ±1,076.6; 95% CI: –2,033.5–2,215.2, or 93.8 ±1076.6; 95% CI: –2,030.6–2,218.2, respectively). Interestingly, culture conditions (IVIG, LPS, or IVIG+LPS) or 24 h with stimulation did not induce changes in the percentage of phagocytic monocytes in NP or P women, but the phagocytic ability of N was severely compromised after 24 h of culture, independently of stimulus.

Regarding neutrophils, their intrinsic ability to phagocytose bacteria was reduced in N compared to NP and P women but did not change by stimulus or incubation time (Table 2). In addition to evaluating the percentage of cells that phagocytosed bacteria, we quantified the relative amounts of intracellular bacteria. According to the reduced percentage of monocytes that phagocytosed bacteria at 24 h of incubation, intracellular bacteria was only reduced in N, but not in NP or P women. For neutrophils, the number of phagocytosed bacteria was similar among the 3 groups based on stimulus and incubation time (Supplementary Table 2).

Cytokine response to intravenous immunoglobulin

Figure 1 shows the cytokine response to IVIG, LPS and IVIG+LPS challenge in WB cultures of adults and newborns. As expected, all groups had a cytokine response after the LPS stimulus, and the IL-1β, IL-6 and IL-8 concentrations were higher than in the WB cultured alone. However, the IL-6 response was lower in P than in NP (Fig. 1D, $p < 0.001$) and N (Fig. 1F, $p < 0.001$) after 12 h and 24 h of LPS challenge (Fig. 1D–F, $p < 0.001$ for both). Furthermore, IVIG alone did not evoke an IL-1 or IL-6 in response in NP or P women, but it did for IL-8 in N after 12 h and 24 h of culture (Fig. 1I, both $p < 0.001$). The IVIG+LPS challenge led to similar IL-1β, IL-6 and IL-8 kinetics in adults and newborns. In addition, the cytokine response elicited by IVIG+LPS was similar to the cytokine response to LPS alone in all groups.

Discussion

Intravenous immunoglobulin is an immunomodulator used to treat various diseases. However, since non-pregnant women, pregnant women and newborns have different regulatory and ontogeny statuses, the IVIG immunomodulation mechanism could also differ in each group. The current work presents the basal differences between groups and the effect of IVIG on myeloid cells under LPS stimulus in NP, P and N.

Since IVIG can stimulate the synthesis of cytokines and promote phagocytosis, among other effects, we explored whether it could change the expression of FcγRs (CD16, CD32 and CD64) over 24 h. Before the stimulus, consistent with other reports, our data showed differential expression of FcγRs at baseline in adults and newborns (Table 1),[14] but when used alone or in combination, IVIG only reduced monocyte and neutrophil CD16 expression in newborns after 24 h. Furthermore, CD16 only reached statistical significance in newborns, even though adults and newborns tended to express lower levels. These results suggest that adults and newborns could express differential biological activity elicited by IVIG through varied CD16 expression.

Among FcγRs, the CD32b isoform (FcγRIIb) expresses immunoreceptor tyrosine-based inhibitory motifs[13,17] and could support an inhibitory IVIG-mediated response. Intravenous immunoglobulin has been reported to increase CD32b expression in myeloid cells, maintaining

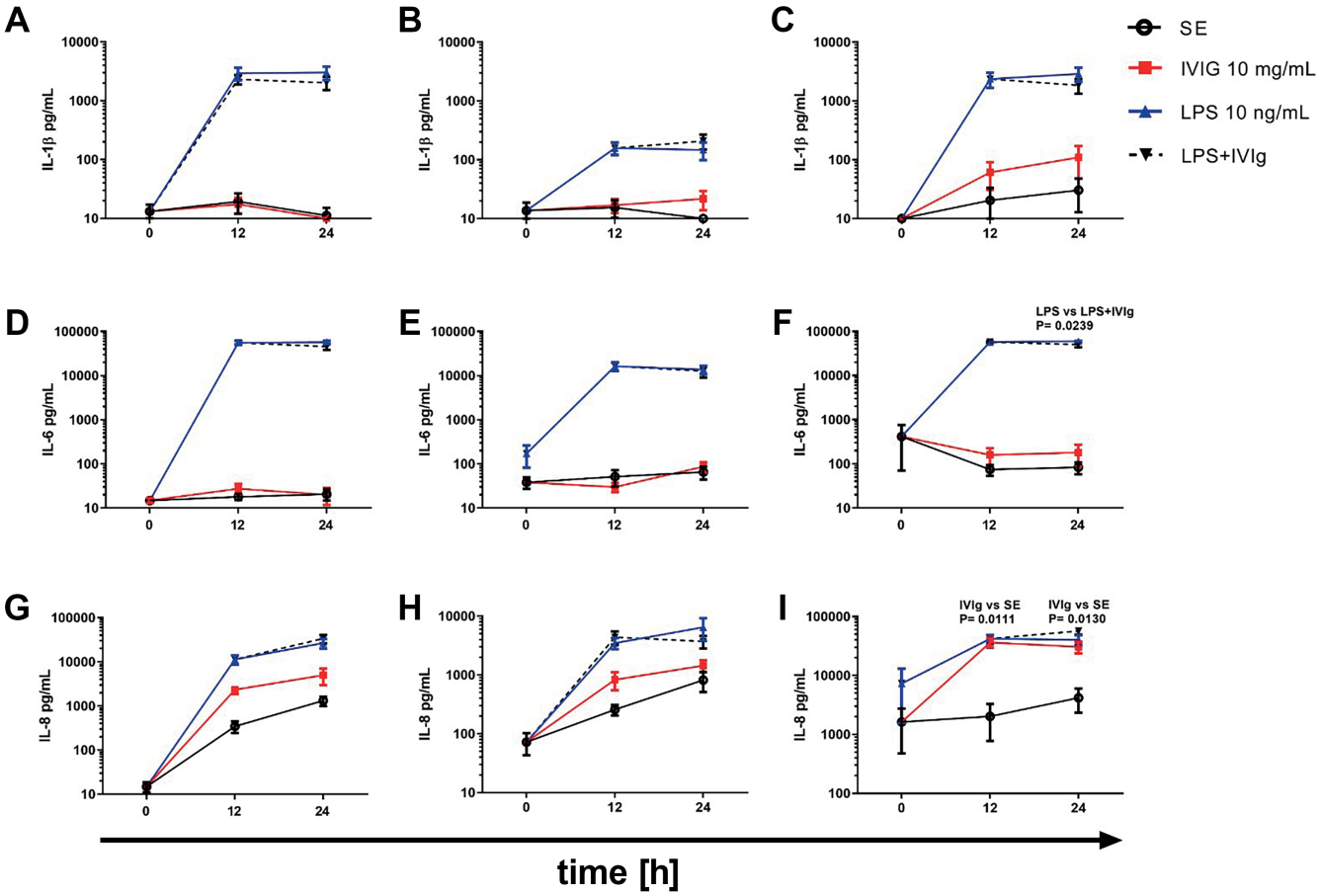

Fig. 1. Cytokine response after lipopolysaccharide (LPS) and intravenous immunoglobulin (IVIG) challenge. Whole blood was cultured for 0 h, 12 h or 24 h in the presence or absence of LPS and IVIG, after which the plasma was collected, and cytokine levels were determined. Ten pairs of mothers and neonates are shown, including non-pregnant women (NP, n = 9), pregnant women (P, n = 9) and neonates (N, n = 9). Results are expressed as mean ± standard deviation (M ±SD) with 95% confidence interval (95% CI) and were analyzed with two-way analysis of variance (ANOVA) with Tukey´s multiple comparisons and significance set at $p < 0.05$

an anti-inflammatory response.[10,12,18] Therefore, we analyzed the CD32 index in monocytes and neutrophils in response to IVIG, but its expression was similar in P and N, with an increased index only observed in NP women (Supplementary Table 1). The study did not analyze the expression of CD32b. As such, future studies must be conducted to determine whether IVIG could overexpress CD32b.

Kozicky et al.[19] reported that IVIG skews monocytes to an anti-inflammatory response. Even when the expression level of FcγR is similar among non-pregnant women, pregnant women and newborns, the transduction signal induced by IVIG after engaging the FcγR could be differential and lead to an inflammatory or anti-inflammatory response. Regarding CD64 expression, Maeda et al.[14] reported that neonatal granulocytes expressed higher levels of CD64, and Luppi et al.[20] and Davis et al.[21] showed that CD64 expression in polymorphonuclear cells gradually increased in the 3rd trimester of pregnancy. Our data show that the monocyte CD64 index was consistently higher in non-pregnant women than in newborns (Table 1), though this was not the case for the neutrophil CD64 index, which remained similar between groups and over time (Table 1 and Supplementary Table 1). In summary, FcγR expression in non-pregnant or pregnant women and newborns was differential, and IVIG only increased the monocyte and neutrophil CD32 index in non-pregnant women.

Phagocytosis could be regulated by IVIG through FcγR. However, contrary to Gille et al.,[22] we did not observe an increase in phagocytosis after IVIG treatment. Mononuclear cells were used in the study by Gille et al. (in vitro model), while we used WB (ex vivo model). We argue that our ex vivo model is closer to the response that could be observed in vivo. Our findings showed that both adult groups (non-pregnant and pregnant) expressed more than 80% of pHrodo *E. coli*-positive monocytes after 24 h in culture. In contrast, only 75% of neonatal monocytes had this ability at the beginning of the culture, which was less than 20% after 24 h. Such a functional limitation in newborns could be due to the physiologic leukocytosis. Despite this condition, IVIG did not change the response to phagocytosis in adults or newborns. Monocytes and neutrophils are fundamental phagocytic cells in peripheral blood that support many aspects of inflammatory immune responses.[23] In particular, they produce large amounts of IL-1β, IL-6 and IL-8.[24–26]

Intravenous immunoglobulin could limit the production of inflammatory cytokines, such as TNF-α, IL-1β and

IL-6 in patients with sepsis.[27–29] However, some studies found that IVIG could have inhibitory or enhancing effects on IL-6 concentration.[30,31] Our results showed that IVIG increased IL-8 concentration in newborns but not adults, suggesting that it may be especially important for modulating the inflammatory response in newborns. Interestingly, we observed a low IL-8 response in the mother, indicating that the pregnant state could modulate the effects of IVIG. Such an IL-8 response could promote neutrophil migration and support inflammation in newborns; however, limited phagocytosis by neutrophils could limit the effect in the neonate. On the other hand, higher production of IL-8 in newborns could compensate for the lower phagocytic capacity.

Although IVIG has been used in adults and newborns with sepsis,[18,32,33] contradictory results have been reported. Indeed, since IVIG did not improve fatal outcomes in newborns, its use is not recommended in this group.[34,35] In contrast, some authors report prevention of early sepsis through IVIG treatment,[36] though its use does not prevent fatal outcomes. More studies are needed to determine if the clinical condition improves with this immunomodulatory drug. Furthermore, it should be elucidated if IVIG could substantially support the treatment of newborns.

Limitations

Our study had several limitations, such as small sample sizes and short-term kinetics. Nonetheless, the study showed differential IL-8 responses between groups, indicating that IVIG could up-regulate different mechanisms in adults and newborns. We infer that the hormonal status in pregnant women and newborns is similar since steroids can pass through the placenta, as are some of the characteristic tolerogenic and immunoregulatory responses. However, we did not analyze the hormonal status in any of the groups and cannot determine if they regulate the response to IVIG.

Our model was limited to the UCB response after birth, and newborn peripheral blood can express different responses. In addition, the response to IVIG can vary in newborns if they have an infection at birth. More studies are needed to determine the responses to IVIG in adults and newborns.

Conclusions

Intravenous immunoglobulin induced a strong IL-8 response in the cord blood of newborns that could lead to improved immunity.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10407063. The package includes the following files:

Supplementary Table 1. FcγR Index on monocytes or neutrophils of non-pregnant women, pregnant women and neonates during 48 h of culture, and the bootstrap RM ANOVA test for Table 1.

Supplementary Table 2. Mean fluorescence intensity of bacteria phagocyted by monocytes or neutrophils of non-pregnant women, pregnant women and neonates, and the bootstrap RM ANOVA test for Table 2.

PROIVIg Cytokine Normality test dic2023. Excel archive with the normality test of cytokine.

PROIVIg cytokine Tukey's test dic2023. Excel archive with the Tukey test of cytokine.

PROIVIg Fcgamma R monocyte normality test dic2023. Excel archive with the normality test of FcgammaR on monocyte.

PROIVIg Fcgamma R PMN normality test dic2023. Excel archive with the normality test of FcgammaR on PMN.

PROIVIg phagocytosis monocyte normality test dic2023. Excel archive with the normality test of phagocytosis on Monocyte.

PROIVIg phagocytosis PMN normality test dic2023. Excel archive with the Normality test of phagocytosis on PMN.

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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References

1. Shankar-Hari M, Spencer J, Sewell WA, Rowan KM, Singer M. Bench-to-bedside review: Immunoglobulin therapy for sepsis – biological plausibility from a critical care perspective. *Crit Care*. 2012;16(2):206. doi:10.1186/cc10597
2. Yang X, Meng T. Is there a role of intravenous immunoglobulin in immunologic recurrent pregnancy loss? *J Immunol Res*. 2020;2020:6672865. doi:10.1155/2020/6672865
3. Branch DW, Porter TF, Paidas MJ, Belfort MA, Gonik B. Obstetric uses of intravenous immunoglobulin: Successes, failures, and promises. *J Allergy Clin Immunol*. 2001;108(4 Suppl):S133–S138. doi:10.1067/mai.2001.117821
4. Rendell V, Bath NM, Brennan TV. Medawar's paradox and immune mechanisms of fetomaternal tolerance. *OBM Transplant*. 2020;4(1):26. doi:10.21926/obm.transplant.2001104

5. PrabhuDas M, Bonney E, Caron K, et al. Immune mechanisms at the maternal–fetal interface: Perspectives and challenges. *Nat Immunol*. 2015;16(4):328–334. doi:10.1038/ni.3131
6. Walsh SW, Stanczyk FZ, Novy MJ. Daily hormonal changes in the maternal, fetal, and amniotic fluid compartments before parturition in a primate species. *J Clin Endocrinol Metab*. 1984;58(4):629–639. doi:10.1210/jcem-58-4-629
7. Walsh SW, Ducsay CA, Novy MJ. Circadian hormonal interactions among the mother, fetus, and amniotic fluid. *Am J Obstet Gynecol*. 1984;150(6):745–753. doi:10.1016/0002-9378(84)90679-3
8. Raghupathy R, Szekeres-Bartho J. Progesterone: A unique hormone with immunomodulatory roles in pregnancy. *Int J Mol Sci*. 2022; 23(3):1333. doi:10.3390/ijms23031333
9. Piccinni MP, Raghupathy R, Saito S, Szekeres-Bartho J. Cytokines, hormones and cellular regulatory mechanisms favoring successful reproduction. *Front Immunol*. 2021;12:717808. doi:10.3389/fimmu.2021.717808
10. Tha-In T, Bayry J, Metselaar HJ, Kaveri SV, Kwekkeboom J. Modulation of the cellular immune system by intravenous immunoglobulin. *Trends Immunol*. 2008;29(12):608–615. doi:10.1016/j.it.2008.08.004
11. Gelfand EW. Intravenous immune globulin in autoimmune and inflammatory diseases. *N Engl J Med*. 2012;367(21):2015–2025. doi:10.1056/NEJMra1009433
12. Nagelkerke SQ, Kuijpers TW. Immunomodulation by IVIg and the role of Fc-gamma receptors: Classic mechanisms of action after all? *Front Immunol*. 2015;5:674. doi:10.3389/fimmu.2014.00674
13. Rosales C. Fcgamma receptor heterogeneity in leukocyte functional responses. *Front Immunol*. 2017;8:280. doi:10.3389/fimmu.2017.00280
14. Maeda M, Van Schie RCAA, Yüksel B, et al. Differential expression of Fc receptors for IgG by monocytes and granulocytes from neonates and adults. *Clin Exp Immunol*. 1996;103(2):343–347. doi:10.1046/j.1365-2249.1996.d01-615.x
15. Flores-Mejía LA, Cabrera-Rivera GL, Ferat-Osorio E, et al. Function is dissociated from activation-related immunophenotype on phagocytes from patients with SIRS/sepsis syndrome. *Shock*. 2019;52(5):e68–e75. doi:10.1097/SHK.0000000000001314
16. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science*. 2001; 291(5503):484–486. doi:10.1126/science.291.5503.484
17. Smith KGC, Clatworthy MR. FcγRIIB in autoimmunity and infection: Evolutionary and therapeutic implications. *Nat Rev Immunol*. 2010; 10(5):328–343. doi:10.1038/nri2762
18. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: How does IgG modulate the immune system? *Nat Rev Immunol*. 2013;13(3):176–189. doi:10.1038/nri3401
19. Kozicky LK, Menzies SC, Zhao ZY, et al. IVIg and LPS co-stimulation induces IL-10 production by human monocytes, which is compromised by an FcγRIIA disease-associated gene variant. *Front Immunol*. 2018;9:2676. doi:10.3389/fimmu.2018.02676
20. Luppi P, Haluszczak C, Betters D, Richard CAH, Trucco M, DeLoia JA. Monocytes are progressively activated in the circulation of pregnant women. *J Leukoc Biol*. 2002;72(5):874–884. PMID:12429709.
21. Davis D, Kaufmann R, Moticka EJ. Nonspecific immunity in pregnancy: Monocyte surface Fcγ receptor expression and function. *J Reprod Immunol*. 1998;40(2):119–128. doi:10.1016/S0165-0378(98)00076-X
22. Gille C, Dreschers S, Spring B, et al. Differential modulation of cord blood and peripheral blood monocytes by intravenous immunoglobulin. *Cytometry B Clin Cytom*. 2012;82(1):26–34. doi:10.1002/cyto.b.20609
23. Venet F, Monneret G. Advances in the understanding and treatment of sepsis-induced immunosuppression. *Nat Rev Nephrol*. 2018;14(2):121–137. doi:10.1038/nrneph.2017.165
24. Kwiatkowska K, Ciesielska A. Lipid-mediated regulation of pro-inflammatory responses induced by lipopolysaccharide. *Postepy Biochem*. 2018;64(3):175–182. doi:10.18388/pb.2018_129
25. Geng Y, Zhang B, Lotz M. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J Immunol*. 1993;151(12):6692–6700. PMID:8258685.
26. Lakshmikanth CL, Jacob SP, Chaithra VH, De Castro-Faria-Neto HC, Marathe GK. Sepsis: In search of cure. *Inflamm Res*. 2016;65(8):587–602. doi:10.1007/s00011-016-0937-y
27. Domínguez-Soto Á, Simón-Fuentes M, De Las Casas-Engel M, et al. IVIg promote cross-tolerance against inflammatory stimuli in vitro and in vivo. *J Immunol*. 2018;201(1):41–52. doi:10.4049/jimmunol.1701093
28. Murakami K, Suzuki C, Kobayashi F, et al. Intravenous immunoglobulin preparation attenuates LPS-induced production of pro-inflammatory cytokines in human monocytic cells by modulating TLR4-mediated signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol*. 2012;385(9):891–898. doi:10.1007/s00210-012-0765-8
29. Kasztalska K, Ciebiada M, Górski P. Mechanism of action of immunoglobulin applied intravenously [in Polish]. *Pol Merkur Lekarski*. 2010; 29(172):263–268.
30. Aukrust P, Müller F, Frøland SS. Elevated serum levels of interleukin-4 and interleukin-6 in patients with common variable immunodeficiency (CVI) are associated with chronic immune activation and low numbers of $CD4^+$ lymphocytes. *Clin Immunol Immunopathol*. 1994;70(3):217–224. doi:10.1006/clin.1994.1032
31. Ling ZD, Yeoh E, Webb BT, Farrell K, Doucette J, Matheson DS. Intravenous immunoglobulin induces interferon-gamma and interleukin-6 in vivo. *J Clin Immunol*. 1993;13(5):302–309. doi:10.1007/BF00920238
32. Hamano N, Nishi K, Onose A, et al. Efficacy of single-dose intravenous immunoglobulin administration for severe sepsis and septic shock. *J Intensive Care*. 2013;1(1):4. doi:10.1186/2052-0492-1-4
33. Nimmerjahn F, Ravetch JV. Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol*. 2008;26:513–533. doi:10.1146/annurev.immunol.26.021607.090232
34. Ohlsson A, Lacy JB. Intravenous immunoglobulin for suspected or proven infection in neonates. *Cochrane Database Syst Rev*. 2015;(3):CD001239. doi:10.1002/14651858.CD001239.pub5
35. INIS Collaborative Group; Brocklehurst P, Farrell B, King A, et al. Treatment of neonatal sepsis with intravenous immune globulin. *N Engl J Med*. 2011;365(13):1201–1211. doi:10.1056/NEJMoa1100441
36. Jenson HB, Pollock BH. Meta-analyses of the effectiveness of intravenous immune globulin for prevention and treatment of neonatal sepsis. *Pediatrics*. 1997;99(2):E2. doi:10.1542/peds.99.2.e2

High glucose regulates the cells dysfunction of human trophoblast HTR8/SVneo cells by downregulating *GABRP* expression

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Abstract

Background. In response to the high-glucose environment in patients with gestational diabetes mellitus (GDM), trophoblast cells undergo a series of pathological changes. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) is involved in the development of pregnancy-related diseases and regulation of blood glucose.

Objectives. To explore the relationship between *GABRP* and hyperglycemia stimulation in GDM patients, and to provide preliminary experimental evidence for whether *GABRP* has the potential as a molecular target for the treatment of GDM.

Materials and methods. Within 30 min after birth, placental samples were taken from 20 GDM patients and 20 pregnant women without GDM. Human chorionic trophoblast HTR-8/SVneo cells were utilized for in vitro experimental investigation. We explored changes in *GABRP* expression in placental samples and HTR-8/Svneo cells using western blot and quantitative reverse transcription polymerase chain reaction (RT-qPCR). Cells in the high-glucose treatment group were exposed to medium containing 25 mM glucose. To explore the relationship between *GABRP* and high-glucose stimulation, *GABRP* was overexpressed in HTR-8/SVneo cells. We monitored the cell viability, invasion and migration abilities using Cell Counting Kit-8 (CCK-8), transwell and scratch assays, respectively.

Results. We found that *GABRP* expression was significantly reduced in placental samples from GDM patients. Furthermore, high-glucose treatment decreased the expression level of *GABRP* in HTR-8/SVneo cells. High-glucose stimulation reduced the cell viability, invasion and migration abilities. *GABRP* overexpression reversed the biological dysfunction of the cells induced by high-glucose stimulation.

Conclusions. Hyperglycemia in GDM patients downregulates the expression of *GABRP*, and overexpression of *GABRP* promotes the viability, migration and invasive ability of HTR8-/SVneo cells.

Key words: gestational diabetes mellitus, *GABRP*, HTR8/SVneo trophoblast cell, cellular function

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Background

Gestational diabetes mellitus (GDM), defined as abnormal glucose tolerance that is first detected during pregnancy, is a common disease. The development of GDM has short- and long-term adverse effects on maternal and fetal health.[1,2] The placenta is a temporary but extremely important organ formed during pregnancy, which serves as the only nutrient transport channel between the mother and the fetus.[3] A healthy maternal environment promotes the growth, development and maturity of the placenta, thereby ensuring its normal function. Trophoblast cells are important constituent cells of the placenta.[4] During placental development, extravillous trophoblast cells with multiple cellular functions migrate and invade into the maternal decidua. Trophoblast cells are in direct contact with maternal tissues and are the first to be affected by changes in maternal blood glucose. In response to the high-glucose environment, trophoblast cells undergo a series of pathological changes. The impairment in trophoblast cells can also cause pathological changes in the placenta.

There are abnormally expressed genes in the placental tissue of GDM patients, and these genes may lead to placental dysfunction by regulating the function of trophoblast cells. Gamma-aminobutyric acid type A receptor subunit pi is the pi subunit of the inhibitory neurotransmitter gamma-aminobutyric acid (*GABA*) A receptor (*GABRP*). In addition to being expressed in nervous system, *GABRP* is also expressed in peripheral tissues, such as digestive tract, uterus and ovary.[5] Previous studies have found that *GABRP* is upregulated in the placenta during the first trimester.[6,7] Lu et al. found that upregulating the expression of *GABRP* in cells can significantly affect the invasion and apoptosis of placental trophoblast cells.[7] These results suggest that *GABRP* may participate in the occurrence and development of pregnancy-related diseases by regulating trophoblast function. Purwana et al. found that *GABA* increases β-cell mass and improves glucose homeostasis by binding to *GABRP*.[8]

In this study, we hypothesized that there might be a potential association between changes in *GABRP* expression and GDM. We investigated the expression level of *GABRP* in the placental tissue from patients with GDM. The HTR-8/SVneo trophoblast cells were cultured in vitro, and the effects of high-glucose culture on the expression level of *GABRP* and their cell functions were detected.

Objectives

The purpose of this study was to determine the relationship between *GABRP* and hyperglycemia stimulation in GDM patients, and to provide preliminary experimental evidence for whether *GABRP* has the potential as a molecular target for the treatment of GDM.

Materials and methods

Clinical samples

From January 2019 to May 2020, 20 GDM patients (GDM group) were admitted to the Department of Obstetrics at the Second Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). Another 20 healthy pregnant women without GDM were used as the control group. In this study, GDM cases were diagnosed according to the recommended guidelines for the diagnosis of GDM in China.[9] Placental tissue was collected from GDM patients and healthy pregnant women within 30 min of delivery. Tissue samples were cleaned with phosphate-buffered saline (PBS) and then kept in liquid nitrogen for subsequent use. The differences in age, gestational weeks, body mass index (BMI), and fasting blood glucose between the 2 groups are summarized in Table 1. The average fasting blood glucose in the GDM group was 5.04 mmol/L, which was higher than 4.16 mmol/L in the control group. The research protocol was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (approval No. LCKY2019-287). All participants signed informed consent prior to the study.

Cell culture and treatment

Human villous trophoblasts HTR-8/SVneo cells were purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). HTR-8/SVneo cells were grown adherently in 10% fetal bovine serum (FBS) Dulbecco's modified Eagle's medium (DMEM) medium (Life Technology, Guangzhou, China), and incubated in a humidity-saturated incubator containing 5% CO_2 at 37°C. Twenty-four hours after plating, cell culture media were starved for 24 h. Cells were divided into 2 groups: high-glucose

Table 1. Demographic and clinical characteristics of the study subjects

Parameter	Control group (n = 20)	GDM group (n = 20)	Mann–Whitney U test	p-value
Age [years]	30.58 ±3.73	31.60 ±4.09	223.000	0.547
Gestational age [weeks]	38.15 ±1.33	38.02 ±1.74	189.000	0.779
BMI [kg/m^2]	24.13 ±1.92	24.03 ±2.21	188.000	0.758
Fasting blood glucose [mmol/L]	4.16 ±0.49	5.04 ±1.09	312.500	0.002

Data were expressed as mean ± standard deviation (M ±SD); BMI – body mass index.

induction group (HG) and normal concentration glucose group (control). Cells in the HG group were exposed to medium containing 25 mM glucose, while cells in the control group were normally cultured in medium containing 5 mM glucose.

Cell transfection

To overexpress *GABRP*, HTR-8/SVneo cells were subjected to transient transfection. The *GABRP* gene was cloned into the pcDNA3.1 plasmid (Gene Pharma, Shanghai, China). Cells were divided into 3 groups: the overexpression (OE) of *GABRP* group (transfected with the pcDNA3.1-*GABRP* plasmid), the negative control (NC) group (transfected with the empty pcDNA3.1 plasmid) and the blank control group (untransfected cells). The manufacturer's instructions for Lipofectamine 3000 (Invitrogen Life Technologies, Carlsabad, USA) were properly followed throughout the transfection process. After transfection, the cell culture medium of each group was replaced. After 48 h culture, total cell RNA and protein were collected to evaluate transfection efficiency.

Cell migration assay

HTR-8/SVneo cells were seeded on the 6-well plates for cell scratch test. When the cells reached >80% confluency, the scratch line was gently scraped out with a 1 mL pipette tip, and the exfoliated cells were removed. The culture media was switched out for one that contained a high concentration of glucose. Scratch images were photographed at 0 h and 24 h using an inverted microscope (Axiovert 40 CFL; Carl Zeiss AG, Jena, Germany) and the cell migration rate was calculated.

Transwell invasion assay

An amount of 50 µL of diluted Matrigel (BD Biosciences, San Jose, USA) was evenly and carefully spread on the bottom surface of each upper chamber, and then air-dried. Cells were resuspended in FBS-free DMEM medium. Then, 200 µL of cell suspension and 700 µL of culture medium was added to the upper and lower chambers, respectively. After continuing to culture for 24 h, the transwell chamber was removed for the next experiment. The transwell chamber was fixed with 4% paraformaldehyde for 5 min and finally stained with 0.1% crystal violet for 1 min. After natural drying, 3 fields of view were randomly selected and photographed at a ×100 magnification to count the number of migrated cells. The average number of migrated cells was calculated and the above experiment was repeated 3 times.

Western blot analysis

After treatment, the cells were washed and lysed. Protein sample concentrations were measured to adjust loaded samples to contain the same concentration of protein. Protein samples were added to the wells of the gel for separation, and the separated proteins are transferred to polyvinylidene difluoride (PVDF) membranes by electrotransfer. The washed membrane was blocked in blocking buffer for 2 h at room temperature (RT). The corresponding primary antibodies were added to the washed membrane and incubated overnight at 4°C. The following primary antibodies were used in this study: *GABRP* (1:1000; Invitrogen) and *β-actin* (1:1000; Beyotime Biotechnology, Shanghai, China). After washing, the appropriate secondary antibody (1:2000; Boster, Wuhan, China) was added to the membrane and incubated at room temperature for 1 h. The fluorescent signal of the bands was detected and the gray value was analyzed using ImageJ software (National Institutes of Health, Bethesda, USA).

Quantitative real-time PCR

Placental tissue samples and cell plates were taken out, and the total RNA was isolated using Trizol method. The following tests were conducted strictly in line with the kit's manufacturer's instructions (Takara, Shiga, Japan). Beta-actin served as an internal benchmark. The relative mRNA expression of *GABRP* was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences relevant to the study are included in Table 2.

Table 2. Sequences of quantitative reverse transcription polymerase chain reaction (RT-qPCR) primers

Gene name	Primer	Sequence 5′ > 3′
β-actin	forward	GCTGTGCT ATCCCTGTACGC
	reverse	TGCCTCAGGGCAGCGGAACC
GABRP	forward	TTTCTCAGGCCCAATTTTGGT
	reverse	GCTGTCGGAGGTATATGGTGG

Statistical analyses

The experimental data were expressed as mean ± standard deviation (M ±SD). IBM SPSS v. 25.0 statistical software IBM Corp., Armonk, USA)was used for statistical analysis. The student's t-test or one-way analysis of variance (ANOVA) was used for comparison between groups. A p-value <0.05 indicates that the difference is statistically significant.

Results

GABRP expression decreased in placental tissues of GDM patients

As shown in Fig. 1, the expression levels of *GABRP* protein and mRNA in the placenta of GDM patients were lower than those of healthy pregnant women ($p < 0.001$).

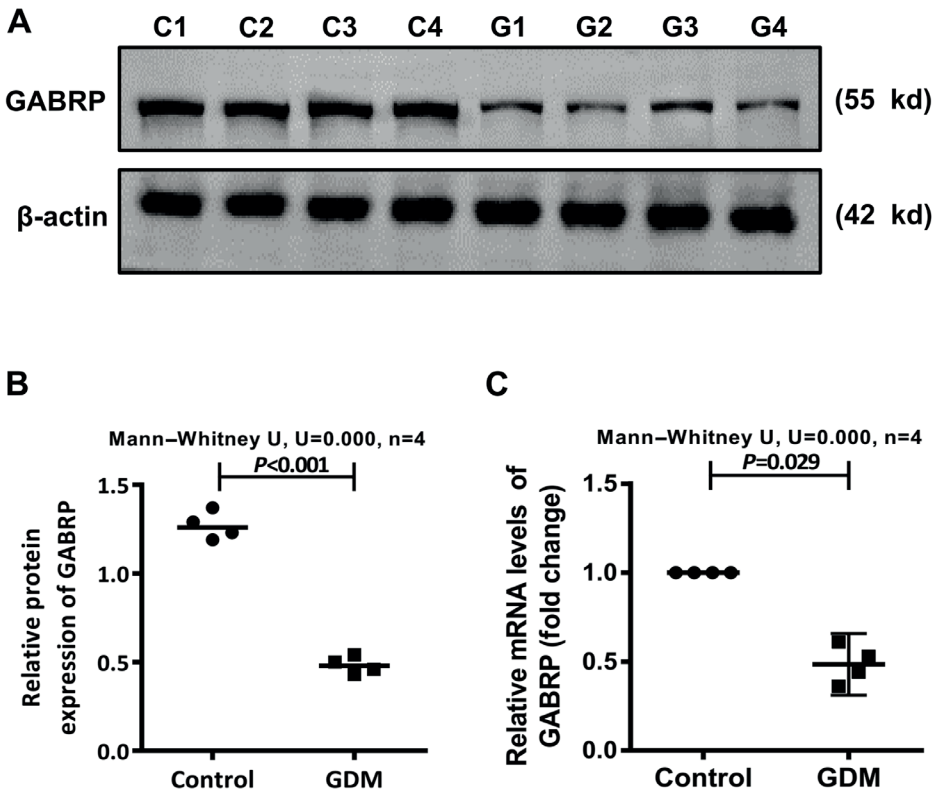

Fig. 1. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) expression decreased in placental tissues of gestational diabetes mellitus (GDM) patients. A. Representative western blot bands of *GABRP*. Beta-actin served as an internal control; B. The ratio of *GABRP/β-actin* was used to quantify protein levels; C. The mRNA level of *GABRP* in placental tissue was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Each experiment was repeated 3 times and the data were expressed as mean ± standard error of the mean (M ±SEM)

***p < 0.001 compared to control.

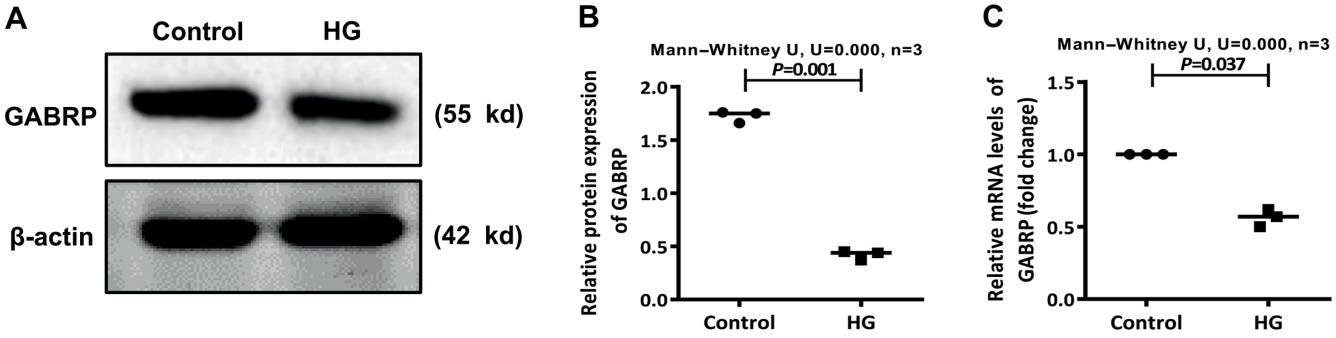

Fig. 2. High-glucose stimulation reduced the expression of gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) in HTR-8/SVneo cells. A. Representative western blot bands of *GABRP*. Beta-actin served as an internal control; B. The ratio of *GABRP/β-actin* was used to quantify protein levels; C. The mRNA level of *GABRP* in cells was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR)

**p < 0.01; ***p < 0.001 compared to control.

That is, the expression of *GABRP* in the placental tissue samples of GDM patients is reduced.

High-glucose stimulation reduced the expression of *GABRP* in HTR-8/SVneo cells

After 25 mM glucose treatment, the expression of *GABRP* protein was lower than that of the control group ($p < 0.01$; Fig. 2A,B). Similarly, the expression of *GABRP* mRNA was decreased in HTR-8/SVneo cells cultured with high-glucose ($p < 0.001$; Fig. 2C). Overall, high-glucose (25 mM) treatment decreases *GABRP* expression in cells.

High-glucose stimulation inhibited cell viability, invasion and migration

To investigate whether high-glucose stimulation can modulate cellular function, we examined the cell viability, invasion and migration abilities using Cell Counting Kit-8 (CCK-8), transwell and scratch assays. As shown in Fig. 3A, when compared to the control group, there was no difference in the cell viability in the 24 h group ($p > 0.05$), whereas it decreased in the 48-h and 72-h groups ($p < 0.001$). From the results of tranwell and scratch assays ($p < 0.01$; Fig. 3B–E), it can be seen that after high-glucose culture, the invasion and migration ability decreased. The above results indicate that high-glucose stimulation can affect the cellular function.

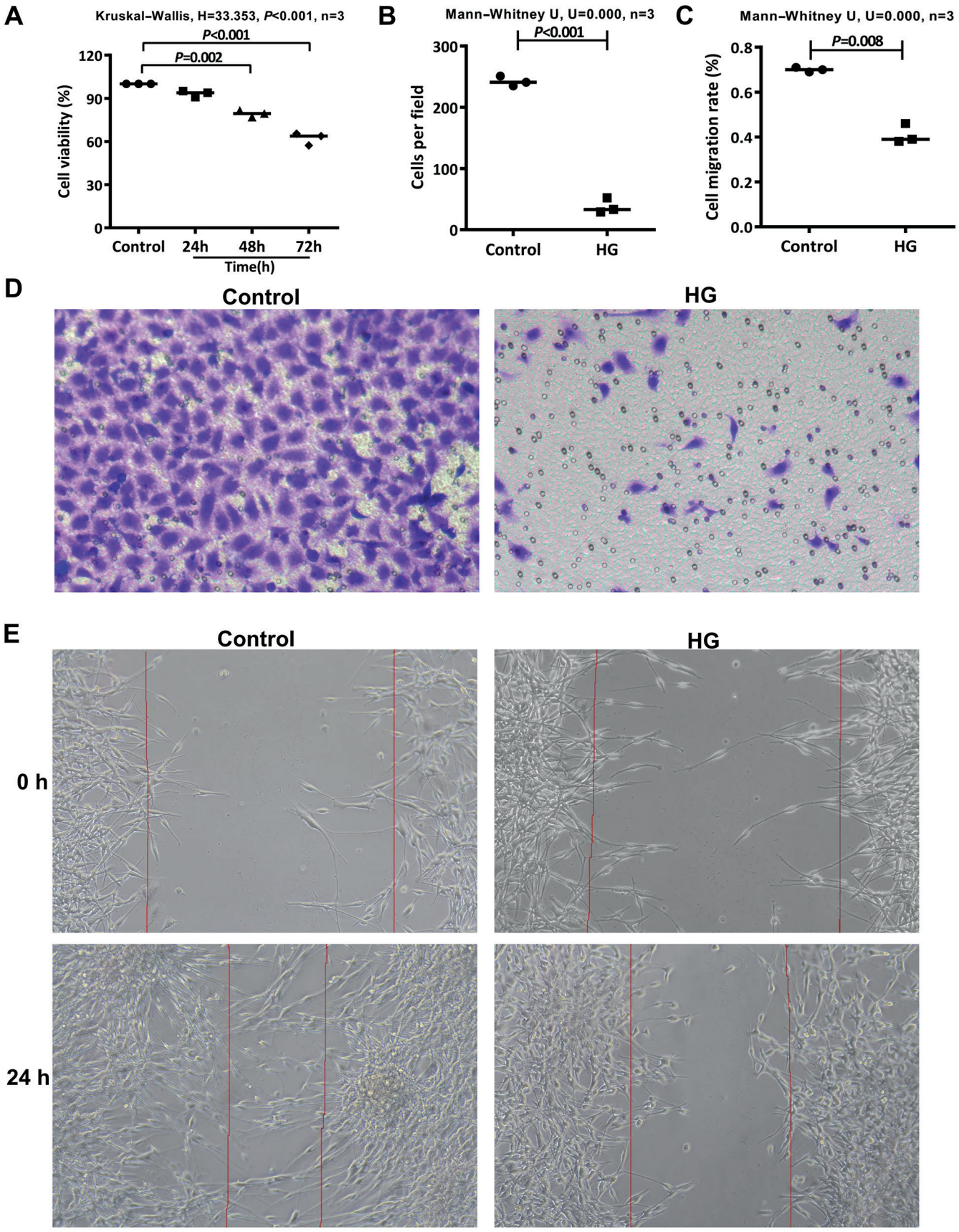

Fig. 3. High-glucose stimulation inhibited cell viability, invasion and migration of HTR-8/SVneo cells. A. The cells viability of HTR-8/SVneo cells at different timepoints (24 h, 48 h or 72 h) cultured in high glucose was detected with Cell Counting Kit-8 (CCK-8) assay; B. Quantification of invasive cell number; C. Graph indicating the rate of cell migration; D. Images of the transwell invasion assay; E. Representative photographs of scratch wounds at point 0 and 24 h of the cell migration assay (magnification ×100). Red lines depict the edges of the wound

**p < 0.01; ***p < 0.001 compared to control.

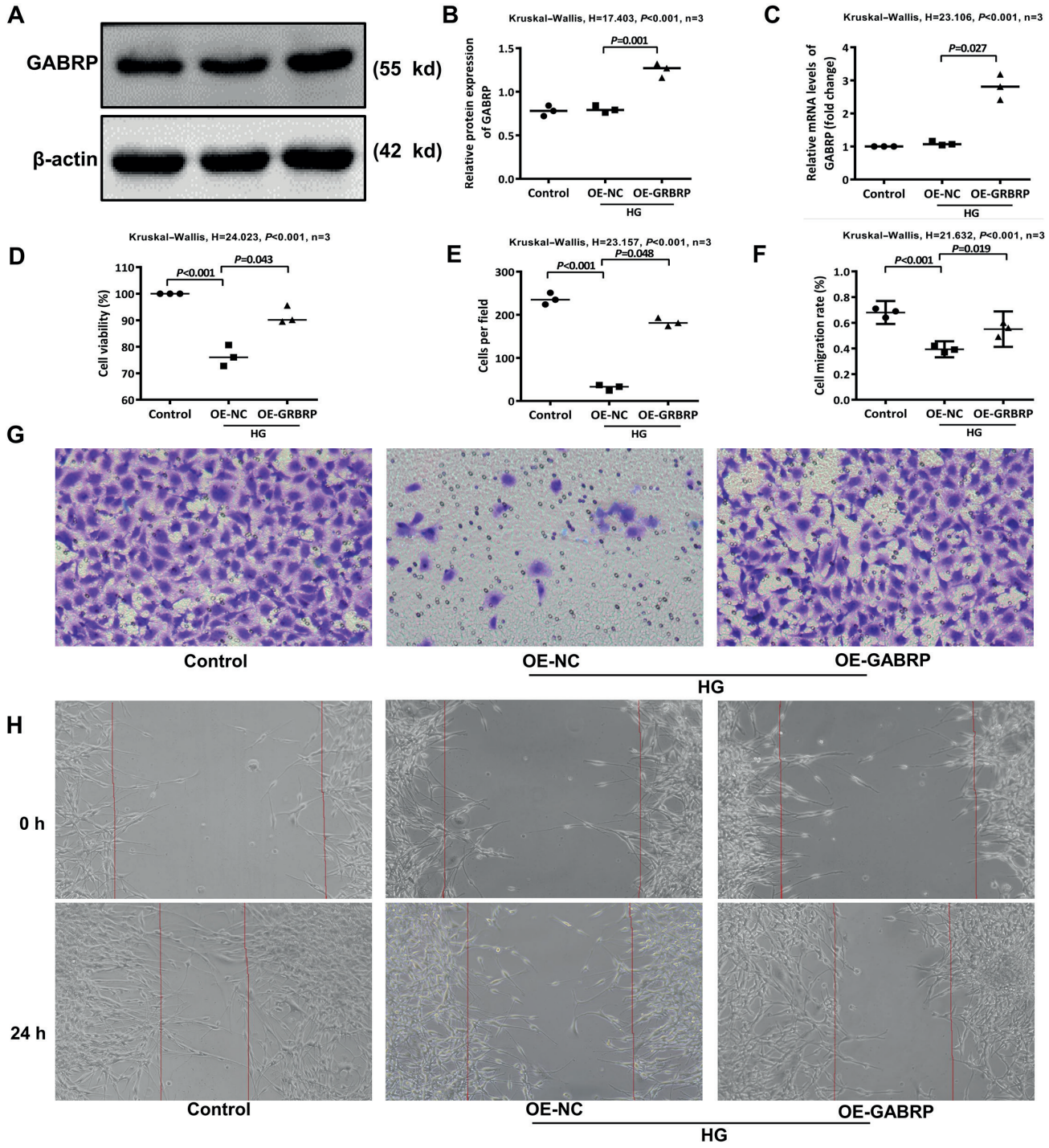

Fig. 4. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) overexpression reversed the effect of high glucose stimulation on the biological function of HTR-8/SVneo cells. A. Representative protein bands showing the protein expression of *GABRP*. Beta-actin was used as a loading control; B. The ratio of GABRP/β-actin was used to quantify protein levels; C. The relative mRNA expression level of *GABRP*; D. Cell viability of each group detected using the Cell Counting Kit-8 (CCK-8) assay; E. Number of transwell cells was quantified; F. Cell migration rates were assessed using the scratch test method; G. Representative image of transwell; H. Representative images of the scratch test

**p < 0.01; ***p < 0.001 compared to control; ##p < 0.01; ###p < 0.001 compared to control.

GABRP overexpression reverses the effects of high-glucose stimulation on cellular function

To explore the relationship between *GABRP* and high-glucose stimulation, *GABRP* was overexpressed by transfection. As shown in Fig. 4A–C, the levels of *GABRP* protein and mRNA expression were considerably higher in the OE-*GABRP* group compared to the OE-NC group (p < 0.001). The cell viability, invasion and migration abilities were altered after *GABRP* overexpression. Comparing with the HG+OE-NC group, the cellular function of HG+OE-*GABRP* group was

improved ($p < 0.01$; Fig. 4D–H). In conclusion, overexpression of *GABRP* reverses the functional impairment induced by high-glucose stimulation.

Discussion

Before the fetus matures, the placenta is an important place for the fetus to absorb nutrients from the mother. In addition, the placenta is also a crucial endocrine organ, and the development of the placenta affects the outcome of pregnancy.[10,11] Trophoblast cells are the main constituent cells of the placenta and serve important functions.[12] During placental development, trophoblast cells secrete a large number of hormones and cytokines.[13,14] When the cellular function of trophoblasts is abnormal, it will cause a variety of pregnancy diseases. The biological behavior of trophoblasts and related regulatory mechanisms are key physiological events to maintain placental homeostasis.[15] However, an imbalance of these processes can impair placental function under pathological conditions. In this study, the cell viability, invasion and migration abilities were inhibited after high-glucose stimulation.

Patients with GDM are have hyperglycemia, which increases the incidence of macrosomia.[16] When hyperglycemia is severe, it is more likely to lead to early miscarriage, defects in placenta formation, intrauterine growth retardation, and malformations.[17] This study found that the viability of trophoblast cells cultured in high glucose in vitro decreased. The results are consistent with the study by Zhang et al.[18] High-glucose-induced reduction in viability of trophoblast cells is involved in the pathogenesis of GDM. However, in clinical practice, the majority of GDM patients exhibit excessive placental tissue growth and give birth to macrosomic infants. It is reasonable to speculate that this difference may be related to the concentration of glucose used to culture cells in vitro. The concentration of glucose used in this experiment is 25 mmol/L, which is mainly used for in vitro cellular studies of type 2 diabetes.[19,20] Patients with GDM are usually considered to have mild type 2 diabetes.[21] People with type 2 diabetes before pregnancy are more likely to have adverse pregnancy outcomes.[22,23] There is no clear cutoff value for hyperglycemia leading to adverse pregnancy outcomes, but it is positively correlated with the degree of elevated blood glucose.[24] Therefore, the conclusions of this study are more applicable to GDM patients with poor blood sugar control.

Islet cells secrete *GABA* at a relatively constant rate, which is regulated by the metabolic state of the cells.[25] Purwana et al. showed that *GABA* participates in the improvement of human β-cell mass by combining with *GABRP*, which may be beneficial for the treatment of diabetes.[8] In the reproductive system, there are many studies on the role of *GABRP* in the uterus. Lu et al. found that changing the expression of *GABRP* in cells can significantly affect the invasion function and apoptosis behavior of trophoblast cells.[7] In this study, by comparing the expression of *GABRP* in the placenta of GDM and normal pregnant women, it was found that the expression of *GABRP* in the former was abnormally reduced. Meanwhile, the expression level of *GABRP* was decreased in HTR-8/SVneo cells cultured under high-glucose conditions. After overexpressing *GABRP*, the biological behavior of trophoblast cells altered. The results showed that *GABRP* could reverse the functional impairment of HTR-8/SVneo cells induced by high-glucose stimulation.

Limitations

We speculated that the high-glucose environment may inhibit cellular function by downregulating the expression of *GABRP*. It is suggested that *GABRP* differentially expressed in clinical samples of GDM patients may be involved in adverse pregnancy outcomes. The upstream and downstream signaling molecules of *GABRP* are not clear. Therefore, further mechanistic exploration and etiological studies are urgently needed to confirm.

Conclusions

The state of hyperglycemia in GDM patients downregulates the expression of *GABRP*, and overexpression of *GABRP* promotes the viability, migration and invasive ability of HTR8-SV/neo cells.

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References

1. Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS, Robinson JS. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. *N Engl J Med*. 2005;352(24):2477–2486. doi:10.1056/NEJMoa042973
2. Catalano PM, McIntyre HD, Cruickshank JK, et al. The Hyperglycemia and Adverse Pregnancy Outcome Study. *Diabetes Care*. 2012;35(4):780–786. doi:10.2337/dc11-1790
3. Maltepe E, Fisher SJ. Placenta: The forgotten organ. *Annu Rev Cell Dev Biol*. 2015;31(1):523–552. doi:10.1146/annurev-cellbio-100814-125620
4. Rossant J, Cross JC. Placental development: Lessons from mouse mutants. *Nat Rev Genet*. 2001;2(7):538–548. doi:10.1038/35080570
5. Hedblom E, Kirkness EF. A novel class of GABAA receptor subunit in tissues of the reproductive system. *J Biol Chem*. 1997;272(24):15346–15350. doi:10.1074/jbc.272.24.15346
6. Karvas RM, McInturf S, Zhou J, et al. Use of a human embryonic stem cell model to discover GABRP, WFDC2, VTCN1 and ACTC1 as markers of early first trimester human trophoblast. *Mol Hum Reprod*. 2020;26(6):425–440. doi:10.1093/molehr/gaaa029
7. Lu J, Zhang Q, Tan D, et al. GABA A receptor π subunit promotes apoptosis of HTR-8/SVneo trophoblastic cells: Implications in preeclampsia. *Int J Mol Med*. 2016;38(1):105–112. doi:10.3892/ijmm.2016.2608
8. Purwana I, Zheng J, Li X, et al. GABA promotes human β-cell proliferation and modulates glucose homeostasis. *Diabetes*. 2014;63(12):4197–4205. doi:10.2337/db14-0153

9. Obstetrics Subgroup, Chinese Society of Obstetrics and Gynecology, Chinese Medical Association, Group of Pregnancy with Diabetes Mellitus, Chinese Society of Perinatal Medicine, Chinese Medical Association, Obstetrics Subgroup Chinese Society of Obstetrics and Gynecology Chinese Medical Association, Group of Pregnancy with Diabetes Mellitus Chinese Society of Perinatal Medicine Chinese Medical Association. Diagnosis and therapy guideline of pregnancy with diabetes mellitus [in Chinese]. *Zhonghua Fu Chan Ke Za Zhi*. 2014;49(8):561–569. PMID:25354853.
10. Martino J, Sebert S, Segura MT, et al. Maternal body weight and gestational diabetes differentially influence placental and pregnancy outcomes. *J Clin Endocrinol Metab*. 2016;101(1):59–68. doi:10.1210/jc.2015-2590
11. Parikh RM, Joshi SR, Menon PS, Shah NS. Intensive glycemic control in diabetic pregnancy with intrauterine growth restriction is detrimental to fetus. *Med Hypotheses*. 2007;69(1):203–205. doi:10.1016/j.mehy.2006.10.020
12. Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage TKJB, James J. Human placenta and trophoblast development: Key molecular mechanisms and model systems. *Cell Mol Life Sci*. 2019;76(18):3479–3496. doi:10.1007/s00018-019-03104-6
13. Malassine A, Cronier L. Hormones and human trophoblast differentiation. *Endocrine*. 2002;19(1):3–12. doi:10.1385/ENDO:19:1:3
14. Sun Y, Wu S, Zhou Q, Li X. Trophoblast-derived interleukin 9 mediates immune cell conversion and contributes to maternal–fetal tolerance. *J Reprod Immunol*. 2021;148:103379. doi:10.1016/j.jri.2021.103379
15. Aires MB, Dos Santos A. Effects of maternal diabetes on trophoblast cells. *World J Diabetes*. 2015;6(2):338. doi:10.4239/wjd.v6.i2.338
16. Billionnet C, Mitanchez D, Weill A, et al. Gestational diabetes and adverse perinatal outcomes from 716,152 births in France in 2012. *Diabetologia*. 2017;60(4):636–644. doi:10.1007/s00125-017-4206-6
17. Plows J, Stanley J, Baker P, Reynolds C, Vickers M. The pathophysiology of gestational diabetes mellitus. *Int J Mol Sci*. 2018;19(11):3342. doi:10.3390/ijms19113342
18. Zhang C, Wang L, Chen J, Song F, Guo Y. Differential expression of miR-136 in gestational diabetes mellitus mediates the high-glucose-induced trophoblast cell injury through targeting E2F1. *Int J Genomics*. 2020;2020:3645371. doi:10.1155/2020/3645371
19. Hsieh CF, Liu CK, Lee CT, Yu LE, Wang JY. Acute glucose fluctuation impacts microglial activity, leading to inflammatory activation or self-degradation. *Sci Rep*. 2019;9(1):840. doi:10.1038/s41598-018-37215-0
20. Ying C, Wang S, Lu Y, et al. Glucose fluctuation increased mesangial cell apoptosis related to AKT signal pathway. *Arch Med Sci*. 2019;15(3):730–737. doi:10.5114/aoms.2019.84739
21. Catalano PM. Trying to understand gestational diabetes. *Diabet Med*. 2014;31(3):273–281. doi:10.1111/dme.12381
22. Schmidt CB, Voorhorst I, Van De Gaar VHW, et al. Diabetes distress is associated with adverse pregnancy outcomes in women with gestational diabetes: A prospective cohort study. *BMC Pregnancy Childbirth*. 2019;19(1):223. doi:10.1186/s12884-019-2376-6
23. Deputy NP, Kim SY, Conrey EJ, Bullard KM. Prevalence and changes in preexisting diabetes and gestational diabetes among women who had a live birth: United States, 2012–2016. *MMWR Morb Mortal Wkly Rep*. 2018;67(43):1201–1207. doi:10.15585/mmwr.mm6743a2
24. Coustan DR, Lowe LP, Metzger BE. The hyperglycemia and adverse pregnancy outcome (HAPO) study: Can we use the results as a basis for change? *J Matern Fetal Neonatal Med*. 2010;23(3):204–209. doi:10.3109/14767050903550667
25. Bhandage AK, Jin Z, Korol SV, et al. GABA regulates release of inflammatory cytokines from peripheral blood mononuclear cells and $CD4^+$ T cells and is immunosuppressive in type 1 diabetes. *EBioMedicine*. 2018;30:283–294. doi:10.1016/j.ebiom.2018.03.019

Hesperetin affects osteoclast differentiation via MAPK signaling pathway

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Abstract

Background. The number and activity of osteoblasts and osteoclasts play an important role in skeletal biology, especially in bone reconstruction. Scientific and rational regulation of osteoclast formation and activity has become a critical strategy aimed at inhibiting the loss of bone mass in the body and alleviating the occurrence of bone diseases. Currently, there are only a few reports related to hesperetin-regulated osteoclast differentiation.

Objectives. To investigate the influence of hesperetin on osteoclast-like cell differentiation and formation, and determine whether the MAPK signaling pathway is involved in the differentiation process.

Materials and methods. The RAW264.7 cells were induced and cultured in vitro to promote their differentiation into osteoclast-like cells. Tetrazolium bromide was utilized to determine the effects of different concentrations (100, 200, 400, and 600 μM) of hesperetin on the proliferation of osteoclast-like cell precursors. Osteoclast-like cell differentiation was conducted using tartrate-resistant acid phosphatase (TRAP) staining assay. The status of nuclei and actin filaments of differentiated osteoclast-like cells was observed with the use of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and actin-tracker green staining experiments. Changes in key proteins of the MAPK signaling pathway were detected using western blot.

Results. The results of TRAP staining experiments showed that the number of osteoclast-like cells decreased with the increase in hesperetin concentration. The DAPI and actin-tracker green staining demonstrated that the nuclei of differentiated osteoclast-like cells reduced in size with the increase in hesperetin concentration, and the osteoclast-like cells became smaller. Western blot for key MAPK signaling pathway proteins revealed that phospho-ERK and phospho-p38 protein levels were not significantly inhibited, but phospho-JNK protein levels were reduced.

Conclusions. Hesperetin inhibits the differentiation of osteoclast-like cells. Further studies revealed that hesperetin also affects the activation level of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in the induced differentiation of osteoclast-like cells.

Key words: MAPK signaling pathway, hesperetin, osteoclast-like cell

Background

The maintenance of the total skeletal mass of the body depends on the dynamic balance between new bone formation mediated by osteoblasts and the resorption of old bone mass mediated by osteoclasts.[1–5] Osteoblasts are specialized mesenchymal cells responsible for bone matrix production and mineralization, with both the receptors and catabolic enzymes required to internalize and utilize circulating lipids. Disruption of these receptors or enzymes could impair osteoblast function and lead to bone defects.[6] Osteoclasts are multinucleated macrophage lineage cells found uniquely in the bone.[7] They interact with the extracellular matrix of fibronectin, collagen, bone salivary protein, and bone bridging proteins to adhere to the bone surface and migrate, which degrades the bone matrix components. Osteoclasts may be the only cells in the body with bone resorption activity, and they are essential for the establishment and maintenance of bone homeostasis and repair after bone injury.[8] A growing number of studies have shown that abnormal osteoclast activity is an important factor in various bone diseases, such as osteoporosis, osteosclerosis, osteoarthritis, and Paget's disease. Scientific and rational regulation of osteoclast formation and activity has become a key strategy to inhibit the loss of bone mass in the body and alleviate the occurrence of bone diseases.[9] The currently available clinical drugs for the treatment of skeletal disorders, such as bisphosphonates and vitamin D, have not achieved satisfactory results.[10] Natural herbs are expected to be potential therapeutic agents for many diseases, such as those related to the skeletal system, due to their high activity and low side effects.[11] Some flavonoids, such as quercetin, epimedoside, naringin, ephedrine, and gerberoside, have been reported to be used, or attempted to be used, in the prevention and treatment of abnormal bone metabolism diseases.[12] Hesperetin also belongs to the flavonoid family of compounds and is one of the main active ingredients of traditional Chinese medicine tangerine peel and it is found in high levels in the pulp and peel of citrus plants of the *Rutaceae* family. Hesperetin has been shown to significantly inhibit the fibrotic process in the lung, liver, kidney, and heart muscle,[13–16] while another study found that hesperetin could promote osteogenesis.[17] However, few reports on the regulation of osteoclast differentiation by hesperetin are available, and no cases of its application in the prevention and treatment of clinical skeletal diseases have been reported. The tertiary members of the mitogen-activated protein kinase (MAPK) cascade pathway are MAPK, MAPKK, and MAPKKK. These 3 kinases are activated sequentially and simultaneously regulate various important physiological/pathological effects, such as cell growth, differentiation, stress, and inflammatory responses. Previous reports have suggested that the MAPK signaling pathway is involved in osteoclast differentiation and activation.[18] Reports also indicated that hesperidin inhibits RANKL-induced osteoclast formation.[19,20] However, only low concentrations (0–150 μM) of hesperetin were used in the above study to explore the effects of hesperetin on osteoclast differentiation and its potential mechanisms. Therefore, the effects of high concentrations of hesperetin on osteoclast differentiation and its mechanisms need to be further investigated. Herein, we investigated whether high concentrations of hesperetin affect osteoclast differentiation and whether the MAPK signaling pathway plays a role in this process. The results may provide a theoretical reference for the prevention and treatment of skeletal diseases caused by abnormal osteoclast activity in clinical settings.

Objectives

This study aimed to investigate the effects of hesperetin on osteoblast differentiation and whether the MAPK signaling pathway is involved in this process.

Materials and methods

Materials

RAW264.7 cells were purchased from the Shanghai Institute of Life Sciences of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium alpha (MEM-α) were obtained from Hyclone Laboratories, Inc. (Logan USA). RANKL (PeproTech, Thermo Fisher Scientific, Waltham, USA), tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, St. Louis, USA), fetal bovine serum (FBS; GE Healthcare, Chicago, USA), hesperetin (MedChemExpress, Monmouth Junction, USA), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), actin-tracker green, FR180204, SP600125, and SB203580 were bought from Beyotime Biotechnology (Shanghai, China). p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2), SAPK/JNK antibody, phospho-SAPK/JNK antibody, p38 MAPK antibody, and phospho-p38 MAPK were obtained from Cell Signaling Technology (Danvers, USA).

Determination of the effects of hesperetin on precursors of osteoclast-like cell proliferation using MTT method

RAW264.7 cells were inoculated into 96-well plates (3,000/well) and incubated at 37°C and 5% CO_2. RANKL cytokines were added following cell wall attachment. At the end of the induction culture, 100, 200, 400, and 600 μM of hesperetin were added to each experimental group, and each group contained 5 replicate wells. Dimethyl sulfoxide (DMSO) without hesperetin was used as the control group. After 24 h of hesperetin treatment, MTT (100 μL/well) was added, and the MTT solution was aspirated and discarded after 4 h. The DMSO solution was added and incubated overnight at 37°C in a 5% CO_2 incubator and the optical density (OD) values were measured.

TRAP staining to observe the effects of hesperetin on osteoclast-like cell formation

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and RANKL cytokine was added after cell wall attachment. At the end of the induction culture, 100, 200, 400, and 600 μM of hesperetin were added to each group and incubated overnight, followed by TRAP staining. Next, 5 mL of ultrapure water was added to a 15 mL centrifuge tube heated in a 37°C water bath. The medium was aspirated, and 4% paraformaldehyde solution was added for 10 min to fix the cells. Then, 50 μL of freshly prepared fast garnet GBC base solution and 50 μL sodium nitrite solution were added in a 1.5 mL Eppendorf (EP) tube and incubated at room temperature for 2 min. The cells were fixed and washed twice with ultrapure water. All the prepared reagents were transferred to the pre-warmed ultrapure water. Thereafter, 50 μL NA-B solution, 200 μL AS solution, and 100 μL tartrate solution reagent were added and mixed well with 150 μL staining solution per well. The plate was incubated at 37°C for 60 min. The staining solution was then aspirated and discarded, and 100 μL ultrapure water was added per well to prevent drying. Then, the samples were observed with a light microscope and images were acquired (model IX71; Olympus Corp., Tokyo, Japan).

Effects of DAPI and actin-tracker green staining on osteoclast-like cell formation by hesperetin

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and RANKL cytokine was added after cell wall attachment. A total of 100, 200, 400, and 600 μM of hesperetin were added to each cell well at the end of the induction culture. The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, and washed thrice with PBS containing 0.1% Triton X-100 (Beyotime Biotechnology). After actin-tracker green staining solution was added, the sample was incubated for 60 min at room temperature in the dark, washed 3 times with PBS containing 0.1% Triton X-100 for 5 min each time, and finally, DAPI was added (working concentration 6 μg/mL) and incubated for 15 min in the dark. The staining solution was discarded, washed twice in PBS for 3–5 min each time, observed, and images were acquired under a fluorescent microscope (model IX71; Olympus Corp.).

Western blot for detection of protein changes

RAW264.7 cells were inoculated in 6-well cell culture plates (800,000/well), and RANKL cytokine was added after cell apposition. A total of 100, 200, 400, and 600 μM of hesperetin were added to each group at the end of the induction culture, and the total protein was extracted at the end of the cell culture. After the protein was quantified, it was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to a nitrocellulose (NC) filter membrane. The NC membrane was then blocked using Tris-buffered saline with Tween (TBST) containing 5% skim powdered milk for 90 min by shaking at room temperature. Next, the primary antibody was added, followed by incubation on ice overnight. The sample underwent TBST washing 3 times, for 5 min each time, followed by the secondary antibody incubation for 90 min at room temperature. The sample was then washed in TBST 3 times for 10 min each time. Finally, enhanced chemiluminescence (ECL) detection was performed.

Effects of key protein inhibitors of MAPK signaling pathway on osteoclast-like cell differentiation

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and FR180204 (ERK inhibitor, 20 mM), SP600125 (JNK inhibitor, 20 mM), and SB203580 (p38 inhibitor, 20 mM) were added to each group after cell wall attachment. RANKL cytokines were added to each experimental group at the end of drug treatment. The TRAP staining was performed at the end of the culture.

Statistical analyses

All data were analyzed using GraphPad Prism v. 8.0 (GraphPad Software, San Diego, USA), and results are displayed as mean ± standard deviation (M ±SD). The Kruskal–Wallis one-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test was used for 3 or more groups of nonparametric data. The experiments were repeated independently 3 times. A value of $p < 0.05$ indicated statistical significance.

Results

Effects of hesperetin on the proliferation of differentiated osteoclast-like cell precursors

The MTT results showed that hesperetin significantly inhibited the proliferation of differentiated osteoclast-like cell precursors. The hesperetin groups of 100, 200, 400, and 600 μM all demonstrated significantly reduced cell proliferation rates compared to the DMSO group in a dose-dependent manner (Fig. 1).

Effects of hesperetin on osteoclast-like cell formation

The TRAP staining showed that the differentiated osteoclast-like cells were large and had many nuclei and a burgundy cytoplasm and colorless nuclei; by contrast,

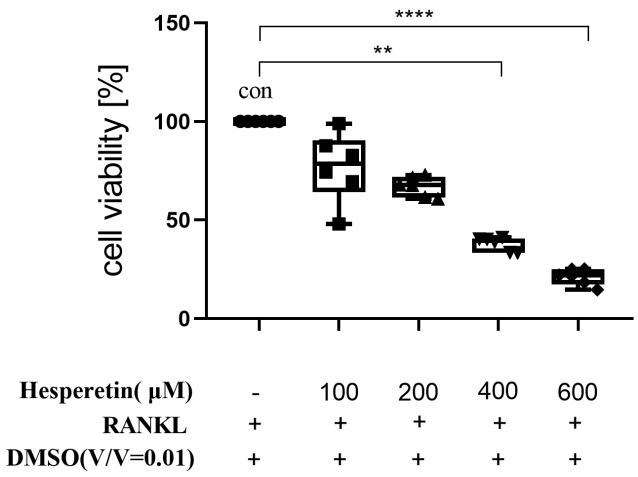

Fig. 1. Effects of hesperetin on the viability of osteoclast-like cell precursors. The results showed that the cell proliferation rates of hesperetin groups of 100, 200, 400, and 600 μM were significantly lower than those of the control group in a dose-dependent manner. The Kruskal–Wallis test (K–W) with Dunn's post hoc test was applied in the statistical analysis

**p = 0.002, 400 μM compared with the control group; ****p < 0.001, 600 μM compared with the control group; K–W, Dunn's post hoc test.

the undifferentiated cells showed mononuclear aggregates (Fig. 2A). The number of TRAP-positive cells indicated that hesperetin affected the differentiation of osteoclast-like cells, with a reduction in TRAP-positive cells with the increase in hesperetin concentration. The number of TRAP-positive cells was significantly reduced in the high-concentration hesperetin groups of 400 (6 ±0.67) and 600 μM (2.67 ±0.19) compared with the control group (Fig. 2B).

Effects of hesperetin on the cytoskeleton and nucleus of osteoclast-like cells

The DAPI staining highlighted that the nuclei of differentiated osteoclast-like cells in the control group were tightly clustered together, forming a relatively independent region. The cytoskeletal microfilament proteins were more widely distributed and exhibited a more complete cell outline. The differentiated cells in the low-concentration hesperetin groups of 100 μM and 200 μM had a higher number of nuclei than those in the control group, and the nuclei in the high-concentration group were also aggregated. However, the results of actin-tracker green staining showed that the aggregated cells in the hesperetin-treated group were not differentiated multinucleated osteoclast-like cells but only single RAW264.7 cells aggregated together, and the number of differentiated osteoclasts decreased with increasing hesperetin concentration. Moreover, the differentiated osteoclasts were larger, with extensive distribution of intracellular actin filaments, whereas the undifferentiated cells were smaller, with clear intercellular boundaries. Hesperetin reduced the size of differentiated osteoclast-like cells and decreased the number of nuclei in a dose-dependent manner compared with controls (Fig. 3).

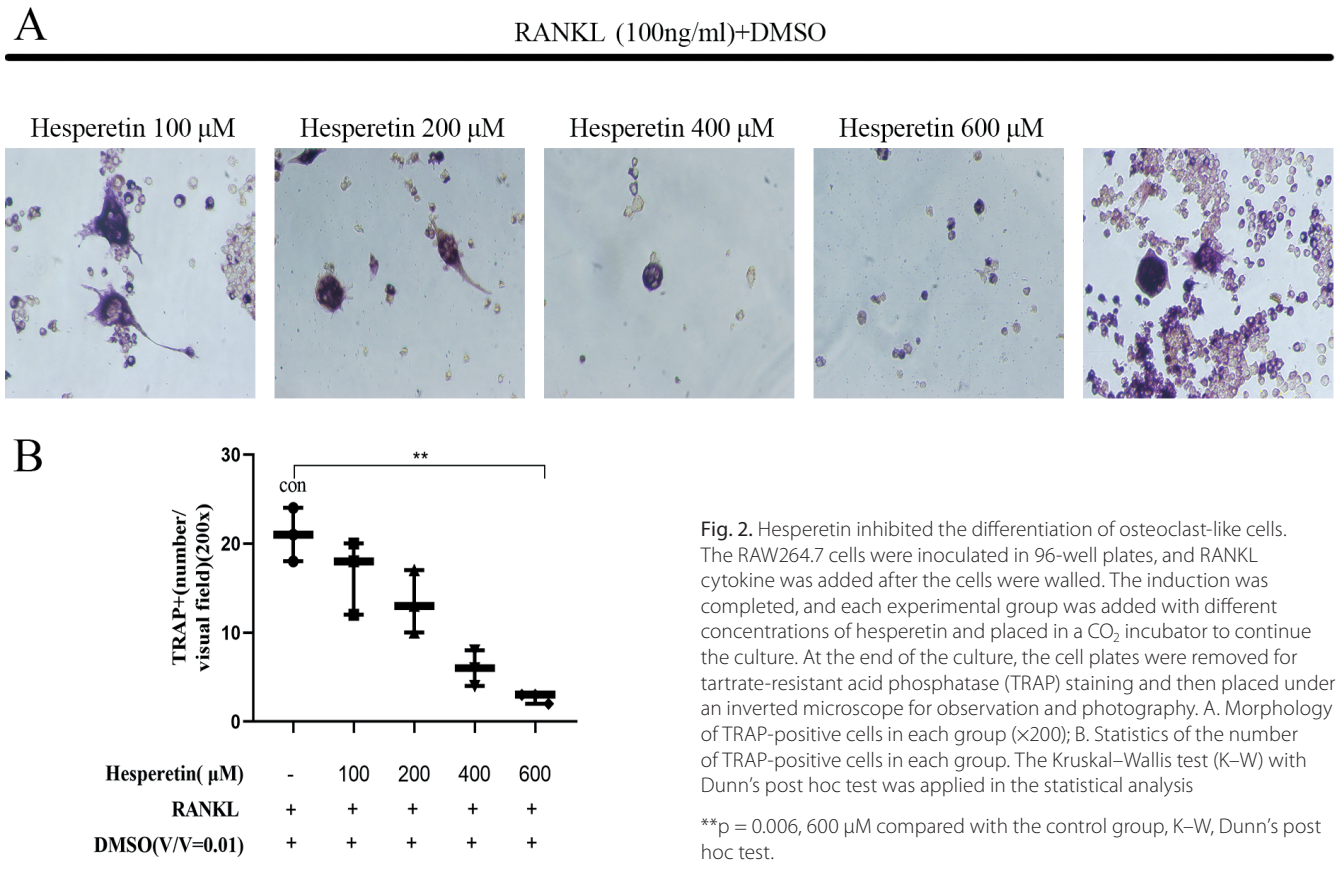

Fig. 2. Hesperetin inhibited the differentiation of osteoclast-like cells. The RAW264.7 cells were inoculated in 96-well plates, and RANKL cytokine was added after the cells were walled. The induction was completed, and each experimental group was added with different concentrations of hesperetin and placed in a CO_2 incubator to continue the culture. At the end of the culture, the cell plates were removed for tartrate-resistant acid phosphatase (TRAP) staining and then placed under an inverted microscope for observation and photography. A. Morphology of TRAP-positive cells in each group (×200); B. Statistics of the number of TRAP-positive cells in each group. The Kruskal–Wallis test (K–W) with Dunn's post hoc test was applied in the statistical analysis

**p = 0.006, 600 μM compared with the control group, K–W, Dunn's post hoc test.

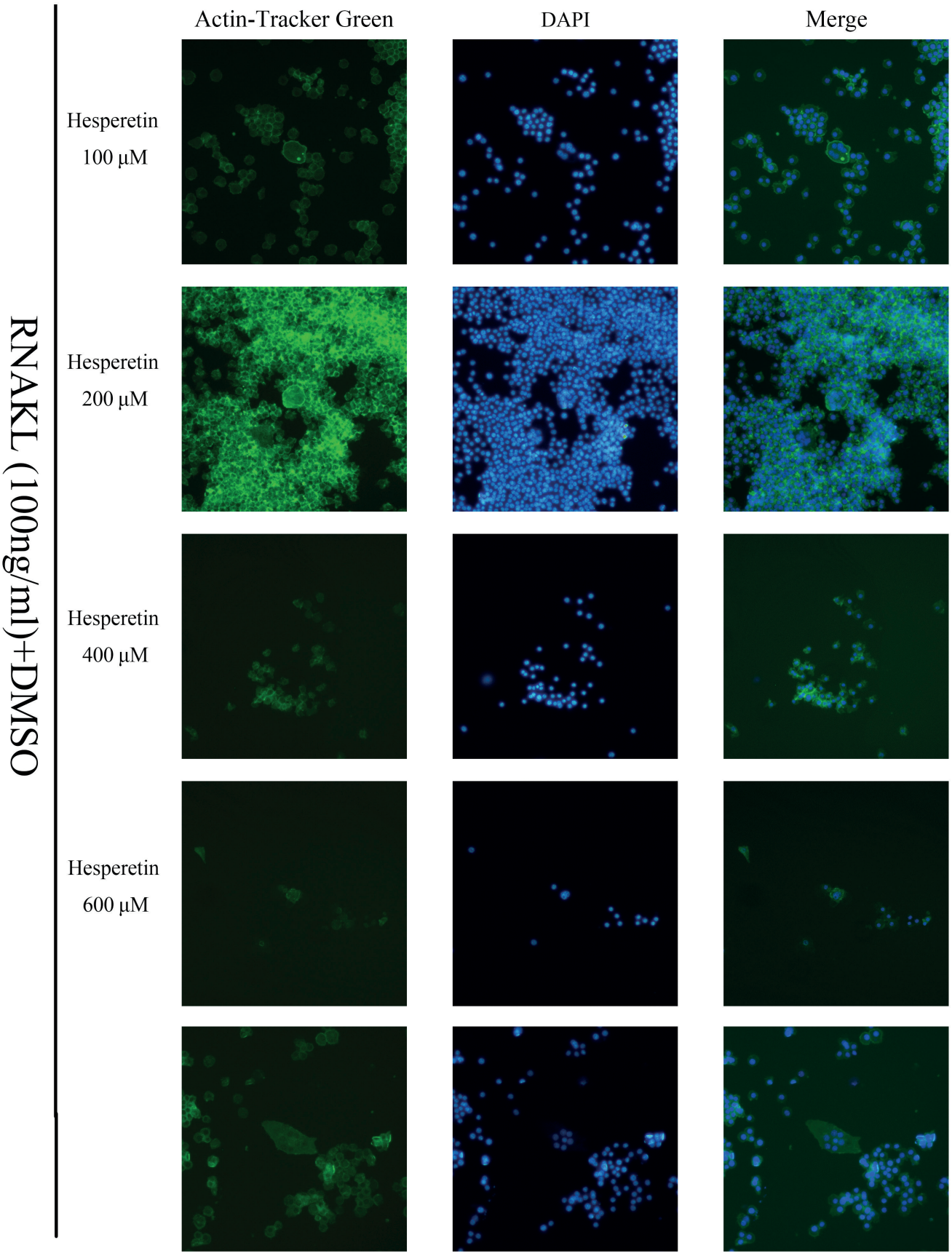

Fig. 3. Hesperetin reduced the number of osteoclast-like nuclei and decreased the cell volume. The RAW264.7 cells were inoculated in 96-well cell plates, cultured overnight, and induced by adding RANKL cytokine. The procedure was followed by the addition of different concentrations of hesperetin for each experimental group. Incubation was continued in a CO_2 incubator. At the end of the culture, the medium was aspirated and discarded to add paraformaldehyde for fixation, followed by actin-tracker green staining and DAPI (4',6-diamidino-2-phenylindole) staining. When the staining was completed, the sample was immediately observed under an inverted fluorescent microscope and photographed (×100)

Effects of hesperetin on the expression of key proteins of MAPK signaling family in osteoclast-like cells

The western blot experiments showed that the expression of phospho-ERK, phospho-JNK, and phospho-p38, which are key proteins of the MAPK signaling family, changed with the increase in hesperetin concentration relative to the DMSO group. The phospho-p38 and phospho-ERK protein levels were not significantly inhibited by the increase in hesperetin concentration, but phospho-JNK protein levels decreased (Fig. 4).

Effects of key protein inhibitors of MAPK signaling pathway on osteoclast-like cell differentiation

The TRAP staining showed that the formation of osteoclast-like cells was significantly inhibited in the groups treated with the MAPK signaling pathway protein inhibitors FR180204 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) (Fig. 5A). The number of TRAP-positive cells was significantly reduced in the groups treated with FR180204 (ERK inhibitor, 11 ±0.33), SP600125 (JNK inhibitor, 10 ±1), and SB203580 (p38 inhibitor, 9 ±0.33) compared with that in the control group (23.33 ±0.51, Fig. 5B).

Discussion

In this paper, the differentiation of osteoclast-like cells was inhibited with increasing concentrations of hesperetin, as evidenced by a decrease in the number of multinucleated cells. In addition, the number of nuclei in the differentiated osteoclast-like cells was reduced, and the cell size decreased in the high-hesperetin-concentration groups. Further studies revealed that hesperetin also affected the level

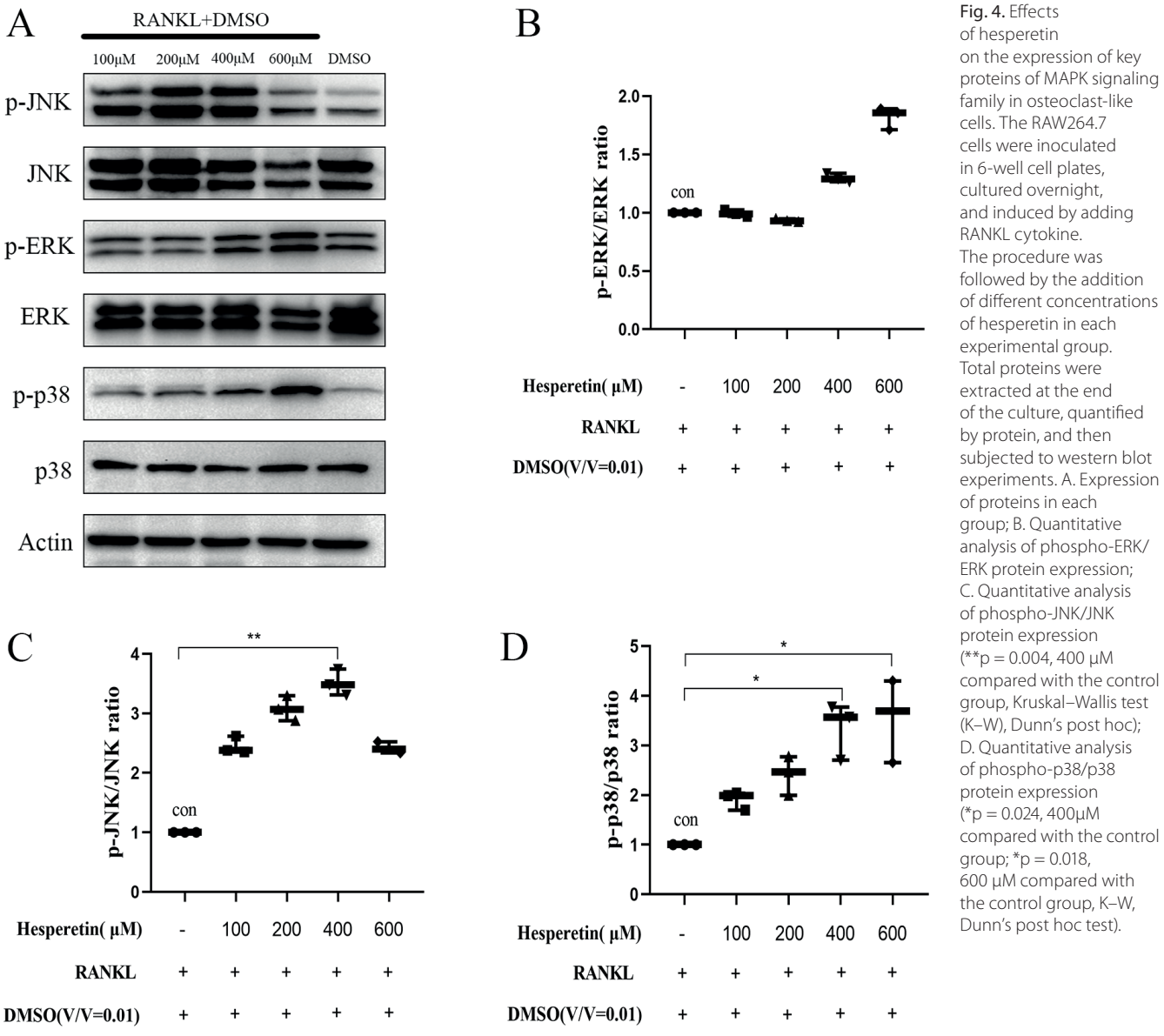

Fig. 4. Effects of hesperetin on the expression of key proteins of MAPK signaling family in osteoclast-like cells. The RAW264.7 cells were inoculated in 6-well cell plates, cultured overnight, and induced by adding RANKL cytokine. The procedure was followed by the addition of different concentrations of hesperetin in each experimental group. Total proteins were extracted at the end of the culture, quantified by protein, and then subjected to western blot experiments. A. Expression of proteins in each group; B. Quantitative analysis of phospho-ERK/ERK protein expression; C. Quantitative analysis of phospho-JNK/JNK protein expression (**p = 0.004, 400 μM compared with the control group, Kruskal–Wallis test (K–W), Dunn's post hoc); D. Quantitative analysis of phospho-p38/p38 protein expression (*p = 0.024, 400μM compared with the control group; *p = 0.018, 600 μM compared with the control group, K–W, Dunn's post hoc test).

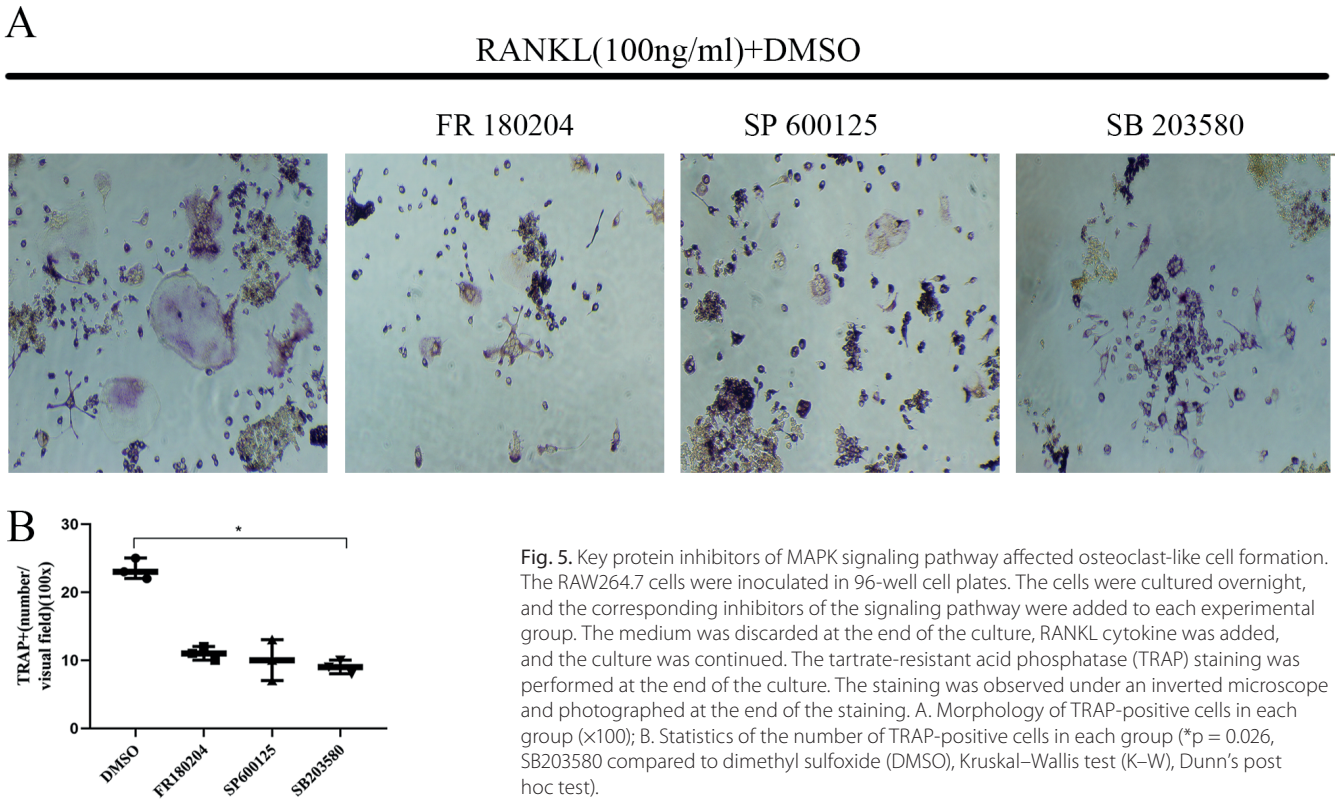

Fig. 5. Key protein inhibitors of MAPK signaling pathway affected osteoclast-like cell formation. The RAW264.7 cells were inoculated in 96-well cell plates. The cells were cultured overnight, and the corresponding inhibitors of the signaling pathway were added to each experimental group. The medium was discarded at the end of the culture, RANKL cytokine was added, and the culture was continued. The tartrate-resistant acid phosphatase (TRAP) staining was performed at the end of the culture. The staining was observed under an inverted microscope and photographed at the end of the staining. A. Morphology of TRAP-positive cells in each group (×100); B. Statistics of the number of TRAP-positive cells in each group (*p = 0.026, SB203580 compared to dimethyl sulfoxide (DMSO), Kruskal–Wallis test (K–W), Dunn's post hoc test).

of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in the differentiated osteoclast-like cells. Hesperetin is a dihydroflavonol compound with molecular formula C16H14O6 and a molecular weight of 302.28 Da. Its structure contains ketocarbonyl, ether, methoxy, and several phenolic hydroxyl groups, which give it a wide range of pharmacological effects. Hesperetin has been shown to have antibacterial, anti-inflammatory, antioxidant, antitumor, and immune-modulating effects.[21] In the present study, the effects of hesperetin on the formation of osteoclast-like differentiation were investigated, and we found that hesperetin affects osteoclast differentiation through the MAPK signaling pathway. The activity of osteoclasts in the organism is mainly reflected by the ability to differentiate bone marrow mesenchymal stem cells into osteoclasts and the strength of their osteolytic function.[22] Osteoclasts are multinucleated giant cells that originate from clones of mononuclear macrophages differentiated from hematopoietic stem cells. They have an abundant intracytoplasmic enzyme system and a series of signature proteins that can be used as markers to identify osteoclasts and determine their differentiation stage. Among them, TRAP is a specific marker for osteoclasts, and TRAP staining that shows TRAP activity in the cytoplasm of osteoclasts is a specific staining method for identifying these cells.[23] Our TRAP staining results highlighted that the formation of osteoclast-like cells was inhibited with the increase in hesperetin concentration. The cytoskeleton is a three-dimensional (3D) network structure composed of microtubules, microfilaments, and intermediate fibers in eukaryotic cells. Their main functions are to maintain the structure and morphology of cells, influence cell motility,[24] and play an important role in proliferation, differentiation, and apoptotic activities. Moreover, microfilaments play a key role in the attachment of osteoclasts to the bone matrix, and the microenvironment formed by the adhesion of osteoclasts to the bone matrix is a prerequisite for osteoclasts to undergo bone resorption. Therefore, the dynamic changes in microfilaments could be used as an indicator of osteoclast activity. This activity could be determined by observing the microfilament-mediated attachment, migration, and the "osteolytic" function of osteoclasts on the bone matrix.[25] In the present study, actin-tracker green staining revealed that hesperetin disrupted the cytoskeleton (the cell outline maintained by microfilaments) in osteoclast-like cells, which then reduced the cell size and inhibited their differentiation into osteoclast-like cells. Reports have shown that the actin ring is a cytoskeletal protein unique to osteoclasts that undergo bone resorption.[26] Future investigations will likely focus on whether hesperetin could disrupt the structure of the actin ring and pseudopods in osteoclasts, thereby affecting bone resorption activity.

We found that RANK within osteoclasts and their precursors could activate 3 signaling pathways of the MAPK family, namely, ERK, JNK, and p38, which promote osteoclast differentiation and activation.[18] TAK1/TAB2, which is a downstream protein of TARF6 in the RANK signaling pathway, activates JNK with p38 protein.[27] Furthermore, downstream signaling of the ERK and JNK pathways include AP-1 transcription factors, Fos family

dimers (c-Fos, FosB, Fra-1, and Fra-2) and Jun family (c-Jun, JunB, and JunD).[28] ERK could induce and activate c-Fos, while JNK could enhance the transcriptional activity of AP-1 through the phosphorylation of c-Jun. AP-1 could also initiate the encoding of genes, such as matrix metalloproteinases (MMPs), which promote the differentiation, survival, and fusion of osteoclast precursors and the activation of mature osteoclasts. Activated ERK enters the nucleus and stimulates the transcription factor Elk, which binds to a sequential regulatory element in the c-Fos gene promoter and controls the transcription of the c-Fos gene. This results in mature macrophages being converted into osteoclast precursors.[29] In the present study, the phospho-p38 and phospho-ERK protein levels were not significantly inhibited by the increase in hesperetin concentration, but the phospho-JNK protein levels were reduced. Further studies revealed that the inhibitors of the key proteins of the MAPK signaling pathway could inhibit the differentiation and formation of osteoclasts. These results suggest that the MAPK signaling pathway is involved in regulating osteoclast-like cell formation, and that hesperetin could affect this process by downregulating the phosphorylation level of JNK.

Limitations

This study only focused on the in vitro cellular model, making the results and findings relatively limited. In vivo animal experiments are needed to further validate the effects of hesperetin on osteoclast differentiation.

Conclusions

Hesperetin inhibits the differentiation process of osteoclast-like cells, and further studies revealed that hesperetin could also affect the activation level of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in differentiated osteoclast-like cells.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10020626. The package contains the following files:

Supplementary Fig. 1. Detailed results of MTT experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 2. Detailed results of TRAP experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 3. Detailed results of Western blot experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 4. Detailed results of Effects of key protein inhibitors of MAPK signaling pathway data analyzed with Kruskal-Wallis method.

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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References

1. Tresguerres FGF, Torres J, López-Quiles J, Hernández G, Vega JA, Tresguerres IF. The osteocyte: A multifunctional cell within the bone. *Ann Anat*. 2020;227:151422. doi:10.1016/j.aanat.2019.151422
2. Yahara Y, Nguyen T, Ishikawa K, Kamei K, Alman BA. The origins and roles of osteoclasts in bone development, homeostasis and repair. *Development*. 2022;149(8):dev199908. doi:10.1242/dev.199908
3. Ambrosi TH, Marecic O, McArdle A, et al. Aged skeletal stem cells generate an inflammatory degenerative niche. *Nature*. 2021;597(7875):256–262. doi:10.1038/s41586-021-03795-7
4. Kim JM, Lin C, Stavre Z, Greenblatt MB, Shim JH. Osteoblast–osteoclast communication and bone homeostasis. *Cells*. 2020;9(9):2073. doi:10.3390/cells9092073
5. Cawley KM, Bustamante-Gomez NC, Guha AG, et al. Local production of osteoprotegerin by osteoblasts suppresses bone resorption. *Cell Rep*. 2020;32(10):108052. doi:10.1016/j.celrep.2020.108052
6. Alekos NS, Moorer MC, Riddle RC. Dual effects of lipid metabolism on osteoblast function. *Front Endocrinol*. 2020;11:578194. doi:10.3389/fendo.2020.578194
7. Sugiyama T, Nagasawa T. Bone marrow niches for hematopoietic stem cells and immune cells. *Inflamm Allergy Drug Targets*. 2012;11(3):201–206. doi:10.2174/187152812800392689
8. Miyamoto T. Regulators of osteoclast differentiation and cell–cell fusion. *Keio J Med*. 2011;60(4):101–105. doi:10.2302/kjm.60.101
9. Yang N, Liu D, Zhang X, et al. Effects of ginsenosides on bone remodelling for novel drug applications: A review. *Chin Med*. 2020;15(1):42. doi:10.1186/s13020-020-00323-z
10. Maraka S, Kennel KA. Bisphosphonates for the prevention and treatment of osteoporosis. *BMJ*. 2015;351:h3783. doi:10.1136/bmj.h3783
11. Jin H, Wang Q, Chen K, et al. Astilbin prevents bone loss in ovariectomized mice through the inhibition of RANKL-induced osteoclastogenesis. *J Cell Mol Med*. 2019;23(12):8355–8368. doi:10.1111/jcmm.14713
12. Cao L, Wang J, Zhang Y, Tian F, Wang C. Osteoprotective effects of flavonoids: Evidence from in vivo and in vitro studies (Review). *Mol Med Rep*. 2022;25(6):200. doi:10.3892/mmr.2022.12716
13. Li S, Shao L, Fang J, et al. Hesperetin attenuates silica-induced lung injury by reducing oxidative damage and inflammatory response. *Exp Ther Med*. 2021;21(4):297. doi:10.3892/etm.2021.9728
14. Kong R, Wang N, Luo H, Lu J. Hesperetin mitigates bile duct ligation-induced liver fibrosis by inhibiting extracellular matrix and cell apoptosis via the TGF-β1/Smad pathway. *Curr Mol Med*. 2018;18(1):15–24. doi:10.2174/1566524018666180608084947
15. Choi D, Kim CL, Kim JE, Mo JS, Jeong HS. Hesperetin inhibit EMT in TGF-β treated podocyte by regulation of mTOR pathway. *Biochem Biophys Res Commun*. 2020;528(1):154–159. doi:10.1016/j.bbrc.2020.05.087

16. Wang B, Li L, Jin P, Li M, Li J. Hesperetin protects against inflammatory response and cardiac fibrosis in postmyocardial infarction mice by inhibiting nuclear factor κB signaling pathway. *Exp Ther Med*. 2017; 14(3):2255–2260. doi:10.3892/etm.2017.4729
17. Liu L, Zheng J, Yang Y, Ni L, Chen H, Yu D. Hesperetin alleviated glucocorticoid-induced inhibition of osteogenic differentiation of BMSCs through regulating the ERK signaling pathway. *Med Mol Morphol*. 2020;54(1):1–7. doi:10.1007/s00795-020-00251-9
18. Etich J, Rehberg M, Eckes B, Sengle G, Semler O, Zaucke F. Signaling pathways affected by mutations causing osteogenesis imperfecta. *Cell Signal*. 2020;76:109789. doi:10.1016/j.cellsig.2020.109789
19. Liu H, Dong Y, Gao Y, et al. Hesperetin suppresses RANKL-induced osteoclastogenesis and ameliorates lipopolysaccharide-induced bone loss. *J Cell Physiol*. 2018;234(7):11009–11022. doi:10.1002/jcp.27924
20. Zhang Q, Tang X, Liu Z, et al. Hesperetin prevents bone resorption by inhibiting RANKL-induced osteoclastogenesis and Jnk mediated Irf-3/c-Jun activation. *Front Pharmacol*. 2018;9:1028. doi:10.3389/fphar.2018.01028
21. Ortiz ADC, Fideles SOM, Reis CHB, et al. Therapeutic effects of citrus flavonoids neohesperidin, hesperidin and its aglycone, hesperetin, on bone health. *Biomolecules*. 2022;12(5):626. doi:10.3390/biom12050626
22. Moore SC, Matthews CE, Ou Shu X, et al. Endogenous estrogens, estrogen metabolites, and breast cancer risk in postmenopausal Chinese women. *J Natl Cancer Inst*. 2016;108(10):djw103. doi:10.1093/jnci/djw103
23. Lamp EC, Drexler HG. Biology of tartrate-resistant acid phosphatase. *Leuk Lymphoma*. 2000;39(5–6):477–484. doi:10.3109/10428190009113378
24. Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. *Nature*. 2010;463(7280):485–492. doi:10.1038/nature08908
25. Kajiya H, Okabe K, Okamoto F, Tsuzuki T, Soeda H. Protein tyrosine kinase inhibitors increase cytosolic calcium and inhibit actin organization as resorbing activity in rat osteoclasts. *J Cell Physiol*. 2000;183(1):83–90. doi:10.1002/(SICI)1097-4652(200004)183:1<83::AID-JCP10>3.0.CO;2-W
26. Matsubara T, Kinbara M, Maeda T, Yoshizawa M, Kokabu S, Takano Yamamoto T. Regulation of osteoclast differentiation and actin ring formation by the cytolinker protein plectin. *Biochem Biophys Res Commun*. 2017;489(4):472–476. doi:10.1016/j.bbrc.2017.05.174
27. Li YX, Chen FC, Liu T, et al. Pantoprazole (PPZ) inhibits RANKL-induced osteoclast formation and function in vitro and prevents lipopolysaccharide- (LPS-) induced inflammatory calvarial bone loss in vivo. *Stem Cells Int*. 2020;2020:8829212. doi:10.1155/2020/8829212
28. Sun Y, Li J, Xie X, et al. Recent advances in osteoclast biological behavior. *Front Cell Dev Biol*. 2021;9:788680. doi:10.3389/fcell.2021.788680
29. Mun SH, Park PSU, Park-Min KH. The M-CSF receptor in osteoclasts and beyond. *Exp Mol Med*. 2020;52(8):1239–1254. doi:10.1038/s12276-020-0484-z

The roles of autophagy in the treatment of diabetic nephropathy with rapamycin

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Abstract

Background. Rapamycin is known to induce autophagy, promote cell survival and inhibit the progression of diabetic nephropathy (DN).

Objectives. The aim of this study was to examine the role of autophagy in the treatment of DN with rapamycin to provide the basis for the DN treatment with rapamycin.

Materials and methods. Human mesangial cells (HMC) were cultured in a constant temperature incubator with 5% CO_2, at 37°C and saturated humidity. Cells were divided into 5 groups and the 5-ethynyl-2-deoxyuridine (EdU) cell proliferation assay was used to determine cell proliferation. Flow cytometry was used to determine cell apoptosis, while GFP–RFP–LC3 showed autophagy flow. Western blot was employed to detect the expression of autophagy-related proteins LC3-II/LC3-I and P62. Enzyme-linked immunosorbent assay (ELISA) was used to determine the contents of type IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA) in the extracellular matrix (ECM).

Results. Cell proliferation was the lowest in the hyperglycemic group. Additionally, the hyperglycemic group displayed the lowest number of autolysosomes compared to other groups. In contrast, the rapamycin group exhibited the highest number of autolysosomes. The LC3-II/LC3-I ratio was also the lowest in the hyperglycemic group, measuring 0.53 (0.50–0.58), while the expression level of P62 was significantly higher in that group at 0.98 (0.95–1.01) compared to other groups. Upon the introduction of rapamycin, the LC3-II/LC3-I ratio was significantly increased at 2.21 (1.95–2.21), and P62 was significantly decreased 0.38 (0.38–0.39) compared to the hyperglycemic group. Both changes were statistically significant, with p-values of 0.034 and 0.010, respectively. Enzyme-linked immunosorbent assay was employed to detect Col4, HA and LA content. The study findings demonstrated significantly higher levels of glucose in the hyperglycemic group in comparison to other groups. In contrast, the rapamycin group exhibited significantly lower levels of glucose than the hyperglycemic group, yet the difference was not statistically significant.

Conclusions. Hyperglycemic can inhibit the autophagic activity of HMC, promote cell apoptosis, enhance ECM accumulation, and facilitate the DN progression. In contrast, rapamycin can elicit autophagy, decrease mesangial matrix proliferation, and therefore impede DN progression.

Key words: diabetic nephropathy, autophagy, human mesangial cells

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Background

At present, diabetic nephropathy (DN) has become the leading cause of end-stage renal disease (ESRD) in China. The treatment of DN is difficult, and there is no recognized specific drug that can cure DN. It is an urgent problem to further understand the pathogenesis of DN and seek effective treatment. Mesangial cells are extremely important target cells and effector cells in the development of DN, participating in the process of glomerular injury and repair, and promoting inflammation and the development of glomerular sclerosis together with extracellular matrix (ECM). At the same time, oxidative stress, inflammation and autophagy are also involved in various pathological processes of DN.

Autophagy refers to the formation of autophagosomes in the cytoplasm to degrade damaged organelles, proteins and other components to meet the metabolic needs of the cells themselves and help the cells survive. According to the different forms, autophagy can be divided into macroautophagy, microautophagy and chaperone-mediated autophagy, among which macroautophagy is the most common. Autophagy is a continuous dynamic process that involves 5 distinct stages: initiation, nucleation, expansion and elongation, closure and fusion, and cargo degradation The autophagy process is regulated by several autophagy-related genes (ATG). Autophagy is activated under stress, such as hypoxia, amino acid deficiency or low glucose, and forms autophagosomes after activation to remove damaged cell components and ensure cell survival. It is the process of fusing macromolecular proteins, mitochondria, ribosomes, and organelle fragments with autophagosomes to break them down into small molecules.[1]

Autophagy and proteasome pathways belong to 2 intracellular degradation pathways, but the contents degraded by the 2 are different. The latter specifically degrades ubiquitinated small protein molecules, while the denatured, misfolded large protein molecules and aging and deteriorated organelles are degraded by autophagy. Therefore, cells generally maintain some level of autophagy activity, and the level of autophagy varies depending on the tissue type. Under the condition of hyperglycemia, autophagy activity of mesangial cells is inhibited, but with the increase of intracellular advanced glycation end products (AGEs), a large number of proteins are inactivated, and organelle function is impaired. At this point, both protein degradation pathways may be activated. Although autophagy changes at different stages of DN are still a controversial issue, most evidence supports the fact that the process of renal autophagy is blocked in DN, promoting apoptosis and the occurrence of disease. As an inducer of autophagy, rapamycin enables cells to survive and resist external damage.

This study will further verify the autophagy of cells under the condition of high glucose, whether high glucose has an effect on cell survival, and the effect of rapamycin on the autophagy activity of cells, providing evidence for the application of rapamycin in diabetic nephropathy.

Objectives

To study the role of autophagy in the treatment of DM with rapamycin and to provide the basis for the treatment of DN with rapamycin.

Materials and methods

Materials

Human mesangial cells (HMC) were purchased from the Shanghai Fuheng Biology (Shanghai, China; cat. No. FH0241) and cell culture flasks were purchased from FALCON (Chongqing, China; cat. No. 353014).

Culture and grouping of mesangial cells

Human mesangial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin in a constant temperature incubator with 5% CO_2, at 37°C and saturated humidity. The HMC group was cultured in the original medium component without glucose. The hypoglycemic group was cultured in medium containing 5 mmol/L glucose. In the hyperglycemic group, HMCs were cultured in medium containing 30 mmol/L glucose. In isotonic mannitol group HMCs were cultured in medium containing 24.5 mmol/L mannitol and 5.5 mmol/L glucose. In the rapamycin group, HMCs were cultured in the medium containing 30 mmol/L glucose and 1 μM rapamycin.

Proliferation of 5-ethynyl-2-deoxyuridine (EdU) cells

The cells were treated with 2 × EdU working solution for 2 h, fixed with 4% paraformaldehyde and incubated for 30 min. The cells were cultured using conventional methods. After discarding the fixed solution in the culture medium, the cells were washed 3 times and incubated with a transparent solution at room temperature for 20 min. Following this, the cells were incubated at room temperature away from light for 30 min before being observed under a fluorescence microscope (Olympus IX71; Olympus Corp., Tokyo, Japan).

Apoptosis cells were determined with flow cytometry

After digesting the HMC with trypsin, they were collected by centrifugation at 2,000 rpm for 5–10 min and then washed again. Following this, 300 µL of buffer suspension cells were added, and 5 µL of Annexin V-FITC was mixed in. The resulting mixture was kept away from light. Before loading the sample, 5 µL of propidium iodide (PI) staining was added and allowed to bind for 5 min. Finally, after adding 200 µL of buffer, the results were observed.

GFP–RFP–LC3 labeling autophagy flow

The HMC were inoculated into 24-well plates, The amount of GFP–RFP–LC3 adenovirus in each group was within the range of multiplicity of infection (MOI) = 20. After 2 h, the medium was changed, and the cells were washed with culture medium or buffer. After the cleaning solution was carefully absorbed, the cells were fixed with a freshly prepared and pre-heated buffer containing 4% paraformaldehyde for 10 min at room temperature. The fixative was removed, and the cells were rinsed several times with an appropriate buffer. Nuclear staining was performed in a dark room to avoid light. Fluorescent dye was applied diluted (DAPI (4',6-diamidino-2-phenylindole, 1:1,000)), washed cells were applied and incubate at room temperature for 7–10 min, and then wash 3 times for 5 min with phosphate-buffered saline (PBS); finally, the liquid was discarded. The film was sealed and observed with a confocal laser microscope (Leica TCS SP8; Leica Camera AG, Wetzlar, Germany).

Protein concentrations of LC3-II/LC3-I and P62 were detected with western blot

Cell pellets from each group were collected. A total of 150 µL of lysate was added to each sample, and gel plates were prepared in the following manner: 1. The polyvinylidene fluoride (PVDF) membrane was sealed with a sealing solution containing 5% skim milk powder at room temperature for 2 h. 2. The corresponding primary antibody (rabbit anti-human LC3, P62) was diluted with blocking solution. The PVDF membrane was then immersed in the primary antibody incubation solution and incubated overnight at 4°C. 3. Excess primary antibodies were removed by washing the PVDF membrane thoroughly in Tris-buffered saline with Tween (TBST) and repeating the process 3–4 times for 15 min each time. 4. The secondary antibody was applied (goat anti-rabbit IgG antibody) and the PVDF membrane was soaked in the solution for secondary antibody incubation and incubated at 37°C for 2 h. 5. Excess secondary antibodies were washed away by fully washing the PVDF membrane in TBST 3–4 times for 15 min each time. 6. Color exposure was performed.

The content of Col4 HA, and LA of cells was determined by ELISA

Cells digested with trypsin were harvested and the resulting supernatant was collected. Biotinylated antibody working solution (100 µL) was added to each well, followed by incubation at 37°C for 1 h after covering the plate with film. Enzyme conjugate working solution (100 µL) was then added to each well, followed by substrate (tetramethylbenzidine) solution (90 µL). The enzyme plate was covered with film and incubated away from light. Stop solution (50 µL) was added to each well and the optical density (OD) was immediately measured at 450 nm using a microplate reader (Multiskan Mk3 Microplate Reader; Thermo Fisher Scientific, Waltham, USA).

Statistical analyses

IBM SPSS v. 26.0 (IBM Corp., Armonk, USA) software was utilized for statistical analysis. The experimental data were replicated 3 times for each group. All tested variables were expressed as medians with interquartile ranges (IQRs; 25^{th} to 75^{th} percentiles). We assessed statistical differences with the Kruskal–Wallis test, followed by Dunn's test for multiple comparisons. We adjusted p-values using the Bonferroni correction. We considered a p-value below 0.05 statistically significant. Bootstrap medians and 95% confidence intervals (95% CIs) have been reported.

Results

EdU cell proliferation under different conditions

Hoechst 33342 stimulated the nuclei to emit blue fluorescence, while Azide 488 caused proliferation of cells resulting in green fluorescence. Figure 1 shows a significant number of cells in HMC group, hypoglycemic group and isotonic mannitol group, which were 948.5 (881.75–1008.5), 1019.5 (938.5–1055.5) and 920.5 (808.5–954.5), respectively. Additionally, there were a considerable number of proliferating cells: 463.0 (416.75–514.5), 512.5 (462.25–525.25 and 444.5 (411.75–513.25) were the measured values for total number of cells in hyperglycemic group. The group contained 912.0 (860.5–969.5) cells in total. Among them, only 141.0 (130.0–163.25) were found to be proliferating. The proliferation rate of cells in hyperglycemic group was statistically significant compared to HMC group (p-value 0.017), with a rate of 17.5% (13.75–19.75%). Table 1 displays the results, while Table 2 and Table 3 show the nonparametric test results and post hoc analysis, respectively. Hoechst 33342 induced blue fluorescence in all nuclei, while Azide 488 induced green fluorescence in proliferating cells. Figure 1A,B,D illustrates a significant number of cells: 948.5 (881.75–1008.5), 1019.5 (938.5–1055.5)

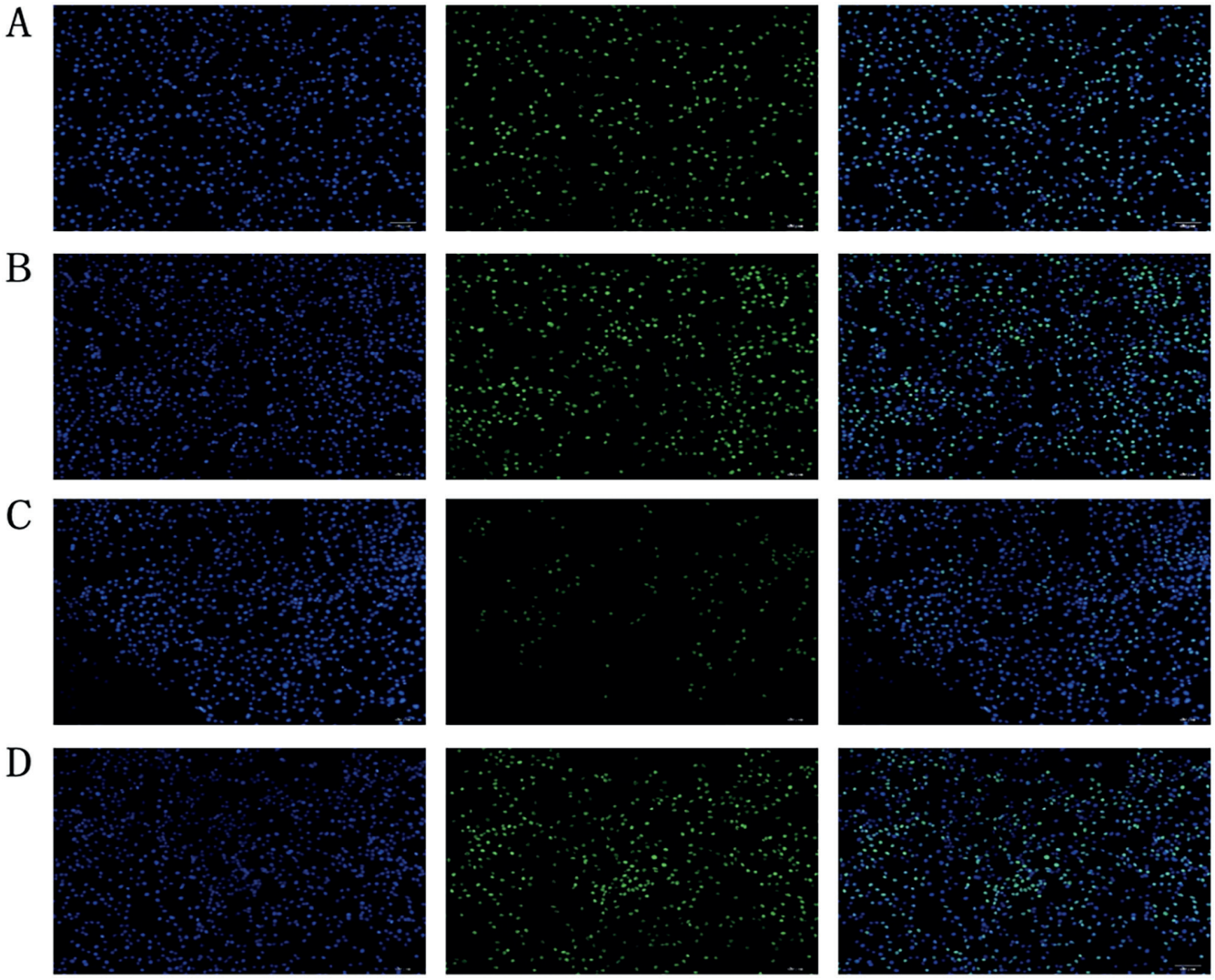

Fig. 1. Proliferation of 5-ethynyl-2-deoxyuridine (EdU) cells under fluorescence microscope. A. Human mesangial cells (HMC); B. Hypoglycemic group; C. Hyperglycemic group; D. Mannitol isotonic group. The blue fluorescence represents nucleated cells and the green fluorescence represents proliferating cells. The number of cells in group C was 912 (860.5–969.5), and in groups A, B and D it was 948.5 (881.75–1008.5), 1019.5 (938.5–1055.5) and 920.5 (808.5–954.5), respectively. The cell proliferation rate of group C was 17.5% (13.75–19.75%), which was statistically significant compared with group A (p-value at 0.017)

and 920.5 (808.5–954.5), respectively. Furthermore, there was a considerable number of proliferating cells: the total cell count for hyperglycemic group was 912.0 (860.5–969.5) and the number of proliferating cells was 141.0 (130.0–163.25). The proliferation rate of cells in group C was 17.5% (13.75–19.75%), indicating significant statistical difference from HMC group with a p-value of 0.017.

Flow cytometry detection of cell apoptosis

Flow cytometry was utilized to evaluate cell apoptosis in various conditions, as depicted in Fig. 2. The proportion of apoptotic cells in HMC group, hypoglycemic group and isotonic mannitol group were 2.6% (2.52–2.65%), 3.86% (3.77–3.87%) and 3.41% (3.37–3.53%), respectively, based on the data presented in Table 4. Notably, these rates were significantly lower than the 16.04% (16.03–16.55%) proportion in hyperglycemic group. The survival rates of cells in HMC group, hypoglycemic group and isotonic mannitol group were 93.0% (92.75–93.55%), 93.2% (92.4–93.25%) and 93.8% (93.25–94.0%), respectively. The survival rate of cells in group C was 79.0% (78.55–79.4%). The number of apoptotic cells in hyperglycemic group was statistically significant (p-value = 0.013) compared to HMC group. The nonparametric test results and post hoc analysis are shown in Table 5 and Table 6.

GFP-RFP-LC3 dual fluorescence labeling of autophagy flow

The red fluorescent protein (RFP) emitted red fluorescence, while the green fluorescent protein (GFP) emitted green fluorescence. Yellow spots represented autophagosomes, while red spots represented autolysosomes. As shown in Fig. 3, hyperglycemic group had the lowest number of autolysosomes, while HMC group, hypoglycemic

Table 1. General statistical description of 5-ethynyl-2-deoxyuridine (EdU) cell proliferation tests

Group	Median (25–75% quartile)	Lower 95% CI	Upper 95% CI
Number of proliferating cells			
HMC	463 (514.5–416.75)	399	523.38
Hypoglycemic group	512.5 (525.25–462.25)	399	618.5
Hyperglycemic group	141 (163.25–130)	113	156.433
Mannitol isotonic group	444.5 (513.25–411.75)	348.175	529.5
Total number of cells			
HMC	948.5 (1008.5–881.75)	831	1089.528
Hypoglycemic group	1019.5 (1055.5–938.5)	908.5	1199
Hyperglycemic group	912 (969.5–860.5)	828	1047.5
Mannitol isotonic group	920.5 (954.5–808.5)	862.5	1129.151
Proliferation rate			
HMC	50.5 (53.5–47.5)	45.027	57.5
Hypoglycemic group	50 (51.75–48.25)	47	54
Hyperglycemic group	17.5 (19.75–13.75)	15	22
Mannitol isotonic group	51.5 (53–47)	48.5	58

All tested variables were expressed as medians with interquartile ranges (IQRs) (25^{th}–75^{th} percentiles). Bootstrap medians 95% confidence intervals (95% CIs) were reported in the table; EdU – 5-ethynyl-2-deoxyuridine; HMC – human mesangial cells.

Table 2. Nonparametric test results of 5-ethynyl-2-deoxyuridine (EdU) cell proliferation tests

Variables	Statistics	p-value	df
Number of proliferating cells	13.253	0.004	3
Total number cells	3.427	0.330	3
Proliferation rate	13.059	0.005	3

The Kruskal–Wallis test was used. The significance level of the mean difference was 0.05.

group and isotonic mannitol group had more autolysosomes than hyperglycemic group, with rapamycin group having the highest number of autolysosomes.

Expression levels of autophagy markers LC3 and P62 were detected with western blotting

The results indicate that in hyperglycemic group, there was a significant increase in the expression of P62 and an apparent decrease in the expression of autophagy marker LC3-II, while the expression of LC3-I was apparently increased, as compared to HMC group, hypoglycemic group and isotonic mannitol group. These results are presented in Fig. 4 and Fig. 5. When rapamycin was added to the sample (rapamycin group), the expression level of LC3-II increased significantly, while the expression levels of LC3-I and P62 decreased significantly. The LC3-II/LC3-I ratio was 2.21 (1.95–2.21), which was significantly higher than that of the other groups. Also, the expression

Table 3. The multiple comparison of post hoc analysis of 5-ethynyl-2-deoxyuridine (EdU) cell proliferation tests

Variables	Comparison	Statistics	p-value	p-value adjustment
Number of proliferating cells	A–B	– 0.470	0.639	1
	A–C	2.777	0.005	0.033
	B–C	3.247	0.001	0.007
	A–D	– 0.020	0.984	1
	B–D	0.449	0.653	1
	C–D	– 2.798	0.005	0.031
Total number cells	A–B	– 0.898	0.369	1
	A–C	0.653	0.514	1
	B–C	1.551	0.121	0.725
	A–D	0.735	0.462	1
	B–D	1.633	0.102	0.615
	C–D	0.082	0.935	1
Proliferation	A–B	0.184	0.854	1
	A–C	2.984	0.003	0.017
	B–C	2.800	0.005	0.031
	A–D	– 0.061	0.951	1
	B–D	–0.245	0.806	1
	C–D	–3.045	0.002	0.014

Multiple comparisons were detected with Dunn's test and p-values were adjusted using the Bonferroni correction; A – HMC (human mesangial cells); B – hypoglycemic group; C – hyperglycemic group; D – mannitol isotonic group; the significance level of the mean difference was 0.05.

Table 4. General statistical description of cell apoptosis under different conditions was detected using flow cytometry

Groups	Median (25–75% quartile)	Lower 95% CI	Upper 95% CI
Q2 + Q3			
HMC	2.6 (2.515–2.645)	2.51	2.77
Hypoglycemia group	3.86 (3.77–3.87)	3.84	4.04
Hyperglycemia group	16.04 (16.03–16.55)	15.02	16.06
Mannitol isotonic group	3.41 (3.37–3.525)	3.18	3.49
Q4			
HMC	93 (92.75–93.55)	91.9	93.5
Hypoglycemia group	93.2 (92.4–93.25)	93.1	94.8
Hyperglycemia group	79 (78.55–79.4)	78.2	79.9
Mannitol isotonic group	93.8 (93.25–94)	93.4	94.9

All tested variables were expressed as medians with interquartile ranges (IQRs) (25^{th}–75^{th} percentiles). Bootstrap medians 95% confidence intervals (95% CI) were reported in the table; Q2 and Q3 – apoptotic cells; Q4 – living cells; HMC – human mesangial cells.

level of P62 was 0.38 (0.375–0.39), which was significantly lower than that of the other groups. The levels of P62 expression and the ration of LC3-II/LC3-I in hyperglycemic group significantly differed from those in rapamycin group, with p-values of 0.01 and 0.03, respectively, as displayed in Table 7. Table 8 and Table 9 illustrate the nonparametric test results and post hoc analysis.

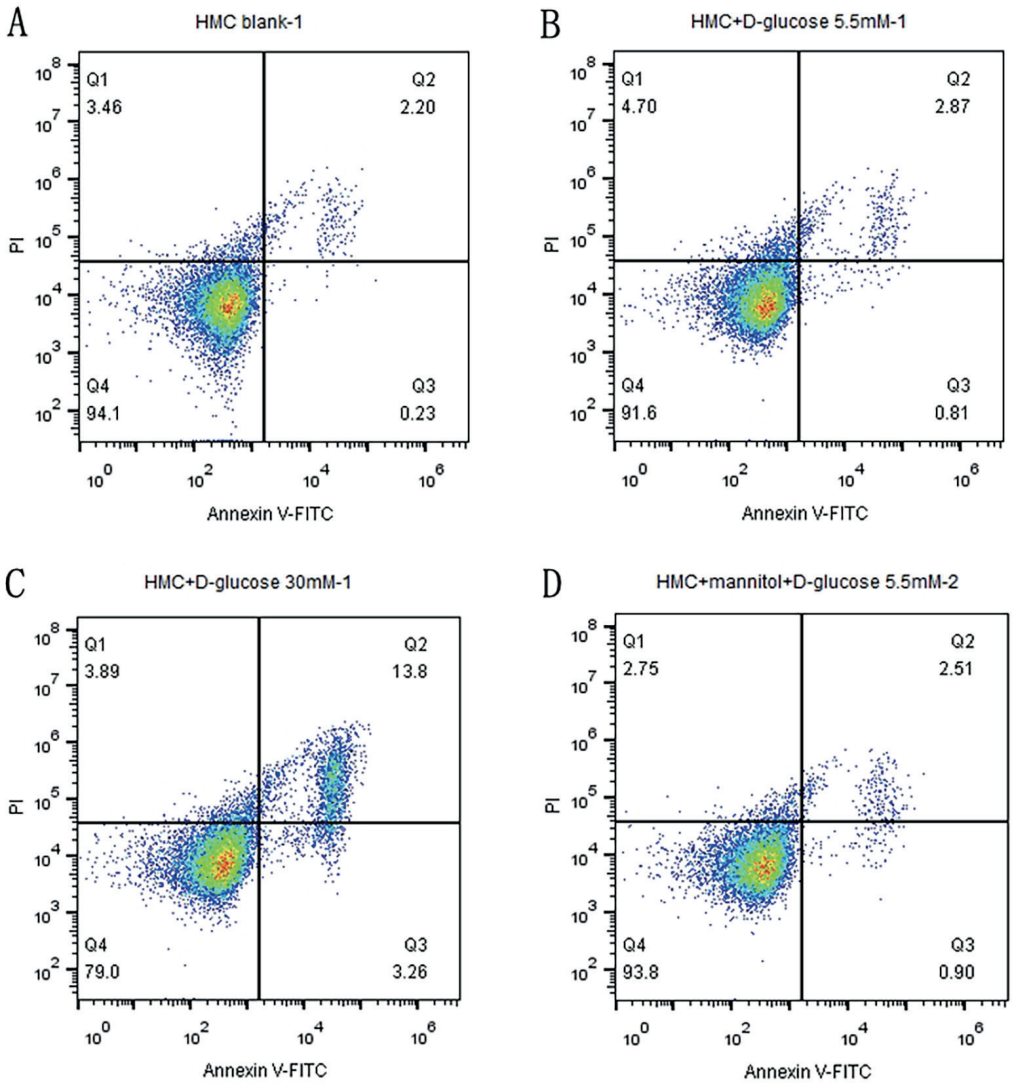

Fig. 2. Cell apoptosis under different conditions was detected with flow cytometry. A. Human mesangial cells (HMC); B. Hypoglycemic group; C. Hyperglycemic group; D. Mannitol isotonic group; Q1 – cell debris; Q2 and Q3 – apoptotic cells; Q4 – living cells. The proportion of apoptotic cells in groups A, B and D were 2.6% (2.52–2.65%), 3.86% (3.77–3.87%) and 3.41% (3.37–3.53%), respectively, which was significantly lower than that in group C: 16.04% (16.03–16.55%). The survival rates of cells in group C was the lowest and amounted to 79.0% (78.55–79.4%). Compared with group A, the number of apoptotic cells in group C was statistically significant (p = 0.013)

Table 5. Nonparametric test results of apoptosis test

Variables	Statistics	p-value	df
Q2	8.929	0.030	3
Q3	9.462	0.024	3
Q4	6.897	0.075	3
Q2 + Q3	10.385	0.016	3

The Kruskal–Wallis test was used. df – degrees of freedom; Q2 and Q3 – apoptotic cells; Q4 – living cells; the significance level of the mean difference was 0.05.

Table 6. Multiple comparison of apoptotic cells post hoc analysis

Variables	Comparison	Statistic	p-value	p-value adjustment
Q4	A–B	0.226	0.821	1
	A–C	1.925	0.054	0.325
	B–C	1.698	0.089	0.537
	A–D	–0.566	0.571	1
	B–D	–0.793	0.428	1
	C–D	–2.491	0.013	0.076
Q2 + Q3	A–B	–2.038	0.042	0.249
	A–C	–3.057	0.002	0.013
	B–C	–1.019	0.308	1
	A–D	–1.019	0.308	1
	B–D	1.019	0.308	1
	C–D	2.038	0.042	0.249

Multiple comparisons were detected with Dunn's test and p-values were adjusted using the Bonferroni correction; A – HMC (human mesangial cells); B – hypoglycemic group; C – hyperglycemic group; D – mannitol isotonic group; Q2 and Q3 – apoptotic cells; Q4 – living cells; the significance level of the mean difference was 0.05.

The contents of Col4, HA and LA in cells were detected with ELISA

The main elements of ECM were detected using ELISA and displayed in Fig. 6. In the hyperglycemic group, the levels of IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA) were significantly higher compared to those in the HMC, hypoglycemic and mannitol isotonic groups. The levels were as follows: 18.65 (17.04–19.43) ng/mL, 89.17

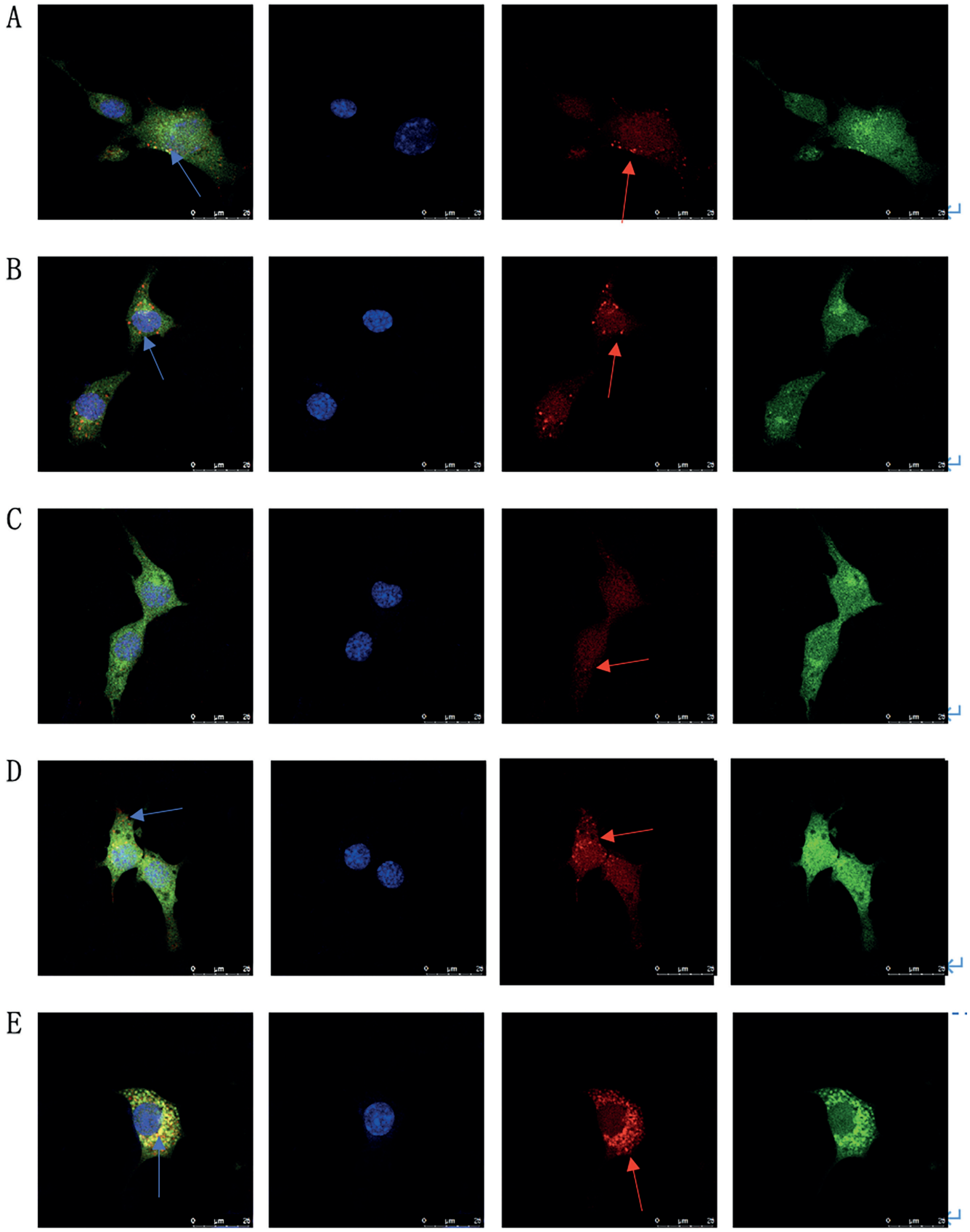

Fig. 3. GFP-RFP-LC3 labeled autophagy flow. A. Human mesangial cells (HMC); B. Hypoglycemic group; C. Hyperglycemic group; D. Mannitol isotonic group; E. Rapamycin group. Yellow spots represent autophagosomes and red spots represent autolysosomes. Group C had the least number of autolysosomes, while groups A, B, D and E had more autolysosomes than group C, among which group E had the most autolysosomes

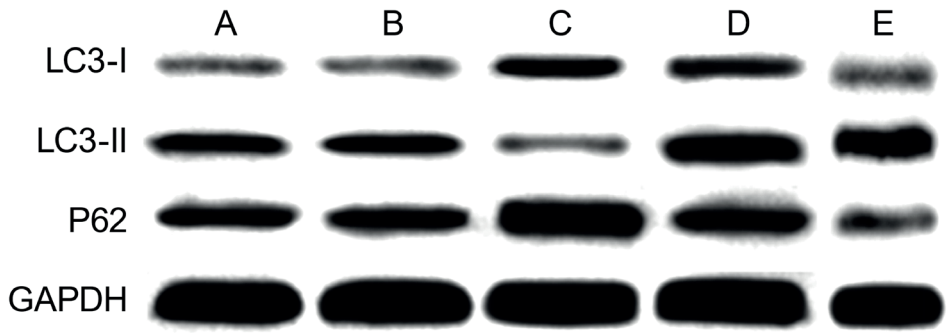

Fig. 4. The expression levels of LC3-II, LC3-I and P62 in mesangial cells were detected with western blotting; A – human mesangial cells (HMC); B – hypoglycemic group; C – hyperglycemic group; D – mannitol isotonic group; E – rapamycin group; GAPDH – control group. In group C, the expression of autophagy marker LC3 was the lowest, while the expression of LC3-I and P62 was the highest, but they had no difference in groups A, B, D, and E.

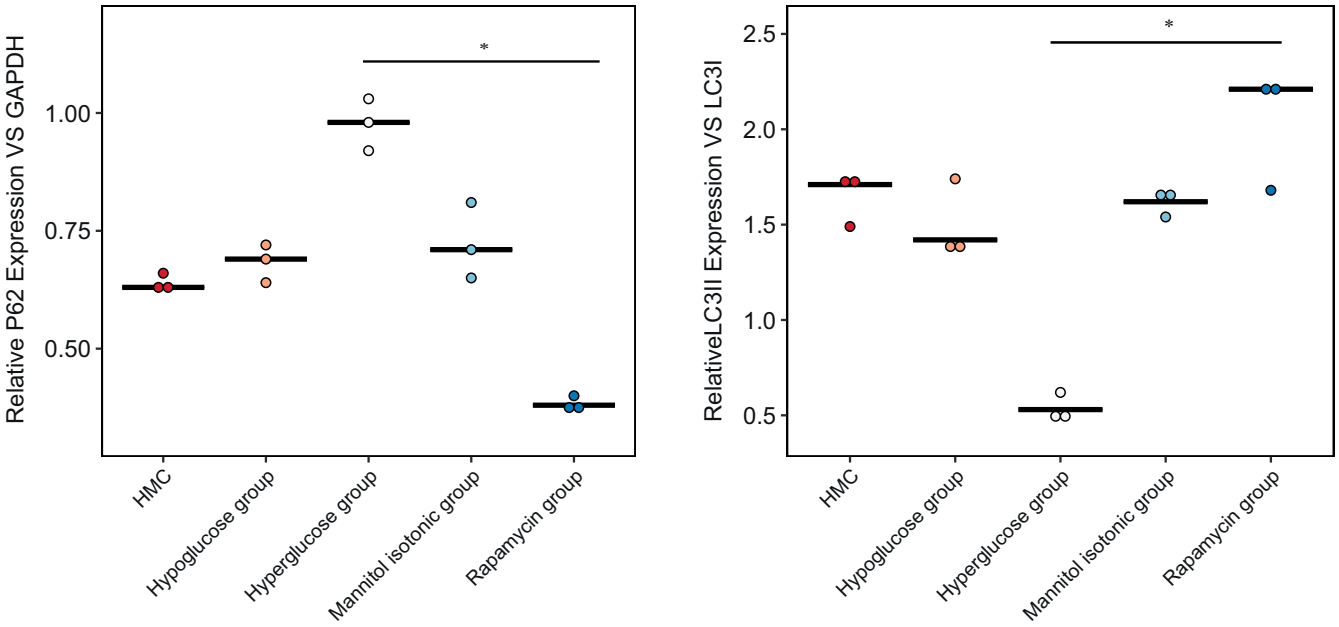

Fig. 5. Expression levels of LC3-II/LC3-I ratio and P62 in cells were detected with western blotting. Point means the raw data values and black lines mean median of each group. The Kruskal–Wallis test was applied to test differences between 5 compared group. Multiple comparisons were detected with Dunn's test and p-values were adjusted using the Bonferroni correction. In the hyperglycemic group, the LC3-II/LC3-I ratio was lower, P62 was increased, but in the rapamycin group, the expression of LC3-II/LC3-I ratio and P62 was different compared to hyperglycemic group

*hyperglycemic group was significantly different from rapamycin group; p-values of 0.03 and 0.01, respectively.

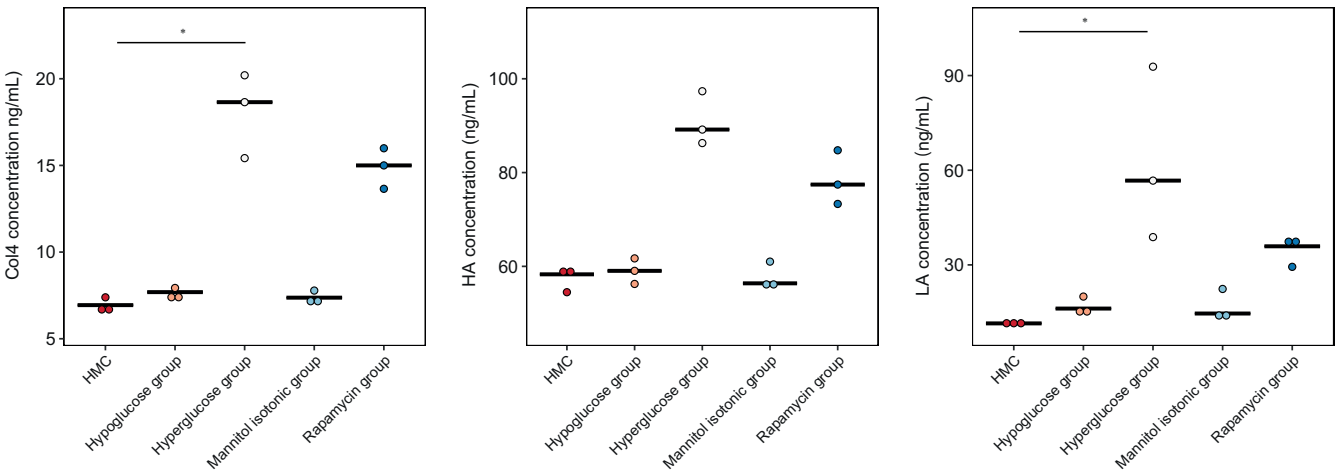

Fig. 6. The levels of type IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA) in cells were detected with enzyme-linked immunosorbent assay (ELISA). Points mean the raw data values and black lines mean median of each group. The Kruskal–Wallis test was used to test differences between 5 compared group. Multiple comparisons were detected with Dunn's test, and p-values were adjusted using Bonferroni correction

* human mesangial cells (HMC) group was significantly different from hyperglycemic group.

Table 7. General statistical description of western blotting detection of p62 and LC3-II/LC3-I

Group	P62			LC3-II/LC3-I		
	median (25–75% quartile)	lower 95% CI	upper 95% CI	median (25–75% quartile)	lower 95% CI	upper 95% CI
HMC	0.63 (0.645–0.63)	0.6	0.63	1.71 (1.725–1.6)	1.68	1.93
Hypoglycemic group	0.69 (0.705–0.665)	0.66	0.74	1.42 (1.58–1.385)	1.1	1.49
Hyperglycemic group	0.98 (1.005–0.95)	0.93	1.04	0.53 (0.575–0.495)	0.44	0.6
Mannitol isotonic group	0.71 (0.76–0.68)	0.61	0.77	1.62 (1.655–1.58)	1.55	1.7
Rapamycin group	0.38 (0.39–0.375)	0.36	0.39	2.21 (2.21–1.945)	2.21	2.74

All tested variables were expressed as medians with interquartile ranges (IQRs) (25^{th}–75^{th} percentiles). Bootstrap medians 95% confidence intervals (95% CIs) were reported in the table. HMC – human mesangial cells.

Table 8. Nonparametric test results of LC3-II/LC3-I and P62 in western blotting detection

Variables	Statistics	p-value	df
P62	12.122	0.016	4
LC3	9.325	0.053	4

The Kruskal–Wallis test was used. The significance level of the mean difference was 0.05; df – degrees of freedom.

Table. 9. Expression levels of LC3-II/LC3-I and P62 in western blotting multiple comparisons post hoc analysis

Variables	Comparison	Statistics	p-value	p-value adjustment
P62	A–B	–0.822	0.411	1
	A–C	–2.284	0.022	0.224
	B–C	–1.462	0.144	1
	A–D	–1.096	0.273	1
	B–D	–0.274	0.784	1
	C–D	1.188	0.235	1
	A–E	1.005	0.315	1
	B–E	1.827	0.068	0.676
	C–E	3.289	0.001	0.010
	D–E	2.101	0.036	0.356
LC3-II/LC3-I	A–B	0.732	0.464	1
	A–C	2.149	0.032	0.316
	B–C	1.417	0.156	1
	A–D	0.412	0.681	1
	B–D	–0.320	0.749	1
	C–D	–1.736	0.082	0.823
	A–E	–0.777	0.437	1
	B–E	–1.509	0.131	1
	C–E	–2.926	0.003	0.034
	D–E	–1.189	0.234	1

Multiple comparisons were detected with Dunn's test and p-values were adjusted using the Bonferroni correction; A – HMC (human mesangial cells); B – hypoglycemic group; C – hyperglycemic group; D – mannitol isotonic group; E – rapamycin group. The significance level of the mean difference was 0.05.

Table 10. Type IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA) contents of cells in different states were detected using enzyme-linked immunosorbent assay (ELISA)

Group	Median (25–75% quartile)	Lower 95% CI	Upper 95% CI
Col4			
HMC	6.94 (7.165–6.69)	6.49	7.44
Hypoglycemic group	7.69 (7.81–7.395)	7.45	8.28
Hyperglycemic group	18.65 (19.425–17.035)	17.1	21.88
Mannitol isotonic group	7.37 (7.575–7.165)	6.96	7.78
Rapamycin group	15 (15.495–14.325)	14.01	16.35
HA			
HMC	58.3 (58.875–56.395)	57.15	62.11
Hypoglycemic group	59.04 (60.37–57.645)	56.38	61.83
Hyperglycemic group	89.17 (93.255–87.73)	81	92.05
Mannitol isotonic group	56.38 (58.695–56.15)	51.75	56.84
Rapamycin group	77.44 (81.095–75.375)	70.13	81.57
LA			
HMC	11.46 (11.83–11.14)	10.72	12.1
Hypoglycemic group	16.16 (18.06–15.225)	12.36	18.03
Hyperglycemic group	56.71 (74.76–47.76)	20.61	74.61
Mannitol isotonic group	14.59 (18.48–13.965)	6.81	15.84
Rapamycin group	35.89 (37.35–32.635)	32.97	42.4

All tested variables were expressed as medians with interquartile ranges (IQRs) (25^{th}–75^{th} percentiles). Bootstrap medians 95% confidence intervals (95% CIs) were reported in the table. HMC – human mesangial cells.

(87.73–93.23) ng/mL and 56.71 (47.76–74.76) ng/mL, respectively. Compared to the HMC group, there was a significant difference in Col4 and LA levels with p-values of 0.035 and 0.012, respectively. Upon the addition of rapamycin, the hyperglycemic group demonstrated a significant decrease in the levels of Col4, HA and LA, although no statistical significance was observed, as indicated in Table 10. The nonparametric test results and post hoc analysis are presented in Table 11 and Table 12. Under hyperglycemic conditions, the concentrations of Col4, HA and LA were significantly elevated in comparison to the other groups (represented by the hyperglycemic group as compared to HMC; $p < 0.05$). Although there was

Table 11. Nonparametric test results of type IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA)

Variables	Statistics	p-value	df
Col4	11.567	0.021	4
HA	11.033	0.026	4
LA	12.714	0.013	4

The Kruskal–Wallis test was used. The significance level of the mean difference was 0.05; df – degrees of freedom.

Table 12. Type IV collagen fiber (Col4), hyaluronic acid (HA) and laminin (LA) multiple comparisons post hoc analysis

Variables	Comparison	Statistics	p-value	p-value adjustment
Col4	A–B	–1.004	0.315	1
	A–C	–2.921	0.003	0.035
	B–C	–1.917	0.055	0.552
	A–D	–0.639	0.523	1
	B–D	0.365	0.715	1
	C–D	2.282	0.022	0.225
	A–E	–2.282	0.022	0.225
	B–E	–1.278	0.201	1
	C–E	0.639	0.523	1
	D–E	–1.643	0.100	1
HA	A–B	–0.456	0.648	1
	A–C	–2.647	0.008	0.081
	B–C	–2.191	0.028	0.284
	A–D	–0.091	0.927	1
	B–D	0.365	0.715	1
	C–D	2.556	0.011	0.106
	A–E	–1.826	0.068	0.679
	B–E	–1.369	0.171	1
	C–E	0.822	0.411	1
	D–E	–1.734	0.082	0.828
LA	A–B	–1.279	0.201	1
	A–C	–3.244	0.001	0.012
	B–C	–1.964	0.0495	0.495
	A–D	–1.188	0.235	1
	B–D	0.091	0.927	1
	C–D	2.056	0.040	0.398
	A–E	–2.512	0.012	0.120
	B–E	–1.233	0.217	1
	C–E	0.731	0.465	1
	D–E	–1.325	0.185	1

Multiple comparisons were detected with Dunn's test and p-values were adjusted using the Bonferroni correction; A – HMC (human mesangial cells); B – hypoglycemic group; C – hyperglycemic group; D – mannitol isotonic group; E – rapamycin group; the significance level of the mean difference was 0.05.

no significant difference between the rapamycin group and the hyperglycemic group, the concentrations of Col4, HA and LA were significantly reduced in the rapamycin group.

Discussion

Diabetic nephropathy has become the predominant cause of chronic kidney disease (CKD) in China.[2] Under high glucose conditions, mesangial cells experience abnormal proliferation, resulting in the secretion and deposition of a considerable amount of ECM in the mesangial region.[3] Furthermore, high-glucose conditions also impair autophagy activity in glomerular and renal tubular cells.[4–6] An inhibition of such activity reduces ECM degradation and facilitates DN progression. In many studies on the mechanism of mesangial cells and matrix proliferation, autophagy in mesangial cells has received little consideration.[7]

Autophagy is an intracellular self-degradation process that degrades and recycles misfolded proteins and damaged organelles to maintain cell homeostasis.[8,9] This process is realized through the transport of cytoskeletal microtubule network system, and its molecular mechanism involves essential proteins, such as LAMP1, LAMP2, Rab7, and UVRAG.[10] The mTOR signaling pathway is the main regulatory pathway of autophagy.[11] It contains at least 2 protein complexes with different functions: mTORC 1 and mTORC 2.[12,13] Of these, mTORC 1, that is a rapamycin sensitive protein complex, is one of the main regulatory factors controlling the activity of ULK1 complex. It can modulate autophagy by phosphorylating and dephosphorylating related components within the ULK1 complex, specifically ULK1 and Atg13.

This study aimed to elucidate the impact of high-glucose condition on HMC. The 5-ethynyl-2-deoxyuridine is a nucleoside analogue of thymine (T) and an effective replacement for T in replicating DNA during cell proliferation. The green fluorescence of newly proliferated cells is observed under a fluorescence microscope after staining with Azide 488, with excitation wavelength falling between 495–519 nm. After performing DNA staining with Hoechst 33342, all cells emit blue fluorescence at an excitation wavelength of 346–460 nm, including newly proliferated cells and previously undivided proliferated cells. The results of EdU cell proliferation experiment showed that the number of nuclei and cell proliferation in hyperglycemic group were significantly reduced compared with the other 3 groups (912.0 (860.5–969.5) and 141.0 (130–163.25, respectively), and the proliferation rate was the lowest: 17.5 (13.75–19.75).

The flow cytometry results indicated that the percentage of mesangial cell apoptosis was 16.04% (16.03–16.55%) under high-glucose conditions, surpassing that of the other groups. The high-glucose conditions significantly affected cell survival rates, while the proliferation rate and apoptosis number of cells in the mannitol isotonic group did not differ significantly from those in the HMC group. This finding further demonstrates that high-glucose conditions or its glucose metabolites can impede cell proliferation and promote cell apoptosis, but this effect is unaffected by high

osmotic state. Second, this study confirmed that high-glucose conditions inhibited HMC proliferation and autophagy activity. Autophagy flow detection can determine autophagy state. Changes in autophagy flow are primarily monitored using GFP–RFP–LC3 tandem fluorescent protein labeling, which has sensitivity different to lysosomal acidic microenvironment.[14]

The strength of autophagy can be assessed through red and yellow fluorescence intensity. This study utilized a GFP–RFP–LC3-labeled autophagy flow experiment to demonstrate that the hyperglycemic group had significantly diminished autolysosome count compared to the HMC group, indicating subdued autophagy activity. Previous research has demonstrated that inhibition of autophagy exacerbates oxidative damage in cells. Damaged and aging organelles and macromolecules within cells may not be eliminated in a timely manner, resulting in their accumulation and worsening of cellular damage, aging, and eventual promotion of cell apoptosis. This process could potentially be one of the mechanisms behind the development of DN.[15–17]

LC3 and P62 are proteins commonly used as markers for autophagy, reflecting both its expression and intensity. LC3 is found in 2 forms, LC3-I and LC3-II. Initially, Atg4 cleaves the precursor LC3 to produce LC3-I, which later, under the activation of Atg7, generates its membrane binding form (LC3-II), which locates at autophagosome and autolysosome membranes[18]. The changes in LC3-II protein content can offer insight into the changes in autophagic structures, such as autophagosomes and autolysosome.[19]

P62, a protein that binds to ubiquitin, is essential for autophagy and serves as a selective autophagy receptor that links LC3 to the ubiquitinated substrate targeted for degradation.[20] Western blotting analysis revealed that the expression of LC3-II was significantly lower in the hyperglycemic group compared to the HMC group. Additionally, the LC3-II/LC3-I ratio was reduced, while P62 expression levels were elevated, strongly indicating that hyperglycemia significantly inhibits cellular autophagy activity.

Under high-glucose conditions, inhibition of autophagy decreases ECM degradation, resulting in an ECM synthesis and degradation imbalance. This leads to the accumulation of ECM components such as Col4, HA and LA in the mesangial region of the kidney. Significant increases in Col4, HA and LA contents were observed in the hyperglycemic group compared to other groups.

Rapamycin is a known inducer of autophagy. The study revealed that the addition of rapamycin into the HMC of the hyperglycemic group resulted in a significant increase in the expression of LC3-II compared to other groups. Additionally, the expression of LC3-I and P62 decreased significantly, indicating that rapamycin could activate inhibited autophagy. The levels of Col4, HA and LA of ECM components were reduced in the rapamycin group, as confirmed with ELISA. This indicates that after autophagy was activated, the accumulation of ECM decreased significantly, leading to an improvement in the condition. Nonetheless, no statistical difference was observed between the rapamycin group and the hyperglycemic group, which may be due to the small sample size.

Limitations

The study had a small sample size and an imperfect test grouping. At the outset, the rapamycin group did not undergo cell proliferation or apoptosis tests, and the ELISA test did not yield statistically significant differences in HA, resulting in poor persuasiveness. Additionally, no experiments were conducted to verify rapamycin's autophagy induction effect.

Conclusions

Autophagy activity of glomerular mesangial cells is significantly inhibited under high-glucose conditions, but rapamycin can induce cell autophagy, improve cellular metabolic processes, and reduce mesangial cell proliferation and matrix hyperplasia, thereby inhibiting the progression of DN. This provides a theoretical basis for the clinical application of rapamycin in DN treatment.

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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References

1. Ding Y, Kim JK, Kim SI, et al. TGF-β1 protects against mesangial cell apoptosis via induction of autophagy. *J Biol Chem*. 2010;285(48):37909–37919. doi:10.1074/jbc.M109.093724
2. Zhang L, Long J, Jiang W, et al. Trends in chronic kidney disease in China. *N Engl J Med*. 2016;375(9):905–906. doi:10.1056/NEJMc1602469
3. Wu Z, Yin W, Sun M, Si Y, Wu X, Chen M. BK_{Ca} mediates dysfunction in high glucose-induced mesangial cell injury via TGF-β 1/Smad2/3 signaling pathways. *Int J Endocrinol*. 2020;2020:3260728. doi:10.1155/2020/3260728
4. Kitada M, Ogura Y, Suzuki T, et al. A very-low-protein diet ameliorates advanced diabetic nephropathy through autophagy induction by suppression of the mTORC1 pathway in Wistar fatty rats, an animal model of type 2 diabetes and obesity. *Diabetologia*. 2016;59(6):1307–1317. doi:10.1007/s00125-016-3925-4
5. Takahashi A, Takabatake Y, Kimura T, et al. Autophagy inhibits the accumulation of advanced glycation end products by promoting lysosomal biogenesis and function in the kidney proximal tubules. *Diabetes*. 2017;66(5):1359–1372. doi:10.2337/db16-0397
6. Zhou D, Zhou M, Wang Z, et al. Progranulin alleviates podocyte injury via regulating CAMKK/AMPK-mediated autophagy under diabetic conditions. *J Mol Med*. 2019;97(11):1507–1520. doi:10.1007/s00109-019-01828-3

7. Lin YC, Chang YH, Yang SY, Wu KD, Chu TS. Update of pathophysiology and management of diabetic kidney disease. *J Formos Med Assoc*. 2018;117(8):662–675. doi:10.1016/j.jfma.2018.02.007
8. Cheng Z. The FoxO–autophagy axis in health and disease. *Trends Endocrinol Metab*. 2019;30(9):658–671. doi:10.1016/j.tem.2019.07.009
9. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008;132(1):27–42. doi:10.1016/j.cell.2007.12.018
10. Alessandrini F, Pezzè L, Ciribilli Y. LAMPs: Shedding light on cancer biology. *Semin Oncol*. 2017;44(4):239–253. doi:10.1053/j.seminoncol.2017.10.013
11. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett*. 2010;584(7):1287–1295. doi:10.1016/j.febslet.2010.01.017
12. Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*. 2004;6(11):1122–1128. doi:10.1038/ncb1183
13. Loewith R, Jacinto E, Wullschleger S, et al. Two TOR complexes, only one of which is rapamycin-sensitive, have distinct roles in cell growth control. *Mol Cell*. 2002;10(3):457–468. doi:10.1016/S1097-2765(02)00636-6
14. Jin L, Qian Y, Zhou J, et al. Activated CRH receptors inhibit autophagy by repressing conversion of LC3BI to LC3BII. *Cell Signal*. 2019;58:119–130. doi:10.1016/j.cellsig.2019.03.001
15. Mammucari C, Milan G, Romanello V, et al. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab*. 2007;6(6):458–471. doi:10.1016/j.cmet.2007.11.001
16. Xie Z, Klionsky DJ. Autophagosome formation: Core machinery and adaptations. *Nat Cell Biol*. 2007;9(10):1102–1109. doi:10.1038/ncb1007-1102
17. Kondo-Okamoto N, Noda NN, Suzuki SW, et al. Autophagy-related protein 32 acts as autophagic degron and directly initiates mitophagy. *J Biol Chem*. 2012;287(13):10631–10638. doi:10.1074/jbc.M111.299917
18. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol*. 2007;8(9):741–752. doi:10.1038/nrm2239
19. Kadowaki M, Karim MdR. Cytosolic LC3 ratio as a quantitative index of macroautophagy. *Methods Enzymol*. 2009;452 Pt B:199–213. doi:10.1016/S0076-6879(08)03613-6
20. Boyle KB, Randow F. The role of 'eat-me' signals and autophagy cargo receptors in innate immunity. *Curr Opin Microbiol*. 2013;16(3):339–348. doi:10.1016/j.mib.2013.03.010

Fixed appliances orthodontic therapy as a risk factor for caries development: Systematic review

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Abstract

Orthodontic treatment is often mandatory to improve the patient's health condition. However, the fixed appliance can create additional plaque retention areas, which can increase the risk of caries development. Clinically, one can observe various effects of fixed appliance treatment on caries prevalence. This study aims to analyze to what extent orthodontic therapy with fixed appliances is a risk factor for developing caries in pediatric and adult patients. The keywords used in the search strategy were as follows: ("caries" AND "caries risk" AND "caries experience" AND" "orthodontic treatment" OR "fixed appliance" ") and ("caries experience" AND "orthodontic treatment").

From 808 potential articles, 15 were included in the review. In individuals undergoing fixed orthodontic therapy, several factors can increase the risk of caries during fixed orthodontic treatment, such as salivary composition, oral dysbiosis and plaque accumulation. On the other hand, factors that reduce caries risk are, i.e., oral hygiene self-awareness and previous orthodontic treatment. In most studies which used the Decayed, Missing, and Filled Teeth (DMFT) index, there were no significant differences between the values obtained before orthodontic treatment and after the treatment. Moreover, it is easier for a patient with aligned teeth to remove plaque.

In the young population, fixed orthodontic treatment appears to reduce the incidence of caries. In the adult population, fixed orthodontic treatment increases the risk of dental caries. However, education on proper oral hygiene during orthodontic treatment can reduce the risk of dental caries. The study protocol was registered in the PROSPERO database (PROSPERO CRD42022356628).

Key words: risk factor, prophylaxis, orthodontics, caries, fixed appliances

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Introduction

Caries is a multifactorial disease affecting the tooth's hard tissues, resulting from the demineralization of enamel and dentin. Even today, it is one of the most common diseases, especially in the pediatric population.[1] In addition to the well-known factors that can affect the promotion of demineralization of the tooth's hard tissues, such as the presence of bacteria,[2] the presence of a substrate for bacterial metabolism (carbohydrates),[3] host susceptibility,[3,4] and time,[2,3] many patients undertake orthodontic treatment, especially to resolve functional and/or cosmetic problems, which can cause psychosocial distress and affect their quality of life and self-esteem.[5,6] Therefore, orthodontic treatment is often mandatory to improve the patient's health condition. However, fixed appliances can create additional plaque retention areas, which can cause various spots of demineralization around the bracket.[7] Initially, the carious lesion is reversible, without loss of substance, devoid of painful symptomatology, and presents clinically on the enamel as a chalky-white area of decalcification called a white spot. Although it is a non-cavitated lesion, if not diagnosed and treated with an appropriate prevention plan, it can further progress and turn into an irreversible cavitated lesion, which requires invasive treatment.[8] Clinically, one can observe various effects of fixed appliance treatment on the prevalence of caries. The factors that may influence the occurrence and progression of caries are: patient's hygiene and dietary habits, patient's cooperation with both the orthodontist and dental hygienist, and self-awareness.[9] Therefore, an interesting question arises: How does orthodontic treatment affects the prevalence of caries?

Objectives

This study aims to analyze to what extent orthodontic therapy with fixed appliances is a risk factor for developing caries in growing and adult patients.

Materials and methods

Search strategy

The study protocol was registered after the screening phase in the PROSPERO database (PROSPERO CRD42022356628). The review process was conducted in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement[10,11] and PRISMA reporting guidelines.[10,11] Literature searches of free text and MeSH terms were conducted using MEDLINE (PubMed) and Google Scholar (2000 to 2022). All searches were conducted using a combination of thematic titles and accessible text terms. The final search strategy was determined through several pre-selections. The keywords used in the search strategy were as follows: ("caries" AND "caries risk" AND "caries experience" AND" "orthodontic treatment" OR "fixed appliance" ") and ("caries experience" AND "orthodontic treatment"). The search string presented here refers to the MEDLINE (PubMed) search engine. The search strings associated with the search engines used in this systematic review are shown in Fig. 1. The PICO (Problem/Population, Intervention, Comparison, Outcome) question was: How does orthodontic treatment with fixed appliances influence the risk of caries?[12]

Inclusion criteria

The following inclusion criteria were applied for this systematic review: clinical studies on growing and adult subjects with fixed orthodontic therapy. The following were the exclusion criteria: 1) in vitro studies, 2) animal studies, 3) randomized clinical trials (RCTs), and 4) case-control studies. There was no language restriction applied.

Data extraction

After retrieving the results from the search engines to create a database, the duplicates were removed. Literature was selected following the inclusion criteria by reading the titles and abstracts by 2 authors (SP and MM). The full text of each identified article was then read to determine if it was suitable for inclusion. Whenever a disagreement occurred, it was resolved by the study supervisor (AN). Data were sought regarding the changes in care experience before and after therapy with fixed orthodontic appliances. The authors extracted the values regarding caries severity and dental hygiene from the papers included to find the ones that were used in most of the studies and thus could be compared. The Cohen's K coefficient for the agreement between the authors in study selections indicates a high agreement between the authors, as it was equal to 0.98. Authorship, year of publication, type of each eligible study, and the main results regarding the caries occurrence were extracted by one author (SP) and examined by another author (MM). The data sought were different oral hygiene and caries prevalence indices which reflect changes that would promote the development of caries.

Quality assessment

According to the PRISMA statement,[11] the assessment of methodological quality indicates the strength of the evidence provided by the study, as methodological flaws can cause bias. The Newcastle-Ottawa Scale (NOS) has been used for quality assessments of cohort, case-control, and cross-sectional studies. Each study type has its own, specific criteria. This scale has 7 items and a maximum of 10 stars in cases involving cross-sectional

studies, and 9 in cases involving cohort studies can be distributed. In the cohort studies spreadsheet, the quality of the selection of groups under study (4 questions), comparability of the groups (1 question) and outcomes of interest (2 questions) are assessed. Up to 5 stars can be given to the selection section, a maximum of 2 for comparability, and 3 stars for the outcome of interest section.[13] In the cross-sectional studies spreadsheet, there are 3 main categories: selection (5 questions and 5 stars maximum), comparability (1 question and 2 stars maximum) and outcome (2 questions and 3 stars maximum).[14] The Jadad scale was used to assess the quality of RCTs. There are 5 questions, and the first 3 are characterized by a binary response (yes = 1 point; no = 0 points), and cover details on the randomization of subjects, description of patients and operator blinding, and description of the proportion of subjects lost to follow-up. One point will be subtracted or added from the total of the first 3 questions for each of these questions on the appropriateness of randomization and double-blinding. This procedure provides a total score ranging from 0 to 5, where 0 indicates a low-quality study and 5 indicates the highest possible quality. A study is considered good quality when it scores at least 3.[15]

Results

Search results

A systematic literature review was conducted for studies published from 2010 to 2022. The search strategy identified 808 potential articles from PubMed and Google Scholar. After the removal of duplicates, 538 articles were analyzed.

Afterward, 201 articles were excluded because they were not relevant to the topic, author debates, lack of effective statistical analysis, case reports, incomplete studies, reviews, and in vitro studies. Of the remaining 337 articles, 322 were excluded because they were not relevant to the full-text analysis. The remaining 15 articles were included in the review, and are represented in Table 1. Figure 1 presents the search strategy and the final number of studies included.

Extracted data

The data extracted from the included articles are presented as follows: authors, year, location, study setting, number of subjects studied, duration of therapy, and

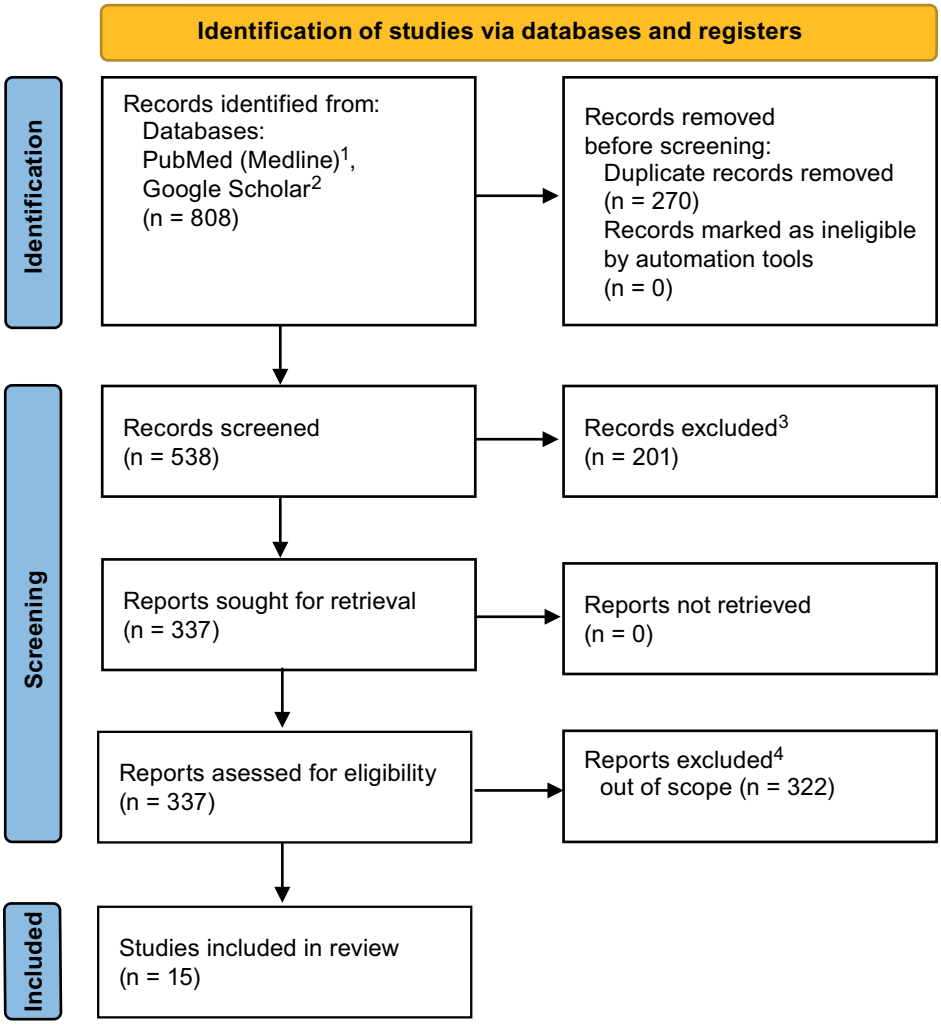

Fig. 1. Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 flow diagram

[1] search string "caries experience"[All Fields] AND "orthodontic treatment"[All Fields]
[2] search string: "caries" AND "caries risk" AND "caries experience" AND" "orthodontic treatment" OR "fixed appliance" "
[3] because not relevant to the subject, author's debates, lack of effective statistical analysis, case reports, incomplete studies, reviews, in-vitro studies
[4] because not relevant to the full-text analysis

indices used. Table 1 presents the summary of data provided from the included studies.

The included studies were published between 2010 and 2022 and were conducted in North America (n = 2), South America (n = 1), Asia (n = 7), Europe (n = 4), and Australia and Oceania (n = 1). Thirteen studies were conducted at universities, 3 in hospitals (1 both at a university and in a hospital), and 1 of them was partially conducted in private practice.

The total number of subjects examined was 23,943, with an average of 1,596 subjects per study. The total number of growing subjects was a clear minority of the total, n = 1,633 (6.8%). The total number of adult subjects was 22,310 (93.2%). The average age of the total number of subjects examined was 20.15. The average age of growing subjects was 13.37, while the average age of adult subjects was 33.7. The average duration of orthodontic therapy was approx. 23 months.

The indices used to detect caries were Decayed Teeth (DT), Filled Teeth (FT), Decayed Surfaces (DS), Filled Surfaces (FS), Decayed and Filled Teeth (DFT), Nyvad, Decayed, Missing and Filled Teeth (DMFT), Decayed and Filled Surfaces (DFS), Decayed, Missing and Filled Surfaces (DMFS), and White Spot Lesions (WSL) indices. In addition, periodontal indices such as gingival index (GI) and plaque index (PI) were also used. The main outcomes included in studies were mean DMFT in growing subjects before therapy, mean DMFT in growing subjects after therapy, mean DMFT in adult participants before therapy, and mean DMFT in adult subjects after therapy. In summary, the DMFT index was used on a total of 13,503 patients and in a total of 15 studies.

No specific information was provided regarding the type of brackets used, except for in the study conducted by Sanpei et al.[30]

Quality assessment

The evaluation of the RCT with the use of the Jadad scale is presented in Table 2. Moreover, the cohort and cross-sectional studies were evaluated with the use of the corresponding type of NOS, as presented in Table 3,4.

From the conducted risk of bias assessment, it could be stated that a RCT is of low quality, while 4 cohort studies are of high quality and 4 others of medium quality. All cross-sectional studies are of high quality.

Discussion

Influence of fixed orthodontic therapy on the prevalence of caries

This systematic review sought to comprehensively show the available evidence on caries risk during fixed orthodontic therapy in the growing and adult population.

In growing patients undergoing fixed orthodontic treatment, Cardoso et al.[17] showed that salivary composition plays an essential role in the development of active carious

Table 1. Summary of findings from included studies

Authors, year, reference	Study location	Study setting	Total number of subjects	Age range [years]	Type of orthodontic treatment	Observation time	Indices used	Results
Alsulaima and Brennann[16] 2021	all states in the USA	hospital setting	9,486	24–39	not specified	72 months	DT, FT, DFT	Patients who underwent orthodontic treatment before being characterized by lower values of all indices.
Cardoso et al.[17] 2020	Piracicaba (Brazil)	university setting	22	11–22	fixed orthodontic treatment	6 months	Nyvad	During follow-up, 59% of patients developed at least 1 white spot lesion, but no cavities. The concentration of Ca^{2+} ions in saliva changes during treatment. An increase in their concentration indicates an increased risk of white spot lesion.
Choi[18] 2020	Uijeongbu (South Korea)	university setting	11,732	>19	fixed orthodontic treatment	36 months	DMFT pre and post orthodontic treatment	DMFT pre: 7.27; DMFT post: 7.55 People who have had orthodontic treatment are less likely to have untreated dental caries than those who have not had orthodontic treatment.
Zabokova-Bilbilova et al.[19] 2020	Skopje (North Macedonia)	university setting	60	young adults (age not specified)	fixed orthodontic treatment	whole treatment time (not specified)	DMFT pre and post orthodontic treatment	DMFT pre: 6.45; DMFT post: 7.50 All patients experienced an increase in DMFT. The introduction of professional fluor prophylaxis did not cause significant changes in the increase of carious lesions.
Baeshen et al.[20] 2019	Gothenburg (Sweden)	university setting	171	11.2–17.3; mean: 14.8	fixed orthodontic treatment	no follow-up	DFS, SD, FS	FS and DS study group: 0.57 ±1.41; FS and DS control group: 0.65 ±1.38 Adolescents who suffered from different type of malocclusion have higher prevalence of carious lesions than the healthy control group.

Table 1. Summary of findings from included studies – cont.

Authors, year, reference	Study location	Study setting	Total number of subjects	Age range [years]	Type of orthodontic treatment	Observation time	Indices used	Results
Doğramaci[21] 2019	Adelaide (Australia)	university and hospital setting	632	not specified; mean: 30	fixed orthodontic treatment	12 months	DMFT pre and post orthodontic treatment	DMFT pre: 1.00; DMFT post: 1.12 Orthodontically treated participants had a lower DMFT but statistically insignificant.
Enerbäck et al.[22] 2019	Gothenburg (Sweden)	university setting	255	12–20; mean: 15.4	fixed orthodontic treatment	whole treatment time (not specified)	DMFT pre and post orthodontic treatment	DMFT: 0.89; DMFT post: 0.86 To reduce the likelihood of developing caries while undergoing orthodontic treatment, it is recommended to use toothpaste with a high fluoride content (5,000 ppm F) or a mouth rinse containing 0.2% NaF with regular toothpaste on a daily basis.
Almosa et al.[23] 2018	Riyadh (Saudi Arabia)	university setting and private clinic	40	mean: 26.4	fixed orthodontic treatment	48 months after the treatment	DMFS and PI	Mean DMFS increased during follow-up from 1.8 to 2.3. Both plaque index and the risk of caries were reduced steadily during the follow-up time.
Morgenstern et al.[24] 2018	Chapel Hill (USA)	university setting	10	13–15	fixed orthodontic treatment	3 months	GI, PI	Both indices during the first 3 months of treatment increased – GI PL.I. by several times.
Karabekiroğlu et al.[25] 2017	Konya (Turkey)	university setting	178	14–20	fixed orthodontic treatment	3 months	DMFT after finishing the orthodontic treatment	DMFT pre: 5.8; DMFT post: 7.4 The frequent use of CPP-ACP toothpaste did not improve the appearance of white spot lesions significantly better than regular toothpaste only.
Chen and Zhou[26] 2015	Wenzhou (China)	university setting	60	11–13	fixed orthodontic treatment	18 months	DMFS pre and post treatment	DMFS pre: 139; DMFS post: 158 for 3240 surfaces of treated group; DMFS pre: 149; DMFS post: 179 for 3240 surfaces of control group.
Lucchese and Gherlone[27] 2013	Milan (Italy)	hospital setting	191	8–12	fixed orthodontic treatment	13 ±0.9 months	WSL index	WSL index scored 28 in study group compared to 9 in control group. The scores did not differ significantly between 1st (6 months) and 2nd (12 months) check-up.
Borzabadi-Farahani et al.[28] 2011	Isfahan (Iran)	university setting	748	11–20; mean age: 15.11	fixed orthodontic treatment	whole treatment time (not specified)	DMFT <8 and >8	The formation of carious lesions is influenced by the size of the family (more than 6 members have a negative impact), as well as lower age.
Tufekci et al.[29] 2011	Richmond (USA)	university setting	100	>12; mean age: 17.4 ±1.3	fixed orthodontic treatment	6 months and 12 months	WSL index	In the group that was observed for 6 months, 38% of individuals had at least 1 visible white spot lesion. In the group that was observed for 12 months, this percentage was 46%. However, in the control group, it was only 11%. The groups that were observed for 6 and 12 months were significantly different from the control, but there was no significant difference between them.
Sanpei et al.[30] 2010	Niigata (Japan)	university setting	42	mean age: 8.8 ±0.92	fixed orthodontic treatment with sectional appliances	8 months	DMFT	DMFT pre: 4.53; DMFT post: 4.41 The concentration of ***Lactobacillus*** was found to be of special importance while monitoring the risk of caries in pediatric patients.

PI – plaque index; GI – gingival index; DT – Decayed Teeth; FT – Filled Teeth; DS – Decayed Surfaces; FS – Filled Surfaces; DFT – Decayed and Filled Teeth; DMFT – Decayed, Missing and Filled Teeth; DFS – Decayed and Filled Surfaces; DMFS – Decayed, Missing and Filled Surfaces; WSL – White Spot Lesions.

Table 2. randomized controlled trial (RCT) evaluation using the Jadad scale

Jadad scale for reporting randomized controlled trials	
Author	Enerbäck et al.[22]
1) Is the study described as randomized?	*
2) Is the study described as double blind?	–
3) Is there a description of withdrawals and dropouts?	1 point
4) Is the method of randomization appropriate?	1 point
5) Is the method of blinding appropriate?	–
Total score	2

lesions, and with proper composition monitoring, it is possible to accurately assess the risk of caries development. Moreover, results from the by Morgenstern et al.[24] showed that fixed orthodontic appliances could lead to dysbiosis, and those alterations in the oral microbiome are correlated with the development of caries. This relates directly to the results of the research by Lucchese et al.[27] and Tufekci et al.[29] Both of them found that fixed orthodontic appliances are a risk factor for plaque accumulation and the development of white spots. Both studies also proved that white spot lesions form primarily at the beginning of treatment (in the first 6 months), and their formation rate significantly decreases after this time. The distribution of white spot lesions is not equal, and a small percentage of patients in the study groups suffered from a large number of them on many teeth.

The study conducted by Enerbäck et al.[22] shows that the risk of caries during orthodontic treatment is significantly increased due to the increased number of cariogenic bacteria. However, the regular use of toothpaste with a high fluoride content (5000 ppm F) or a mouth rinse containing 0.2% NaF together with standard toothpaste (1450 ppm) on a daily basis significantly reduces the risk of caries development. What is more, a study by Zabokova-Bilbilova et al.[19] found that the introduction of professional fluoride-containing prophylaxis, as the only additional fluoride agent besides standard toothbrushing, did not cause a significant protective effect in subjected patients. Thus, professional application of fluoride, during follow-up visits only, does not protect the patient from lesion formation.

Another important factor is the type of orthodontic equipment applied by the clinician. Elastic ligatures showed a significantly lower susceptibility to plaque adhesion, in comparison to the stainless steel in the metallic ligatures.[31] Elastic ligatures, on the other hand, occupy more space around the bracket, therefore requiring the patient

Table 3. Evaluation of cohort studies

Newcastle-Ottawa quality assessment scale cohort studies		Authors							
		Alsulaiman and Brennann[16] 2021	Cardoso et al.[17] 2020	Doğramacı[21] 2019	Almosa et al.[23] 2018	Morgenstern et al.[24] 2018	Karabekiroğlu et al.[25] 2017	Chen and Zhou[26] 2015	Sanpei et al.[30] 2010
Selection: (max. 4 stars)	1) Representativeness of the exposed cohort	*	*	*	*	–	*	*	*
	2) Selection of the non-exposed cohort	*	*	*	*	–	*	*	*
	3) Ascertainment of exposure	*	*	*	*	*	*	*	*
	4) Demonstration that outcome of interest was not present at start of study	–	–	–	–	–	*	–	–
Comparability: (max. 2 stars)	5) Comparability of cohorts on the basis of the design or analysis	*	*	*	*	*	*	*	*
Outcome: (max. 3 stars)	6) Assessment of outcome**	*	*	*	*	*	*	*	*
	7) Was follow-up long enough for outcomes to occur	*	*	*	*	*	*	*	*
	8) Adequacy of follow up of cohorts	*	*	–	*	*	–	–	–
Total score		7	7	6	7	5	7	6	6

Table 4. Evaluation of cross-sectional studies

Newcastle-Ottawa Scale adapted for cross-sectional studies		Authors					
		Choi[18] 2020	Zabokova-Bilbilova et al.[19] 2020	Baeshen et al.[20] 2019	Lucchese and Gherlone[27] 2013	Borzabadi-Farahani et al.[28] 2011	Tufekci et al.[29] 2011
Selection (max. 5 stars)	1) Representativeness of the sample	*	*	*	*	*	*
	2) Sample size	*	*	*	*	*	*
	3) Non-respondents		*		*		*
	4) Ascertainment of the exposure (risk factor)	*	*	*	*	*	*
Comparability (max. 2 stars)	5) The subjects in different outcome groups are comparable, based on the study design or analysis. Confounding factors are controlled.	*	*	*	*	*	*
Outcome (max. 3 stars)	6) Assessment of the outcome**	**	**	**	**	**	**
	7) Statistical test	*	*	*	*	*	*
Total score		7	8	7	8	7	8

to cooperate reasonably and not avoid follow-up visits. For this reason, in a number of studies, greater amounts of microorganisms are found in patients with elastic ligatures than in those who have arches fixed with metal ligatures.[32] Therefore, it can be deduced that the type of ligature applied should be appropriately selected for patients according to their compliance. In addition, it is important to note that an orthodontic band would hold bacterial plaque more quickly than a bracket, which should also be taken into account when planning the placement of the appliance.[33]

Phenomena observed in growing patients

The clinical outcomes of the experience of caries in the growing patient are not unequivocal; in fact, Chen and Zhou[26] observed a decrease in interproximal caries in patients undergoing fixed orthodontic treatment. However, Sanpei et al.[30] did not find a significant change in DMFT during the treatment, but an accumulation of lactobacilli, which is considered a risk factor for caries. In both cases, there was a lack of analysis of potential confounding factors, such as the degree of crowding prior to orthodontic fixed treatment, or subjective factors, such as individual caries risk assessment on a genetic and exposomal basis. Therefore, it is not possible to draw a definitive conclusion on the relationship between fixed appliances and variation in caries experience in growing patients.

Phenomena observed in adult patients

In the study carried out by Choi,[18] it was shown that orthodontic treatment is considered a risk factor for dental caries since fixed orthodontic appliances increase the surface area on which plaque can adhere. Moreover, the irregular shape of the brackets makes it almost impossible to remove the plaque altogether. Thus, oral hygiene education during orthodontic treatment is vital to reduce the risk of caries. According to Doğramacı et al.,[21] orthodontic treatment is not considered a risk factor for dental caries in the long term. Furthermore, a relationship between DMFT and orthodontic treatment has not been found. The study by Alsulaiman and Brennann[16] found that patients who have already undergone orthodontic treatment have better hygiene habits, which reduces the risk of caries. What is more, it is easier to remove plaque with properly aligned teeth.

Limitations

From the risk of bias assessment, it can be stated that the results from the studies included are more than likely to translate into actual clinical conditions. The limitation of this study is the fact that there was a limited number of RCTs and time restriction for article search. The other problem is the fact that factors that appear to be most important – patient cooperation and hygiene – are purely behavioral. Therefore, it should be underlined that the patients included in this study may behave differently than the ones clinicians are cooperating with on an everyday basis.

Summary

The studies included convey a distinct message. In most studies in which DMFT was used to assess caries risk, there were no significant differences between the value

obtained before orthodontic treatment and the post-treatment value. Although orthodontic therapy with fixed appliances is commonly associated with the incidence of caries, the crucial factor is proper dental hygiene, which, when adequately maintained by the patient, can exclude the formation of any carious lesions during that time. Doctors and hygienists must constantly support, remind, and encourage proper hygiene practices so that the patient gets through the orthodontic treatment unaffected. From the recent study on the hygiene status of patients undergoing orthodontic treatment, it is known that this applies to all kinds of orthodontic therapy and removable appliances, e.g., aligners.[34]

Conclusions

Based on the results of this systematic review, it can be concluded that several factors can increase the risk of caries during fixed orthodontic treatment, such as salivary composition, oral dysbiosis and plaque accumulation. It was not possible to establish a direct correlation between the fixed multi-bracket appliance and caries experience in the developmental patient, whereas this is confirmed in the adult patient. Therefore, both age groups need to be addressed to reduce the causative factors of caries risk during treatment, with the benefit of making patients more aware of the importance of biofilm removal, even after the conclusion of fixed therapy.

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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References

1. Kazeminia M, Abdi A, Shohaimi S, et al. Dental caries in primary and permanent teeth in children's worldwide, 1995 to 2019: A systematic review and meta-analysis. *Head Face Med*. 2020;16(1):22. doi:10.1186/s13005-020-00237-z
2. Uerlich MF, Baker SR, Day PF, Brown L, Vettore MV. Common determinants of dental caries and obesity in children: A multi-ethnic nested birth cohort study in the United Kingdom. *Int J Environ Res Public Health*. 2021;18(23):12561. doi:10.3390/ijerph182312561
3. Sabharwal A, Stellrecht E, Scannapieco FA. Associations between dental caries and systemic diseases: A scoping review. *BMC Oral Health*. 2021;21(1):472. doi:10.1186/s12903-021-01803-w
4. Guerra F, Mazur M, Corridore D, Pasqualotto D, Nardi GM, Ottolenghi L. Evaluation of the esthetic properties of developmental defects of enamel: A spectrophotometric clinical study. *ScientificWorldJournal*. 2015;2015:878235. doi:10.1155/2015/878235
5. Raji AlRwuili M, Jamal Alwaznah F, Ahmed R, Anwar S, Shaikh Omar FA, Hadi Tairan E. A detailed correlation of oral-health-related quality of life of patients undergoing fixed orthodontic therapy. *Cureus*. 2023;15(1):e33854. doi:10.7759/cureus.33854
6. Sarul M, Antoszewska-Smith J, Park HS. Self-perception of smile attractiveness as a reliable predictor of increased patient compliance with an orthodontist. *Adv Clin Exp Med*. 2019;28(12):1633–1638. doi:10.17219/acem/110320
7. Marincak Vrankova Z, Rousi M, Cvanova M, et al. Effect of fixed orthodontic appliances on gingival status and oral microbiota: A pilot study. *BMC Oral Health*. 2022;22(1):455. doi:10.1186/s12903-022-02511-9
8. Mazur M, Westland S, Ndokaj A, Nardi GM, Guerra F, Ottolenghi L. In-vivo colour stability of enamel after ICON® treatment at 6 years of follow-up: A prospective single center study. *J Dent*. 2022;122:103943. doi:10.1016/j.jdent.2021.103943
9. Cruz CL, Edelstein BL. Linking orthodontic treatment and caries management for high-risk adolescents. *Am J Orthod Dentofacial Orthop*. 2016;149(4):441–442. doi:10.1016/j.ajodo.2015.12.007
10. Beller EM, Glasziou PP, Altman DG, et al. PRISMA for Abstracts: Reporting systematic reviews in journal and conference abstracts. *PLoS Med*. 2013;10(4):e1001419. doi:10.1371/journal.pmed.1001419
11. Rethlefsen ML, Kirtley S, Waffenschmidt S, et al. PRISMA-S: An extension to the PRISMA Statement for Reporting Literature Searches in Systematic Reviews. *Syst Rev*. 2021;10(1):39. doi:10.1186/s13643-020-01542-z
12. Straus SE, Glasziou P, Richardson WS, Haynes RB, eds. *Evidence-Based Medicine: How to Practice and Teach EBM*. 5th ed. Amsterdam, the Netherlands-New York, USA: Elsevier; 2019. ISBN:978-0-7020-6296-4.
13. Wells G, Shea B, O'Connell D, et al. The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses. Ottawa, Canada: Ottawa Hospital Research Institute; 2014. https://www.ohri.ca/programs/clinical_epidemiology/oxford.asp. Accessed August 27, 2023.
14. Modesti PA, Reboldi G, Cappuccio FP, et al. Panethnic differences in blood pressure in Europe: A systematic review and meta-analysis. *PLoS One*. 2016;11(1):e0147601. doi:10.1371/journal.pone.0147601
15. Jadad AR, Enkin M. *Randomized Controlled Trials: Questions, Answers, And Musings*. 2nd ed. Malden, USA: Blackwell Publishing; 2007. ISBN:978-1-4051-3266-4.
16. Alsulaiman AA. Orthodontic treatment as a protective factor for dental caries experience and severity: A population-based study. *Int J Dent*. 2021;2021:9926069. doi:10.1155/2021/9926069
17. Cardoso A, De Sousa E, Steiner-Oliveira C, Parisotto T, Nobre-dos-Santos M. A high salivary calcium concentration is a protective factor for caries development during orthodontic treatment. *J Clin Exp Dent*. 2020;12(3):e209–e214. doi:10.4317/jced.56331
18. Choi YY. Relationship between orthodontic treatment and dental caries: Results from a national survey. *Int Dent J*. 2019;70(1):38–44. doi:10.1111/idj.12515
19. Zabokova-Bilbilova E, Sefanovska E, Mijoska A, Kokoceva-Ivanovska O. Effect of fixed-appliance orthodontic treatment on DMFT-index. *Maced Pharm Bull*. 2021;66(2):81–86. doi:10.33320/maced.pharm.bull.2020.66.02.008
20. Baeshen H, Rangmar S, Kjellberg H, Birkhed D. Dental caries and risk factors in Swedish adolescents about to start orthodontic treatment with fixed appliances. *J Contemp Dent Pract*. 2019;20(5):537–542. doi:10.5005/jp-journals-10024-2553
21. Doğramacı EJ, Brennan DS. The influence of orthodontic treatment on dental caries: An Australian cohort study. *Community Dent Oral Epidemiol*. 2019;47(3):210–216. doi:10.1111/cdoe.12446
22. Enerbäck H, Möller M, Nylén C, Ödman Bresin C, Östman Ros I, Westerlund A. Effects of orthodontic treatment and different fluoride regimens on numbers of cariogenic bacteria and caries risk: A randomized controlled trial. *Eur J Orthod*. 2019;41(1):59–66. doi:10.1093/ejo/cjy025
23. Almosa NA, Lundgren T, Al-Mulla A, Birkhed D, Kjellberg H. Caries risk profiles in orthodontic patients: A 4-year follow-up study using the Cariogram model in governmental vs. private clinics. *Saudi Dent J*. 2018;30(2):166–174. doi:10.1016/j.sdentj.2018.02.001

24. Morgenstern A. Microbiome Shifts in the Supragingival Biofilm in Patients Undergoing Orthodontic Treatment with Fixed Appliances: A Pilot Study. Chapel Hill, USA: University of North Carolina at Chapel Hill; 2018. https://cdr.lib.unc.edu/concern/dissertations/2f75r919r?locale=en. Accessed June 30, 2022.
25. Karabekiroğlu S, Ünlü N, Küçükyilmaz E, Şener S, Botsali MS, Malkoç S. Treatment of post-orthodontic white spot lesions with CPP-ACP paste: A three year follow up study. *Dent Mater J*. 2017;36(6):791–797. doi:10.4012/dmj.2016-228
26. Chen W, Zhou Y. Caries outcomes after orthodontic treatment with fixed appliances: A longitudinal prospective study. *Int J Clin Exp Med*. 2015;8(2):2815–2822. PMID:25932240. PMCID:PMC4402887.
27. Lucchese A, Gherlone E. Prevalence of white-spot lesions before and during orthodontic treatment with fixed appliances. *Eur J Orthod*. 2013;35(5):664–668. doi:10.1093/ejo/cjs070
28. Borzabadi-Farahani A, Eslamipour F, Asgari I. Association between orthodontic treatment need and caries experience. *Acta Odontol Scand*. 2010;69(1):2–11. doi:10.3109/00016357.2010.516732
29. Tufekci E, Dixon JS, Gunsolley JC, Lindauer SJ. Prevalence of white spot lesions during orthodontic treatment with fixed appliances. *Angle Orthod*. 2011;81(2):206–210. doi:10.2319/051710-262.1
30. Sanpei S, Endo T, Shimooka S. Caries risk factors in children under treatment with sectional brackets. *Angle Orthod*. 2010;80(3):509–514. doi:10.2319/072909-431.1
31. Condò R, Casaglia A, Condò SG, Cerroni L. Plaque retention on elastomeric ligatures. An in vivo study. *Oral Implantol (Rome)*. 2013;5(4):92–99. PMID:23741603. PMCID:PMC3671820.
32. Akgun OM, Altug H, Karacay S, Guven Polat G, Duyan S, Bedir O. Effect of 2 elastomeric ligatures on microbial flora and periodontal status in orthodontic patients. *Am J Orthod Dentofacial Orthop*. 2014;145(5):667–671. doi:10.1016/j.ajodo.2014.01.018
33. Opsahl Vital S, Haignere-Rubinstein C, Lasfargues JJ, Chaussain C. Caries risk and orthodontic treatment. *Int Orthod*. 2010;8(1):28–45. doi:10.1016/j.ortho.2009.12.003
34. Chhibber A, Agarwal S, Yadav S, Kuo CL, Upadhyay M. Which orthodontic appliance is best for oral hygiene? A randomized clinical trial. *Am J Orthod Dentofacial Orthop*. 2018;153(2):175–183. doi:10.1016/j.ajodo.2017.10.009

Chronic acid sphingomyelinase deficiency diagnosed in infancy/childhood in Polish patients: 2024 update

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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. Acid sphingomyelinase deficiency (ASMD) is an autosomal recessive lysosomal storage disease (LSD) associated with biallelic pathogenic variants in the sphingomyelin phosphodiesterase 1 (*SMPD1*) gene.

Objectives. The aim of this study was to provide the 2024 update on chronic visceral and neurovisceral ASMD diagnosed in the infancy/childhood in Polish patients.

Materials and methods. All the patients diagnosed in the pediatric age (0–18 years) with ASMD, both chronic neurovisceral and visceral type, and then systematically followed up, were enrolled into the study.

Results. A total number of 7 patients were enrolled into the study. Four patients were previously reported. Two patients were newly recognized with ASMD – 1 with chronic visceral and 1 with chronic neurovisceral ASMD. Splenomegaly was noted in all the patients while a mild liver enlargement was observed in 4 of 7 patients. All patients presented with decreased high-density lipoprotein cholesterol (HDL-C) and decreased serum 25-hydroxy-vitamin D concentration while almost all (6 of 7) with hypercholesterolemia. Cherry-red spot was observed in 5 of 7 patients, including 1 patient with neurovisceral type. Seven various *SMPD1* gene variants were identified and missense variants were the most common types of genetic lesions, comprising 71% of all alleles. In all the screened patients, lyso-sphingomyelin (lyso-SM) in dried blood spot (DBS) was found elevated; however, the greater values were observed for patients with chronic neurovisceral type.

Conclusions. Chronic acid sphingomyelinase deficiency (ASMD) is a slowly progressive disease. Pediatric ASMD is characterized by spleno-hepatomegaly, dyslipidemia (with decreased HDL-C as the most characteristic) and infiltrative (interstitial) lung disease. Both visceral and neurovisceral chronic ASMD patients could present with cherry-red spot. Both acid spingomyelinase activity and lyso-spingomyelin concentration in DBS should be regarded as a first-tier screening method into ASMD.

Key words: children, lysosomal storage disease, acid sphingomyelinase deficiency, lyso-spingomyelin, dried blood spot

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Background

Acid sphingomyelinase deficiency (ASMD), due to biallelic pathogenic variants in the sphingomyelin phosphodiesterase 1 (*SMPD1*) gene (MIM *607608), is an autosomal recessive lysosomal storage disease (LSD) associated with accumulation of lysosomal sphingomyelin.[1]

The original Niemann–Pick type A (MIM # 257200) disease is currently classified as the infantile neurovisceral ASMD, while Niemann–Pick type B (# 607616) disease is referred to as the chronic visceral type, and Niemann–Pick type A/B as the chronic neurovisceral type.[2,3] Infantile neurovisceral ASMD constitutes a neuronopathic (neurodegenerative), rapidly progressing, and fatal disorder while chronic visceral ASMD is a non-neuronopathic, slowly progressive and visceral disorder.[2,3] Infants with neurovisceral ASMD typically present with delayed psychomotor development followed by its regression (clinically noted in the 2nd 6 months of life). Neurological features are accompanied by massive hepatosplenomegaly, failure to thrive, and recurrent respiratory tract infections. Patients with visceral ASMD do not present neurological features, but the somatic phenotype is very heterogeneous. The most common signs and symptoms include hepatosplenomegaly (sometimes associated with thrombocytopenia), elevated serum transaminases (mildly-to-moderately), dyslipidemia, and interstitial lung disease (based on radiological features). An intermediate neurological phenotype (slowly progressive neurological disease) with somatic (visceral) manifestations similar to type B, underlines the chronic neurovisceral ASMD.[2–4] In this group of ASMD patients, the onset of neurological symptoms occurs later in life than in patients with infantile neurovisceral form, but is usually noted in childhood. The most commonly reported symptoms include a mild hypotonia and/or hyporeflexia.

In December 2020, enzyme replacement therapy (ERT) with recombinant human ASM (olipudase alfa) was approved by the European Medicines Agency (EMA) for the treatment of the non-neurological manifestations of ASMD.[5] Enzyme replacement therapy has become available in Poland since April 2024 (as part of a drug program financed by the National Health Fund). The eligibility criteria for children with ASMD in the drug program, in addition to those included in the EMA registration, include the need for a spleen volume that is at least 5 times the normal volume as measured by a magnetic resonance imaging (MRI) scan.

Objectives

In 2018, we published a single-center study comprising 16 patients (both children and adults) with chronic visceral ASMD (formerly known as Niemann–Pick type B) who were diagnosed and followed up at the Children's Memorial Health Institute (Warsaw, Poland).[6] Since then, several novel patients, including 2 children, have been diagnosed. In April 2024, an enzyme replacement therapy with olipudase alfa became available for patients with ASMD in Poland.

The aim of this study was to provide the 2024 update on chronic visceral and neurovisceral ASMD diagnosed in the infancy/childhood in Polish patients.

Patients and methods

Study design, setting and participants

All the patients diagnosed in the pediatric age (0–18 years) with ASMD, both chronic neurovisceral and visceral type, and then systematically followed up, were enrolled into the study. A retrospective chart review of the patients' medical records was performed.

Variables: ASM activity, *SMPD1* gene sequencing and biomarkers

Acid sphingomyelinase deficiency was diagnosed through the demonstration of reduced ASM activity in peripheral blood leukocytes or dried blood spot (DBS) and confirmed by identification of *SMPD1* pathogenic variants. Acid sphingomyelinase activity in leukocytes was measured with the 2-N-hexadecanoylamino-4-nitrophenylphosphorylcholine as a substrate.[7] Chitotriosidase (ChT) activity was measured in plasma samples using a spectrofluorometric method as presented by Hollak et al.[8] Dried blood spot tests were performed as suggested by the producer (ARCHIMED Life Science GmbH, Vienna, Austria). ARCHIMEDlife laboratory has been certified with ISO 15189 (Medical Laboratory* – Clinical Chemistry to Genetics), ISO 9000 (Quality Management System), ISO 13485 (Medical Devices – IVD Development and Production) and GLP-lab certificate fully integrated for clinical studies. According to the laboratory information, the sample was analyzed as previously described.[9]

Results

A total number of 7 patients (3 men and 4 women) were enrolled into the study. The characteristics of individual patients are presented in Table 1. Four patients were previously reported.[6] Two patients were newly recognized with ASMD – 1 with chronic visceral type (Patient (Pt) 3) and 1 with chronic neurovisceral type (Pt 2). The last patient (Pt 6) was not previously (2018 year) reported due to a diagnosis of chronic neurovisceral type of ASMD.

Three patients from 1 family were of Romani descent (currently living in foster care) and were diagnosed through family screening (at 12 months, 1.5 years and 5 years of age,

Table 1. Clinical, biochemical and molecular characteristics of the study patients

Patient No.	Genotype	Age [years]	Lyso-SPM [ng/mL] <70	ChT [nmol/mL/h] <150	Liver length in midclavicular line [mm]	Spleen longitudinal length [mm]	Platelets [1000/μL] <150	AST [IU/L] <45	ALT [IU/L] <40	TC [mg/dL] <200	LDL-C [mg/dL] <115	HDL-C [mg/dL] 32–63	TG [mg/dL] 44–197	25-OH-D [ng/mL] >30
1	Hmz; c.880C>A, p.Gln294Lys	5	490.8	620	100	110	161	48	18	175	123	27	125	24
2	c.748A>C, p.Ser250Arg/ c.1092-1G>C, p.?	1	294.9	960	80	110	121	212	160	235	149	19	331	24
		2	813.5	1080	125	165	118	130	150	210	125	17	288	15
3	Hmz; c.1177T>G, p.Trp393Gly	1	n.a.	36	50	85	255	160	138	220	130	20	350	n.a.
		3	n.a.	2048	110	130	300	49	46	208	140	21	230	17
		5	279	1540	130	180	236	44	32	213	156	20	182	22
4	Hmz; c.1177T>G, p.Trp393Gly	1.5	n.a.	n.a.	110	90	330	1,200	580	186	120	9	300	36
		3	n.a.	488	120	115	230	470	370	206	138	18	250	24
		8	367	360	150	210	153	46	30	219	158	17	220	9
5	Hmz; c.1177T>G, p.Trp393Gly	5	n.a.	296	100	120	256	77	65	243	145	45	270	12
		13	303	n.a.	160	150	259	95	25	220	160	39	106	18
		15	378	n.a.	190	180	238	37	23	192	120	34	191	10
6	c.880C>A, p.Gln294Lys/ c.1758_1786del, p.Ala597Profs*7	2.5	n.a.	2000	135	165	191	57	17	121	93	13	75	21
		6	n.a.	3500	150	190	162	64	35	128	87	18	113	21
		10	756	n.a.	160	200	97	42	39	150	90	17	124	17
7	c.581dup, p.Ala195Serfs*14/ c.1829_1831del, p.Arg610del	3	n.a.	n.a.	10 cm below costal margin	5 cm below costal margin	222	38	20	253	n.a.	n.a.	242	n.a.
		13	n.a.	n.a.	8 cm below costal margin	10 cm below costal margin	240	35	30	270	n.a.	n.a.	220	n.a.
		20	n.a.	908	145	165	180	20	22	n.a.	186	n.a.	162	n.a.
		35	n.a.	n.a.	150	180	138	25	30	235	205	20	195	n.a.
		45	309	n.a.	180	180	127	35	30	278	194	25	298	13.4

n.a. – not analyzed; hmz – homozygote; ChT – chitotriosidase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; TC – total cholesterol; TG – triglicerides.

respectively) – 2 older brothers had died (the primary cause of death was not related to ASMD).[6] Four other patients were of Polish origin and were diagnosed at 12 months, 2.5 years, 5 years, and 3 years of age, respectively. Six of the 7 patients underwent routine clinical evaluation. Follow-up of the study patients ranged from 1 to 42 years.

On the basis on clinical and molecular characteristics, the chronic visceral type of ASMD was diagnosed in 5 patients, while 2 others (Pt 2 and Pt 6) were diagnosed with chronic neurovisceral ASMD. Patient 2 presented with a slightly delayed psychomotor development and based on genetic results – 2 *SMPD1* variants (in-trans): c.748A>C (p.Ser250Arg) and c. c.1092-1G>C associated with Niemann–Pick type A disease (Table 2). Patient 6 presented with psychomotor regression since the age of 3 years and developed epilepsy at the age of 6–7 years. He was found to be a homozygote for c.880C>A (p.Gln294Lys) variant associated with the intermediate type.

Splenomegaly was noted in all the patients, while a mild liver enlargement was observed in 4 of 7 patients at the time of ASMD diagnosis. A gradual enlargement of both organs, spleen and liver, was observed during clinical monitoring (Table 1). Elevated serum aspartate aminotransferase (AST) was observed in 6 of 7 patients while elevated serum alanine aminotransferase (ALT) was observed in 3 of 7 patients at the time of diagnosis. The value of serum transaminases was found to be several-fold above the upper limit of normal values (Table 1). A decrease (to normalization) of serum transaminases was observed during follow-up.

Only 1 patient (Pt 2 with chronic neurovisceral type) presented with thrombocytopenia (defined as platelets below 150,000/μL) at the time of diagnosis. Two other patients (Pt 6 with chronic neurovisceral type and Pt 7 with chronic visceral type) developed thrombocytopenia at the age of 10 years and 35 years, respectively.

Table 2. *SMPD1* variants in the study group according to ClinVar

SMPD1 variant	Molecular consequence	Protein change	Condition
c.1177T>G	missense variant	p.Trp393Gly	Niemann–Pick disease, type B
c.880C>A	missense variant	p.Gln294Lys	Niemann–Pick disease, intermediate, protracted neurovisceral
c.1092-1G>C	splice acceptor	Non applicable	Niemann–Pick disease, type A
c.1785_1786del	frameshift variant	p.Ala597fs	Niemann–Pick disease, type A
c.748A>C	missense variant	p.Ser250Arg	Niemann–Pick disease, type A
c.581dup	frameshift variant	p.Ala195Serfs*14	Niemann–Pick disease, type A; Niemann–Pick disease, type B
c.1829_1831del	inframe deletion	p.Arg610del	Niemann–Pick disease, type B

Elevated total serum cholesterol and LDL cholesterol as well as triglycerides were noted in 6 of 7 patients, while decreased HDL cholesterol was observed in all patients. Decreased serum 25-hydroxyvitamin D concentrations were also observed in all patients (Table 1). Cherry-red spot was described in 5 of 7 patients at the time of ASMD diagnosis, including 1 patient with neurovisceral type.

All patients were diagnosed with interstitial lung disease by chest X-ray or computed tomography (CT). Except for frequent respiratory tract infections in infancy/early childhood reported in all patients, no clinical consequences (normal spirometry in all 3 diagnosed patients) of pulmonary macrophage involvement in childhood/adolescence were observed. The last patient (Pt 7), who was followed for almost 42 years, was diagnosed with restrictive lung disease at 20 years of age.

Data on ASM activity were available for all (7) patients (5 in peripheral blood leukocytes and 2 in DBS). In case of 1 patient (Pt 6), ASM activity in leukocytes was found normal; however, due to strong clinical suspicion (and excluding Gaucher disease; GD), the final diagnosis of ASMD was established by molecular analysis of the *SMPD1* gene. This patient was found to be heterozygous for Q292K *SMPD1* variant (and deletion on the 2nd allele). Seven various *SMPD1* gene variants (Table 2) were identified in the study group, and missense variants were the most common types of genetic lesions, comprising 71% of all alleles.

At the time of diagnosis, the lyso-sphingomyelin (lyso-SM) value in DBS was available only for 2 patients, while 6 patients underwent follow-up assessment (Table 1). In all patients with ASMD, lyso-SM was found elevated, however, the greater values were observed in patients with chronic neurovisceral type.

Chitotriosidase activity in serum was available for all 6 patients, however, in 4 of them subsequent analyses were performed. It decreased in 2 of them and increased in 2 others (neurovisceral type).

Discussion

The paper described the 2024 update on ASMD in Poland from pediatric perspective. It provides a clinical, biochemical and molecular characteristics of 7 Polish patients with chronic visceral and neurovisceral ASMD who were diagnosed in the infancy/childhood and systematically followed up.

With the growing awareness of rare diseases, including inherited metabolic diseases (IMD), and the greater availability of diagnostic methods, including routine assessment of lyso-spingolipids as biomarkers in LSD, but most importantly next-generation sequencing (NGS) technology, new patients with known diseases and novel diseases are being discovered.[10] Only last year (2023), 2 pediatric patients with chronic ASMD were diagnosed in Poland. This is of great importance in the context of enzyme replacement therapy that has only recently become available in Poland.

The clinical and biochemical features of the presented cohort of pediatric patients with ASMD are consistent with those reported in the literature. Children with ASMD typically present with hepatosplenomegaly accompanied by elevated serum transaminases, dyslipidemia and radiological features of interstitial lung disease.

Based on the data presented and those from the literature, we recommend that all children with splenomegaly (with or without hepatomegaly) of unknown cause should undergo ASM analysis. Thrombocytopenia was not a frequent abnormality in chronic ASMD as opposed to GD, in which it is usually found in almost all patients.[11] The characteristic feature of pediatric patients with ASMD, especially in infancy and early childhood, is elevated serum transaminases activity, with a tendency to normalize in older children. The exact cause of normalization of serum transaminases is not known; however, progression to hepatic fibrosis is observed in the natural history of liver disease in ASMD.[12] Dyslipidemia was also found to be a unique biochemical feature of chronic ASMD; however, the similar pattern is observed in lysosomal acid lipase

deficiency.[11,13] A quite interesting abnormality was a decreased 25-hydroxy-vitamin D level found in all patients with ASMD. It is a well-known phenomenon observed in GD patients (and other LSD), with no exact explanation.[14] Radiographic evidence of infiltrative (interstitial) lung disease was noted in all children with chronic ASMD. Besides frequent respiratory tract infections in early childhood, no other clinical symptoms of pulmonary disease were observed in childhood/adolescence. However, based on the long-term follow-up of 1 study patient (child), there is a risk for restrictive lung disease development in adulthood.

The presence of macular cherry-red spot was noted in both visceral and neurovisceral chronic ASMD patients. This observation is noteworthy since it was believed that the presence of cherry-red spot is associated with a neurological phenotype.[15]

The gold standard in the diagnosis of ASMD was (and still is) a method based on measuring acid sphingomyelinase (ASM) activity in peripheral blood leukocytes followed by *SMPD1* gene sequencing. Recently, a DBS test is in common use, utilizing the same diagnostic protocol with addition of biomarkers (lyso-sphingolipids) assessment.[9] In 2010, lyso-glucosylceramide (lyso-Gb1), a newly introduced biomarker, was expected to provide breakthrough into GD field.[16] An alternative metabolic pathway favored in state of β-glucocerebrosidase deficiency has been identified, where loads of Gb1 undergo deacylation due to an acid ceramidase activity, producing glucosylsphingosine (lyso-Gb1). The quantification of a de-acylated form of sphingomyelin (lyso-SM) and its carboxylated counterpart (lyso-SM-509) has been shown extremely useful in the diagnosis and monitoring of patients with ASMD and Niemann–Pick disease type C (NPC).[17–19] The combined determination of both, lyso-SM and lyso-SM509 allowed the discrimination of ASMD from NPC, as ASMD patients having elevated levels mainly of lyso-SM, while both ASMD and NPC patients exhibit elevated levels of lysoSM-509.[20] However, some adults with attenuated chronic ASMD could present with normal lyso-SM but elevated lyso-SM509 (personal observation). Thus, both these biomarkers should be routinely used as a screening method for ASMD. Unfortunately, lyso-SM509 analysis is not available in Poland, while lyso-SM analysis has been used routinely for about 1 year. Despite the small number of patients and multiple assessments of lyso-SM, there is a noticeable difference between chronic visceral and neurovisceral ASMD patients, with the latter having higher lyso-SM levels.

Increased levels of ChT activity have been reported in several LSD, especially high for GD, due to macrophage activation.[21–23] Mildly elevated ChT activity could be observed in chronic ASMD patients, as presented in our study. This biomarker should not be forgotten due to novel biomarkers development, especially lyso-SM.

The method of collecting and assessing ChT activity is relatively simple and remained the same for over 20 years, which guarantees comparability of results between years.[23] For these reasons, it is justly considered as a useful tool for clinical practice and management of GD patients and probably for ASMD.

Limitations

The study was limited by its retrospective nature and a relatively small number of patients, but given the rarity of ASMD, the paper is important in the field of inherited metabolic diseases.

Conclusions

1. Chronic ASMD is a slowly progressive disease.
2. Pediatric ASMD is characterized by spleno-hepatomegaly, dyslipidemia (with decreased HDL cholesterol as the most characteristic) and infiltrative (interstitial) lung disease. Both visceral and neurovisceral chronic ASMD patients may present with cherry-red spot.
3. Both acid spingomyelinase activity and lyso-SM concentration in DBS should be regarded as a first-tier screening method for ASMD.

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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References

1. Schuchman EH, Desnick RJ. Types A and B Niemann-Pick disease. *Mol Genet Metab*. 2017;120(1–2):27–33. doi:10.1016/j.ymgme.2016.12.008
2. Geberhiwot T, Wasserstein M, Wanninayake S, et al. Consensus clinical management guidelines for acid sphingomyelinase deficiency (Niemann–Pick disease types A, B and A/B). *Orphanet J Rare Dis*. 2023;18(1):85. doi:10.1186/s13023-023-02686-6
3. McGovern MM, Avetisyan R, Sanson BJ, Lidove O. Disease manifestations and burden of illness in patients with acid sphingomyelinase deficiency (ASMD). *Orphanet J Rare Dis*. 2017;12(1):41. doi:10.1186/s13023-017-0572-x
4. Pavlů-Pereira H, Asfaw B, Poupčtová H, et al. Acid sphingomyelinase deficiency. Phenotype variability with prevalence of intermediate phenotype in a series of twenty-five Czech and Slovak patients: A multi-approach study. *J Inherit Metab Dis*. 2005;28(2):203–227. doi:10.1007/s10545-005-5671-5
5. Jones SA, McGovern M, Lidove O, et al. Clinical relevance of endpoints in clinical trials for acid sphingomyelinase deficiency enzyme replacement therapy. *Mol Genet Metab*. 2020;131(1–2):116–123. doi:10.1016/j.ymgme.2020.06.008

6. Lipiński P, Kuchar L, Zakharova EY, Baydakova GV, Ługowska A, Tylki-Szymańska A. Chronic visceral acid sphingomyelinase deficiency (Niemann–Pick disease type B) in 16 Polish patients: Long-term follow-up. *Orphanet J Rare Dis*. 2019;14(1):55. doi:10.1186/s13023-019-1029-1
7. Gal AE, Fash FJ. Synthesis of 2-N-(Hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide, a chromogenic substrate for assaying sphingomyelinase activity. *Chem Phys Lipids*. 1976;16(1):71–79. doi:10.1016/0009-3084(76)90015-3
8. Hollak CE, Van Weely S, Van Oers MH, Aerts JM. Marked elevation of plasma chitotriosidase activity: A novel hallmark of Gaucher disease. *J Clin Invest*. 1994;93(3):1288–1292. doi:10.1172/JCI117084
9. Verma J, Thomas DC, Kasper DC, et al. Inherited metabolic disorders: Efficacy of enzyme assays on dried blood spots for the diagnosis of lysosomal storage disorders. *JMID Rep*. 2016;31:15–27. doi:10.1007/8904_2016_548
10. Ferreira CR, Rahman S, Keller M, Zschocke J; ICIMD Advisory Group. An International Classification of Inherited Metabolic Disorders (ICIMD). *J Inherit Metab Dis*. 2021;44(1):164–177. doi:10.1002/jimd.12348
11. Lipiński P, Tylki-Szymańska A. The liver and lysosomal storage diseases: From pathophysiology to clinical presentation, diagnostics, and treatment. *Diagnostics (Basel)*. 2024;14(12):1299. doi:10.3390/diagnostics14121299
12. Wasserstein M, Dionisi-Vici C, Giugliani R, et al. Recommendations for clinical monitoring of patients with acid sphingomyelinase deficiency (ASMD). *Mol Genet Metab*. 2019;126(2):98–105. doi:10.1016/j.ymgme.2018.11.014
13. Lipiński P, Ługowska A, Zakharova EY, Socha P, Tylki-Szymańska A. Diagnostic algorithm for cholesteryl ester storage disease: Clinical presentation in 19 Polish patients. *J Pediatr Gastroenterol Nutr*. 2018;67(4):452–457. doi:10.1097/MPG.0000000000002084
14. Stirnemann J, Belmatoug N, Camou F, et al. A review of Gaucher disease pathophysiology, clinical presentation and treatments. *Int J Mol Sci*. 2017;18(2):441. doi:10.3390/ijms18020441
15. Chen H, Chan AY, Stone DU, Mandal NA. Beyond the cherry-red spot: Ocular manifestations of sphingolipid-mediated neurodegenerative and inflammatory disorders. *Surv Ophthalmol*. 2014;59(1):64–76. doi:10.1016/j.survophthal.2013.02.005
16. Daykin EC, Ryan E, Sidransky E. Diagnosing neuronopathic Gaucher disease: New considerations and challenges in assigning Gaucher phenotypes. *Mol Genet Metab*. 2021;132(2):49–58. doi:10.1016/j.ymgme.2021.01.002
17. Polo G, Burlina AP, Kolamunnage TB, et al. Diagnosis of sphingolipidoses: A new simultaneous measurement of lysosphingolipids by LC-MS/MS. *Clin Chem Lab Med*. 2017;55(3):403–414. doi:10.1515/cclm-2016-0340
18. Piraud M, Pettazzoni M, Lavoie P, et al. Contribution of tandem mass spectrometry to the diagnosis of lysosomal storage disorders. *J Inherit Metab Dis*. 2018;41(3):457–477. doi:10.1007/s10545-017-0126-3
19. Voorink-Moret M, Goorden SMI, Van Kuilenburg ABP, et al. Rapid screening for lipid storage disorders using biochemical markers: Expert center data and review of the literature. *Mol Genet Metab*. 2018;123(2):76–84. doi:10.1016/j.ymgme.2017.12.431
20. Kubaski F, Burlina A, Pereira D, et al. Quantification of lysosphingomyelin and lysosphingomyelin-509 for the screening of acid sphingomyelinase deficiency. *Orphanet J Rare Dis*. 2022;17(1):407. doi:10.1186/s13023-022-02560-x
21. Kadali S, Kolusu A, Sunkara S, Gummadi MR, Undamatla J. Clinical evaluation of chitotriosidase enzyme activity in Gaucher and Niemann–Pick A/B diseases: A retrospective study from India. *Clin Chim Acta*. 2016;457:8–11. doi:10.1016/j.cca.2016.03.004
22. Szymańska-Rożek P, Czartoryska B, Kleinotiene G, Lipiński P, Tylki-Szymańska A, Ługowska A. A 20-year longitudinal study of plasma chitotriosidase activity in treated Gaucher disease type 1 and 3 patients: A qualitative and quantitative approach. *Biomolecules*. 2023;13(3):436. doi:10.3390/biom13030436
23. Tylki-Szymańska A, Szymańska-Rożek P, Hasiński P, Ługowska A. Plasma chitotriosidase activity versus plasma glucosylsphingosine in wide spectrum of Gaucher disease phenotypes: A statistical insight. *Mol Genet Metab*. 2018;123(4):495–500. doi:10.1016/j.ymgme.2018.02.004