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Pursuant to the ordinance of the Rector of Wroclaw Medical University No. 12/XVI R/2023, from February 1, 2023, authors are required to pay a fee for each manuscript accepted for publication in the journal Advances in Clinical and Experimental Medicine. The fee amounts to 990 EUR for original papers and meta-analyses, 700 EUR for reviews, and 350 EUR for research-in-progress (RIP) papers and research letters.

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Types of articles in *Advances in Clinical and Experimental Medicine* in 2021 and 2022: Editors' perspective

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

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Abstract

The present editorial summarizes the last 2 calendar years of *Advances in Clinical and Experimental Medicine* (ACEM) publication (2021 and 2022). The specific aims were: 1) To clarify the classification of papers published in ACEM; 2) To present motivations behind choosing this classification; 3) To show how this classification is reflected in citations. Six categories of papers published in ACEM are presented: editorials, meta-analyses, reviews (including systematic reviews), multicenter studies, research-in-progress studies, and research letters; lack of clear definitions for editorials, research letters and research-in-progress studies is discussed. Thematic fields covered by all categories in 2021 and 2022 are presented and differences in this regard between 2021 and 2022 are highlighted. Reasons for not publishing case reports (CRs) are discussed, with some of the debate on this issue in medical literature summarized. The article type classification used in ACEM in only one of many possible solutions and may be modified in the future – it should be both clear for the authors and allow for orientation in the journal's content. The motivation for choosing the employed categories stem both from their position on the accepted levels of evidence in evidence-based medicine (EBM) and their potential to be cited.

Key words: editorial, case report, meta-analysis, article type, research letter

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Introduction

Genology is a branch of literary theory that is concerned with the study of artistic genres, e.g., types of novels (mystery, science-fiction, etc.). Similar studies are well established in film theory. While scholars, specialists in the fields mentioned above, reflect on evolution of different genres across time and frequently express doubt whether genres as a mode of classifying narrative works of art should still be seen as something more than mere convention, readers or viewers still intuitively use terms like 'horror' or 'comedy' to have some basic orientation in literature, cinema and television. In scientific publishing, there are also analogues of genres, called "types" or "categories" of scientific papers. This editorial focuses exclusively on these types/categories of medical articles and is an attempt of self-reflection of *Advances in Clinical and Experimental Medicine* (ACEM) editors on the classification of papers employed in this journal from submission by authors to publishing.

There is no universal list of scientific medical article types, as evidenced by the editorial policy documents or instructions for authors disseminated by respected journals, e.g., guidelines listed by the *Yale Journal of Biology and Medicine*,[1] *New England Journal of Medicine*,[2] *The BMJ*,[3] *Frontiers in Medicine*,[4] *The Medical Journal of Australia*,[5] and Springer Publishing.[6] The above guidelines are examples of editorial practices. However, the scientific discussion regarding this topic has taken 2 different directions. A similar classification was proposed by Peh and Ng,[7] organizing the medical research into introduction, methods, results, and discussion (IMRAD). Röhrig et al.,[8] on the other hand, classify medical research into primary research (including basic medical research, clinical research, and epidemiological research) and secondary research (including meta-analyses and reviews).

The declared scope of ACEM includes articles that deal with all clinical and experimental medicine. The journal's scope is deliberately broad and general, since "advances" occur constantly in all fields of medicine and related sciences, and the leading research areas change as medical knowledge progresses. However, this does not mean that all types of articles are accepted. Since 2021, ACEM has been following a new classification of academic papers: 1) original papers (including randomized and nonrandomized clinical trials, retrospective studies, animal and cell line studies, and others); 2) meta-analyses; 3) reviews (including systematic reviews, scope, narrative, and other reviews); 4) multicenter studies; 5) research-in-progress (encompassing all types of experimental research in medicine); 6) research letters; and 7) editorials. In 2023, ACEM introduced a new category – editorial commentary. However, the present article covers the years 2021 and 2022, so it will not be discussed. Of other frequently encountered types of scientific medical papers, case reports and case series are not accepted in ACEM, which will be explained later in the text.

The classification used and the order of papers assigned to different categories in each issue reflect the hierarchy of evidence (levels of evidence) in evidence-based medicine (EBM): 1) editorials; 2) meta-analyses; 3) original papers; 4) reviews; and 5) research letters. Editorials opens an issue because they offer a more personal perspective and can serve as a guide from an experienced investigator to other, more focused studies. Editorials are followed by meta-analyses because, in clinical research, the best evidence of treatment efficacy comes mainly from meta-analyses of randomized controlled trials (RCTs).[9,10] Meta-analytic results are considered the most trustworthy source of evidence by the EBM literature.[11,12] Original papers are the primary source of new knowledge and represent the majority of each issue, with reviews offering theoretical summaries of complex issues, and research letters provide concise reports of original research findings.

Objectives

The specific aims of this editorial are as follows:

1) to present the classification of papers published in ACEM;

2) to clarify definitions of specific categories of papers which may not be entirely clear to all readers, and to provide context for these definitions from selected literature;

3) to outline the role played in ACEM by papers of different categories;

4) to present motivations behind choosing this classification (the scope of the journal is broad, but types of papers accepted are limited);

5) to explain why certain types of papers are not considered for publication in ACEM;

6) to provide recommendations for the Editorial Board of ACEM concerning possible modifications in classification used.

Since original papers constitute the broadest and largest group of papers, they will be analyzed later in another editorial by the same authors. Presenting papers of this type requires a different approach, focusing not on definitions but on thematic patterns observed in different years. Employing such an approach in the present paper would deviate from the main discourse and deform the structure of the paper.

Editorials

Similar to research letters (discussed in one of the following sections of this paper), editorials are often a source of confusion regarding their definition; however, while research letters are confused with other, distinct types of texts published in scientific journals, the concept of an editorial is so vague that each perspective seems to be equally deeply rooted in both tradition and practice.

The first concept stems from the popular press, especially from prestigious newspapers and weekly journals of opinion, and views an editorial as mean of presenting the stance of the whole journal concerning a specific problem; in modern scientific journals, editorials understood as "content provided by the editors" can also direct readers' attention to certain papers published in a given issue, announce calls for submissions or other initiatives, offer personal or more generalized reflection on a topic related to editing a scientific journal (e.g., propose definitions of different types of papers or instruct authors on how to prepare them), describe journal achievements, plans or policies, as well as present various guidelines and other similar documents. The second concept sees an editorial as an opinion piece that expresses reflections on topical issues, explains complex research, and/or highlights strengths, weaknesses and alternative interpretations of studies. The 2 above views are not mutually exclusive because editorials of both types can appear in a single journal, sometimes even in a single issue. Papers within this category are usually by-invitation only, although there are journals (e.g., *British Journal of Anaesthesia*) that accept unsolicited editorials after a pre-submission approval process.

The literature on editorials in scientific journals is diverse. Nundy et al. authored a chapter explaining the basic concepts of this scientific literary genre, presenting both of the above views and focusing of the role of an editorial as an opinion maker.[13] This perspective was shared by Singh and Singh, who offered a more personal view and concentrated on expressiveness of opinions and style of formulating them.[14] Van Teijlingen et al.[15] provided more specific advice and examples of appropriately prepared editorials for prospective authors, while Gray concisely described 5 rules of writing and editing an editorial.[16] However, the paper by Leslie and Hemmings Jr. had the broadest scope – they discussed how an editorial can be defined and described the structure of an excellent paper of this type, while also providing examples and more literature on the topic.[17] Guidelines for authors publishing in Springer Open journals[18] should also be mentioned – several journals established clear stipulations regarding word and reference count in an editorial; there can also be more or less severe limitations concerning tables and/or figures.

Editorials were introduced in ACEM in 2021 and are solicited from members of the Editorial Board and Scientific Committee, as well as from seasoned researchers among journal's experienced peer-reviewers. Only editorials submitted by invitation are considered for publication. This category of papers was introduced to provide authors with an opportunity to present their own opinions, commentaries and perspectives concerning selected issues within a selected research field. Editorials are meant to provide readers with context, analysis and reflection regarding specific subjects or problems. The popularity of this type of paper in ACEM is increasing: 6 editorials were published in 2021 and 12 in 2022.

In 2021, the solicited editorials addressed a specific topic or issue in the field of medicine, research, or healthcare. They explored different aspects of medical research, clinical systems, and health-related concerns. For instance, some editorials discussed the issues of monitoring the kynurenine system[19] and validating medical equipment.[20]

In 2022, these articles reflected the ongoing advances in the field of clinical and experimental medicine, addressing current issues, innovative treatments, and the impact of global events like the COVID-19 pandemic on medical research. The articles covered several problems, including the neurobiological advances of learned fear in humans[21] and the comorbidities of depressive and anxiety disorders.[22]

Editorials provide a valuable forum for expressing viewpoints, shaping discussions, and guiding researchers in their work. Among the top 10 most cited works in the ACEM for the 2021–2022 period are 4 editorials, namely Tanaka and Vécsei[19] (2nd most cited), Battaglia[21] (3rd most cited) and Chen[22] (4th most cited; all citation data as of September 30, 2023). Such high citation rates raise the question why this category of papers receives so much attention in the scientific community. In the opinion of the ACEM editorial staff, these reasons differ regarding specific papers. Below are the assumed reasons divided into categories for clarity.

Perspective and opinion: Editorials often present the perspectives, opinions, and insights of experts or key figures in a particular field. Researchers may cite editorials to support or align their own viewpoints or arguments.

Guidance and recommendations: Editorials may provide guidance, recommendations, or commentary on emerging trends, methodologies, or practices within a field. Researchers may cite editorials when discussing best practices or adopting recommended approaches.

Critical Analysis: Editorials provide a critical analysis of current research, policies, or developments. Therefore, researchers may cite editorials to support their evaluations or to acknowledge influential critiques.

Historical context: Editorials can provide a historical context for a particular field or topic. Citing editorials helps researchers anchor their work within the historical evolution of ideas, theories, or practices.

Debates and controversies: Editorials often focus on debates or address controversial topics within a field. Researchers may cite editorials to highlight or participate in ongoing discussions, acknowledging the influence of these debates on the field of study.

Expert commentary: Editorials may include expert commentary on scientific breakthroughs. Researchers may cite editorials to include authoritative perspectives and acknowledge the influence of recognized experts.

Editorial policies and journal direction: Editorials can discuss changes in editorial policies, journal direction, or broader trends in publishing. Researchers citing this type of editorials may refer to shifts in the academic landscape or to the direction of a particular journal.

Meta-analyses

Meta-analyses (MAs) are considered the most trustworthy source of evidence by the EBM literature.[11,12,23] They have the capacity to compare and contrast results from different studies, and identify both patterns and sources of disagreement among study results, or other relationships highlighted by multiple studies.[24] Therefore, MAs are considered the most valuable papers in ACEM and are always placed at the beginning of each issue, immediately following editorials. Moreover, the editors of ACEM aim to publish at least 1 paper from this category in every issue. In 2021, a decision was made to classify MAs separately from reviews, but due to complex resources and demanding research process involved in their preparation, first 6 MAs were published in ACEM only in 2022. Two thematic fields were prominent in these papers:

Diagnostic and therapeutic efficacy: Several of the articles focused on assessing the accuracy and efficacy of various diagnostic and therapeutic approaches. This included evaluating the accuracy of machine learning algorithms for the assessment of upper-limb motor impairments in patients with post-stroke hemiparesis[25] and the efficacy of home-based exercise programs in knee osteoarthritis treatment.[26]

Disease comorbidity and risk factors: Another common theme was the exploration of how certain conditions or factors influence the risk and outcomes of specific diseases; e.g., the impact of diabetes on patients receiving reperfusion therapy for acute ischemic stroke,[27] along with the role of tumor necrosis factor alpha promoter polymorphism in susceptibility to sepsis.[28]

Meta-analysis is one of the most thoroughly theoretically discussed type of papers – its complex methodology requires deep understanding in order to be properly translated into practice (i.e., a meta-analytic article fit for publication). Shorten and Shorten[29] provided a precise definition and explanation of the term 'meta-analysis', while Haidich[30] and Stangl and Berry[31] presented it in the context of medical research. Egger and Smith[32] and Egger et al.[33] discussed its theoretical premises, and Levi et al.[23] provided exhaustive reflection on both MAs and systematic reviews. Ahn and Kang[34] and Uman[35] discussed the common characteristics and mutual relations between systematic reviews and MAs, and explained the importance of articles of both types among all scientific medical papers in the context of hierarchy of scientific evidence.

There are several methodological problems regarding MAs, with publication bias and other types of bias being the most significant. These issues were discussed by Sterne et al.[36] and Sterne and Egger,[37] who used funnel plots to detect bias in MAs. Measuring inconsistency in papers from this category was reflected upon by Higgins,[38] while Higgins and Thomas further discussed quantifying heterogeneity in a MA.[39] Some limitations of MAs were pointed out by Naylor.[40]

Numerous guidelines concerning MAs have been released: among others, Hansen et al.[41] who offered a general guide in 8 steps, while Herrera Ortiz et al.[11] provided a guide concerning both systematic reviews and MAs. A useful tool in preparing MAs is the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) checklist that was presented and explained by Liberati et al.,[42] with an updated version of this checklist provided by Page et al.[43] Cochrane Handbook for Systematic Reviews of Interventions is also an important resource of information and guidelines concerning this issue.[44]

Many otherwise diligently prepared papers in this category have more or less serious flaws in the statistical analyses. This cannot be overlooked since proper statistics are a crucial part of each MA, and methodological errors in this regard can disqualify the entire work from publication. Harrer et al.[45] and Schwarzer et al.[46] wrote 2 fundamental papers on how to conduct a MA using statistical methods and the R language. Other important sources on statistical methods in MAs are: Borenstein et al.[47] (*Introduction to Meta-Analysis*), Borenstein[48] (*Common Mistakes in Meta-analysis and How to Avoid Them*), Cooper et al.[49] (*The Handbook of Research Synthesis and Meta-analysis*), Hedges and Olkin[50] (*Statistical Methods for Meta-analysis*), Cooper[51] (*Research Synthesis and Meta-analysis: A Step-by-step Approach*), Lipsey and Wilson[52] (*Practical Meta-analysis*), Littell et al.[53] (*Systematic Reviews and Meta-analysis*), and Pigott[54] (*Advances in Meta-analysis*). Resources presenting the above issues in a specific medical context are (among others): Egger et al.[55] (*Systematic Reviews in Health Care: Meta-Analysis in Context*), Sutton et al.[56] (*Methods for Meta-analysis in Medical Research*) and Whitehead[57] (*Meta-Analysis of Controlled Clinical Trials*). Rothstein et al.[58] (*Publication Bias in Meta-Analysis: Prevention, Assessment and Adjustments*) and Hunter and Schmidt[59] (*Methods of Meta-analysis: Correcting Error and Bias in Research Findings*) presented the issues of bias and error in a statistical context. Finally, Brockwell and Gordon[60] provided a comparison of statistical methods used in MAs.

Reviews and systematic reviews

The number of reviews published annually in ACEM remains stable. In 2021, there were 15 reviews of various methodologies and 2 systematic reviews (SRs), compared to 16 reviews complemented by 6 MAs (presented above) in 2022. There were no SRs in 2022 and no MAs in 2021. Systematic reviews often include a MA component, which involves using statistical techniques to synthesize the data from several studies into a single quantitative estimate or summary effect size.[23,35] In 2021, Ambros-Antemate et al.[25] published a combined SR and MA to assess the accuracy of machine learning algorithms for the assessment of upper-limb motor impairments in patients with post-stroke hemiparesis; that paper has been classified as a MA. While the concept of review seems to be understood

intuitively in the scientific community, SR is a much more clearly defined concept and there is an abundance of literature clarifying it. The Centre for Reviews and Dissemination at the University of York in the UK provided a detailed guide[61] on how to prepare SRs; a book covering this issue in detail was also published by Purssell and McCrae.[62] An overview of SRs was prepared by Silva et al.,[63] while Higgins et al.[64] analyzed the process of synthesizing quantitative evidence in SRs of complex health interventions, and Munn et al. offered guidance for authors when choosing between a systematic or scoping review approach.[65]

Finally, the resource utilized by many authors of reviews and MAs (and frequently referred to in such papers) is the Cochrane Handbook for Systematic Reviews of Interventions helmed by experts from the Cochrane network led by Higgins et al.[44]

Two SRs published in ACEM in 2021 discussed 2 different topics. The article by Czubak et al.[66] compared the clinical differences among COVID-19, SARS, influenza, and the common cold, while Springer et al.[67] investigated whether the choice of a drug in pharmacologic cardioversion correlates with national and international guidelines pertaining this matter.

Reviews published in ACEM in 2021 covered a wide range of medical topics, including, among others: hematological manifestations and complications of COVID-19 (Erdinc et al.[68]) and machine learning in orthodontics (Liu et al.[69]).

The thematic scope of reviews published in ACEM in 2022 was more diverse and can be categorized as follows:

Medical conditions and their management: Many articles focused on various medical conditions and their diagnosis and management, e.g., chronic kidney disease.[70]

Transitional care and adolescents: Some articles discussed the transition of care, particularly in adolescents with specific urological conditions. This indicates a focus on the healthcare journey and needs of this specific age group.[71]

Biomarkers and diagnostics: Some articles focused on new biochemical, immune, and molecular markers for diagnostic and prognostic purposes in various medical conditions, including lung cancer.[72]

It should be emphasized that the most-cited article published in ACEM in the discussed period is a review. An article titled "Cerebral small vessel disease: A review" by Chojdak-Łukasiewicz et al.[73] has been cited 22 times, while the SR by Czubak et al.[66] gained 7 citations and achieved the 6th position in the citation ranking for the years 2021–2022 (as of September 30, 2023).

Multicenter studies

In contrast to other types of articles discussed further in this editorial, there is no ambiguity in defining a multicenter study (MS), that is, a clinical trial conducted at more than 1 medical center or clinic. The benefits of MSs include a larger number of participants, different geographic locations, the possibility of inclusion of a wider range of population groups, and the ability to compare results among centers, all of which increase the generalizability of the study. In light of these advantages, it is understandable that in several scientific medical journals (e.g., *Frontiers in Public Health*, *BMJ Open* and journals specializing in publishing clinical trials, e.g., *Trials* and *Clinical Trials*), MSs are a separate category of published papers. This is also the case in ACEM, in which such category was introduced in 2017.

The literature on MSs is not abundant, but there are several papers offering advice to researchers who are not entirely familiar with this concept. Das[74] prepared an in-deep study on relevance, design and implementation of MSs, while Serra-Aracil et al.[75] provided a guide on how to start and develop a multicenter prospective RCT. Marsh and Hawkins[76] described statistical techniques most useful in analyzing data harnessed when performing such studies. More advanced knowledge was provided by Lane et al.[77], who outlined approaches for enhancing the informativeness and quality of multicenter trials as formulated by the Trial Innovation Network. Bourin[78] described specific requirements regarding multicenter trials, while Chung et al.[79] focused on stipulations of state agencies in this context; basic principles in this regard were also summarized by Aisen and Schafer.[80]

A cross-sectional study assessing multicenter clinical trial protocols was published by Zhang et al.,[81] while Seifirad and Alquran[82] pointed out several shortcomings of MSs with large samples. A chapter in *Handbook for Clinical Research: Design, Statistics, and Implementation* by Hammond et al. is also devoted to this issue.[83] Finally, there are 3 checklists which can be used for preparing MSs:

1) Consolidated Standards of Reporting Trials (CONSORT),[84] designed for reporting randomized controlled trials, with several specialized versions;

2) Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT),[85] a guideline for the minimum content of a clinical trial protocol; 33-item SPIRIT checklist applies to protocols for all clinical trials and focuses on content rather than format;

3) Transparent Reporting of Evaluations with Nonrandomized Designs (TREND)[86] for reporting intervention evaluation studies using nonrandomized designs.

Two MSs were published in ACEM in the period 2021–2022, both in 2021 and both pertained to the COVID-19 pandemic. The study by Arnabat-Dominguez et al.[87] concerned laser dentistry in daily practice during the COVID-19 pandemic, while a paper by Kusztal et al.[88] inquired whether home-based therapy in Fabry disease is the answer to compelling patients' needs during the COVID-19 pandemic. Both articles explored the benefits, risks, and recommendations for safe treatment in the context of the pandemic, and how patient needs are addressed.

Research-in-progress

Although research-in-progress (RiP) type of article is widely used in scientific publishing, it lacks clear definition. Only Heinrich et al. published an editorial summing up a debate among members of the editors of *Advancing Scholarship and Research in Higher Education*.[89] Four participants did not offer a definition per se, but proposed 4 characteristics of RiP:

1) It cannot only present the research question and methodology, but must at least give some insights to answer the question, or at least to start answering the question;

2) It identifies opportunities to collaborate and indirectly or directly invites other researchers to join the presented research project, or at least to offer their suggestions regarding how to proceed;

3) It needs to have a meticulously prepared Discussion section – the readers have to learn why the incomplete research presented is actually important;

4) It has to include a Limitations section clearly stating that the main limitation of the presented research is that it is still in progress.

In ACEM, the second characteristic is not considered as a requirement. The other 3 characteristics outline how RiP paper is understood in this journal, especially regarding the first characteristic. If a RiP submission lacks adequate data, there is no sufficient evidence to warrant any judgments about the next steps that might be taken to conduct further research.

Two RiP articles were published in ACEM in 2021, and none in 2022. The paper by Dejnek et al.[90] was an analysis and comparison of autologous platelet-rich plasma preparation systems used in the treatment of enthesopathies, while the study by Rudno-Rudzińska et al. analyzed effects of calcium electroporation, electrochemotherapy, and irreversible electroporation on quality of life and progression-free survival in patients with pancreatic cancer.[91]

Research letters

Research letters (RLs) were introduced in ACEM in April 2021. Although editorials and RiP papers also lack a clear definition, it is essential to note that RLs should never be mistaken for a "letter to the editor." While the former one is a concise rendition of an original academic paper, reflecting original research findings, the latter one is a brief commentary on previously published academic papers in scholarly journals.

This view is neither universally accepted nor obvious. For example, Rutkowski and Dohan Ehrenfest mentioned a concept of RL as an autonomous format for the rapid publication of data, with a three-part structure (introduction, description of the methods and results, and discussion), with a word limit of 1,500 and 25 references.[92] Research letters written in this way can disclose and discuss an innovative concept before its full demonstration in the following publications. There are even entire scientific journals devoted only to RLs understood as above, e.g., *Nanoletters*. In ACEM, a RL is defined as a brief, but scientifically important study which is basically a shorter form of an original paper. The text structure (Background – Objectives – Materials and methods – Discussion – Limitations – Conclusions) remains the same as in an original work. Research letters are peer reviewed and subject to a stringent editorial review. Moreover, RL are indexed in all databases, including PubMed and Scopus, and the number of points assigned for such publication is identical as for original papers. However, a RL contains a maximum of 1,500 words, as opposed to 3,500 words in an original article, and may contain at most 2 tables and 2 figures. The number of references is not limited. Authors of original papers considered by the editors too brief for publication will be asked to change the category of the manuscript into a RL.

This concept has one element in common with the idea of Rutkowski and Dohan Ehrenfest[92] – the speed of publication (RLs are published ahead of print online faster than original papers – but it is important to note that they are peer-reviewed like any other paper). A definition more similar to this from ACEM's was provided by Kukafka et al. when RLs were introduced in the *Journal of Medical Internet Research* in 2022 – in their view, they should convey "[…] new, early, or sometimes preliminary research findings, including interesting observations from ongoing research with significant implications that justify concise and rapid communication".[93] Conversely, the *Canadian Journal of Kidney Health and Disease* defines a RL not only regarding its size (500–1,500 words, up to 10 references and 1 figure or 1 table) but also its scope – RL in this journal is a " […] publication of results which deserve dissemination, usually because they may be useful to others, but do not advance the field to the same extent as a full original clinical research paper".[94] Finally, the editors of the *Chest* point out that RLs should be "of high quality, be novel, or have potential clinical impact, but should not be advanced or large enough to warrant publication of a complete original research manuscript", limit the article parameters to 1,000 words, 10 references and 2 tables and/or figures, and stipulate a structure with Introduction, Methods, Discussion and Conclusions, which is, at least in broad terms, in concert with the definition adopted in ACEM.[95]

There were 5 RLs published in ACEM in 2021 and 14 in 2022 – this type of papers has been introduced in this journal in April 2021, but nevertheless these numbers show its growing popularity. The RLs released in 2021 dealt with diverse research problems: 27-gauge sutureless vitrectomy under topical anesthesia,[96] the influence of comorbidities on mortality in bronchiectasis,[97] and ST-segment depression in atrioventricular nodal reentrant tachycardia.[98] They included a pilot study,[96] a prospective observational study,[97] and a presentation of preliminary results.[98]

In 2022, RLs in ACEM covered a much broader field – the following 3 categories dominated:

The health impact of COVID-19: Several articles addressed concerns like ocular complaints in students during the pandemic,[99] and differences in the incidence of symptoms among medical staff working with COVID-19 patients.[100]

Women's health: Some articles focused on women's health issues, e.g., changes in the stomatognathic system in women working with COVID-19 patients.[100]

Orthopedic and musculoskeletal studies: There have been studies related to orthopedic and musculoskeletal conditions, such as distal radius fractures in the elderly.[101]

It is important to note that these RLs fulfilled the role of rapid research dissemination – findings regarding COVID-19 pandemic are a good example.

Case reports: Why are they not published in ACEM?

In January 2021, ACEM implemented a policy precluding publication of case reports (CRs) of any type, while case series are considered for publication only very rarely – in situations when choosing this type of scientific article is the sole way to tackle, e.g., an issue related to an orphan disease or a very rare tumor. Of note, in the 2 discussed years (2021–2022), no case series has been published. Such tendency is observed in several other scientific medical journals, e.g., *PLoS One*, *Anesthesiology*, *Anesthesia and Analgesia*, and *Journal of Stomatology* do not accept CRs at all. Although Dikensoy et al.[102] stated that the majority of high-impact journals adopted such policy, there are no data to support this claim. There are also examples of a more nuanced stance: For example, the *New England Journal of Medicine* (NEJM) does not accept stand-alone CRs; however, articles of other types in this journal may include CRs.

In the literature, it is contented (but not proven) that the vast majority of CRs are very rarely cited or not cited at all (Edelmayer et al. introduced the term "case report classics" to denote the relatively small minority of frequently cited CRs, but noted that most of them were published before 1990s).[103] This has been shown, i.a., by Erivan et al., who performed a retrospective bibliometric analysis of all articles affecting the impact factor (IF) calculated for the year 2017 published in 77 journals covering the field of orthopedics.[104] Individually, in 2017, each CR was cited 0.86 times ±1.4 (0–13) on average. Case reports published in 2015 had a mean of 0.96 ±1.49 (0–13) citations, while those published in 2016 had a mean of 0.76 ±1.29 (0–13) citations ($p = 0.002$). Of all the CRs, 571 (30%) were not cited at all in 2017. After a statistical analysis, Erivan et al. concluded that in 69 instances, the IF would have increased if the journal had not published any CR. Conversely, the IF improved in 8 instances by publishing CR. Papers which are not cited or very scarcely cited increase the denominator (number of papers) but not the numerator (number of citations) in the calculation of IF. This issue was also analyzed by Nabil and Samman in oral and maxillofacial surgery journals.[105] They revealed that CRs had a low citation rate with an average of less than 1. There were 38 (7.2%) CRs with more than 5 citations and 30% of the citing articles were also CRs. The publication of CRs negatively affected journal's impact factor, which correlated directly with the percentage of CRs published in a journal.

In the opinion of the ACEM editorial staff, the research discussed above cannot serve as an argument supporting the decision of excluding CRs from papers considered for publication in a given journal. The key mission of every scientific journal is to disseminate research results. All bibliometric measures such as IF, Scopus CiteScore or others were created to assess the efficiency of such dissemination, and not to become an objective in themselves. Therefore, excluding specific types of papers from a journal to artificially boost citation statistics could be considered ethically questionable.

However, there are other reasons for repudiating CRs. The debate whether CRs should still be an important part of scientific medical publishing is still ongoing and no consensus has been reached so far. They are certainly not without scientific merit, as argued, among others, by Firat et al.,[106] Bhattacharrya et al.,[107] Nieder et al.,[108] Yitschaky et al.,[109] Carey,[110] Carey,[111] Albrecht et al.,[112] and Edelmayer et al.[103] It is important to note that the above studies discussed this issue regarding several different fields of medical research and not only in general terms. However, as stated by Greenhalgh,[113] CRs are placed at the bottom of the hierarchy of clinical evidence, along with case series, because of their inherent methodological limitations, including lack of statistical sampling.

In 2021, the Institutional Review Board of the Johns Hopkins University School of Medicine stated that "[a] case report is a medical/educational activity that does not meet the Department of Health and Human Services (DHHS) definition of «research», which is a «systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge»".[114] Case reports are not based on controlled, experimental research and present only an individual clinical picture of a given patient, while results of RCTs or MAs are considered more reliable and influential in the field of medicine they belong to.

Given the controversial perception of CRs in the medical literature, the Editorial Board of ACEM on the one hand is not interested in publishing them, but on the other hand has no intention of discouraging authors from utilizing this form of scholar contribution. In the last 30 years, with the emergence of digital publishing, a considerable number of journals have appeared that are dedicated solely to disseminating valuable CRs (e.g., *BMJ Case Reports*, *Journal of Medical Case Reports*, and *American Journal of Case*

Reports). Several authors offered valuable advice on where to direct studies of such type for publication. Rison et al.[115] provided detailed criteria for informed choice (there are journals of debatable value operating in this field), while Akers[116] compiled an exhaustive list of journals with such a profile, and the library of the George Washington University (Washington, D.C., USA) established a detailed website on this topic, including an up-to-date list of journals that peer-review and publish CRs.[117]

Effectiveness of the article categorization in ACEM and standardization of papers in ACEM

The types of papers distinguished in ACEM have proved to be a useful tool, since they are deliberately broadly defined and therefore relatively capacious – among them, only the MS, MS and SR types have strict definitions that entail specific stipulations. The presented classification encompasses most of the scientific medical papers of value met in other journals, even if the categorization used in the most respected ones is more detailed. We purposefully avoid formulating subcategories, e.g., regarding different types of reviews – only a SR is discerned because of clear delimitations of this type of paper, while every other type of review article is simply a review in light of the ACEM rules, and no specific stipulations are made for specific subtypes of reviews. Every paper which includes original experimental or clinical research can be submitted as an original paper; should it be too brief for a full-fledged article; it will be reclassified as a RL – if the authors consent. This is to avoid bias and to minimize the possibility that authors will abstain from submitting a manuscript to ACEM because their paper does not fit into any category.

The only limitation observed concerns RLs. Authors of short papers receive a proposal to reclassify them as RLs – before or after the initial acceptance for publication. Since the introduction of RLs in ACEM, several authors (we are unable to provide an exact figure) decided to withdraw their papers in response to such proposal, citing either stipulations made by granting or evaluating institutions (which may only recognize original papers as fulfilling their assessment criteria), or the lower prestige that RLs would, in their opinion, have in their resumes. Such cases are evaluated individually – while the former argument is often a basis for reconsidering the matter, the latter still poses a challenge since – while holding professional opinion of a researcher in high regard – we have not yet been able to find any research results that would substantiate claims of lower prestige of RLs compared to original papers.

Within the scope of this paper, it is impossible to authoritatively state whether the described classification is optimal to reflect the breadth and depth of research in clinical and experimental medicine. However, one argument can be based on the checklists introduced as mandatory for all authors submitting manuscripts to ACEM since January 2021. These tools have been proposed during the last 30 years by various collective bodies within the scientific community and pooled together in the EQUATOR (Enhancing the QUAlity and Transparency Of health Research) network website (https://www.equator-network.org). The most commonly used checklists are:

– Consolidated Standards for Reporting Trials (CONSORT) checklist for clinical trials;

– STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) checklist for observational studies;

– Critical Appraisal Skills Programme (CASP) checklist for qualitative studies;

– Animal Research: Reporting of In Vivo Experiments (ARRIVE) checklist be followed in the preparation of studies involving live animals, from mammals to fish and invertebrates.

– Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist for all MAs and reviews.

There are several other tools of this kind, not presented here, which can be accessed through the EQUATOR website. The authors assume that these checklists reflect the aforementioned breadth and depth of research in clinical and experimental medicine – and because the authors of papers published in ACEM choose various checklists, this broadness of scope translates also to the contents of each category in the employed classification. The checklists also allow for the standardization of the papers' structure and other features – to a reasonable extent – to facilitate better conformity with established standards in scientific medical publishing. These checklists have been extensively discussed in a paper by Misiak and Kurpas.[118]

Comparison to other journals

A comparative study of the classification strategy employed in different journals could be useful in assessing the efficacy and reliability of such categorization. However, differences in scope and editorial and marketing strategy between different journals could make such comparison biased or otherwise unreliable. A large number of different types of papers can usually be found in journals published by enormous transnational entities like, e.g., Taylor & Francis, Springer Nature or Elsevier, which aim to standardize such classification across several titles owned by a respective publisher (at least concerning the most prestigious journals in the portfolio of a given publisher). Even the nomenclature used varies significantly between journals – Editor Resources by Taylor & Francis state that there are more than 1,400 (sic!) different article type headings in use in that publisher's journals alone.[119]

The same publisher provided a glossary of article types for early career researchers, which, while relatively concise, provides a classification that: 1) is more detailed than that used in ACEM (it includes, e.g., method articles); 2) is not compatible with categorization used in several journals owned by this publisher (e.g., *Scandinavian Journal of Gastroenterology*); and 3) uses nomenclature different from ACEM (e.g., an original paper is called a research paper).[120] In a single journal, such a detailed categorization could lead to confusion among authors regarding which type of paper their manuscript should be assigned to during submission. Therefore, we reasoned that the categories used in ACEM should be as broad as possible to reflect only the general types of manuscripts – based on the methodological approach (original papers, reviews, MAs that need to be discerned from reviews, RiP papers and MSs), to emphasize papers written by invitation and providing an opinion (editorials), and to set aside articles shorter than others, but nonetheless important (RLs). Our point is that any subdivision within these broad categories would not serve any purpose worth the effort in a single journal of broad scope (all clinical and experimental medicine).

The policy of each journal regarding 1) what classification of papers is used, and 2) which categories from this classification are considered for review and which are excluded and desk-rejected from a given journal, stems from the scope of a journal and the strategy of journal development adopted by its Editorial Board. The stipulations of institutions that evaluate the work of researchers heavily influences both policies of editors and authors' choices of specific article types (e.g., multiple evaluating bodies require scientists to publish full-text original papers in the first place). Therefore, differences in the employed categorizations may result from strictly scientific, editorial, or external factors, which are not always possible to identify.

To sum up: Such a comparison would provide valuable arguments for shaping journal's policy in this respect, but performing it with due diligence and adequately broad scope would require a separate study of comparable, if not larger, size; otherwise, superficial conclusions based on a skewed selection of the materials might be misleading.

Promoting open science

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Ensuring the public availability of raw data facilitates the transparency of research and enables its replicability. Several papers published in ACEM include a disclaimer in which authors offer to share raw data upon request. Papers published online as ahead of print since November 2023 include a mandatory disclaimer at the end of the main body of the article as follows: "The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request". Supplementary material is a peer-reviewed material directly relevant to the conclusion of a paper that cannot be included in the printed version for reasons of space or medium limitations (e.g., movie clips or sound files). If a paper accepted for publication in ACEM is accompanied by supplementary data of any kind (in many cases, these are statistical data), the Editorial Office requires that all such files be deposited in the Zenodo repository (https://zenodo.org) and that a single DOI be obtained for the entire package. Each file should be numbered (Supplementary Table 1, Supplementary Fig. 1, etc.) and a one-sentence description of its contents should be provided. Both the description and the DOI are placed in a separate subsection titled "Supplementary data" at the end of the manuscript. Neither ACEM nor the journal's publisher (Wroclaw Medical University) are in any way associated with the Zenodo repository, either financially or organizationally. We are simply recommending this entity as a reliable and cost-free third-party data storage opportunity. This policy was introduced in January 2022 and so far, 17 papers published in 2022 and 25 until the September 2023 issue have had the supplementary materials made available as described above. This issue will be covered in another editorial by the same authors.

Limitations

First and foremost, we provided no description of practical application of the discussed theoretical concepts, i.e., case studies showing how the presented article types translate into concrete papers published in ACEM. As mentioned above, the categories used in ACEM are deliberately

broad and presented on the journal's website[121] only in general terms (RiP) or only by their names, because ACEM editors assume that the understanding of an original paper or a MA is rather obvious for a professional researcher. The only types referred to more extensively are RLs (because of the terminological confusion discussed above) and reviews (due to several types of reviews discussed in the literature, a link to a paper by Grant and Booth[122] presenting a typology of reviews – 14 review types and associated methodologies – is provided as a reference and guide). Therefore, there are no strict definitions per se stated as stipulations for the authors that can later be translated into practice. However, one can observe such translation regarding checklists discussed above, which predefine especially the structure of the paper, and within in – particularly the Materials and methods section. Moreover, the choice of the papers considered for publication from all submitted manuscripts and the final form of the chosen papers (following all revisions) are to a greater extent the result of the professional experience, journal's policy, and the decision of Section Editors than the employed paper classification. The stipulations stemming from this categorization and those found in the checklists play more a gatekeeping than shaping role. Manuscripts that do not conform to these rules, or that do not fit into any of the categories used, are desk-rejected during the initial verification by the Managing Editor or the preliminary review by the Section Editor before they can be sent to peer-review.

Second, no suggestions were formulated for an eventual modification of the article type classification utilized in ACEM in the future. We only became more aware that we should conduct a study on this issue in the future.

Third, meaningful comparisons with paper category policies in other scientific medical journals were deemed to be outside the scope of the present study and should be the subject of future studies.

Fourth, original papers were not reflected upon for the reasons stated in objectives, which renders the picture of paper categorization in ACEM more cohesive, but also incomplete. This issue will be addressed in further papers by the same authors.

Finally, the potential influence of publications in ACEM on medical research or policy changes has not been discussed. The Editorial Office of ACEM does not have any tools that would allow for tracking such influence. However, the section editors of our journal attempt to shape the distribution of topics in the journal through calls for submissions. In 2021 and 2022, there were 4 calls for submissions: 1) Heart failure: recent advances and future perspectives in diagnosing, prognosticating and management; 2) Gastroenterology in the face of local and global challenges; 3) Nephrology in the face of local and global challenges; and 4) Metabolic pathways in carcinogenesis: Mechanism, diagnosis and treatment. The response to each call was clearly visible in the stream of manuscripts submitted to the journal, and 14 of these submissions were published – 2 in 2021 (both from call No. 4) and 12 in 2022 (from 3 other calls – 6 from No. 3, 3 from No, 2 and 3 from No. 1). This topic will be covered in another editorial by the same authors.

Recommendations for the ACEM Editorial Board

Although, as stated above, we think that the categorization we use covers the scope of ACEM, even a brief comparison with other journals (as in the "Comparison with other journals" section) shows that there may be paper categories/types that could prove useful in achieving an even broader diversification of content. Exploring a wider range of medical topics and specialties would allow to cater to a more diverse readership. Such broadening should mean incorporating types of articles as for now explicitly (CR) or implicitly (book reviews) excluded from ACEM. A change in this respect could facilitate the inclusion of multidisciplinary perspectives by encouraging submissions that integrate insights from various medical disciplines to provide a more holistic view of healthcare. Therefore, we propose to prepare a report on the chosen article categories employed in different scientific medical of comparable (i.e., more general) scope to identify promising new types of articles to be introduced in ACEM and assess them in the light of ACEM's to-date policies and needs as defined by the Editorial Board. Such a report would not necessarily be a full-fledged comparative study as discussed in the "Comparison with other journals" section, nor should it pretend to be a complete view of the issue of paper classification in medical literature as a whole. However, even a partial view could provide valuable suggestions. We will never achieve an ideal classification, but we should not refrain from modifications – ACEM must evolve along with all other scientific medical journals.

Conclusions

The article type classification used in ACEM in only one of many possible solutions and may be modified in the future – it should be both clear for the authors and allow easy orientation in the journal's content for the readers. The motivation for choosing the employed categories stems from 3 reasons: 1) their position on the accepted levels of evidence in EBM and their potential to be cited; 2) the usefulness and comprehensibility of the chosen categorization for authors, who should be able to assign their paper to an appropriate category without delving into theoretical considerations. It is also obvious that the presented classification of papers is to some extent arbitrary – the important thing is not to exclude any types of papers from consideration based on arbitrary decisions.

It turned out that in the case of 2 types of articles – editorials and RLs – formulating definitions by the authors was necessary because opinions expressed in the literature as well as practice among editors of other journals varied and were sometimes contradictory. Editorials as understood in ACEM proved to be a powerful category of paper, attracting both renowned researchers and high number of citations; reflection on them revealed their diverse roles and strengthened the dedication of ACEM editors to promote this form of scientific publication. Research letters seem still not widely known in the researcher community and are often confused with other types of papers, like short communications or letters to the editor. Although the authors sustain their decision to exclude case reports from consideration in ACEM, an overview of the literature showed that the debate regarding importance of this category is far from over; the hierarchy of evidence in medicine is not put to question, but a low position in this hierarchy is not an argument for abandoning this type of scientific medical publication altogether.

Moreover, certain types of papers are related to specific sources of problems – while RLs are perceived as controversial by some authors due to their purported inferiority in comparison to original papers, MAs often show deficiencies in statistical analyses of the data; to provide guidance to the readers, we have dedicated an entire paragraph to a presentation of the most important publications on this topic.

Future editorials will explore 3 issues:

1) Original papers as the type of article forming the bulk of manuscripts both submitted to and published in ACEM – their diversity and thematic patterns over the years;

2) Preprints as a mode of promoting open science – and ACEM's policy in this regard;

3) Public availability of raw data as a way to facilitate the transparency of research and enable its replicability – and ACEM's policy in this regard.

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MDR3 rs2109505 and *rs1202283* polymorphisms are associated with susceptibility to intrahepatic cholestasis of pregnancy: A meta-analysis

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Abstract

Background. Many studies have assessed the relationship between gene polymorphisms in multidrug resistance protein 3 (MDR3) and the risk of intrahepatic cholestasis of pregnancy (ICP); however, there are many conflicting narratives.

Objectives. This meta-analysis was conducted to assess the association between *MDR3* gene polymorphisms and ICP.

Materials and methods. A multi-database search was conducted using Web of Science, Embase, PubMed, and Chinese Biomedical Literature (CBM) databases. Eleven eligible studies focusing on 4 single nucleotide polymorphisms (SNPs) in the *MDR3* gene were selected for analysis. A fixed- or random-effects model was utilized for allelic, dominant, recessive, and superdominant genes.

Results. The pooled results indicated a statistically significant association between *MDR3* polymorphism *rs2109505* and an increased risk of ICP in both the general population and the Caucasian population. No statistically significant associations were found between *MDR3* polymorphism *rs2109505* and ICP in Italian or Asian populations for the 4 genetic models. The *MDR3* polymorphism *rs1202283* was associated with susceptibility to ICP in both the general and Italian populations.

Conclusions. The *MDR3 rs2109505* and *rs1202283* polymorphisms are associated with ICP susceptibility: however, they displayed no correlation with an increased risk of ICP.

Key words: meta-analysis, intrahepatic cholestasis of pregnancy, SNP, susceptibility, *MDR3* gene polymorphism

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Introduction

Intrahepatic cholestasis of pregnancy (ICP), a pregnancy-related liver disorder, is characterized by maternal pruritus without a skin rash in the 3rd trimester. The disorder is recognized as a reversible form of cholestasis in late pregnancy. In addition, patients have varying degrees of elevated total serum bile acids, with or without elevated serum transaminases.[1] The ICP is associated with an increased risk of adverse perinatal outcomes, including stillbirth, preterm delivery, fetal staining of amniotic fluid, fetal distress, or asphyxia.[2] Elevated total serum bile acids caused by ICP are associated with adverse perinatal outcomes.

It has been acknowledged that genetic factors are closely related to the pathogenesis of ICP.[3] For instance, multidrug resistance protein 3 (MDR3), serving as ATP-binding cassette subfamily B member 4 (ABCB4),[2] was reportedly associated with the pathogenesis of ICP. The MDR3 protein serves as a phosphatidylcholine flippase, transporting phosphatidylcholine from the lumen of the hepatocyte to the lumen of the biliary canaliculi.[4] Moreover, MDR3 dysfunction and failure of phosphatidylcholine secretion triggered by *ABCB4* mutations lead to luminal epithelial cell damage and subsequent biochemical abnormalities, which play a key role in the pathogenesis of ICP.[2] However, a study based on ICP patients from west Sweden showed no association between *MDR3* gene mutations and the pathogenesis of ICP.[5,6] On this basis, we assume that there are conflicting views regarding the roles of *MDR3* gene mutations in the pathogenesis and patients' susceptibility to ICP.

Objectives

This meta-analysis was conducted to assess the relationship between *MDR3* gene polymorphisms and ICP susceptibility.

Materials and methods

Search strategy

The protocols used in the search strategy were described according to the previous methods (PROSPERO ID: CRD42020164230). A systematic search was conducted and focused on the studies regarding variants of the *MDR3* gene in ICP patients published in PubMed, Web of Science, Embase, and Chinese Biomedical Literature (CBM) databases until January 2021. There were no language restrictions in the search strategy. Following keywords were used in the search: "intrahepatic cholestasis of pregnancy" OR "recurrent intrahepatic cholestasis of pregnancy" OR "obstetric cholestasis" OR "cholestasis", AND "multidrug resistance protein 3" OR "ABCB4 protein, human" OR "MDR3 protein."

Studies meeting the following inclusion criteria were eligible to be included in this meta-analysis: 1) studies which examined *MDR3* gene polymorphisms in patients with or without ICP; 2) case-control studies in which the controls had experienced physiological pregnancies; 3) in cases of 2 or more studies from the same cohort, the most integrated study was included; 4) studies which reported the number of genotype variants related to ICP susceptibility. Studies with the following conditions were excluded: 1) the literature revealed familial ICP or other cholesteric liver diseases; 2) studies enrolling nonpregnant women; 3) research or experimental studies that were not conducted on animals; 4) case reports or meeting abstracts; 5) studies lacking particular genotypic data or sequence analysis of *MDR3* polymorphisms.

Data extraction

Data were extracted by 2 investigators independently, including author's name, year of publication, nationality, race, sample size, patient characteristics, risk gene polymorphisms, and method of genotyping. In controversial cases, a thorough discussion was conducted with a 3rd investigator until a consensus was reached.

Statistical analyses

The association of single nucleotide polymorphism (SNP) in the *MDR3* gene with the risk of ICP was estimated by combining pooled odds ratios (ORs), 95% confidence intervals (95% CIs) and Z-test results in the allelic, dominant, recessive, and superdominant gene models. The simplest case was represented by a polymorphism with 2 alleles (A – mutant-type, B – wild-type), one of which (A) is thought to be associated with the disease. Association analysis collects information about the numbers of disease-free and diseased subjects with each of the 3 genotypes (AA, AB and BB). A recessive model compares AA with BB+AB, a dominant model compares AB+AA with BB, and a superdominant model compares AA+BB with AB.

For appropriate continuity correction, a value of 0.5 was substituted in the presence of 0 in the number. Meta-analysis was performed on the 4-gene models using the fixed-effects model. The random-effects model was used to evaluate the OR if there was heterogeneity ($I^2 > 50\%$, $p < 0.05$) among the studies. The publication bias was evaluated using Egger's test. The STATA v. 12.0 software (StataCorp LLC, College Station, USA) was used for all analyses, and all tests were two-sided. A value of $p < 0.05$ was considered statistically significant.

Results

Characteristics of studies

Initially, a total of 291 articles were extracted from PubMed, Web of Science, Embase, and CBM databases. From these articles, 133 were excluded due to duplication, and 113 were excluded after reading the titles and/or abstracts. Subsequently, full texts of 45 articles were accessed, from which 31 articles were excluded, including 2 case reports, 1 conference article, 13 studies focusing on familial ICP or cholesteric disease, 5 review articles, 5 studies reporting no information on the controls, and 5 articles with no analysis of *MDR3* mutations. Afterwards, we identified 14 eligible studies involving 83 SNPs in *MDR3*, including 4 studies focused on the same SNP (Fig. 1). Finally, 11 eligible studies investigating 4 SNPs (i.e., *rs2109505*, *rs1202283*, *rs2302387*, and *rs2230028*) in *MDR3* were included.[7–17]

Meta-analysis

A meta-analysis was performed based on the *rs2109505* (involving 967 cases and 1149 controls), *rs1202283* (179 cases and 415 controls), *rs2302387* (168 cases and 390 controls), and *rs2230028* (542 cases and 714 controls) (Table 1).

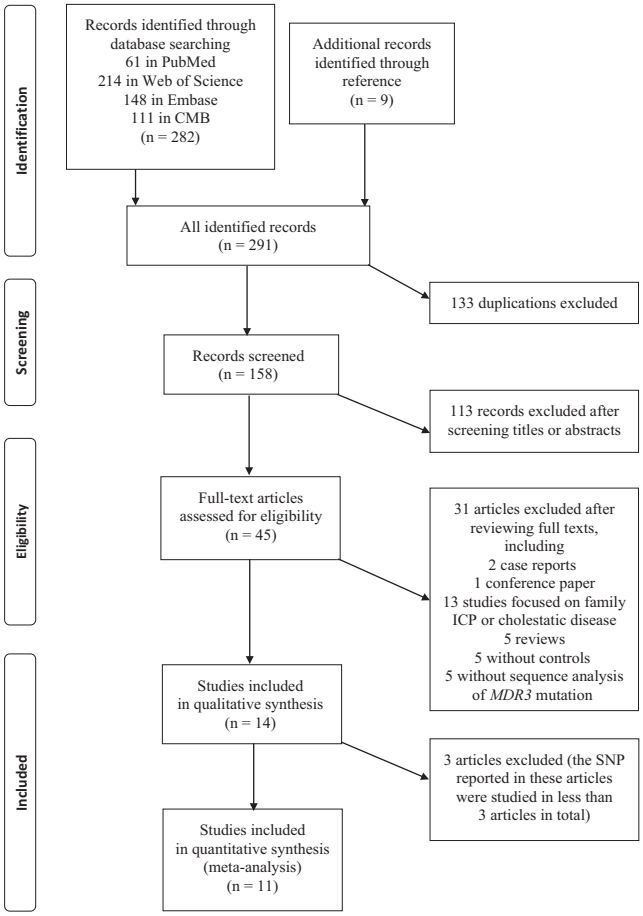

Fig. 1. Study selection process

ICP – intrahepatic cholestasis of pregnancy; MDR3 – multidrug resistance protein 3; SNP – single nucleotide polymorphism; CMB – Chinese Biomedical Literature.

The pooled results indicated a relationship between the *MDR3 rs2109505* polymorphism and increased risk of ICP in both the general (dominant model: OR = 0.72, 95% CI: 0.34–1.53, Z = 1.99, p = 0.047; recessive model: OR = 0.68, 95% CI: 0.33–1.40, Z = 2.23, p = 0.026; superdominant model: OR = 1.38, 95% CI: 0.66–2.90, Z = 2.10, p = 0.036) and Caucasian (allelic model: OR = 1.87, 95% CI: 1.46–2.40, Z = 6.64, p = 0.000; dominant model: OR = 0.49, 95% CI: 0.38–0.63, Z = 6.56, p = 0.000; recessive model: OR = 0.47, 95% CI: 0.22–0.99, Z = 2.74, p = 0.006; superdominant model: OR = 1.92, 95% CI: 1.52–2.44, Z = 5.72, p = 0.000) populations. However, *MDR3 rs2109505* polymorphism was not correlated with the pathogenesis of ICP in Italian or Asian populations under the 4 genetic models (all p > 0.05) (Table 2, Fig. 2A–D).

We found limited evidence that *MDR3 rs1202283* was associated with ICP susceptibility in general (superdominant model: OR = 0.42, 95% CI: 0.12–1.42, Z = 2.43, p = 0.015), Italian (superdominant model: OR = 0.09, 95% CI: 0.00–5.79, Z = 2.44, p = 0.015) and Asian (allelic model: OR = 0.27, 95% CI: 0.16–0.44, Z = 5.27, p = 0.000) populations. No significant correlation was found between the *MDR3 rs1202283* polymorphism and susceptibility to ICP in Greek or Caucasian population (all p > 0.05) (Table 2, Fig. 3A–D), and *MDR3 rs2302387* or *MDR3 rs2230028* showed no correlation with the risk of ICP (all p > 0.05) (Table 2, Fig. 4A–D and Fig. 5A–D).

Publication bias analysis

Egger's test showed no evidence of publication bias for *MDR3 rs1202283* or *rs2302387* and ICP risk (p > 0.05). There was a publication bias for *rs2109505* in the allelic (t = −6.95, p < 0.01) and dominant (t = −5.84, p = 0.001) gene models, as well as for *rs2230028* in the superdominant model (t = 2.98, p = 0.025) (Table 2).

Discussion

The *MDR3* polymorphisms have been reported to play a role in the pathogenesis of ICP. As a transporter for phospholipids, the MDR3 protein mediates the transmission of phosphatidylcholine into the bile capillary, which deactivates toxic bile salts to protect the epithelial lining.[18] The MDR3 dysfunction has been linked to hormonal and environmental factors, which may contribute to the pathogenesis of ICP.[19] This meta-analysis was conducted to further explore the relationship between *MDR3* gene polymorphisms and the pathogenesis of ICP.

Many studies have previously investigated the relationship between ICP and *MDR3* dysfunction, but there are still disputes regarding the conclusions. In a previous study, Anzivino et al. explored *ABCB4* (*MDR3*) and *ABCB11* mutations in ICP patients in an Italian population. The authors concluded that *ABCB4* mutations were

Table 1. Characteristics of studies included in the meta-analysis

SNP	Author	Year	Country	Race/ethnicity	Sample size		Genotype						Allele			
							case			control			case		control	
					case	control	BB	BA	AA	BB	BA	AA	B	A	B	A
rs2109505 (c.711A>T)	Wasmuth et al.[15]*	2007	Sweden	Caucasian**	52	52	42	9	1	33	16	3	93	11	82	22
	Dang et al.[8]*	2015	China	Asian	54	100	30	21	3	67	29	4	81	27	163	37
	Dixon et al.[9]	2014	UK	Caucasian	563	642	446	110	7	411	205	26	1002	124	1027	257
	Anzivino et al.[10]	2013	Italy	Italian	33	100	25	7	1	62	36	2	57	9	160	40
	Tavian et al.[12]	2009	Italy	Italian#	10	43	8	2	0	43	0	0	18	2	86	0
	Bacq et al.[13]*	2009	France	Caucasian	50	107	38	10	2	73	30	4	86	14	176	38
	Pauli-Magnus et al.[16]*	2004	Germany	Caucasian	21	40	15	5	1	25	13	2	35	7	63	17
	Müllenbach[17]	2003	UK	Caucasian	184	65	147	33	4	42	21	2	327	41	105	25
rs1202283 (c.504T>C)	Dang et al.[8]*	2015	China	Asian	54	100	7	28	19	10	39	51	42	66	141	59
	Anzivino et al.[10]	2013	Italy	Italian	33	100	6	20	7	36	50	14	32	34	122	78
	Kitsiou-Tzeli et al.[11]	2010	Greece	Greek	11	25	6	5	0	20	5	0	17	5	45	5
	Tavian et al.[12]	2009	Italy	Italian#	10	43	1	6	3	0	0	43	8	12	0	86
	Bacq et al.[13]*	2009	France	Caucasian	50	107	2	28	20	23	53	31	32	68	99	115
	Pauli-Magnus et al.[16]*	2004	Germany	Caucasian	21	40	5	10	6	11	18	11	20	22	40	40
rs2302387 (c.175C>T)	Dang et al.[8]*	2015	China	Asian	54	100	30	19	5	65	31	4	79	29	161	39
	Anzivino et al.[10]	2013	Italy	Italian	33	100	24	9	0	72	26	2	57	9	170	30
	Tavian et al.[12]	2009	Italy	Italian#	10	43	9	1	0	43	0	0	19	1	86	0
	Bacq et al.[13]*	2009	France	Caucasian	50	107	38	10	2	76	28	3	86	14	180	34
	Pauli-Magnus et al.[16]*	2004	Germany	Caucasian	21	40	17	4	0	29	10	1	38	4	68	12
rs2230028 (c.1954A>G)	Wasmuth et al.[15]*	2007	Sweden	Caucasian**	52	52	49	3	0	42	9	1	101	3	93	11
	Piątek et al.[7]	2018	Poland	Caucasian	96	211	75	21	0	174	37	0	171	21	385	37
	Anzivino et al.[10]	2013	Italy	Italian	33	100	29	4	0	86	14	0	62	4	186	14
	Tavian et al.[12]	2009	Italy	Italian#	10	43	9	1	0	43	0	0	19	1	86	0
	Bacq et al.[13]*	2009	France	Caucasian	50	107	47	3	0	97	9	1	97	3	203	11
	Floreani et al.[14]	2008	Italy	Caucasian	96	96	94	2	0	96	0	0	190	2	192	0
	Pauli-Magnus et al.[16]*	2004	Germany	Caucasian	21	40	17	4	0	28	11	1	38	4	67	13
	Müllenbach[17]	2003	UK	Caucasian	184	65	157	27	0	58	6	1	341	27	122	8

SNP – single nucleotide polymorphism; A – mild type; B – mutant type; * SNP respected the Hardy–Weinberg equilibrium; ** intrahepatic cholestasis of pregnancy (ICP) with bile acid levels >40 µmol/L; # ICP with raised gamma-glutamyl transpeptidase (GGT).

Table 2. Meta-analysis of multidrug resistance protein 3 (MDR3) polymorphisms and intrahepatic cholestasis of pregnancy (ICP) susceptibility

SNP	Genetic model		Relevance test			Heterogeneity test			Publication bias	
			OR (95% CI)	Z	p-value	I^2	Q	p-value	p_{egger}	t
rs2109505	general	allelic model	1.31 (0.66–2.60)	1.88	0.060	89.47%	19.27	0.01	0.000	–6.95
		dominant model	0.72 (0.34–1.53)	1.99	0.047	88.30%	18.21	0.01	0.001	–5.84
		recessive model	0.68 (0.33–1.40)	2.23	0.026	23.61%	6.17	0.52	0.516	0.69
		superdominant model	1.38 (0.66–2.90)	2.10	0.036	87.13%	15.04	0.04	0.410	0.89
	Caucasian	allelic model	1.87 (1.46–2.40)	6.64	0.000	17.10%	2.06	0.72	–	–
		dominant model	0.49 (0.38–0.63)	6.56	0.000	9.04%	1.27	0.87	–	–
		recessive model	0.47 (0.22–0.99)	2.74	0.006	13.97%	2.56	0.63	–	–
		superdominant model	1.92 (1.52–2.44)	5.72	0.000	2.53%	0.58	0.96	–	–
	Italian	allelic model	0.37 (0.01–10.21)	0.58	0.564	77.98%	4.97	0.03	–	–
		dominant model	2.63 (0.07–96.09)	0.52	0.605	80.18%	5.51	0.02	–	–
		recessive model	2.01 (0.25–16.41)	0.65	0.518	1.39%	0.18	0.68	–	–
		superdominant model	0.39 (0.01–15.62)	0.49	0.626	80.93%	5.73	0.02	–	–
	Asian	allelic model	0.68 (0.39–1.20)	1.34	0.181	–	0.00	–	–	–
		dominant model	1.62 (0.82–3.20)	1.4	0.162	–	0.00	–	–	–
		recessive model	1.41 (0.30–6.55)	0.44	0.660	–	0.00	–	–	–
		superdominant model	0.64 (0.32–1.29)	1.25	0.213	–	0.00	–	–	–
rs1202283	general	allelic model	0.93 (0.19–4.66)	1.34	0.181	96.67%	22.96	0.00	0.652	0.49
		dominant model	1.61 (0.52–4.93)	1.25	0.212	74.99%	10.86	0.05	0.346	1.07
		recessive model	0.61 (0.12–3.25)	0.52	0.602	92.10%	17.59	0.00	0.422	0.89
		superdominant model	0.42 (0.12–1.42)	2.43	**0.015**	89.25%	9.67	0.09	0.051	2.75
	Caucasian	allelic model	0.66 (0.39–1.12)	1.78	0.076	31.92%	1.23	0.27	–	–
		dominant model	2.66 (0.54–13.13)	1.14	0.254	63.84%	2.96	0.09	–	–
		recessive model	1.45 (0.77–2.72)	1.22	0.222	6.09%	0.39	0.53	–	–
		superdominant model	0.81 (0.46–1.43)	1.9	0.058	0.16%	0.06	0.81	–	–
	Italian	allelic model	6.79 (0.05–1006.26)	0.7	0.486	91.25%	12.15	0.00	–	–
		dominant model	0.64 (0.02–16.32)	0.28	0.770	73.62%	4.14	0.04	–	–
		recessive model	0.12 (0.00–26.41)	0.72	0.469	91.24%	12.14	0.00	–	–
		superdominant model	0.09 (0.00–5.79)	2.44	**0.015**	85.66%	7.55	0.01	–	–
	Greek	allelic model	0.38 (0.10–1.47)	1.4	0.160	–	0.00	–	–	–
		dominant model	3.33 (0.72–15.54)	1.53	0.125	–	0.00	–	–	–
		recessive model	2.22 (0.04–118.82)	0.39	0.695	–	0.00	–	–	–
		superdominant model	0.30 (0.06–1.40)	1.83	0.067	–	0.00	–	–	–
	Asian	allelic model	0.27 (0.16–0.44)	5.27	**0.000**	–	0.00	–	–	–
		dominant model	0.75 (0.27–2.09)	0.56	0.577	–	0.00	–	–	–
		recessive model	0.52 (0.26–1.03)	1.87	0.062	–	0.00	–	–	–
		superdominant model	0.59 (0.30–1.16)	0.89	0.373	–	0.00	–	–	–

closely involved in the onset of ICP.[10] In addition, 6 *MDR3* polymorphisms, namely *rs2097937*, *rs31676*, *rs1149222*, *rs4148826*, *rs2109505*, and *rs2302386*, were significantly associated with an altered risk of ICP, especially *rs2109505* ($p = 4.6\times10^{-7}$).[9] In a case report, Kamimura et al. presented an ICP patient with the *rs1202283* polymorphism, although no strict link between this polymorphism and ICP was observed.[21] Our data confirmed that *MDR3 rs2109505* and *rs1202283* polymorphisms were closely associated with the pathogenesis of ICP. Furthermore, susceptibility to ICP was related to racial and territorial factors, as revealed in our meta-analysis. In contrast, there were no significant associations between *MDR3 rs2302387* or *rs2230028* and the risk of ICP. This makes our data consistent with the findings of a previous study.[13]

The *rs1202283* polymorphism was closely related to the pathogenesis of ICP among the Caucasian population in France. This polymorphism may affect the splicing and stability of *ABCB4* mRNA, as well as the linkage disequilibrium of other SNPs.[13] Additionally, Dixon

Table 2. Meta-analysis of multidrug resistance protein 3 (MDR3) polymorphisms and intrahepatic cholestasis of pregnancy (ICP) susceptibility – cont.

SNP	Genetic model		Relevance test			Heterogeneity test			Publication bias	
			OR (95% CI)	Z	p-value	I^2	Q	p-value	p_{egger}	t
rs2302387	general	allelic model	0.91 (0.39–2.10)	0.51	0.613	75.93%	5.34	0.25	0.507	–0.75
		dominant model	1.10 (0.46–2.59)	0.29	0.775	70.95%	4.65	0.33	0.482	–0.80
		recessive model	1.64 (0.58–4.62)	1.03	0.305	9.20%	1.34	0.85	0.106	2.29
		superdominant model	1.00 (0.66–1.52)	0.01	0.993	68.48%	3.73	0.44	0.406	–0.97
	Caucasian	allelic model	1.27 (0.70–2.33)	0.81	0.420	3.22%	0.28	0.60	–	–
		dominant model	0.73 (0.38–1.42)	0.93	0.351	0.33%	0.08	0.77	–	–
		recessive model	1.17 (0.23–5.88)	0.17	0.864	1.86%	0.20	0.65	–	–
		superdominant model	0.71 (0.35–1.41)	0.99	0.323	0.00%	0.00	1.00	–	–
	Italian	allelic model	0.48 (0.04–5.27)	0.17	0.866	58.35%	2.52	0.11	–	–
		dominant model	2.20 (0.21–23.38)	0.39	0.695	56.00%	2.35	0.12	–	–
		recessive model	1.25 (0.09–16.84)	0.05	0.958	11.57%	0.58	0.45	–	–
		superdominant model	1.30 (0.57–2.97)	0.61	0.539	53.14%	2.18	0.14	–	–
	Asian	allelic model	0.66 (0.38–1.14)	1.48	0.139	–	0.00	–	–	–
		dominant model	1.49 (0.76–2.92)	1.15	0.251	–	0.00	–	–	–
		recessive model	2.45 (0.63–9.53)	1.29	0.196	–	0.00	–	–	–
		superdominant model	1.21 (0.60–2.44)	0.53	0.597	–	0.00	–	–	–
rs2230028	general	allelic model	1.09 (0.50–2.39)	0.3	0.766	70.47%	10.54	0.16	0.880	0.16
		dominant model	0.97 (0.44–2.14)	0.04	0.970	68.02%	10.24	0.18	0.992	–0.01
		recessive model	0.76 (0.20–2.87)	0.62	0.533	10.76%	3.05	0.88	0.794	0.27
		superdominant model	0.96 (0.45–2.07)	0.38	0.706	65.08	9.39	0.23	0.025	2.98
	Caucasian	allelic model	1.25 (0.60–2.59)	0.44	0.661	62.29%	7.97	0.16	–	–
		dominant model	0.86 (0.41–1.82)	0.09	0.930	59.99%	7.72	0.17	–	–
		recessive model	0.51 (0.12–2.18)	1.02	0.309	6.36%	1.58	0.90	–	–
		superdominant model	1.06 (0.51–2.19)	0.27	0.784	56.23%	6.91	0.23	–	–
	Italian	allelic model	0.47 (0.04–5.59)	0.34	0.732	57.40%	2.45	0.12	–	–
		dominant model	2.17 (0.17–27.09)	0.35	0.729	57.48%	2.45	0.12	–	–
		recessive model	3.52 (0.21–57.83)	0.88	0.381	0.01%	0.01	0.91	–	–
		superdominant model	0.46 (0.04–5.78)	0.35	0.729	57.48%	2.45	0.12	–	–

OR – odds ratio; 95% CI – 95% confidence interval. Random-effects Sidik–Jonkman model was used, an allelic model was used for A compared to B, a dominant model was used for AB+AA compared to BB, a recessive model was used for AA compared to BB+AB, and a superdominant model was used for AA+BB compared to AB. Values in bold are statistically significant.

et al. indicated that there was a linkage disequilibrium between *rs2109505* (c.711A>T, p.I237I) polymorphism and *ABCB4* SNPs,[9] which was considered to be associated with the pathogenesis of ICP in a Swedish population. For the *ABCB4* gene, *MDR3 rs2109505* and *rs1202283* were the most common SNPs. They may affect the special conformation and substrate specificity of the transporter, which then affect the transmission of phospholipids through the MDR3 protein.[21] Furthermore, *MDR3 rs2109505* and *rs1202283* mutations could trigger the dysfunction of the MDR3 transporter, which leads to the decline of phospholipid and phosphatidylcholine in the bile. This would result in the proportion of toxic bile acid, which then leads to injuries of the hepatobiliary cells and an increase in circulating bile acids.

To date, many studies have focused on the association between severe ICP and perinatal outcomes. In an excellent study by Ovadia et al., the authors reported an increased risk of stillbirth for women with serum total bile acids of 100 μmol or more.[22] Cui et al. indicated that women with ICP who also showed serum total bile acids of 40 μmol or more were reported to present an increased risk of preterm birth compared to their counterparts with lower bile acids.[23] Moreover, those with severe ICP were associated with fetal cardiac arrhythmia and placental vessel spasms.[24,25] Based on these data, together with our analysis, we speculate that severe ICP may be associated with *MDR3* polymorphisms.

Limitations

There are some limitations to our study. First, there was a publication bias for the *rs2109505* polymorphism in both allelic and dominant gene models, as well as a bias

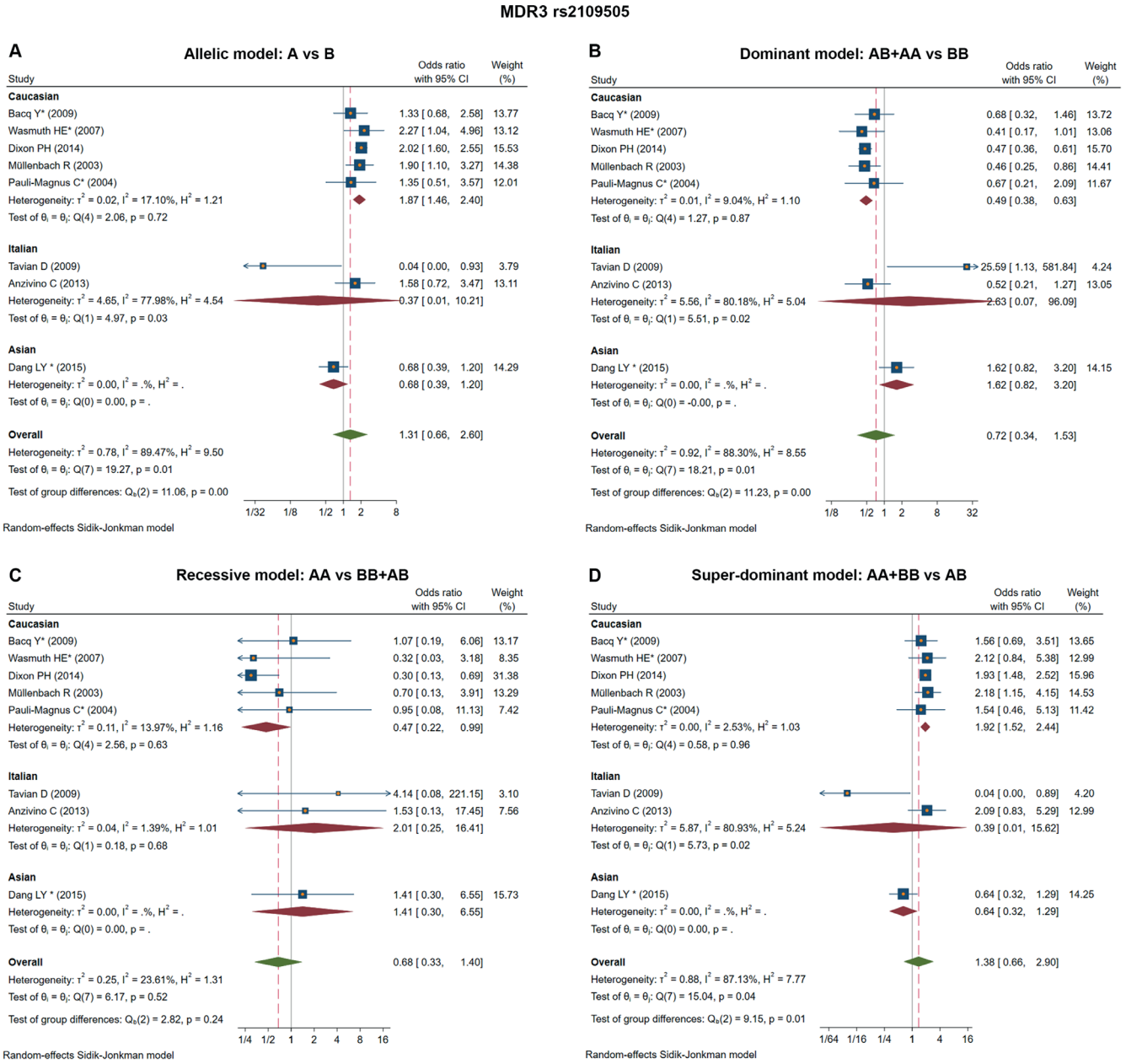

Fig. 2. Forest plot of effect estimates for *MDR3 rs2109505* polymorphism (c.711A>T) compared to intrahepatic cholestasis of pregnancy (ICP) risk in 4 models. A. Allelic model: A vs. B; B. Dominant model: AB+AA vs. BB; C. Recessive model: AA vs. BB+AB; D. Superdominant model: AA+BB vs. AB

95% CI – 95% confidence interval; A – mild type; B – mutant type.

for *rs2230028* in the superdominant model. Second, only 4 studies (40%) on *rs2109505* and 3 (37.5%) on *rs2230028* calculated the Hardy–Weinberg equilibrium (HWE). Moreover, the case groups from 2 gene loci consisted of ICP patients with bile acid levels higher than 40 μmol/L or had elevated levels of gamma-glutamyl transferase (GGT). These 2 conditions were determined as the specific subgroups of ICP, and the rate of *MDR3* mutations was higher than in common ICP patients. We suspect that population bias present in this population, in combination with the violation of the HWE contributed to the publication bias in our meta-analysis. In addition, only 9% of the included studies elucidated the *MDR3 rs1202283* polymorphism and its impact on ICP risk in the Greek population. In the future, more studies are needed to further investigate the impact of racial and regional factors on the relationship between *MDR3* polymorphisms and ICP risk. Furthermore, there was no exploration of the influence of nongenetic factors on ICP susceptibility.

Conclusions

Our meta-analysis indicated that *MDR3 rs2109505* and *rs1202283* gene polymorphisms were associated with ICP

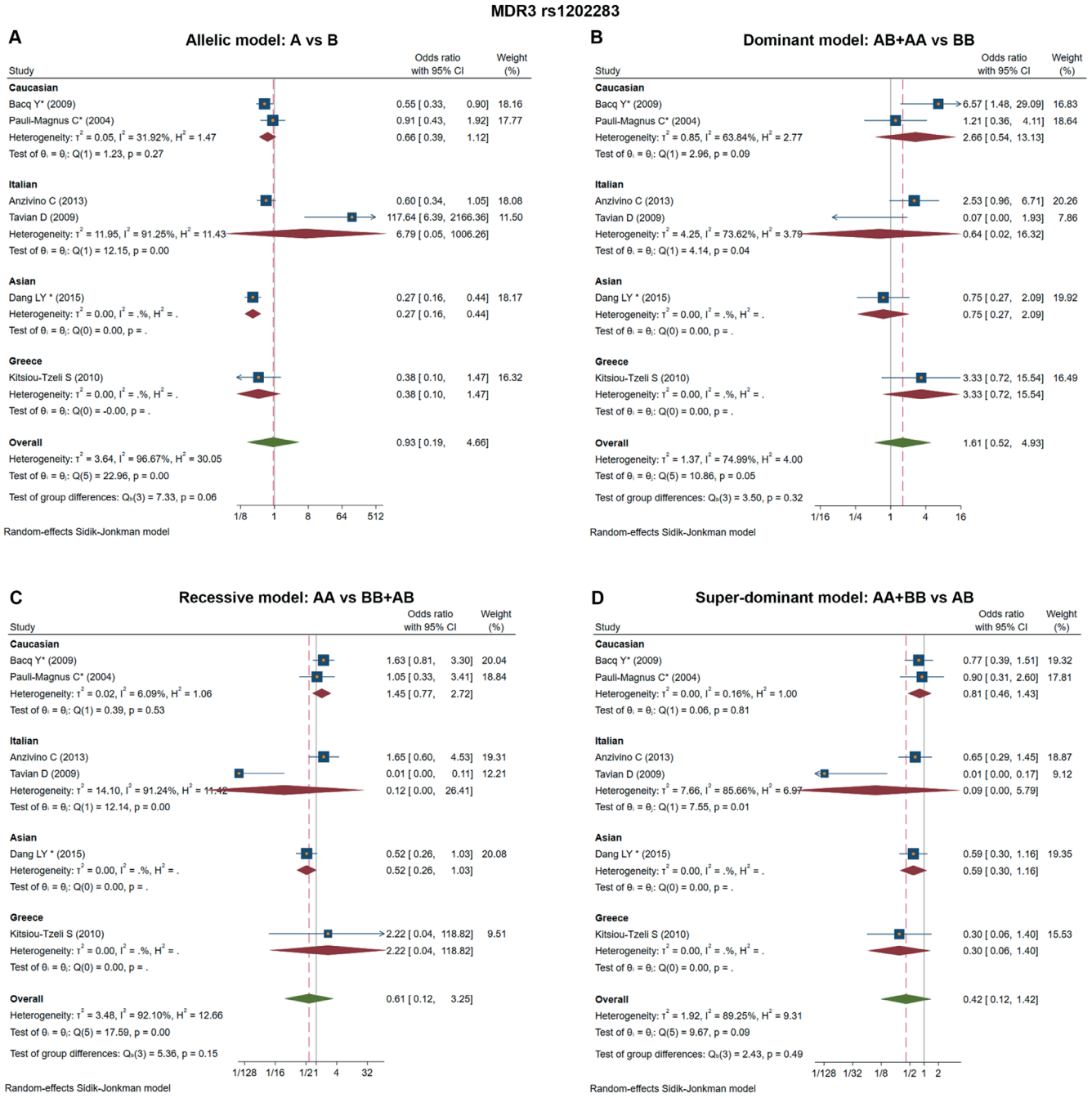

Fig. 3. Forest plot of effect estimates for *MDR3 rs1202283* polymorphism (c.504T>C) compared to intrahepatic cholestasis of pregnancy (ICP) risk in 4 models. A. Allelic model: A vs. B; B. Dominant model: AB+AA vs. BB; C. Recessive model: AA vs. BB+AB; D. Superdominant model: AA+BB vs. AB

95% CI – 95% confidence interval; A – mild type; B – mutant type.

susceptibility. Further studies are required to explore alternative etiologies for ICP susceptibility, such as ethnic, racial, regional, and nongenetic factors.

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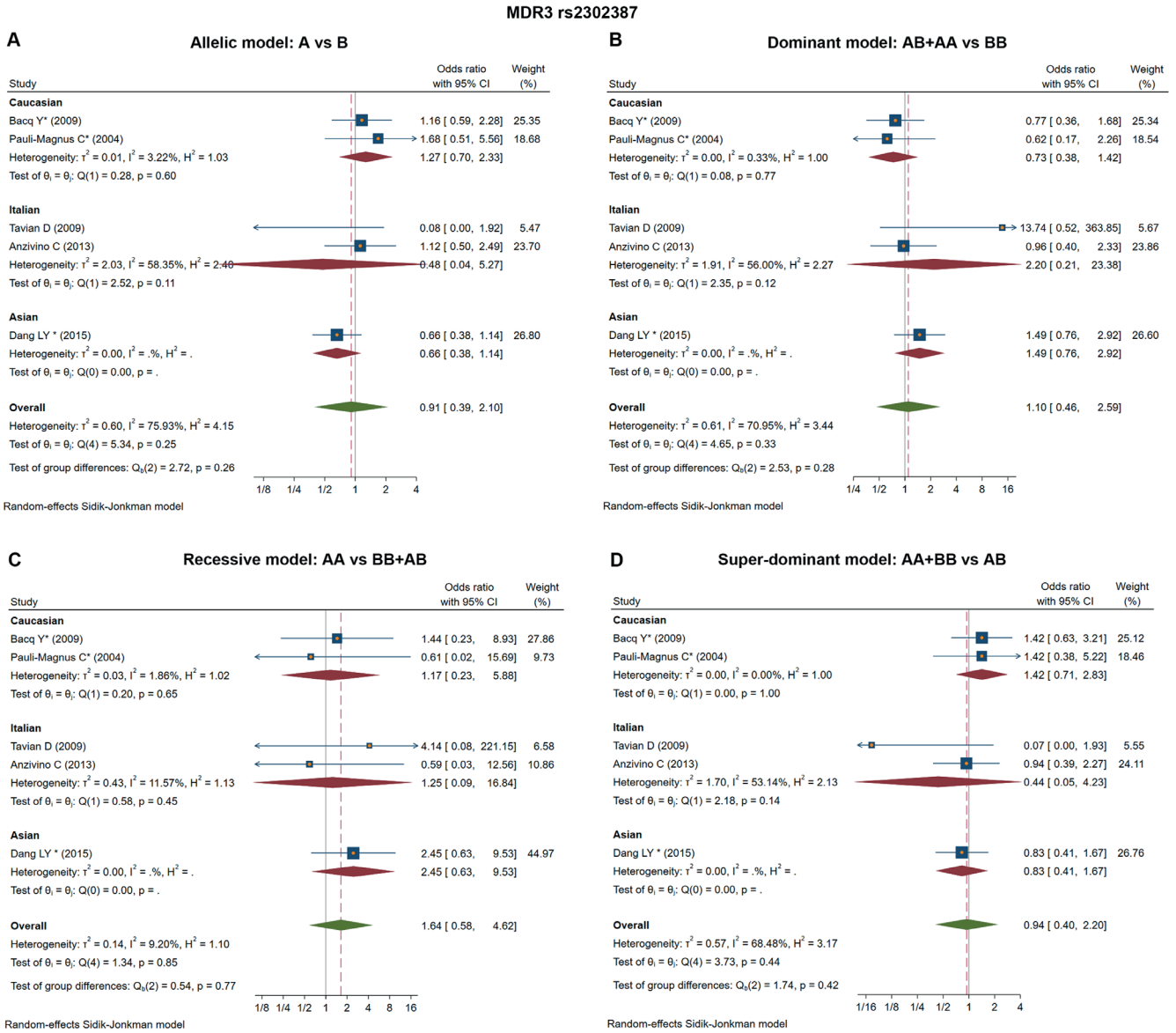

Fig. 4. Forest plot of effect estimates for *MDR3 rs2302387* polymorphism (c.175C>T) compared to intrahepatic cholestasis of pregnancy (ICP) risk in 4 models. A. Allelic model: A vs. B; B. Dominant model: AB+AA vs. BB; C. Recessive model: AA vs. BB+AB; D. Superdominant model: AA+BB vs. AB

95% CI – 95% confidence interval; A – mild type; B – mutant type.

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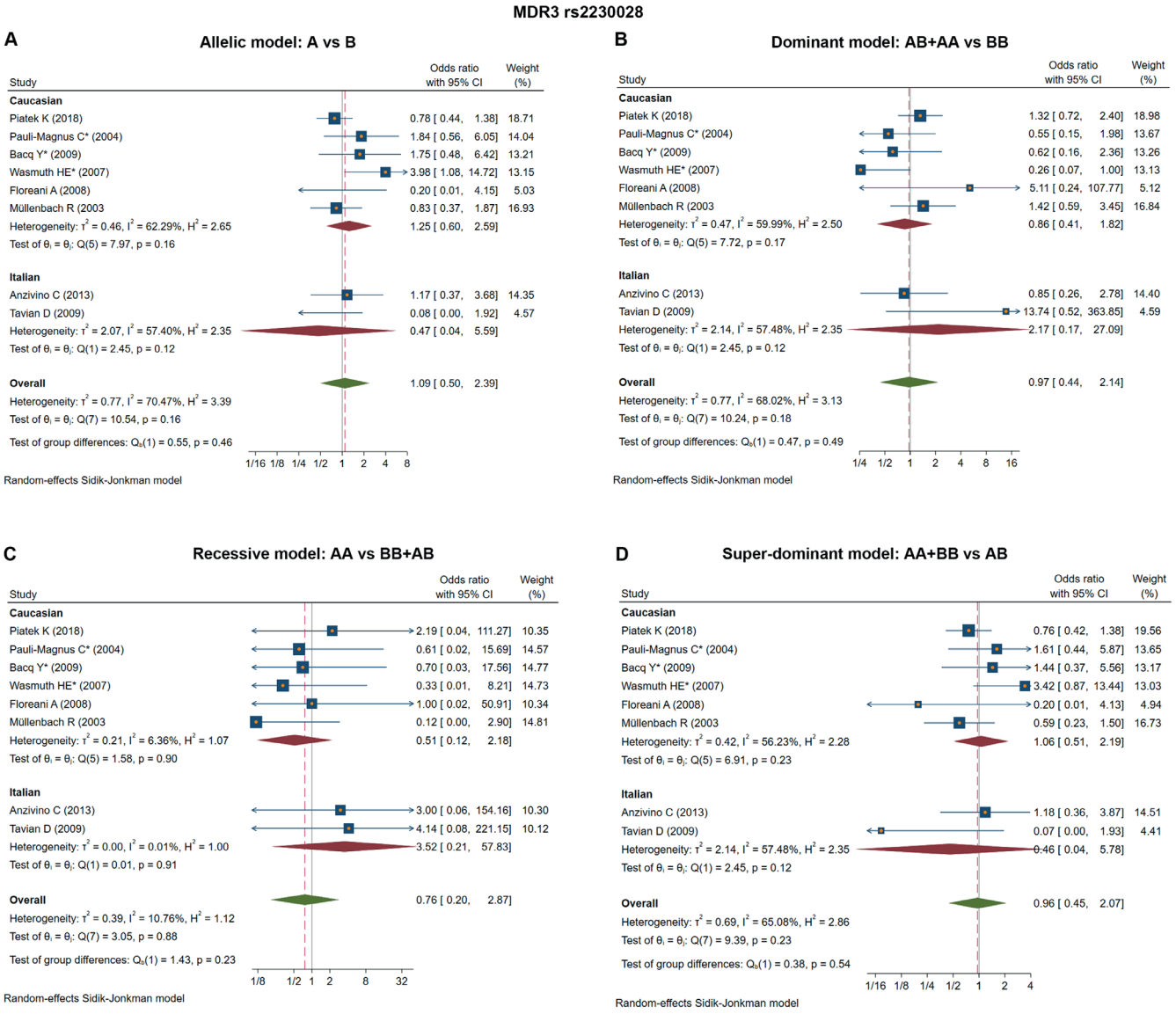

Fig. 5. Forest plot of effect estimates for *MDR3 rs2230028* polymorphism (c.1954A>G) compared to intrahepatic cholestasis of pregnancy (ICP) risk in 4 models. A. Allelic model: A vs. B; B. Dominant model: AB+AA vs. BB; C. Recessive model: AA vs. BB+AB; D. Superdominant model: AA+BB vs. AB

95% CI – 95% confidence interval; A – mild type; B – mutant type.

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Evaluating the influence of health literacy and health-promoting COVID-19 protective behaviors on the spread of infection during the COVID-19 pandemic: A meta-analysis

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Abstract

Background. Health literacy and self-efficacy related to COVID-19 pandemic management are closely linked. Therefore, synthesis of relevant evidence regarding the positive aspects of health literacy and health-promoting protective measures among individuals during COVID-19 pandemic is necessary.

Objectives. To determine the influence of e-health literacy and health-promoting coronavirus disease 2019 (COVID-19) protective behaviors on the spread of infection during the COVID-19 pandemic.

Materials and methods. Following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, PubMed, MEDLINE, PsycINFO, EMBASE, and Cochrane Library databases, as well as PROSPERO and ClinicalTrials.gov registry platforms were searched for eligible literature published from January 2020 to July 2022. Studies were included based on predefined Population, Intervention, Comparison, Outcomes and Study design (PICOS) criteria, and a summary of each study was prepared. To estimate the effect size, the standardized mean difference (SMD) of the evaluated parameters, e-health literacy and health-promoting COVID-19 protective behaviors was extracted. Using RevMan and MedCalc software, a meta-analysis was performed.

Results. Twelve eligible studies involving a total of 9854 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected individuals were included in the meta-analysis. The pooled SMD for e-health literacy was 40.39 (95% confidence interval (95% CI): 28.14–52.63), with the following heterogeneity values: Tau^2 of 396.80, χ^2 of 669.48, degrees of freedom (df) of 11, I^2 of 98%, Z value of 6.47, and $p < 0.001$. Similarly, the pooled SM for COVID-19 protective behaviors was 15.90 (95% CI: 10.96–20.84) with Tau^2 of 55.25, χ^2 of 252.92, df of 11, I^2 of 98%, Z of 6.31, and $p < 0.001$.

Conclusions. This study confirmed that e-health literacy and health-promoting COVID-19 protective behaviors have a strong positive impact on preventing the spread of COVID-19 infection and on its effective management. We recommend that interventions and applicable policies for promoting such e-literacy programs and preventative measures be given a high level of consideration.

Key words: e-health literacy, COVID-19, pandemic, COVID-19 protective behaviors, health-related behavior

Introduction

The nearly 3-year-long coronavirus disease 2019 (COVID-19) pandemic has had a significant impact on societies throughout the world. Globally, approx. 609 million confirmed cases of COVID-19 and approx. 6.5 million deaths have been reported as of September 2022.[1] The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is transmitted primarily through the respiratory droplets of SARS-CoV-2-infected patients or a direct contact with asymptomatic individuals.[2,3] Based on the reported evidence, the World Health Organization (WHO) issued national guidelines in numerous countries recommending the implementation of COVID-19 protective behaviors for droplet and contact precautions.[4,5] These preventative actions were suggested for the effective suppression of virus transmission and flattening of the COVID-19 pandemic curve. Health-promoting COVID-19 protective behaviors or preventative measures include maintaining an appropriate physical distance, regularly washing hands to ensure proper hand hygiene and wearing a face mask to prevent virus entry through nasal routes. These protective measures are highly effective at preventing viral transmission and infection spread.[6,7] Although the COVID-19 vaccine is readily available, it is necessary to engage in these precautionary measures after vaccination to enhance its efficacy.[8,9]

Health-related education or health literacy is required to comprehend the significance of COVID-19 protective behaviors in the prevention and effective management of SARS-CoV-2 infections, as well as how and when to implement them. Health literacy familiarizes an individual with medical terms and enables them to comprehend drug prescriptions, usage instructions and doctor's instructions for the prevention of health problems and better management when the need arises. It allows a person to assess the risks and benefits of a drug, and to navigate the complexities of the healthcare system.[10] Health literacy is termed e-health literacy when it is acquired through the use of various electronic sources such as the Internet, television and social media applications.[11]

According to numerous studies, individuals with high e-health literacy are able to manage their health concerns in a better way and effectively implement COVID-19 protective behaviors. In their systematic review and meta-analysis, Li et al. reported that the preventive behaviors in response to the COVID-19 pandemic and compliance with the security regulations play a significant role in fighting COVID-19 pandemic.[12] Liang et al. also noted that precautionary behaviors are highly effective and advantageous in the effective management of the pandemic.[13] In their research on digital health literacy and online information-seeking behaviors among students during the COVID-19 pandemic, Htay et al. concluded that digital health literacy and online information play an essential role in the management and prevention of COVID-19.[14] In their cross-sectional studies, Riiser et al.[15] and Pechrapa et al.[16] concluded that health literacy and the implementation of health-promoting COVID-19 protective measures are very effective in coping with the pandemic.

Posai et al. evaluated the health-promoting behaviors of hospitalized patients with non-communicable diseases during the 2nd wave of COVID-19 and reported the role of such behaviors in preventing the infection and its spread among patients.[17] Likewise, many researchers conducted cross-sectional studies assessing the influence between e-health literacy and self-efficacy levels on implementing COVID-19 protective behaviors in individuals from different age groups during the pandemic.[18–26] They confirmed the positive impact of effective disease management on preventing the infection.

Objectives

In light of the significance of these parameters, the purpose of the present meta-analysis was to synthesize the relevant evidence regarding the positive results of health literacy and health-promoting protective measures among individuals from various age groups during the COVID-19 pandemic. In addition, the influence of these variables on the spread of infection and the social and cultural behavior of individuals during the COVID-19 pandemic was evaluated.

Materials and methods

Criteria for inclusion and exclusion

This study included articles published between January 2020 and July 2022 that describe the impact of health literacy and health-promoting COVID-19 protective behaviors on individuals during the COVID-19 pandemic.[15–26] Only full-text articles were included, and studies with insufficient data or unrelated to the effect of health literacy and COVID-19 protective behaviors on individuals were excluded.

Information sources

This meta-analysis is based on a comprehensive search based on PRISMA guidelines using, PubMed, MEDLINE, PsycINFO, EMBASE, and Cochrane Library databases, as well as PROSPERO and ClinicalTrials.gov registry platforms, conducted from January 2020 to July 2022.

Search strategy

The following terms were used to search for relevant studies: "COVID-19", "coronavirus infection", "pandemic", "health literacy", "COVID protective behavior", "meta-analysis",

and "health-related behavior". Regardless of language, publication status or study type, all included articles were chosen in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. All studies were chosen randomly based on the Population, Intervention, Comparison, Outcomes and Study design (PICOS) criteria (prospective, retrospective, cross-sectional, or observational study). The inclusion criteria were as follows: 1) randomized controlled trials (RCTs) examining the effect of health literacy on individuals and RCTs that evaluated the effect of health-promoting behaviors during the COVID-19 pandemic; 2) standardized mean differences (SMDs) in COVID-19 management behaviors between a study group and a control group regarding the basis of their health literacy and implication of health-promoting COVID-19 protective behaviors were assessed as the primary outcome. Exclusion criteria encompassed clinical trials with patients already affected by COVID-19 and follow-up periods of less than a month. From the included studies, a concise study summary and event data were extracted.

Selection process

Two authors (LY and CP) independently searched the appropriate sources for related studies. The primary focus was on the full-text articles; abstracts were only used if they contained adequate information for the meta-analysis. Outdated references were removed, and in their place, relevant research was incorporated as required by the inclusion criteria. In addition, 2 researchers (SR and RC) collected event data containing important variables independently from each study.

Data collection process

The methodological validity of the included research was independently assessed by 2 authors (LY and CP). Moreover, they evaluated the heterogeneity of the included experiments. One author (MKS) was responsible for resolving any difference of opinion between the other authors (LY and CP). With the assistance of RevMan software 5 (https://training.cochrane.org/online-learning/core-software/revman/revman-5-download), we were able to calculate Deek's funnel plot, the Cochran's Q statistic and the I^2 index in random bivariate mode to assess the heterogeneity.

The source of heterogeneity

The use of full-text publications as opposed to abstracts was investigated for heterogeneity. Other sources of heterogeneity included differences in the age of patients, number of patients, types of scales used to evaluate infections, and results of the studies.

Risk of bias assessment

The assessment of the potential for bias in the included studies was carried out, and the accompanying risk of bias summary and risk of bias graph were performed using the RevMan software 5.[27]

Statistical analyses

To evaluate the impact of preventive practices and health literacy among people of varying ages on the course of the COVID-19 pandemic, the mean scores of preventive behaviors and health literacy were taken along with their standard deviations (SDs), and the SMD and 95% confidence intervals (95% CIs) were calculated. The mean preventive practice score was calculated for the important preventive behaviors, such as using a tissue to cover one's nose and mouth when sneezing or coughing, disposing of used tissues in the trash, not touching one's eyes, nose or mouth with unwashed hands, and washing one's hands with soap and water as soon as possible after engaging in any of the aforementioned activities. The SMD of the parameters expressing health literacy and health-promoting COVID-19 protective behaviors, as well as their respective 95% CIs, were calculated using the Mantel–Haenszel method[28] with random bivariate effects utilizing RevMan software 5 along with their respective forest plots. This was done in conjunction with the parameters' respective forest plots. The Tau^2, χ^2, I^2, and Z values were utilized in an analysis to determine the degree of heterogeneity present in the included studies. If the p-value was less than 0.05, the study was regarded as statistically significant. The RevMan software 5 was used to perform the meta-analysis. Publication bias in the included studies was analyzed using Begg's and Egger's tests,[29] and a funnel plot was generated using MedCalc software v. 20.218 (MedCalc Software Ltd., Ostend, Belgium) by graphing the proportion of each study against its standard error (SE).[30] Using MedCalc software, the Bland–Altman plot[31] was prepared to examine the level of agreement between the RCTs on health literacy and COVID-19 protective behaviors, and a box and whisker plot[32] was utilized to compare the 2 parameters' respective levels of effectiveness.

Results

Results of a literature search

According to the PICOS criteria[33] listed in Table 1, we identified a total of 1237 studies through electronic searches of various databases. We removed 242 studies as they did not pertain to the topic of interest, leaving 995 entries to be examined. In addition, erroneous references and duplications led to the exclusion of 724 papers, leaving only 271 for final screening. Out of the 271 studies, 215

Table 1. PICOS search

P (patient, problem, population)	individuals from different age groups during COVID-19 pandemic
I (intervention)	evaluation of the impact of health literacy and health protective measures on persons from different age groups during COVID-19 pandemic
C (comparison, control or comparator)	The impact of health literacy and COVID-19 protective behaviors on the individuals from varied age groups during COVID-19 pandemic was assessed on the basis of eHEALS, HELIA scores, PBarS, HPBS, and SSS.
O (outcome/outcomes)	There is a strong correlation between health literacy and COVID-19 protective behaviors. Both factors have a strong positive impact on the prevention and management of COVID-19. E-health literacy provides better access to beneficial healthcare services, preventive material and health information. Likewise, protective behavior helps in the effective management of COVID-19 infection.
S (study type)	cross-sectional studies, randomized controlled trials, observational studies

COVID-19 – coronavirus disease 2019; eHEALS – eHealth Literacy Scale; HELIA – Health Literacy Instrument for Adults; PBarS – Perceived Barrier Scale; HPBS – Health-Promoting Behaviors Scale; SSS – Social Support Scale

were removed based on the inclusion criteria, and the eligibility of the remaining 56 studies was further evaluated. Insufficient evidence and improper comparison criteria to create 2×2 tables for review were the primary reasons for the omission. Figure 1 depicts the study flowchart according to the PRISMA recommendations. Twelve publications that met the inclusion criteria, i.e., were relevant to the issue and supplied sufficient data to produce a 2×2 table, were used in the meta-analysis. The twelve included trials, published from 2020 to 2022, included a total of 9854 participants of various ages during the COVID-19 pandemic, and the effects of health literacy and health-promoting COVID-19 protective behaviors on these individuals were compared. Individuals were assigned to the interventions at random, and the influence of these factors on the prevention of the spread of COVID-19 infections and its successful management were examined and analyzed statistically.

Table 2 provides a concise description of the studies included in this meta-analysis. It contains the study's first author, year of publication, journal of publication, type of study, age of the patients, the purpose of the study, male/female (M/F) ratio, sample size, study location, evaluation scale, intervention results, study conclusion, and p-values indicating statistical significance of the data.

Risk of bias assessment

The assessment of the risk of bias for the included studies is provided in Table 3. The RevMan 5 software was used to conduct the risk of bias analysis, and we determined

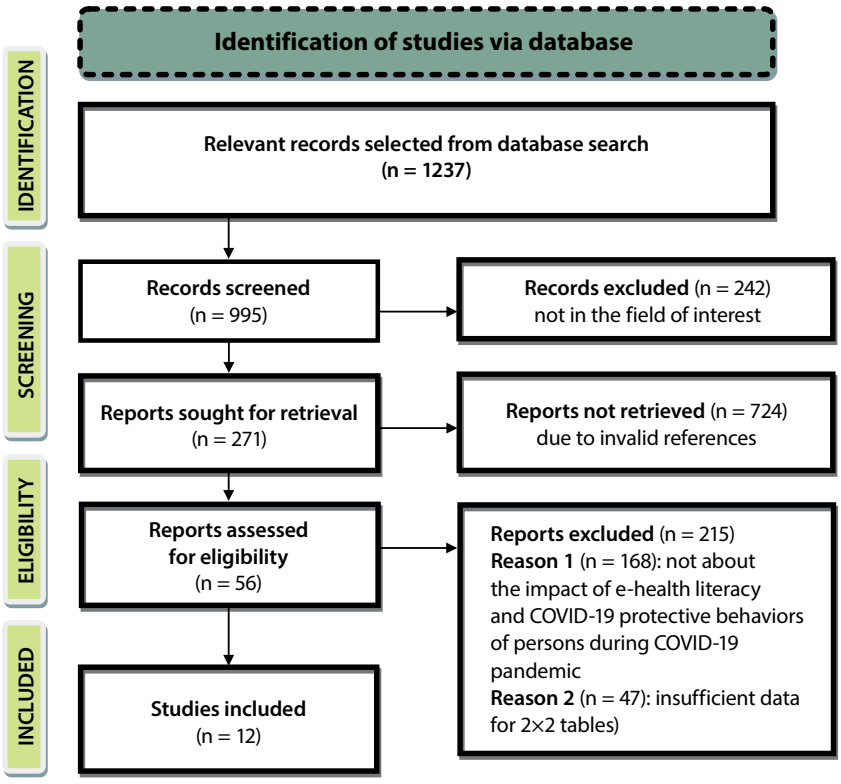

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for the included studies

COVID-19 – coronavirus disease 2019.

Table 2. Brief summary of the included studies

Study ID and year	Journal	Type of study	Sample size	Country	Aim of the study	Evaluation scale	Age of the patients [years]	Gender M/F	Results		Conclusions	p-value
									health literacy	COVID-19 protective behaviors		
Riiser et al., 2020[15]	*PLoS One*	cross-sectional study	2205	Iran	importance of health literacy and health protective measures among adolescents during COVID-19 pandemic	HLSAC scale	16–19	1819/379	14.1 ±1.6	11.9 ±2.6	A positive correlation exists between health literacy and protective measures among adolescents during the COVID-19 pandemic.	<0.05
Pechrapa et al., 2021[16]	*Annals of Geriatric Medicine and Research*	cross-sectional study	421	Thailand	importance of health literacy among older adults during COVID-19 pandemic	5-point Likert scale	60–80	157/264	2.53 ±1.47	2.62 ±1.66	Health literacy is important among older adults in order for them to have good access to healthcare services, preventive material and health information.	<0.001
Posai et al., 2021[17]	*Journal of Multidisciplinary Healthcare*	cross-sectional study	250	Thailand (Bangkok)	evaluation of the health-promoting behaviors of hospitalized patients with non-communicable diseases during the 2nd wave of COVID-19	PBarS, HPBS, SSS	>18	105/145	106.09 ±4.66	16.64 ±1.34	Health literacy and health-promoting behaviors are beneficial for preventing the SARS-CoV-2 infection and COVID-19 spread.	<0.001
Pourfridoni et al., 2022[18]	*Brain and Behavior*	observational study	278	Iran	evaluation of health literacy and fear among patients with COVID-19	HELIA scores	18–30	86/192	128.83 ±18.1	43.78 ±8.56	There is a significant negative association between health literacy and fear of COVID-19 among medical students.	<0.001
Suksatan et al., 2021[19]	*Annals of Geriatric Medicine and Research*	cross-sectional study	415	Thailand	association between health literacy, self-care behavior and blood sugar level among older patients during COVID-19 pandemic	HLS, BSCA	50–80	139/276	2.68 ±0.64	4.0 ±0.33	Increased health literacy in patients with diabetes would improve self-care behavior and, consequently, decrease their blood sugar level.	<0.001
Zahirian Moghadam et al., 2022[20]	*Frontiers in Public Health*	cross-sectional study	380	Iran	to identify the role of e-health literacy and other cognitive factors for developing protective behaviors among COVID-19 patients	eHEALS	20–50	139/244	14.95 ±7.68	5.40 ±3.33	People with high socioeconomic levels had better e-health literacy and COVID-19 protective behaviors.	<0.05
Nakayama et al., 2022[21]	*JMIR Formative Research*	cross-sectional study	3914	Japan	evaluation of effects of COVID-19 protective behaviors and e-health literacy on Japanese adults	HLS	20–69	1953/1961	27.4 ±9.4	4.1 ±1.2	Comprehensive health literacy is necessary for COVID-19 preventive behaviors and making appropriate decisions.	<0.001

Table 2. Brief summary of the included studies – cont.

Study ID and year	Journal	Type of study	Sample size	Country	Aim of the study	Evaluation scale	Age of the patients [years]	Gender M/F	Results		Conclusions	p-value
									health literacy	COVID-19 protective behaviors		
Polat and Karasu, 2022[22]	*Political Research Quarterly*	randomized controlled experimental study	140	Turkey	effect of health promotion training provided to adults on healthy lifestyle behaviors	Health Lifestyle Behavioral Scale-I[34]	>65	80/60	136.17 ±19.60	107.22 ±21.09	Healthy lifestyle behaviors of elderly individuals improved because of the health promotion training.	<0.001
Tesfa et al., 2022[23]	*JMIR Formative Research*	cross-sectional study	383	Ethiopia	evaluation of effects of COVID-19 protective behaviors and e-health literacy among health professionals during the COVID-19 pandemic	eHEALS	20–30	239/144	29.21 ±7.08	3.70 ±1.4	COVID-19 protective behaviors and e-health literacy among health professionals help them to take all the preventive measures and minimize the risk of COVID-19 spread.	<0.05
Wang et al., 2022[24]	*International Journal of Environmental Research and Public Research*	cross-sectional study	425	China	association of e-health literacy with health-promotiong behaviors among older people	eHEALS, GSE	>60	213/212	16.54 ±4.17	60.34 ±6.9	E-health literacy and health promotion behaviors play vital role in self-efficacy and self-care ability; there is a positive correlation between both factors among older adults.	<0.01
Yusefi et al., 2022[25]	*BMC Women's Health*	cross-sectional study	465	Iran	role of health literacy and health-promotiong behaviors among inpatient women during the COVID-19 pandemic	HELIA scores	20–50	465 females	64.41 ±11.31	112.23 ±16.09	Educational planning helps promote health literacy and supports health-promoting behaviors among women in order for them to adopt a healthy lifestyle.	<0.001
Sögüt et al., 2022[26]	*Journal of Nursing Research*	cross-sectional study	578	Turkey	assessment of relationship between e-health literacy and self-efficacy levels in midwifery students during the COVID-19 pandemic	eHEALS, OTSES	<20	578 females	28.44 ±7.13	11.82 ±3.04	E-health literacy and the use of online technologies during midwifery education curriculum enable the midwives to increase the quality of healthcare and improve patient safety.	<0.05

COVID-19 – coronavirus disease 2019; HLSAC – Health Literacy in School-Aged Children; PBarS – Perceived Barrier Scale; HPBS – Health-Promoting Behaviors Scale; SSS – Social Support Scale; HELIA – Health Literacy Instrument for Adults; HLS – Health Literacy Scale; BSCA – blood sugar control assessment; eHEALS – eHealth Literacy Scale; GSE – General Self-Efficacy Scale; OTSES – Online Technologies Self-Efficacy Scale; SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2.

Table 3. Risk assessment for included studies

Study ID and year	Was a consecutive or random sample of patients enrolled?	Did the study avoid inappropriate exclusions?	Did all patients receive the same reference standard?	Were all patients included in the analysis?	Was the sample frame appropriate to address the target population?	Were the study participants sampled in an appropriate way?	Were the study participants and the setting described in detail?	Were valid methods used for the identification of the condition?	Was the condition measured in a standard, reliable way for all participants?	Was there appropriate statistical analysis performed?
Riiser et al., 2020[15]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Pechrapa et al., 2021[16]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Posai et al., 2021[17]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Pourfridoni et al., 2022[18]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Suksatan et al., 2021[19]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Zahirian Moghadam et al., 2022[20]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Nakayama et al., 2022[21]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Polat and Karasu, 2022[22]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Tesfa et al., 2022[23]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Wang et al., 2022[24]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Yusefi et al., 2022[25]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes
Sögüt et al., 2022[26]	yes	yes	yes	no	yes	yes	yes	yes	yes	yes

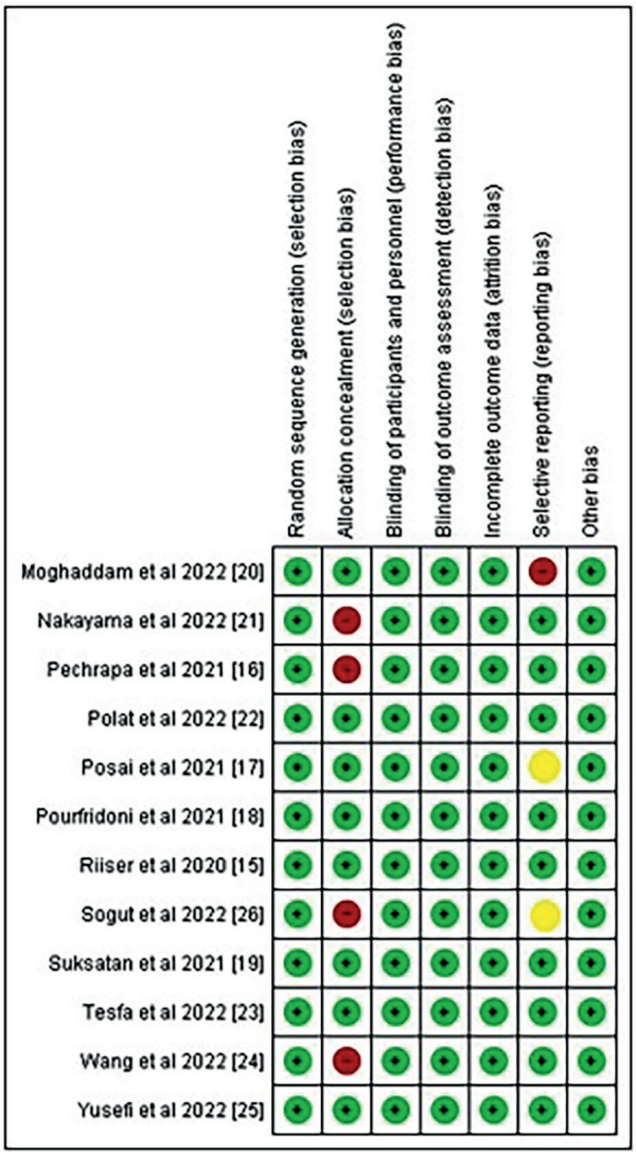

Fig. 2. Risk of bias summary

that the risk of bias was low, as evidenced by the risk of bias summary in Fig. 2 and the risk of bias graph in Fig. 3.

Meta-analysis outcomes

Using RevMan 5 software, the meta-analysis was completed. The publication bias was evaluated with MedCalc software v. 20.218. As indicated by the funnel plot (Fig. 4) and the outcomes of Egger's and Begg and Mazumdar tests, the publication bias risk in the current meta-analysis was low. Egger's regression test reveals the degree of asymmetry of a funnel plot by calculating the intercept of the standard normal regression against the precision. While Begg and Mazumdar rank correlation test explains the relationship between the ranks of effect sizes and their variances, the Pearson's rank correlation test does not. The data were considered statistically significant and at low risk of publication bias for $p < 0.05$. Since the significance threshold or p-value for both statistical tests in our meta-analysis was less than 0.05, i.e., 0.017 for Egger's test and 0.045 for Begg's test, this verifies the minimal likelihood of publication bias.[35]

The pooled SMD for health literacy (Fig. 5) was 40.39 (95% CI: 28.14–52.63), with following heterogeneity values: Tau^2 of 396.80, χ^2 of 669.48, degrees of freedom (df) of 11, I^2 of 98%, Z value of 6.47, and $p < 0.001$. Similarly, the pooled SMD for COVID-19 protective behaviors (Fig. 6) was 15.90 (95% CI: 10.96–20.84) with Tau^2 of 55.25, χ^2 of 252.92, df of 11, I^2 of 98%, Z of 6.31, and $p < 0.001$. A high SMD, greater than 1, for both examined parameters suggests that these factors are likely to have a favorable impact on the prevention and management of COVID-19.[36] Both of these criteria have comparable and substantial effects on the prevention and management of COVID-19, as visible from the size of the boxes and the wide ranges and variability of the whiskers in Fig. 7.[37] The results of all the included studies are in strong alignment, as evident from the Bland–Altman plot shown in Fig. 8. In the Bland–Altman plot, 95% of the data points were lying within ±2 s of the value of the mean differences.[38]

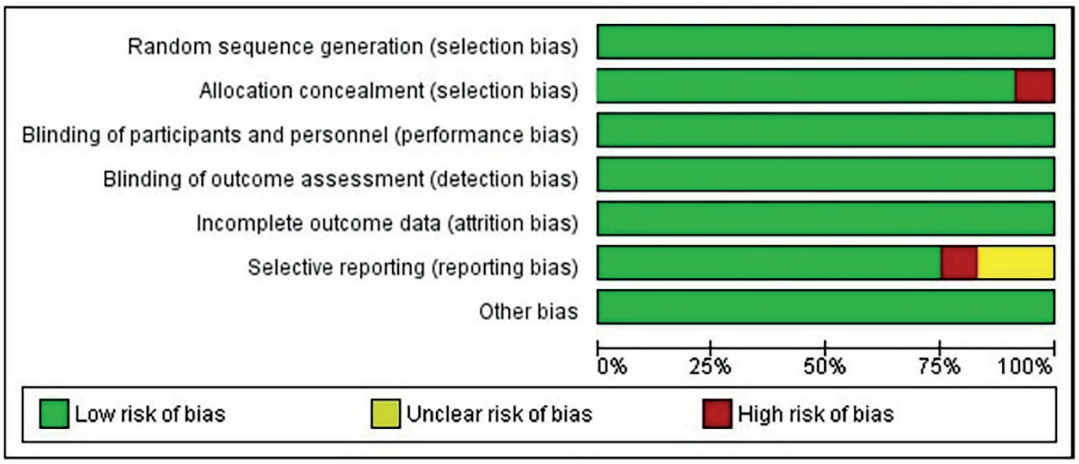

Fig. 3. Risk of bias graph

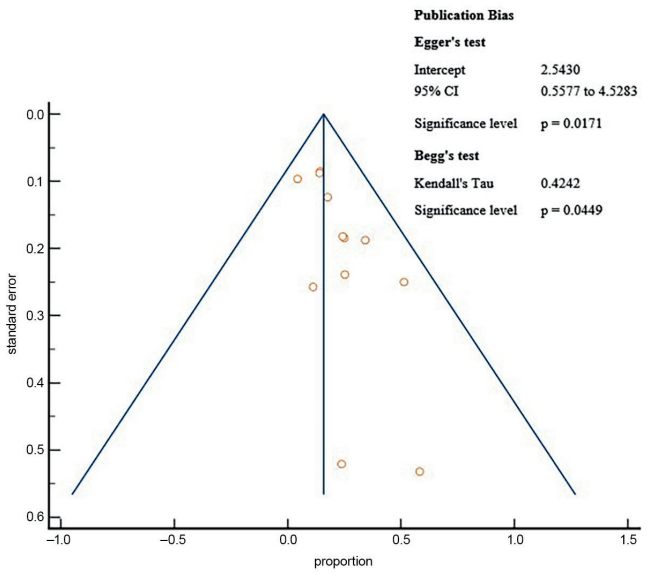

Fig. 4. Funnel plot for publication bias

95% CI – 95% confidence interval.

Taking into account all of these statistically significant meta-analysis results ($p < 0.05$), this study demonstrates that both health literacy and health-promoting COVID-19 protective behaviors contribute significantly to the proper management and prevention of spreading COVID-19 infections.

Discussion

The goal of this meta-analysis was to examine the effects of health literacy and the application of COVID-19 preventative practices among individuals of various age. The recommended preventive behaviors are hand hygiene, the use of a face mask and keeping an appropriate physical distance, in addition to protective measures such as cough covering, house disinfection and daily vitamin C supplementation.[39–41] For keeping the necessary physical distance, individuals are advised to stay at home, avoid

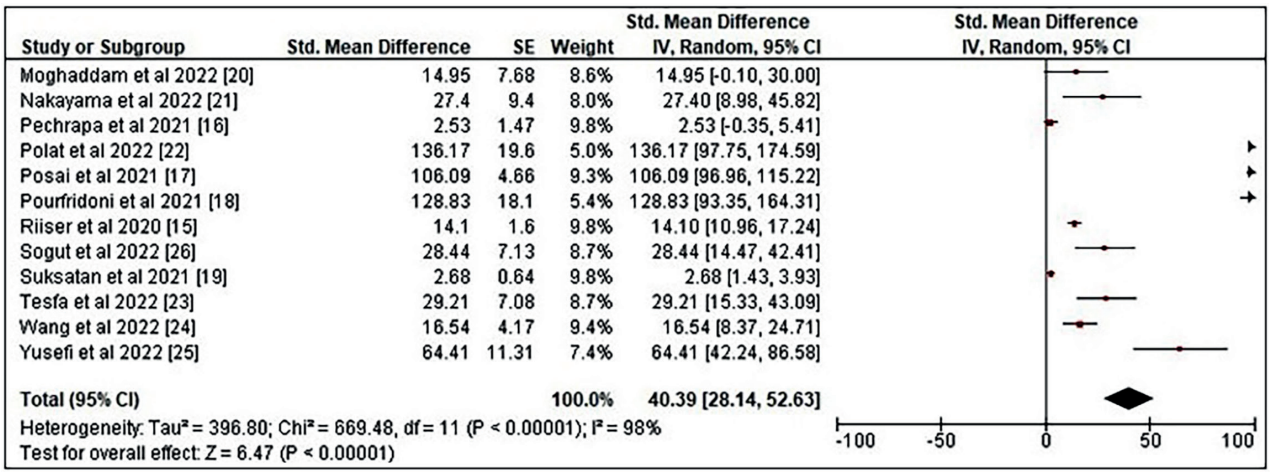

Fig. 5. Forest plot standardized mean difference (SMD) for health literacy

95% CI – 95% confidence interval; SE – standard error; df – degrees of freedom.

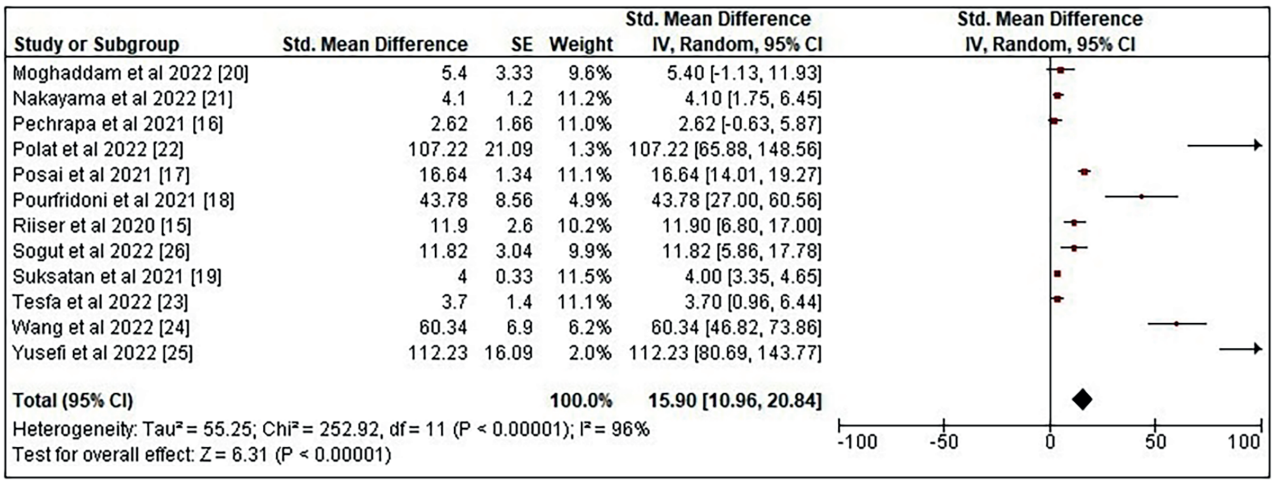

Fig. 6. Forest plot standardized mean difference (SMD) for coronavirus disease 2019 (COVID-19) protective behaviors

95% CI – 95% confidence interval; SE – standard error; df – degrees of freedom.

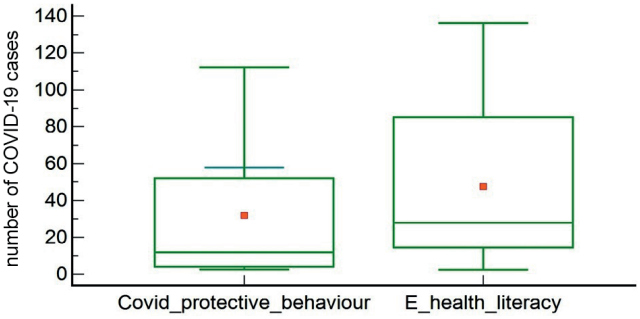

Fig. 7. Box and whisker plot showing effects of health literacy and protective behaviors on coronavirus disease 2019 (COVID-19) prevention and management

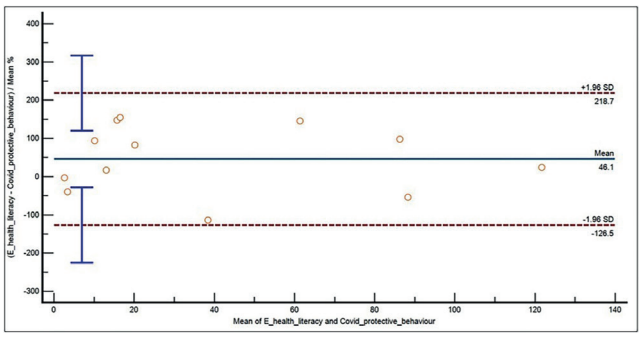

Fig. 8. Bland–Altman agreement analysis

large gatherings and crowded areas, and avoid all personal contact with others, particularly sick individuals.[42,43] Hand hygiene refers to the correct washing of hands with an alcohol-based hand rub, soap and water for a predetermined amount of time in order to remove any viruses that may have been contracted.[44,45] Health literacy is required to understand the advantages of these preventive and protective measures and their effect on health. Health literacy enables an individual to locate, comprehend and assess the value of recommended healthcare information and preventative measures in order to make informed decisions regarding their health and welfare.[46,47] In their cross-sectional study, Duong et al. found that e-health literacy helped in overcoming the dread, stress and despair brought about by the COVID-19 pandemic.[48] Similarly, Seng et al. highlighted in their review study that health literacy has a significant impact on the ability to receive and absorb vital medical information during the COVID-19 pandemic.[49] In their comprehensive review and meta-analysis, Barry et al. found that e-health literacy and the understanding of COVID-19 have a significant impact on the health management of emergency department patients.[50] Similarly, Do et al. showed the positive results of health literacy and health-related behaviors in the management of COVID-19 symptoms during the worldwide pandemic with an odds ratio (OR) of 1.08 (95% CI: 1.04–1.13) and $p < 0.001$.[51]

In the present meta-analysis, we also investigated the influence of health literacy and health-promoting COVID-19 protective behaviors during the COVID-19 pandemic. We achieved statistically significant results with $p < 0.05$ and pooled SMD of 40.39 (95% CI: 28.14–52.63) for health literacy and 15.90 (95% CI: 10.96–20.84) for COVID-19 protective behaviors. Based on these findings, we urge that interventions and related policies encouraging these health literacy programs and preventative measures be given top priority.

Limitations

The present study is limited by the fact that only English-language papers were considered, which may have resulted in a selection bias. In addition, the examination of parameters using different scales also influences outcomes to a degree. For a clearer evaluation of the effects of these parameters, it is also possible to include data from other relevant studies that mention proper documentation regarding the patient's case history, clinical issues during drug administration, post-administration side effects, and associated complications. Similarly, only e-health literacy and health-promoting protective behaviors were examined as predictors of success; however, additional criteria such as the impact of various drugs and other social or psychological problems can be added to improve the results.

Conclusions

Health literacy and health-promoting COVID-19 protective behaviors have substantial favorable effects on the management of SARS-CoV-2 infections during the COVID-19 pandemic. Understanding and implementing these guidelines contributes significantly to the prevention of virus transmission and its effective management, regardless of age or gender. Individuals with a higher level of health literacy were more likely to adhere to recommended preventive measures and sustain healthier behaviors, such as healthy diet, physical activity and timely administering medications. During the COVID-19 pandemic, in-depth understanding of health-related issues considerably increased social and cultural wellbeing, and reduced the incidence of anxiety and sadness. Based on its statistically significant results ($p < 0.05$), the present meta-analysis shows that health literacy as an essential ability that must be acquired by all individuals, and the recommended preventive behaviors must be adopted if the COVID-19 pandemic is to be handled properly.

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The value of mid-upper arm circumference for malnutrition screening in pediatric celiac disease

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Abstract

Background. Malnutrition rates in pediatric celiac disease (CD) patients range from 20.2% to 67.3%.

Objectives. To investigate the prevalence of malnutrition in pediatric CD patients in Turkey using different anthropometric measurements, including mid-upper arm circumference (MUAC).

Materials and methods. This prospective study included 124 patients aged 1–18 years with a diagnosis of CD, admitted to the Pediatric Gastroenterology Outpatient Clinic of Adana City Training and Research Hospital, Turkey. The anthropometric measurements, including weight-for-age (WFA) Z-score, height-for-age (HFA) Z-score, body mass index (BMI)-for-age Z-score, MUAC [cm], and MUAC Z-score were calculated.

Results. The study analyzed 75 female (60.5%) and 49 male (39.5%) patients with a mean age of 9.83 ±4.1 years. While 44 patients (35.5%) had malnutrition according to their BMI Z-scores, 60 patients (48.4%) had malnutrition based on their MUAC Z-scores. The number of patients with stunting (HFA value below –2) was 24 (19.4%), and the WFA value was below –2 in 27 (21.8%) patients. Furthermore, the BMI Z-score failed to identify chronic malnutrition in 70.9% of patients. There was a positive linear correlation ($r = 0.396$) between the BMI value and the MUAC value ($p < 0.001$). However, the degree of agreement between the BMI Z-scores and MUAC Z-scores was weak (κ: 0.300).

Conclusions. The MUAC Z-score successfully detected acute and chronic malnutrition and should be included in standard anthropometric measurements at follow-up nutritional assessments in CD patients.

Key words: pediatric, malnutrition, celiac disease, mid-upper arm circumference (MUAC)

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Background

Celiac disease (CD) is a chronic autoimmune gastrointestinal tract disease characterized by villus atrophy and malabsorption triggered by gluten in genetically susceptible individuals.[1] It is one of the most common genetic diseases, with the global prevalence of 1% in children.[2] Inadequate nutrient, vitamin and mineral absorption in the villi due to ongoing mucosal damage causes severe nutritional deficiencies in CD.[3] Patients with CD may also present with underlying complications such as failure to thrive and weight loss, although 10% of CD patients can be obese, and they should not be overlooked.[4] Furthermore, CD can present with various gastrointestinal complaints, such as diarrhea, cramping, bloating, flatulence, and nausea, along with other non-classical symptoms, such as iron deficiency anemia, elevated transaminases, constipation, ataxia, lethargy, osteoporosis, and dyspepsia.[5] Other complications include developmental delay, stunting, delayed puberty, weight loss, and loss of muscle mass. Due to this wide spectrum of clinical presentations in CD, there can be some delay in its diagnosis, with underdiagnoses increasing the malnutrition risk of patients. Therefore, the malnutrition status of all patients should be assessed at the first presentation and in the follow-up period.

Studies show that malnutrition rates in CD pediatric patients range from 20.2% to 67.3%,[6–8] and malnourished patients, especially those with severe malnutrition, have a higher mortality rate from childhood diseases such as diarrhea and pneumonia.[9] Therefore, accurate and timely diagnosis of children suffering from malnutrition, or those at risk, is essential in all chronic diseases, especially CD. Indeed, it is possible to protect such patients from the deleterious and irreversible effects of undernutrition on growth[10] in order to reduce the 9-fold increased risk of mortality in this population.[11]

Various anthropometric measurements have been used to identify high-risk cases of chronic diseases such as CD. Mid-upper arm circumference (MUAC) is an easy and cheap anthropometric measurement used to identify malnutrition and is a good predictor of survival in children.[12] Since 2009, the World Health Organization (WHO) has recommended using MUAC as anthropometric criterion for admission to nutrition programs.[13]

Objectives

The study aimed to investigate the prevalence of malnutrition in children with CD in Adana, Turkey, and to compare different anthropometric measurements with MUAC.

Materials and methods

Study design

The prospective study assessed the height and weight of each patient with a Harpenden Portable Stadiometer model 603VR (Holtain Ltd, Crymych, UK) and the same healthcare professional performed each measurement. Three measurements of the MUAC of the left arm were simultaneously measured by the same physician using inflexible MUAC bands, and the mean values were used for the analysis.[14]

Study setting

A total of 124 patients diagnosed with CD and aged between 1 and 18 years were followed up at the Pediatric Gastroenterology Outpatient Clinic of Adana City Training and Research Hospital between September 18, 2017 and November 30, 2022.

Participants

A pediatric gastroenterology specialist conducted the follow-up of CD patients, who had to be diagnosed according to the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria using biopsy for inclusion. Patients or legal guardians gave written informed consent to participate in the study, and patient files provided data on age, gender, age at CD diagnosis, disease duration, and laboratory parameters.

Variables

The measured height and weight parameters of the patients were evaluated using the 6D malnutrition awareness application (s://www.malnutrition.com), and weight-for-age (WFA) Z-score, height-for-age (HFA) Z-score, body mass index (BMI)-for-age Z-score, MUAC [cm], and MUAC Z-score values were calculated and recorded.[15,16] Taking into account the recommendations of the WHO, those with a WFA Z-score below –2 were considered underweight, and those with an HFA Z-score below –2 were considered stunted. According to the BMI Z-score, malnutrition was classified as mild malnutrition (from –1 to –1.99), moderate malnutrition (from –2 to –2.99) or severe malnutrition (–3 and below).

Data sources and measurement

The study used 2 versions of the MUAC band, patented in 2019, to determine the MUAC Z-score. One band was designed for infants aged 2–59 months, and the other for children aged 5–18 years. The bands contain markings for different ages, including 5, 5 1/2, 6, 6 1/2, 7, 7 1/2, 8, 8 1/2, 9, 9 1/2, 10, 10 1/2, 11, 12, 13, 14, 15, 16, 17, and 18 years, which indicate malnutrition using colors.[17]

Study size

We assumed that a mean effect size of 0.3 would be accepted as a difference in the mean of the parameters, and the sample size was calculated as 124 patients (95% power analysis) at an α significance level of 0.05. The Ethical Ethics Committee of Adana City Training and Research Hospital approved the study (approval No. 2286 issued during meeting No. 117), which adhered to the principles of the Declaration of Helsinki.

Statistical analyses

Statistical analysis of the data was performed with the IBM SPSS v. 25.0 package (IBM Corp., Armonk, USA). The Shapiro–Wilk test was used to determine whether the parameters evaluated in the study showed a normal distribution. Categorical measurements were summarized as numbers and percentages, and continuous measurements as mean ± standard deviation (M ±SD; median and quarters when the data distribution differed from normal). The χ^2 test compared categorical expressions, and Cohen's κ coefficient evaluated the differences between MUAC and BMI values. Meanwhile, Spearman's rho correlation test determined the relationship between continuous measurements. A value of $p < 0.05$ was considered statistically significant for all tests.

Results

The study included 124 patients, 75 female (60.5%) and 49 male (39.5%), which is in line with the literature on CD predominance in females.[18] The mean age of the patients was 9.83 ±4.1 years, the mean age at diagnosis was 6.39 ±3.7 years and the mean follow-up period was 41.1 ±24.5 months. Forty-four (35.5%) of the patients were malnourished based on BMI Z-score, and 60 (48.4%) had malnutrition according to their MUAC Z-score (Table 1). Furthermore, the number of patients with stunting (HFA Z-score below –2) was 24 (19.4%), and 27 (21.8%) patients obtained WFA Z-scores below –2. Table 2 presents anthropometric measurements of the patients. Twenty-five (20.2%) patients had comorbidities. Guided by the anamnesis and celiac antibody titers, it was noted that 88 patients (71%) showed complete adherence to the celiac diet.

The analysis of the correlation between malnutrition parameters revealed a significant moderate positive linear correlation ($r = 0.396$) between BMI and MUAC ($p < 0.001$) (Fig. 1). The degree of agreement between the Z-scores of BMI and MUAC was weak (κ: 0.300) (Table 3), although the positive linear correlation between HFA Z-score and MUAC values was moderate ($r = 0.268$) and significant ($p = 0.003$). Also, the WFA Z-score and MUAC values showed a moderate positive linear correlation ($r = 0.318$, $p < 0.01$) (Fig. 2,3).

Table 1. Classification of malnutrition according to body mass index (BMI) and mid-upper arm circumference (MUAC) Z-scores

Score	Nutritional status	Number	Percentage
MUAC Z-score	severe malnutrition	3	2.4
	moderate malnutrition	16	12.9
	mild malnutrition	41	33.1
	normal	35	28.2
	overweight	26	21.0
	obese	3	2.4
BMI Z-score	severe malnutrition	1	0.8
	moderate malnutrition	11	8.9
	mild malnutrition	32	25.8
	normal	65	52.4
	overweight	10	8.1
	obese	5	4.0

Table 2. The median values of the anthropometric measurements and laboratory parameters of the patients

Parameter	Median (Q1–Q3)
MUAC [cm]	19.3 (17.02–21.97)
HFA Z-score	–0.91 (–1.75–-0.15)
WFA Z-score	–1.19 (–1.89–-0.45)
BMI Z-score	–0.57 (–1.50–0.25)
Hemoglobin [g/dL]	12.8 (11.8–13.3)
MCV [fL]	79.2 (76–82.5)
Eosinophils [cells/mm^3]	200 (100–400)
PNL [cells/mm^3]	3250 (2500–4400)
Lymphocytes [/mm^3]	2650 (2100–3200)
Albumin [g/mL]	43 (41–45)
Creatine [mg/dL]	0.40 (0.31–0.47)

Q1 – 1st quartile; Q3 – 3rd quartile; MUAC – mid-upper arm circumference; HFA – height-for-age; WFA – weight-for-age; BMI – body mass index; MCV – mean corpuscular volume; PNL – polymorphonuclear leukocytes.

The analysis between laboratory values and the MUAC value showed a positive correlation between hemoglobin (Hb) ($r = 0.572$) and mean corpuscular volume (MCV) ($r = 0.387$) (both $p < 0.01$). However, there was no correlation between the malnutrition rate and gender, according to the MUAC Z-score ($p = 0.933$).

Discussion

This study determined the prevalence of malnutrition in pediatric CD patients using various anthropometric measurements, with malnutrition detected most frequently using the MUAC (48.3%). The malnutrition parameters revealed a significant moderate positive correlation between BMI and MUAC, although WFA, BMI and HFA did not recognize many malnourished patients.

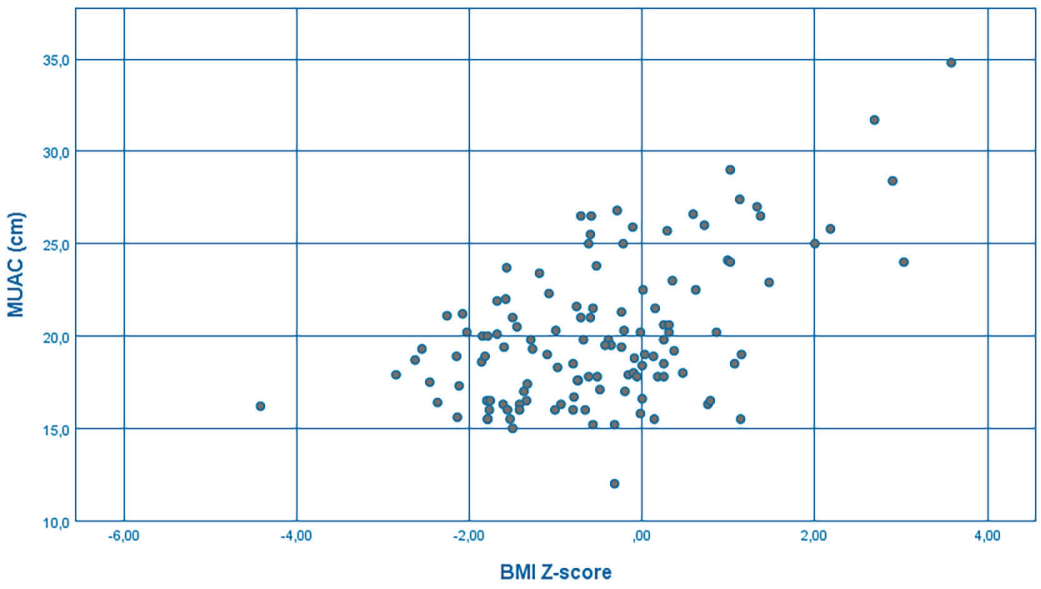

Fig. 1. Correlation between mid-upper arm circumference (MUAC) and body mass index (BMI) Z-score

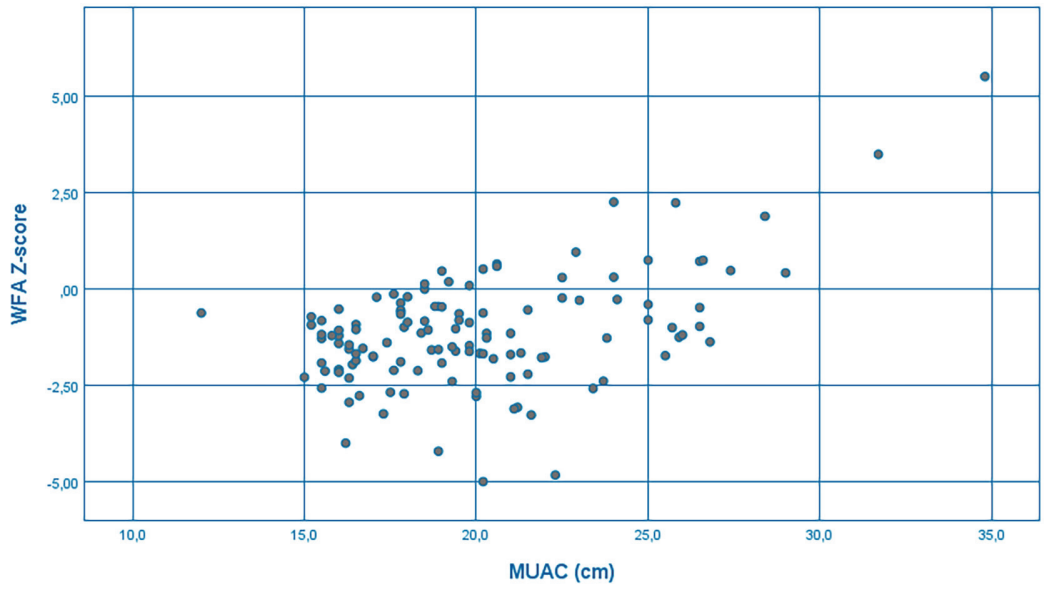

Fig. 2. Correlation between weight-for-age (WFA) Z-score and mid-upper arm circumference (MUAC)

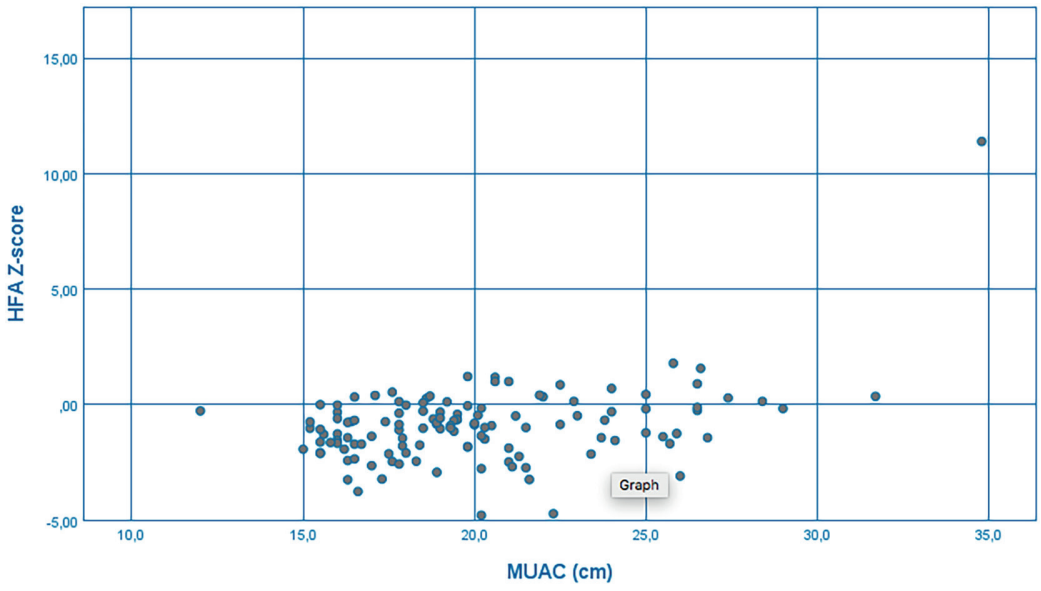

Fig. 3. Correlation between height-for-age (HFA) Z-score and mid-upper arm circumference (MUAC)

The clinical manifestations of CD are diverse, and there has been an increase in the diagnosis rate of patients presenting with subtle clinical findings due to clinicians' awareness. In patients with CD, malabsorption results from damaged villi of the small intestine and constitutes the primary cause of extraintestinal findings.[19] The majority of patients develop malnutrition due to the prolonged time to diagnosis or failure to ensure the necessary protein, vitamin and calorie intake when on a strict celiac diet.[20] The prevalence of malnutrition in children with CD varies by country, but is still high. Indeed, the rate of patients with a WFA Z-score below –2 was 22.4% in an Iranian study,[5] while another study reported low WFA in 30.8% and stunting in 21.5% of patients.[21]

There is no standard anthropometric measure to define malnutrition in the pediatric population older than 5 years, and the classifications proposed by Waterlow and Gomez et al. were not considered during the study design, as they are historical.[22,23] The most commonly used measurement methods in this group of children are WFA, HFA and BMI, which are plotted on standard percentile curves and classified according to the population. However, SD values have been used more recently as they better indicate deviation from the general child population. In 2007, the WHO recommended using the BMI-for-age Z-score, as it is considered the best indicator for identifying malnutrition in school-age children and adolescents.[24] Despite being recommended, it does not seem possible to implement this method for every patient in a standard outpatient setting, since calculating BMI and evaluating BMI Z-scores impose an additional burden on healthcare personnel. Therefore, MUAC has been used to screen for childhood malnutrition and nutritional status, as it is a more rapid and easily measurable parameter, and it was approved as an independent diagnostic criterion for malnutrition in 2007.[25] Indeed, the American Society for Parenteral and Enteral Nutrition (ASPEN) panel determined MUAC to be a more sensitive prognostic indicator for mortality than weight-for-height parameters in malnourished pediatric patients, and recommended that MUAC measurements be part of a full anthropometric assessment.

Previous studies have shown a close relationship between MUAC and BMI values in this age group.[26] Arm measurements such as MUAC and triceps skinfold thickness are better predictors of body composition and malnutrition than WFA, HFA and BMI, which do not make a distinction between muscle and fat mass.[27] The current study is the first to examine malnutrition in children with CD using the MUAC Z-score. However, the frequency of malnutrition detected using MUAC was only 48.3%. Similarly to studies in other patient groups, it was determined that the MUAC value had a moderate positive correlation with the WFA, HFA and BMI Z-scores.[28,29]

Chronic malnutrition in children is recognized using the duration of the symptoms and the HFA Z-score, with a score below –2 considered stunting by the WHO. Evaluation of the HFA Z-score in the current study showed a non-negligible incidence of stunting, as it was found in 24 patients (19%). Stunting is common in patients with CD, although its reasons remain unclear. According to several hypotheses, malnutrition, growth hormone resistance or low levels of insulin-like growth factor 1 (IGF-1) are responsible for stunting.[30–32] An Iranian study found stunting in 10% of patients, based on the WHO criteria. In the current study, patients with comorbidities were not excluded, with 25 patients (20.2%) presenting comorbidities, 10 (41.6%) of whom had stunting, which may explain the increased incidence of chronic malnutrition. Nonetheless, only 7 (29.1%) patients considered to have chronic malnutrition were malnourished according to the BMI Z-score, while 14 (58.3%) were malnourished according to the MUAC Z-score. As such, both measures appear insufficient for defining chronic malnutrition, although it appears that using the MUAC Z-score will lead to better rates of chronic malnutrition diagnosis.

Considering the parameters for classification of malnutrition demonstrate a moderate agreement between the MUAC measurment and BMI Z-scores. But the calculation of the MUAC Z score is weakly correlated with BMI Z scores. (Table 3). This discrepancy is likely due to the fact that the Z-score calculations use American children for reference.[14]

Table 3. Comparison of the nutritional status of the patients according to the MUAC Z-score and BMI Z-score

Parameter	Nutritional status	BMI Z-score (n (%))						p-value and κ
		severe malnutrition	moderate malnutrition	mild malnutrition	normal	overweight	obese	
MUAC Z-score	severe malnutrition	–	1 (9.1%)	2 (6.3%)	–	–	–	p < 0.001 κ = 0.300
	moderate malnutrition	–	6 (54.5%)	8 (25%)	2 (3.1%)	–	–	
	mild malnutrition	1 (100%)	3 (27.3%)	16 (50%)	21 (32.3%)	–	–	
	normal	–	–	4 (12.5%)	29 (44.6%)	2 (20%)	–	
	overweight	–	1 (9.1%)	1 (3.1%)	12 (18.5%)	8 (80%)	4 (80%)	
	obese	–	–	1 (3.1%)	1 (1.5%)	–	1 (20%)	

BMI – body mass index; MUAC – mid-upper arm circumference; p < 0.001, χ^2 test; κ – kappa agreement, reference range for kappa agreement values: <0 is interpreted as "less agreement than would be expected by chance alone", 0.01–0.20 as "slight agreement", 0.21–0.40 as "fair agreement", 0.41–0.60 as "moderate agreement", 0.61–0.80 as "substantial agreement", and 0.81–1.00 as "almost perfect agreement".

Limitations

Study limitations include the lack of MUAC Z-score percentiles of the Turkish population and the absence of most nutritional laboratory parameters. Also, as the study center is a tertiary hospital, CD patients included in the study may have been in worse health condition, which may have skewed the sample.

Conclusions

The prevalence of malnutrition in patients with CD was 35.5% according to the BMI Z-scores, while it was 48.3% according to the MUAC Z-scores. In addition, 19% of the patients had chronic malnutrition based on the HFA measurements, and BMI Z-score failed to identify chronic malnutrition in 70.9% of patients. For this reason, in the group of patients at high risk of malnutrition, nutrition should be evaluated in detail at each follow-up. Moreover, multiple anthropometric measurements should be performed, and the necessary nutritional support should be provided early to prevent complications.

Supplementary data

The supplementary files are available at https://doi.org/10.5281/zenodo.7725284. The package contains the following files:

Supplementary File 1. SPSS data.

Supplementary File 2. Supplementary test of normality and Shapiro–Wilk tests.

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Suicide attempts among children and adolescents admitted to a Polish Emergency Department: Analysis of epidemiology, circumstances and methods of 154 cases

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Abstract

Background. Suicide attempts among pediatric patients are currently one of the most critical issues in modern psychiatry and emergency medicine, and constitute a serious public health problem that affects people of almost every age group. It is often emphasized that attempted suicide is a cry for help, and according to international studies, the pandemic year of 2020 massively impacted the frequency of suicide attempts among children. However, such studies have yet to appear in Poland.

Objectives. To characterize the frequency, circumstances and methods of suicide attempts among children and adolescents, and investigate their relationship with coronavirus disease 2019 (COVID-19).

Materials and methods. The study retrospectively analyzed the medical records of 154 children admitted to the Emergency Department due to suicide attempts between January 2020 and June 2021.

Results. No statistical relationship was found between the direct impact of the pandemic and suicide attempts among children and adolescents. However, age and gender influenced the methods used and the frequency of suicide attempts. Females are more likely to attempt suicide than males, and patients as young as 8 tried to take their own lives.

Conclusions. Due to the increasing frequency of suicide attempts in children and adolescents, people at particular risk of such behaviors should be identified and provided with effective care. Unfortunately, although the vast majority of pediatric patients who attempted suicide had psychiatric consultations in the past, it did not prevent them from actively trying to end their lives. Furthermore, even children of a very young age are at risk of suicide events.

Key words: psychiatry, emergency medicine, pediatrics, self-harm, mental health

Background

Suicide is the 2nd leading cause of mortality among young people aged 10–24 worldwide.[1] A suicide attempt is an independent, potentially lethal act with the intent to take one's own life.[2] Attempted suicide is a serious public health problem, affecting people from almost every age group,[3] and a significant problem in children and adolescents, influenced by complex factors.[3,4] Such factors include, but are not limited to, social and psychological issues, mental diseases and addictions (Fig. 1).[3] Fortunately, death by suicide is a relatively rare event compared to attempted suicide.[4] As such, the number of suicide attempts is incomparably greater than committed (accomplished) suicides, although the frequency of such attempts remains underestimated. Thoughts of suicide and attempted suicide are well-known risk factors for death by suicide, and suicidal thoughts, attempted suicide and death by suicide can create a continuum of suicidality.[5]

In December 2019, the first case of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection was reported in Wuhan, China,[6,7] and on March 11, 2020, the World Health Organization (WHO) announced the outbreak of a global pandemic.[8] To date, 640 million people worldwide have contracted coronavirus disease 2019 (COVID-19), and more than 6.6 million people have died from the infection.[9] The pandemic created new challenges for healthcare, and isolation, quarantine, school closures, as well as high risk of transmission of infections significantly affected the mental health of young people in particular.[10] Indeed, the COVID-19 epidemic significantly impacted the mental state of society, particularly by exacerbating depression and anxiety.[11]

Suicide attempts among pediatric patients are one of the most important issues in modern psychiatry and emergency medicine, and the number of suicide attempts may have increased during the pandemic. Indeed, international studies indicate an increase in the frequency of suicide attempts among children during the pandemic year of 2020.[10,12] Several papers on the risk factors for suicide attempts among children and adolescents during the COVID-19 pandemic demonstrate that they may have been particularly affected by containment measures, such as physical distancing. Furthermore, lack of contact with schools, teachers and peers, difficulties obtaining or continuing psychiatric treatment, a more frequent use of narcotics, and anxiety related to family health and economic problems are the most frequently mentioned reasons for the increased risk of suicide attempts in children and adolescents.[13,14] Moreover, some studies suggest that increased awareness of suicidal thoughts and behaviors by parents, due to spending more time at home, resulted in children being taken to hospital more often.[13] However, such studies have yet to be conducted in Poland.

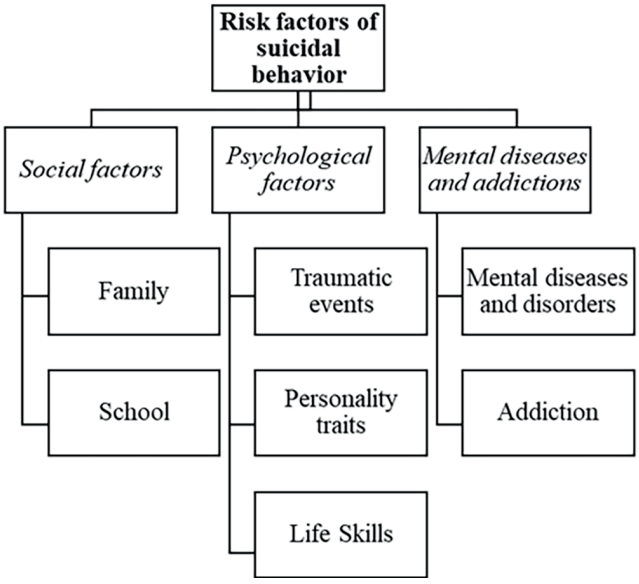

Fig. 1. Risk factors for suicidal behavior. Data are taken from the study by Marzec et al.[3]

Objectives

This study analyzed the frequency, circumstances and methods of suicide attempts among children and adolescents admitted to the Pediatric Emergency Department of the Medical University of Warsaw (MUW), Poland. In addition, the study considered the impact of the COVID-19 pandemic on suicide rates.

Materials and methods

Study design and setting

This single-center retrospective study analyzed medical records of children and adolescents admitted to the Emergency Department of the Pediatric Teaching Clinical Hospital of the University Clinical Center of the MUW. This hospital is the highest referral pediatric center in Poland and one of the 2 psychiatry centers for children up to 14 years of age for the Masovian and Podlaskie Voivodeships. Additionally, as the only children's trauma center in the region, the hospital receives a large proportion of the most seriously injured children.[15] The data analyzed in the study were collected between January 2020 and June 2021.

Ethical approval

The study conformed with the Declaration of Helsinki, European Union directives and the standards required by biomedical journals. The Bioethics Committee of the MUW (approval No. AKBE/108/2021 of July 2, 2021) and the hospital management approved the study.

Participants

Patients admitted to the emergency department requiring psychiatric consultation were selected, with the hospital's electronic medical documentation system used to collect the data, resulting in the inclusion of 154 subjects for whom all necessary data were available. The criteria for patient exclusion were: age >18 years, a suicide attempt in the previous 48 h, incomplete patient data, and any condition preventing psychiatrist examination, such as consciousness disorders or loss of consciousness. The exclusion criterion of a suicide attempt within the last 48 h ensured consistency with other studies.[16,17] A lack of full documentation resulted in exclusion of only 2 patients.

Data sources

Detailed information on the mental state of the child and its psychiatric history was obtained on the basis of a psychiatric consultation conducted by a child psychiatrist. The eligibility criteria for inclusion were complete medical records containing age and gender, details on suicidal behaviors, and thoughts reported by the patients such as talking about wanting to die or commit suicide, looking for suicide methods, talking about feeling hopeless, or having no reason to live. Other required information included details of previous suicide attempts, prior psychiatrist intervention, diagnosis of a mental disorder, family history of mental disorders, and any addictions. Moreover, data included the patient's family situation, such as whether the child came from a complete family, could count on parental support by asking if they rely on their caregivers, and whether the attempted suicide was related to the prevailing COVID-19 pandemic.

The link between the decision to attempt suicide and the COVID-19 pandemic was based on, among others, fear of a pandemic, problems with learning at school, issues with isolation and lack of contact with people outside the immediate family, and exacerbation of financial or family problems due to the pandemic. This information was obtained from the psychiatric consultations and interviews with parents, available in the records. Other data on attempted suicide included the method used, information on whether the child acted to take their life, if the child required hospitalization due to the resulting injuries, and if the child was immediately admitted to a psychiatric department.

Statistical analyses

Statistical analysis employed IBM SPSS software v. 26.0 (IBM Corp., Armonk, USA). Categorical variables were summarized as frequency tables, range and mean ± standard deviation (M ±SD). The relationships between the variables were examined using the χ^2 test, while the strength of dependence was investigated using the Phi–Yule coefficient and the Cramer's V coefficient, the results of which included 95% confidence interval (95% CI). A p-value <0.05 was considered statistically significant.

Results

Table 1 describes the statistical results for the compared values. The study included 123 females (79.9%) and 31 males (20.1%). Participants were divided into age groups of 8–13 years (n = 73) and 14–17 years (n = 81), since patients get admitted to psychiatric youth centers from the age of 14. The mean age for females was 13.8 years (median: 14 years, SD: ±1.64, minimum: 8 years, and maximum: 17 years). The mean age for males was 13.7 years (median: 14 years, SD: ±2.07, minimum: 8 years, and maximum: 17 years). The youngest recorded patient with a suicide attempt was 8 years old (Table 1).

Most of the children investigated were admitted after their first suicide attempt (64.3%, n = 99), with the first attempt being more common in males (77.4%, n = 24) than females (61%, n = 75). The majority of children (81.2%, n = 125) had previously experienced suicidal behavior or thoughts, or both. Suicidal thoughts were more frequent in those aged 14–17 years (85.2%, n = 69) than in patients aged 8–13 years (76.7%, n = 56). Furthermore, suicidal behavior or thoughts, or both, were found more frequently in females (85.4%, n = 105) and males (64.5%, n = 20). Moreover, the relationship between gender and suicidal behavior and thoughts was statistically significant (χ^2 = 7.042, degrees of freedom (df) = 1, p = 0.008). However, the relationship was relatively weak (Phi–Yule coefficient = 0.214).

A large proportion of children (74.7%, n = 115) were previously under the care of a psychiatrist, while some (25.3%, n = 39) had never received such help. Females (76.4% n = 94) visited a psychiatrist more often than males (67.7% n = 21), and those aged 8–13 years (75.3%, n = 55) were under the care of a psychiatrist more often than those aged 14–17 years (74.1%, n = 60). Only 16.2% (n = 25) of children had an addiction, with a large proportion addicted to the Internet/computer (48%, n = 12), 28% (n = 7) to drugs such as cannabis, mephedrone and psychoactive substances, and 24% (n = 6) to other substances such as alcohol and nicotine. Furthermore, addiction differed significantly between males (29%, n = 9) and females (13%, n = 16).

At the time of the suicide attempt, most children (92.9%, n = 143) were not under the influence of intoxicants such as alcohol and psychoactive substances or pharmaceuticals used for nontherapeutic purposes, although 7.1% (n = 11) were. Furthermore, a statistically significant relationship was observed between age and whether the child was under the influence of intoxicants or pharmaceuticals at the time of the suicide attempt (χ^2 = 6.974, df = 1,

Table 1. Characteristics of 154 Polish patients who attempted suicide

Characteristics		Age		p-value	Gender		p-value
		8–13 years (n = 73)	14–17 years (n = 81)		women (n = 123)	men (n = 31)	
Has the child attempted suicide before?	yes	23 (31.5%)	32 (39.5%)	0.301	48 (39%)	7 (22.6%)	0.134
	no	50 (68.5%)	49 (60.5%)		75 (61%)	24 (77.4%)	
Has the child had any previous suicidal behavior or thoughts?	yes	56 (76.7%)	69 (85.2%)	0.179	105 (85.4%)	20 (64.5%)	**0.008**
	no	17 (23.3%)	12 (14.8%)		18 (14.6%)	11 (35.5%)	
Has the child been previously looked after by a psychiatrist?	yes	55 (75.3%)	60 (74.1%)	0.857	94 (76.4%)	21 (67.7%)	0.446
	no	18 (24.7%)	21 (25.9%)		29 (23.6%)	10 (32.3%)	
Is the child addicted?	yes	13 (17.8%)	12 (14.8%)	0.615	16 (13%)	9 (29%)	0.059
	no	60 (82.2%)	69 (85.2%)		107 (87%)	22 (71%)	
Was the child under the influence of intoxicants/pharmaceuticals at the time of the suicide attempt?	yes	1 (1.4%)	10 (12.3%)	**0.010**	8 (6.5%)	3 (9.7%)	0.464
	no	72 (98.6%)	71 (87.7%)		115 (93.5%)	28 (90.3%)	
Can a child count on parental support?	yes	63 (86.3%)	70 (86.4%)	1.000	107 (87.0%)	26 (83.9%)	0.769
	no	10 (13.7%)	11 (13.6%)		16 (13.0%)	5 (16.1%)	
Is the child diagnosed with a mental disorder?	yes	20 (27.4%)	27 (33.3%)	0.424	36 (29.3%)	11 (35.5%)	0.502
	no	53 (72.6%)	54 (66.7%)		87 (70.7%)	20 (64.5%)	
Have any mental disorders occurred in the child's family?	yes	9 (12.3%)	6 (7.4%)	0.312	12 (9.7%)	3 (9.7%)	1.000
	no	51 (69.9%)	69 (85.2%)		97 (78.9%)	23 (74.2%)	
	lack of knowledge	13 (17.8%)	6 (7.4%)		14 (11.4%)	5 (16.1%)	
Method of attempted suicide	poisoning	23 (31.5%)	47 (58%)	**0.007**	59 (48%)	11 (35.5%)	**0.006**
	suicidal self-injury	24 (32.9%)	18 (22.2%)		38 (30.9%)	4 (12.9%)	
	jumping from height	12 (16.4%)	5 (6.2%)		9 (7.3%)	8 (25.8%)	
	other	14 (19.2%)	11 (13.6%)		17 (13.8%)	8 (25.8%)	
Has the child self-harmed before to reduce stress and tension or gain attention?	yes	37 (50.7%)	38 (46.9%)	0.640	68 (55.3%)	7 (22.6%)	**0.002**
	no	36 (49.3%)	43 (53.1%)		55 (44.7%)	24 (77.4%)	
Has the COVID-19 pandemic affected the child's suicide attempt?	yes	17 (23.3%)	20 (24.7%)	0.839	32 (26%)	5 (16.1%)	0.359
	no	56 (76.7%)	61 (75.3%)		91 (74%)	26 (83.9%)	
Did the child require hospitalization as a result of injuries during a suicide attempt?	yes	5 (6.8%)	18 (22.2%)	**0.008**	19 (15.4%)	4 (12.9%)	1.000
	no	68 (93.2%)	63 (77.8%)		104 (84.6%)	27 (87.1%)	

COVID-19 – coronavirus disease 2019. Values in bold indicate statistically significant results.

p = 0.010). However, the relationship was relatively weak (Phi–Yule coefficient = 0.213). Children aged 14–17 years were under the influence of substances more frequently when attempting suicide (12.3%, n = 10). There was n = 1 (1.4%) case in the age group 8–13.

Most hospitalized children came from a complete family (55.8%, n = 86), while 41.6% (n = 64) of children grew up in an incomplete family. Data on the family status of 4 patients (2.6%) could not be obtained. Children aged 14–17 years came from a complete family more often (60.5%, n = 49) than children aged 8–13 years (50.7%, n = 37). Furthermore, 86.4% (n = 133) of children could rely on their parents' support, while 13.6% (n = 21) did not receive such support. The majority of surveyed children have not been previously diagnosed with a mental disorder (69.5%, n = 107), while 30.5% (n = 47) had a history of mental disorder diagnosis. Most children (77.9%, n = 120) had no family history of psychiatric disorders, though 9.7% (n = 15) did, and 12.3% (n = 19) did not have any knowledge on the issue.

The most frequently chosen suicide method was poisoning (45.5%, n = 70), with self-injury ranking 2nd (27.3%, n = 42), followed by jumping from height (11%, n = 17). Other methods used by 16.2% (n = 25) of patients included drowning, jumping under a train and ingesting a corrosive substance. Statistical analysis revealed a significant relationship between gender and the method of suicide attempt ($\chi^2 = 13.267$, df = 3, p = 0.003), and the association was moderate (Cramer's V coefficient = 0.317). Although poisoning was the suicide attempt method used by most children, intoxication was more common in females (48%, n = 59) than in males (35.5%, n = 11) (Fig. 2). Moreover,

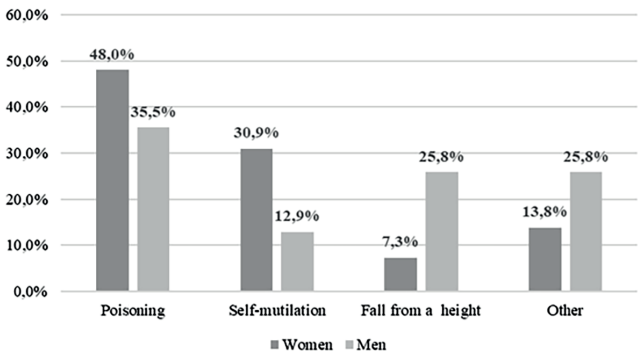

Fig. 2. Relationship between gender and the method of the suicide attempt

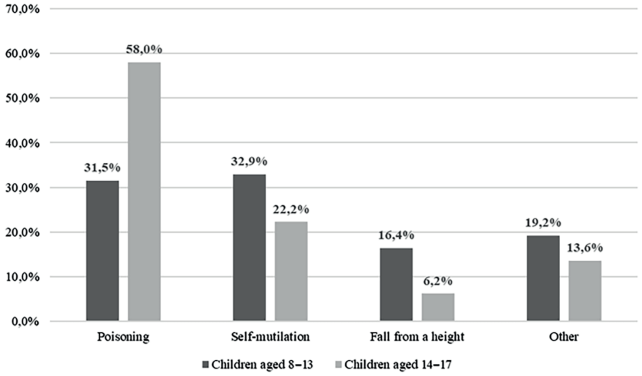

Fig. 3. Relationship between the age and the method of the suicide attempt

self-injury was the most common method among those aged 8–13 years (32.9%), while patients aged 14–17 years favored poisoning (58%) (Fig. 3). A statistically significant relationship was found between the age group and the suicide attempt method (χ^2 (df = 3) = 12.900, p = 0.005), though the relationship was relatively weak (Cramer's V coefficient = 0.295).

In most cases, the children in the study acted to take their own lives, not merely to attract attention. There was a statistically significant relationship between gender and whether the child had self-harmed before to reduce stress and tension or attract attention (χ^2 = 9.331, df =1, p = 0.002), although the relationship was relatively weak (Phi–Yule coefficient = 0.262). Most females (55.3%, n = 68) had self-harmed to reduce stress and tension or to gain attention, and 77.4% (n = 24) of males had not self-mutilated before. Furthermore, 50.7% (n = 37) of children aged 8–13 years had self-harmed previously to reduce stress and tension or to gain attention. On the other hand, most children aged 14–17 years (53.1%, n = 43) did not self-mutilate.

There was no clear link between SARS-CoV-2 and attempted suicide in most cases among children aged 8–13 years (76.7%, n = 56) and 14–17 years (75.3%, n = 61). Also, there was no statistically significant relationship between the age group or gender and the COVID-19 pandemic.

Hospitalization was not required in 93.2% (n = 68) of children aged 8–13 years and 77.8% (n = 63) of those aged 14–17 years as a result of their injuries. However, a weak (Phi–Yule coefficient = 0.215) but statistically significant relationship (χ^2 = 7.142, df = 1, p = 0.008) was observed between the age group and the requirement for hospitalization as a result of self-inflicted injuries.

Discussion

Suicide is a serious global health problem and the 2nd leading cause of death in children and adolescents. The rates of suicidal thoughts among the youth range from 19.8% to 24.0%, while suicide attempts are less frequent, ranging from 3.1% to 8.8%.[2] As far as Polish rates are concerned, 31% of young people aged from 14 to 21 years experienced suicidal ideation, while 8% have attempted suicide.[18] Unfortunately, the trend change is not optimistic, with an increasing number of suicide cases[12,19] and a decreasing age of children attempting suicide.[20] A 20-year analysis of suicide among Polish adolescents showed that suicide attempt rates were increasing, though there was a decrease in suicide-related deaths.[21]

The analysis of the data collected between January 2020 and June 2021 demonstrated a significant predominance of females among reported cases of suicide attempts (79.9%, n = 123). These findings are consistent with well-established knowledge that underage females are more likely than males to have suicidal thoughts and attempts.[22,23] Indeed, a Polish study reported that 10.7% of females and 5.4% of males attempted suicide, giving a ratio of 2:1, while the suicidal ideation ratio was 1.5:1 (37.5% of females and 24.8% of males).[24] However, exceptions exist for Swedish males[19] and very young children.[20] The increased suicidal tendency in females may be connected with a higher risk of depression.[25]

The age of reported individuals ranged from 8 to 17 years, with 1 male and 1 female aged 8 attempting suicide through self-injury. Furthermore, almost half of the cases involved children aged between 8 and 13 years. Research on suicidal behaviors among very young children is insufficient, and little is known about the reasons for such behaviors. However, the frequency of suicidal thoughts and behaviors in preschoolers (children aged 3–6 years) is estimated to range from 4% to 13%.[26] Moreover, high family conflict and low parental supervision seem to be linked with suicidality in children aged 9 and 10.[20] Also, the increased use of social media at a young age and the rise in online bullying in recent years[27,28] may have contributed to the increase in suicides and suicidal self-injury among young children.

Differences exist between the most common methods of suicide across countries. However, contrary to this study, a cross-European comparison of young people showed that the most frequent method of suicide attempt for both genders was hanging, while for males it was jumping from

height and using guns, and for female children it was poisoning by pharmaceuticals and jumping from height.[29] The current study showed that the most frequently chosen suicide method among both genders was poisoning (45.5%, n = 70), followed by suicidal self-injury (27.3%, n = 42). This may be because the consumption of prescribed pharmaceuticals[30] and dietary supplements[31] is prevalent in Poland, with increasing abuse of over-the-counter medicines.[32] The most common substances children ingested in suicidal attempts were acetaminophen and ibuprofen.[33] No case of a gun-related suicide was recorded, which may be due to Poland's restrictive gun laws. In addition, the study involved a highly urbanized area where keeping weapons in the household for purposes such as hunting is rare.

A link was observed between gender and the method of suicide attempt, with more females (50.9%, n = 59) than males (37.9%, n = 11) choosing poisoning, which has a relatively low fatality rate compared to other methods. Indeed, females are more likely to decide on intentional poisoning[34] due to their tendency to choose less lethal methods of suicide.[35] Additionally, the relationship between age and the method of suicide attempt demonstrated that older children, aged 14–17 years (61.8%), were more likely to choose poisoning, while younger children, aged 8–13 years (34.8%), mostly adopted suicidal self-injury. These findings are in line with other studies showing that suicide attempts due to poisoning are most often attempted by adolescents aged 14–18 years.[3] However, data from the USA indicate that younger children aged 5–11 most frequently use hanging or suffocation, followed by using guns, which is contrary to the findings of this study.[36]

Regarding the psychiatric aspect, a large number of children were under the care of a psychiatrist before attempting suicide (74.7%, n = 115), which is higher than prior reports showing that less than half of young people who attempted suicide had previously received psychiatric help.[37] At the same time, approx. 1/3 of the children had a history of mental disorders. The research of Gmitrowicz et al. showed that previous psychiatric treatment and psychotherapy was the most significant risk factor for a suicide attempt.[18] Furthermore, Ong et al. demonstrated that depressive disorders among pediatric patients were associated with a higher likelihood of suicide than attention-deficit disorder, disruptive behavior disorder, bipolar disorder, and schizophrenia.[38]

Addiction was noted in 16.2% (n = 25) of respondents, with the Internet/computer addiction, or a combination of both, being the most common. The link between substance abuse and suicidal behavior has been well documented,[18,39] while little is known about the relationship between the Internet/computer addiction and suicide risk.[40] However, there is evidence that time spent using a computer and social media is harmful to children's mental health.[28,40]

Recently, there has been a strong emphasis on distinguishing between non-suicidal self-injury (NSSI) and suicide attempts.[41] In the current study, almost 80% of children had self-mutilated before the suicide attempt, and NSSI is now believed to be a risk factor for suicide attempts in adolescents,[42,43] as it increases the risk of future suicide attempts.[44]

The COVID-19 pandemic disrupted the lives of people around the world. Furthermore, the mental health of young children and adolescents was affected by the social and psychological effects of the pandemic, which caused distress, and a rise in mental disorders and behavioral issues incidence.[10] However, the impact of the pandemic on the number of suicides among children is unclear. Indeed, some studies demonstrated no relationship,[45] and others reached opposite conclusions.[12,46] Nonetheless, the current study showed no statistically significant relationship between the SARS-CoV-2 pandemic and the suicide rate in Polish children and adolescents.

Limitations

Limitations of the study include the disparity between the number of males and females,[22] though this imbalance may reflect the fact that females are much more likely to attempt suicide. Another constraint is the increased proportion of suicidal self-injuries recorded as suicide attempts, especially in children aged 8–13, which may be due to an overestimation resulting from an insufficient distinction between suicidal behavior and NSSI in Poland. Indeed, NSSI is often considered a suicide attempt, even though self-harm is generally nonlethal. In addition, no link existed between the COVID-19 pandemic and the suicide rate. However, this analysis may have been limited by relying on the psychiatric consultations and parental interviews mentioning the SARS-CoV-2 component. As such, determining the impact of the pandemic on mental health depended on subjective assessment, and healthcare professionals did not explicitly ask every child or caregiver about this element. Therefore, the results may be underestimated.

Conclusions

Suicide among children is a complex issue and a major public health concern, with the number of suicide attempts among pediatric patients steadily increasing. Children and adolescents attempt suicide under the influence of many factors, including mental disorders and addictions. Underage females are significantly more likely to mutilate themselves or attempt suicide. Meanwhile, age and gender impacted the suicide attempt frequency and methods used, with poisoning being the most common method used among examined pediatric patients. Fortunately, most patients attempting suicide had previous contact with a psychiatrist, although this did not prevent the suicide attempt.

The present research did not assess the impact of pandemic-related distress on suicidal behaviors among

the study group. Additional research is needed to evaluate the pattern and protective factors that may be associated with suicide hazards in the context of a global pandemic. It remains the responsibility of healthcare providers to be aware of the risk factors, changing patterns and management of suicide attempts among children.

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Evaluation of the effects of hyperbaric on human attention functions based on eye movements recorded using an infrared camera

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Abstract

Background. This study aimed to assess the influence of elevated atmospheric pressure on the functions of attention of medical personnel working in hyperbaric chambers. We enrolled 15 participants who met the inclusion criteria. The test consisted of performing the same medical procedure under 2 conditions. For each of these test conditions, right eye movements were recorded using an oculograph. The obtained results revealed a relationship between elevated atmospheric pressure and the ability of medical personnel to focus.

Objectives. To assess the influence of hyperbaric oxygen (HBO_2) on visual attention in medical personnel during medical activities performed under normobaric (1 absolute atmosphere (1 ATA)) and hyperbaric (4 ATA) conditions inside a hyperbaric chamber.

Materials and methods. Each participant had a valid license to act as a medical attendant during therapeutic hyperbaric sessions. Fifteen individuals, 10 men and 5 women aged between 28 and 65 years, participated in the study. The participants were asked to perform a medical procedure involving the preparation of a syringe with a drug administered by an infusion pump under 2 test conditions: 1 ATA corresponding to the atmospheric pressure on land, and 4 ATA corresponding to an underwater depth of 30 m. The order of test conditions was random. Both test conditions were performed inside a hyperbaric chamber.

Results. The number of fixations in the area of interest (AOI) varied between stages (1, 2 and 3) and task conditions (1 ATA and 4 ATA), with lower values for the 4 ATA condition. Under 1 ATA, 30% of eye fixations were in the AOI, as compared to only 6% under 4 ATA.

Conclusions. The obtained results indicate that elevated atmospheric pressure has negative effects on the attention of medical personnel.

Key words: hyperbaric therapy, attention, oculography, medical staff

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Background

Although hyperbaric oxygen (HBO2) therapy has been used for decades, it has evolved over the last quarter of a century, with an increase in the number of indications for this treatment modality. Currently, the use of technology based on patient exposure to artificially increased pressure is justified in the emergency and elective treatment of multiple clinical entities. It is employed both as a therapy of choice, e.g., in carbon monoxide poisoning or decompression sickness, and as an adjunctive treatment.[1] Multi-profile indications for the use of HBO2 include such disease entities as burns, difficult-to-heal wounds, osteoarthritis, ischemic skin loss, sudden hearing loss, as well as acute hemorrhagic anemia.[2] In the last 2 decades, the list of indications for HBO2 was extended to include locomotor trauma with severe edema and inflammation – conditions which often reduce proper oxygen saturation in the tissues at the site of injury due to their dynamic nature, compromising the efficacy of surgical treatment.[3]

The growing awareness among clinicians of hyperbaric medicine translates into an increasing recognition of this discipline and its implementation in everyday therapeutic management. It is important to point out that medical personnel accompany the patient in the pressure chamber to guarantee proper therapy and safety during therapeutic compression sessions in the clinical setting. Therefore, the present study shows the impact of increased atmospheric pressure on attention in medical personnel performing their professional duties in pressure chambers that may contribute to therapeutic safety.

Objectives

In a clinical hyperbaric environment, medical personnel are exposed to the same pressure values as patients during a therapy session. Previous studies have shown that oxygen therapy under pressure above 1 atmosphere has a beneficial effect on improved cognitive function in healthy elderly people, as well as in those who have suffered brain damage due to stroke or craniocerebral trauma.[4,5] However, the effect of HBO2 on cognitive functions described in the literature refers to the results obtained after completing a therapeutic compression cycle. Marine literature shows that a hyperbaric atmosphere may have a negative effect on central nervous system (CNS) functions in situ.[6,7] The main objective of our study was to assess the influence of HBO2 on visual attention in medical personnel during medical activities performed under normobaric (1 absolute atmosphere (1 ATA)) and hyperbaric (4 ATA) conditions inside a hyperbaric chamber.

Materials and methods

The study was conducted in 2019–2021 as part of an in-house project, one of the main objectives of which was to evaluate the effect of HBO2 on the attention capacity of medical professionals working in hyperbaric medicine units. The research was initiated after obtaining the approval of the Bioethics Committee at the Military Medical Institute in Warsaw, Poland (approval No. 22/WIM/2018 issued on April 17, 2019).

All participants received oral and written information about the research and provided written informed consent to participate in the study. Data on the participants (medical history, physical examination and results of the tests performed) were collected on forms prepared for this purpose and on electronic devices dedicated only for the purposes of the research. The obtained data were archived at the Clinical Department of Hyperbaric Medicine of the Military Medical Institute in Warsaw in accordance with the current regulations on data anonymity.

Each participant was authorized to act as a medical attendant during therapeutic hyperbaric sessions. Fifteen individuals (13 nurses and 2 medical doctors, including 10 men and 5 women) aged between 28 and 65 years (mean (M) = 38.47; standard deviation (SD) = 5.77), participated in the study (Table 1).

Study inclusion criteria were as follows: 1) written informed consent to participate in the study; 2) medical education and valid license to work in a clinical hyperbaric setting; 3) no significant disease of the right eyeball; 4) no contraindications to the study.

Individuals with one or more of the following criteria prior to the study: pregnancy, otitis media, visual impairment, cardiac arrhythmia, and general malaise, were not allowed to participate in the study. At the time of qualification for the study, no participant met these exclusion criteria. Each participant had a paired visual organ that did not exclude them from professional activities due to dysfunction.

Table 1. Characteristics of study participants

Age	Participants (n = 15)	Male (n = 10)	Female (n = 5)
M	39.33	41.6	34.8
SD	9.32	10.29	5.22
Min	28.0	31.0	28.0
Me	36.0	39.0	36.0
Max	65.0	65.0	42.0

M – mean; SD – standard deviation; Min – minimum; Me – median; Max – maximum.

Methods

The participants were asked to perform a medical procedure involving the preparation of a syringe with a drug

administered by an infusion pump under 2 test conditions: 1 ATA corresponding to the atmospheric pressure only on land or on land and sea level, and 4 ATA corresponding to an underwater depth of 30 m. The order of test conditions was random. Both test conditions were performed inside a hyperbaric chamber.

The medical task for both pressure levels comprised of the same elements. When the participants performed the task, eye movements were recorded using a calibrated head-mounted video-oculography system. During both tests, the participants were in the hyperbaric chamber, directly at the workstation. Before starting the task, participants were required to familiarize themselves with the devices used in the study, which consisted of an eye tracker and the infusion pump used at the Clinical Department of Hyperbaric Medicine. All participants had experience in working with the infusion pump used in the study. To exclude communication errors, safety and communication procedures between the investigator and the participant were discussed prior to the study. The study was divided into 3 stages for each test condition to quantitatively assess the data collected.

Stage I: Preparation of the syringe with the drug – from the start of the task where the participant grasped the syringe until it was filled with the drug.

Stage II: Placing the syringe in the infusion pump – from the moment when the syringe was filled with the drug until the syringe together with the infusion tube were placed in the infusion pump by the participant.

Stage III: Setting the drug flow parameters – from the time the syringe was placed in the infusion pump to the start of the pre-set drug flow parameters expressed in milliliters per hour [mm/h].

A Therapeutic Barox HBO2 Omega chamber (Yaklaşım Makine, Istanbul, Turkey) equipped with 16 seats for HBO2 therapy, including a seat for a medical attendant, was used for the study. The maximum operating pressure of the Barox HBO2 Omega is 10 bar.

Right eye movements were recorded using a configurable oculography device (Pupil Labs GmbH, Berlin, Germany) measuring right eye movements at 120 Hz (up to a maximum of 200 Hz). The image from the ambient camera was sampled at 30 Hz. Eye movements were recorded to assess the level of distraction. The indicator of distraction was the ratio of eye fixations in the area of interest (AOI) to fixations outside this area. Data collection was performed using Pupil Capture Software v. 1.11.4 (Pupil Labs), with a sampling rate of 120 Hz and the camera image resolution 1280 × 720 pixels.

Data fragmentation and analysis were performed with software Mask AOI v. 1.5.1, developed by one of the authors in December 2019 for this research. Each fixation position was recorded and defined as being in/out of the AOI by using the point in polygon algorithm and coded as a binary variable where 0 or 1 meant fixation in or fixation out, respectively. Task completion time was measured as the number of video frames recorded from an ambient camera.

Statistical analyses

Statistical analyses were performed using Python 3.8 language packages: pingouin v0.3.5 (summary statistics), pymer4 v0.7.8 and rpy2 v3.4.5 (generalized linear mixed models (GLMM)). Figures were plotted in seaborn v0.11.2. The significance level was set at a $p < 0.05$.

The data were aggregated for each subject, ATA conditions and stage of the study. The time of completion was calculated as the number of frames for completion of each stage of the task. Fixations were coded as a binomial (0 and 1) variable as the presence or absence of fixations in the central field of view in each frame. The fixations were then analyzed for each of the stages in the study. To estimate the proper number of fixations in/out of the central view, we corrected the fixations by the number of frames and adjusted them for the number of frames due to the difference in the amount of time the participants required to complete each task. Therefore, we have set the completion time as weights. We chose the GLMM model, which allows the analysis of data in a structure that meets the aims of the study. Selected distributions for the completion time were the Poisson distribution and bimodal distribution with weights for the fixations, which corresponded to the characteristics of the dependent variables, as the completion time is composed of numbers starting with 0 and occurring at specific intervals (imposed by the recording resolution), and the fixations are coded as a binomial variable, aggregated and corrected by the number of frames (Python code is included in the Supplementary materials).

Model selection

Due to the lack of normality in both completion time and fixations ($p < 0.05$ for each condition using the Shapiro–Wilk test) and nested nature of repeated measures (1 ATA: stages 1, 2 and 3; 4 ATA: stages 1, 2 and 3), GLMM was used with the individual number of each participant as a random effect, and ATA value and stage as fixed (within) effects. Moreover, in the case of analyzing completion time, GLMM was analyzed using the Poisson distribution model for completion time and underweighted binomial distribution for the fraction of fixations in AOI over all fixations. Outliers have not been removed as there were no apparent effects on residuals during model diagnostics. To test the goodness-of-fit (Akaike criterion (AIC)), we compared 5 models: 1) only with intercept; 2) with the stage as a coefficient; 3) with ATA value as a coefficient; 4) with ATA value and stage without interaction effect; and 5) ATA value, stage and the interaction effect.

The goodness-of-fit test showed that there were differences in fixations in/out of the AOI (each $p < 0.001$) between model 1 (fixations AIC = 6706) and model 2 (fixations AIC = 6049), model 3 (fixations AIC = 7855),

model 4 (fixations AIC = 6041) and model 5 (fixations AIC = 5732). In the completion time, only model 2 (time AIC = 4597) and model 5 (time AIC = 4565) differed significantly from model 1. However, model 5 (with both coefficients and interaction effects) has the lowest AIC for both fixations and completion time and was selected for the analysis. Model diagnostics showed that the residuals are approaching normal distribution, and despite the occurrence of some deviations, the model did not need to be changed due to the resistance of the GLMM to deviations from the normal distribution. More information on the model testing is presented in Fig. 1,2 and in the Supplementary materials.

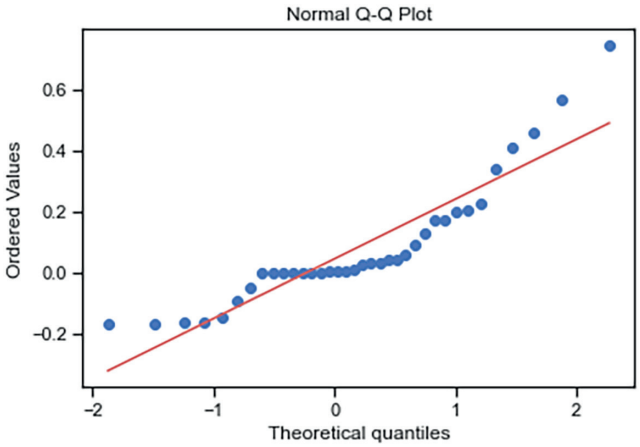

Fig. 1. Diagnostic normal Q-Q plot for the generalized linear mixed models (GLMM) model with fixations as a dependent variable (red line – normal distribution, points – model residuals)

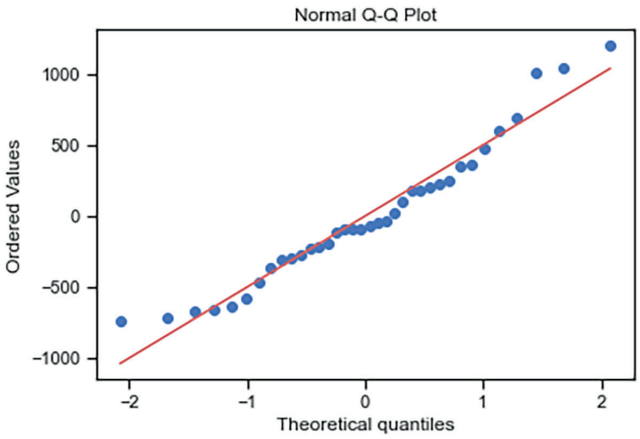

Fig. 2. Diagnostic normal Q-Q plot for generalized linear mixed models (GLMM) model with the completion time as a dependent variable (red line – normal distribution, points – model residuals)

Results

The distributions of the fixations (ratio) and the completion time based on chosen coefficients are presented in Fig. 3.

Task completion time

The camera image was recorded at a resolution of 30 Hz with a median of frames (Me = 2036.5, 1st quartile (Q1) = 1592.75, 3rd quartile (Q3) = 2718.25) within 67.8 s. The longest part of the task was stage 3 when the participants set the drug flow parameters (Me = 2202, Q1 = 1832, Q3 = 3231). The task was on average 78.38 frames longer under 4 ATA, but the median for 4 ATA was lower (1 ATA Me = 2114, Q1 = 1643, Q3 = 3146; 4 ATA Me = 1927, Q1 = 1502, Q3 = 2855).

The task completion time differed between the participants depending on testing conditions (ATA) and stage of task completion. The GLMM model showed that both the conditions (1 ATA and 4 ATA) and stages (1, 2 and 3), were statistically significant predictors of task completion times ($p < 0.001$). The 4 ATA conditions resulted in slightly more time required to complete the task than 1 ATA conditions, and stages 2 and 3 had longer completion times than stage 1. However, although there were differences in the means, they were not commensurate with the differences in the median (1 ATA compared to 4 ATA: Me = 2114 compared to 1927, Q1 = 1643 compared to 1502, Q3 = 3146 compared to 2855). In 4 ATA, stage 2 did not differ from stage 1 ($p = 0.172$), but stage 3 did (Table 2). The analysis used 95% confidence intervals (95% CI).

Table 2 presents the GLMM model with Poisson distribution of the task completion times measured as the number of frames during the completion of the task in each condition.

Analysis of attentional processes

The number of fixations in the AOI varied between stages (1, 2 and 3) and task conditions (1 ATA and 4 ATA), with lower values for the 4 ATA conditions. Under 1 ATA, the mean ratio of eye fixations in the AOI was 0.20 (Me = 0.066, Q1 = 0.013, Q4 = 0.361) as compared to only a 0.6 ratio under ATA 4 (Me = 0.003, Q1 = 0, Q4 =0.036).

The GLMM with weighted binomial link function showed that both the test conditions (ATA) and the test stages (1, 2 and 3) were statistically significant predictors of the fraction of fixations in the region of interest. Furthermore, increases in ATA value decreased the chance of directing eyes on the region of interest (OR = 0.659; 95% CI: 0.607–0.716). On the other hand, during stages 2 and 3, the chance of fixing eyesight on the region of interest increased compared to stage 1 (Table 3).

GLMM post hoc comparisons

Post hoc analysis with the Bonferroni correction was used to test the hypotheses about the influence of predictors (ATA value: 1 and 4; stages: 1, 2 and 3) on the dependent variables (fixations and completion time). Tests showed statistically significant differences between almost all testing

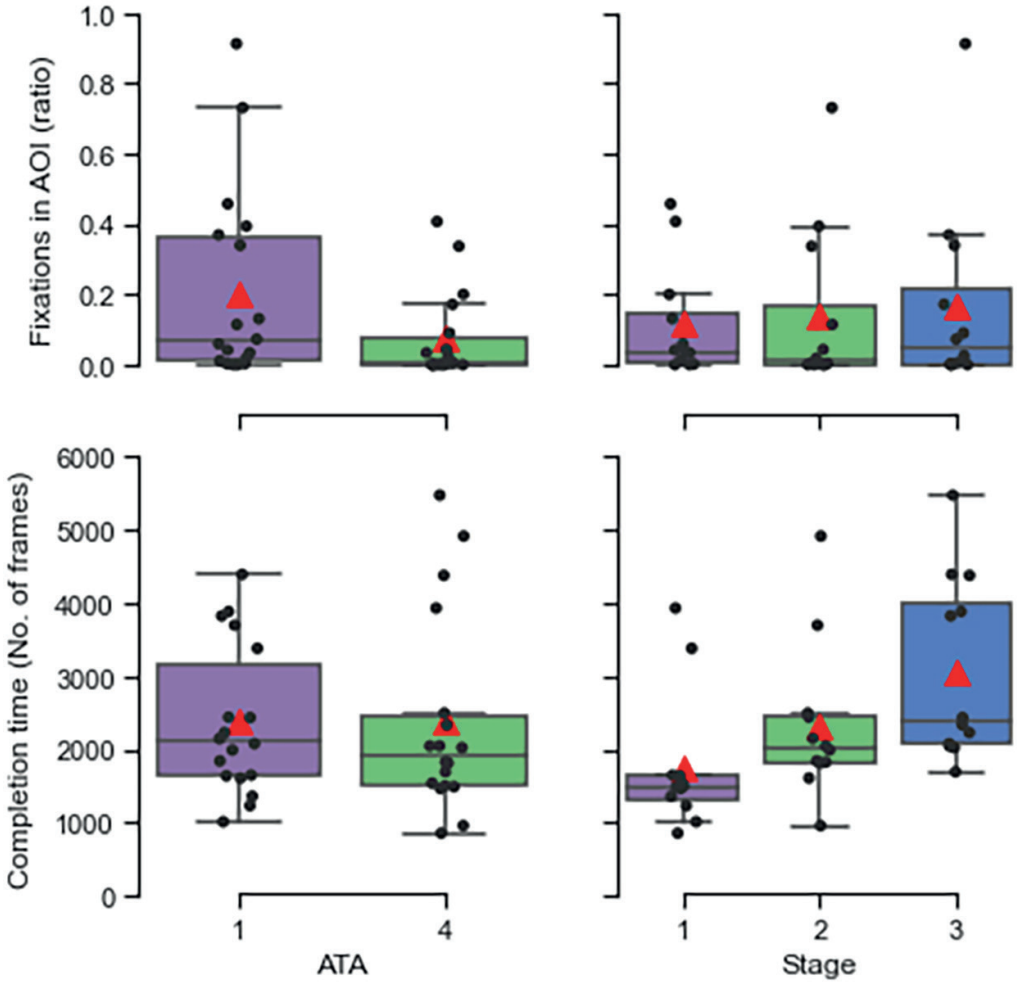

Fig. 3. Distribution of fixation ratios and the completion times depend on ATA value (1 and 4) and stages (1, 2 and 3). Fixations were measured as a fraction of fixations on the area of interest (AOI) from all fixations in each condition; the completion time was measured as the number of frames in each condition. Boxes represent 1st quartile (Q1) and 3rd quartile (Q3), black line represents the median (Me), whiskers represent values outside the middle 50% from minimum (lower whisker) to maximum (upper whisker), and the red triangle represents the mean (M)

Table 2. Generalized linear mixed models (GLMM) model with Poisson distribution of the tasks completion time measured as the number of frames during completing the task in each condition. One absolute atmosphere (1 ATA) and stage 1 were taken as an intercept; therefore, the coefficients were coded as factors, 4 ATA was compared to 1 ATA, and stages 2 and 3 to stage 1

Coefficients	β	CI 2.5%	CI 97.5%	SE	p-value
Intercept	7.393	7.145	7.640	0.127	<0.0001
4 ATA	0.049	0.022	0.076	0.014	0.0004
Stage 2	0.289	0.264	0.315	0.013	<0.0001
Stage 3	0.606	0.582	0.630	0.012	<0.0001
4 ATA × stage 2	–0.025	–0.061	0.011	0.018	0.172
4 ATA × stage 3	–0.097	–0.131	–0.063	0.017	<0.001

β – standardized beta for coefficient; CI – confidence interval; SE – standard error.

Table 3. Generalized linear mixed models (GLMM) model with weighted binomial distribution of the fraction of fixation in the region of interest in relation to all fixations in each condition. One absolute atmosphere (1 ATA) 1 and stage 1 were taken as an intercept; therefore, the coefficients were coded as factors, 4 ATA was compared to 1 ATA, and stages 2 and 3 to stage 1

Coefficients	β	OR	CI 2.5%	CI 97.5%	SE	p-value
Intercept	–2.086	0.124	0.040	0.380	0.482	<0.001
ATA 4	–0.417	0.659	0.607	0.716	0.042	<0.001
Stage 2	0.388	1.474	1.316	1.648	0.057	<0.001
Stage 3	0.553	1.739	1.585	1.908	0.047	<0.001
ATA 4 × stage 2	–1.303	0.272	0.218	0.336	0.110	<0.001
ATA 4 × stage 3	–1.404	0.246	0.203	0.296	0.010	<0.001

β – standardized beta for coefficient; OR – odds ratio; CI – confidence interval; SE – standard error.

conditions, with the biggest difference (Z score = −8.952, 95% CI: −0.178−−0.09, SE = 0.015, p = 0.0067) for the differences during stage 1 under 1 ATA compared to 4 ATA for completion time, except comparisons in stage 2 compared to stage 3 in ATA 1 (Z score = −0.165, 95% CI: −0.341–0.0104, SE = 0.0599, p = 0.086) and under 4 ATA (Z score = −0.065, 95% CI: −0.412–0.283, SE = 0.015, p > 0.999) in fixations and stage 2 under 1 ATA compared to 4 ATA (Z score = 0.108, 95% CI: −0.059, 0.156, SE = 0.024, p = 0.717) for completion time (Fig. 4).

The participants were more likely to fix their eyes away from the AOI (fixations in AOI ratio: M = 0.139, SD = 0.226, Q1 = 0.0004, Me = 0.029, Q3 = 0.182), but more fixations in the AOI were recorded at the final stage of the test under 1 ATA (fixations in AOI ratio: M = 0.283, SD = 0.349, Me = 0.206, Q1 = 0.02, Q3 = 0.36), while the least were recorded at stages 2 (fixations in AOI ratio: M = 0.064, SD = 0.135, Me = 0.002, Q1 = 0, Q3 = 0.033) and 3 (fixations in AOI ratio: M = 0.008, SD = 0.012, Me = 0.003, Q1 = 0, Q3 = 0.01) under 4 ATA.

There was no significant difference between 1 ATA and 4 ATA conditions (Z = 1.560, 95% CI: −0.005–0.042, SE = 0.012, p = 0.253), but comparisons within each stage showed differences in each of them between 1 ATA and 4 ATA conditions (stage 1: M difference = −0.049, p < 0.001; stage 2: M difference = −0.024, p = 0.048; stage 3: M difference = −0.048, p < 0.0001).

Completion time significance was as follows: stage 1 under 1 ATA compared to 4 ATA: p = 0.0067, stage 2 compared to stage 3 under 1 ATA: p = 0.086, stage 2 compared to stage 3 under 4 ATA: p > 0.999, other comparisons: p < 0.001 The fixation significance was as follows: stage 2 under 1 ATA compared to 4 ATA: p = 0.717, other comparisons : p < 0.001.

Post hoc comparisons are presented in Fig. 4.

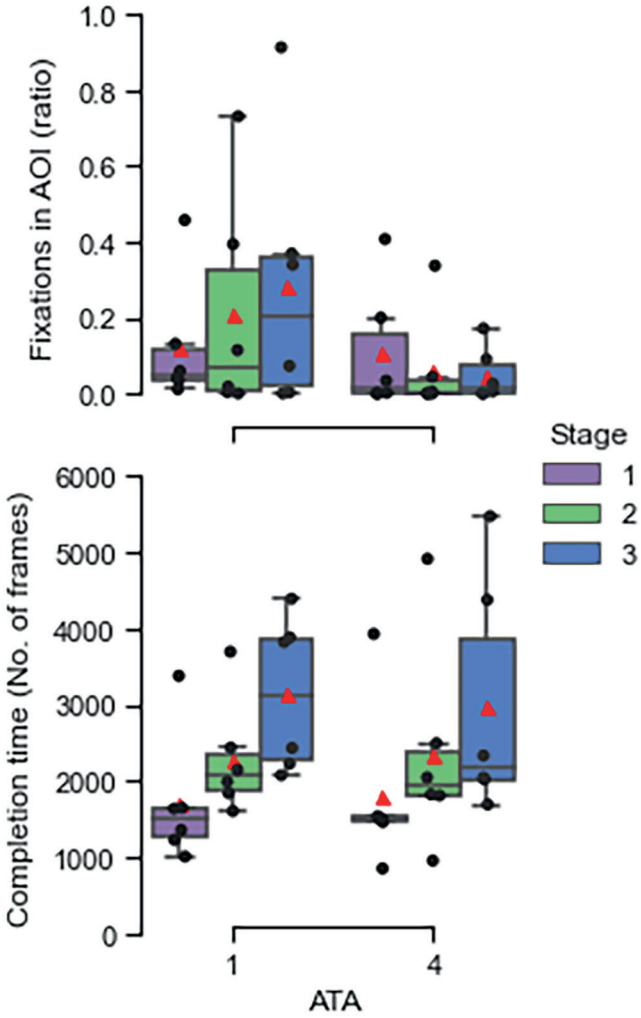

Fig. 4. Post hoc comparisons with Bonferroni correction of the fixation ratios and the completion times. Fixations were measured as a fraction of fixations in are of interest (AOI) from all fixations in each condition (1 ATA: stage 1, 2, or 3; 4 ATA: stage 1, 2 or 3); the completion time was measured as the number of frames in each condition. Boxes represent 1st quartile (Q1) and 3rd quartile (Q3), black line represents the median (Me), whiskers represent values outside the middle 50% from minimum (lower whisker) to maximum (upper whisker), and the red triangle represents the mean (M)

Discussion

The influence of the environment on human behavior is subject to analysis and monitoring at many levels of social activity.[8,9] Particular attention is given to those conditions that are obviously not the natural environment of humans, and which have emerged with the progress of civilization. A common goal of such measures is to minimize the risk. The dynamic development of technology over the last 100 years and the ever-increasing automation, the main purpose of which is to increase productivity, has not removed man from the position of the last decision-making link.[10,11]

Available studies devoted to the problem of professional activities in a pressurized atmosphere mostly refer to the work performed by divers.[12,13] Work in open water and a pressure chamber seem to bear some physical similarities – for example, in both conditions, the increase in pressure of the environment causes an increase in respiratory effort and the toxicity of respiratory gases. Under both conditions, nitrogen is of key importance and is responsible for the development of nitrogen narcosis. Working in open water and a hyperbaric chamber differ mainly in the number of environmental distractors, which can affect the professional operator in different ways. Staying underwater involves exposure to a range of stress-activating stimuli with physical and psychological vectors. Physical stimuli include changes in physicochemical parameters of respiratory gases, space, visibility, and finally, breathing performed with the use of a breathing apparatus.[14] Hypothermia is an important aspect for the cognitive functioning of a diver. Body heat loss is dependent on the form of protection and the duration of exposure to the surrounding environment. The cumulative impact of stress-inducing stimuli has a detrimental effect on the diver's nervous system capacity and compromises their performance.[15] During therapeutic sessions

in pressure chambers, medical personnel together with the patient are subjected, as during diving, to the physical process of compression to the required pressure value, as predetermined in the therapeutic protocol. In our study, we did not record the temperature inside the pressure chamber during the compression of participants to the test conditions of 4 ATA. However, the increase in temperature inside the hyperbaric chamber during this procedure was a subjective feeling reported by each participant. The breathing process in pressure chambers takes place in a gaseous environment. Therefore, patients and the medical staff do not use any breathing devices. Hazards due to visual disturbances do not occur in a therapeutic pressure chamber. Due to patient-oriented environmental conditions, accidental hypothermia does not pose a significant risk to medical staff or patients inside the pressure chamber during a therapy session. Increased ambient absolute pressure and limited space are the main factors potentially adversely affecting a person inside the chamber. The common denominator for the described living environments is the physicochemical changes in the respiratory gases induced by physical changes in the environment and the performance of activities requiring high cognitive performance in an unnatural environment or limited space.

A study conducted in a pressure chamber by Łaszczyńska et al. using the eye-tracking technique revealed a deterioration in the ability of the cohort of divers assessed in the context of performing operator-like activities in simulated diving conditions at depths of 30 m and 50 m. The authors also showed that, based on eye–hand coordination tests, there was a significant increase in the number of errors made by participants under 4 ATA conditions. In the case of exposure to 2 ATA, the effect of "gaining practice" in the given activities appeared in the subjects. The time of saccadic eye movements was also assessed but without separation of gaze fixation in and out of the AOI.[16] Despite the different methods of assessing eye movements, the results obtained in the present paper and in the described study seem to converge. Cognitive improvement with decreasing ambient pressure should be considered the point of convergence here. Taking advantage of the different possibilities of assessing saccadic eye movements during performed activities, we decided to select areas of visual interest at each stage of the test. According to experts, the identification of AOIs in eye-tracking studies carries certain risks (for example risk associated with the selection of specific test conditions) for the obtained measurement results.[17] However, due to the fact that our study had the same test conditions for each measurement and was conducted on a group of experts, i.e., medical professionals, we eliminated the risk raised in the literature related to the lack of precise location of AOI in the environment observed by the researcher, if only by narrowing the operator space for the task performed. Blake et al. investigated the effects of working in hyperbaric therapy chambers on the attentional skills of staff using the Trail Making Test-A (TMT-A). Their study was conducted in a group of 28 volunteers who were either medical professionals or candidates to work in a hyperbaric facility. The test was conducted under 1 ATA, 1.8 ATA and 3 ATA conditions. The time during which the participants performed the task assessed using the TMT-A was considered a predictor at each stage of the study. No statistically significant differences were found regarding the time required for subjects to complete the TMT-A in 1.8 ATA and 3 ATA conditions. After completing the task, the participants noted that they experienced anxiety and some degree of fogginess while under 3 ATA. This suggests that visual testing methods may not be sensitive enough to assess the effects of elevated atmospheric pressure on cognitive functions.[18] In our study, we attempted to assess the level of attentional focus only during the performance of medical tasks in a real-time setting. In our opinion, oculography is a good tool that offers such technical possibilities.

Studies on the influence of elevated atmospheric pressure on human cognitive functions published in medical literature emphasize the importance of nitrogen necrosis. The necrotic properties of nitrogen in humans are revealed when the partial pressure of this gas increases. A depth of 30 meters underwater (muw) is considered to be the point of development of nitrogen necrosis, beyond which the risk of developing nitrogen narcosis increases significantly. At shallower depths, the occurrence of nitrogen necrosis is symptomatically similar to alcohol intoxication and poses a threat to the life of the diver.[19] Karakaya et al. in their study involving the assessment of recorded EEG potentials collected in a hyperbaric chamber under 3 study conditions (pre-dive, deep-dive and post-dive) observed that nitrogen necrosis caused mild and moderate impairment of cognitive functions among air recreational scuba divers at a pressure of 5 ATA. Cognitive impairment was also registered after decompression.[20] We did not examine the full spectrum of cognitive functions in our study, but focused on assessing the function of attention. However, for safety reasons, the protocol of our study included assessment for the development of behavioral symptoms of nitrogen necrosis in participants. We did not observe any overt symptoms of nitrogen narcosis. Although most medical procedures in hyperbaric chambers are performed under the conditions of 2–3 ATA, there are disease entities (e.g., gas embolism and decompression sickness) where the value of 3 ATA is exceeded. It is for this reason we evaluated the effects of elevated atmospheric pressure equal to or greater than 3 ATA on the attention function of medical personnel. In the future, this research may have a significant impact on improving the safety and quality of care for patients requiring hyperbaric therapy.

Limitations

The main limitation of the study was the small size of the cohort. It resulted from technical and logistical dependencies. The study was carried out in a therapeutic

hyperbaric chamber, and the technical aspect of calibrating the eye tracker through the vision window of the chamber turned out to be a big problem. Calibration of the eye tracker took place before the start of the individual test and took longer than the test protocol provided for, which potentially affected the amount of data obtained.

Conclusions

The results we obtained and the test methods are supported by scientific literature. The assessment of eye movements using eye-tracking under hyperbaric chamber conditions was challenging due to its unprecedented nature in logistical and technical terms. Due to the results obtained in this study, we intend to continue further work on the effects of elevated atmospheric pressure on simple cognitive functions in medical personnel.

Supplementary materials

The Supplementary materials are available at https://doi.org/10.5281/zenodo.8234186. The package contains the following files:

Supplementary File 1. Python code with comparisons between models, chosen model results, diagnostics, and visualizations.

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Regionalized analysis of gut barrier components in the small intestine of BALB/c mice following chronic immobilization stress

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Conflict of interest
None declared

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Abstract

Background. Microbiota and tight junction proteins (TJPs) are components of the gut barrier, and are considered stress targets that have deleterious effects on intestinal homeostasis.

Objectives. This study aimed to evaluate the effects of chronic immobilization stress on selected small intestine homeostasis parameters.

Materials and methods. Female BALB/c mice were divided into a stress group that underwent short-term immobilization for 2 h per day for 4 consecutive days, and a non-stressed control group (n = 6 per group). Proximal and distal small intestine samples were excised to assess colony-forming units per gram (CFU/g) of total bifidobacteria in selective agar plates, luminal albumin was assessed using immune-enzymatic assay, pro-inflammatory cytokines were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and TJPs (pore-forming, claudin (Cld)-2; pore-sealing, Cld-4; ambiguous, Cld-7, -12 and -15) were assessed with RT-qPCR and western blotting.

Results. Compared with the control group, the stress group had lower body weight and energy intake. In the distal region, the stressed mice had lower bifidobacteria count and messenger ribonucleic acid (mRNA) expression of Cld-2, Cld-4 and Cld-12, though they had more albumin and higher interleukin (IL)-6 mRNA expression. Within the proximal region, the stressed mice had higher mRNA expression of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-6, Cld-7, Cld-12, and Cld-15, along with lower levels of IL-10 and Cld-4. However, mRNA and protein expression of TJPs were discordant.

Conclusions. These findings indicate divergent stress-induced outcomes in the small intestine, evidenced by the elicitation of a pro-inflammatory response and decreased anti-inflammatory response in the duodenum, and by increased albumin transudation and decreased bifidobacterial growth in the distal region.

Key words: small intestine, tight junction proteins, board immobilization stress, intestinal albumin, intestinal bifidobacteria

Background

Intestinal homeostasis refers to a healthy balance of gut barrier components regarded as markers of intestinal health, including microbiota and tight junction proteins (TJPs) that bind the epithelial monolayer paracellularly.[1] Gut commensals, such as those of the genus *Bifidobacterium*, secrete organic acids that enhance the structural and functional integrity of TJPs and prevent the deleterious effects of the potent pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) on epithelial permeability.[2] Gradients in the density and diversity of such microbiota increase from the proximal to distal small intestine regions, and reach their maximum extent in the colon.[3]

Tight junction proteins, including occludin and claudins (Clds), are expressed at the most apical side of the epithelial cell paracellular membrane and have a regionalized location in each segment of the gastrointestinal tract,[4] with occludin involved in regulating the rate of flux of molecules across the epithelial layer.[5] At the same time, Clds are classified as pore-sealing, pore-forming or ambiguous, with the latter encompassing Cld-7, -12 and -13. The pore-sealing variants, Cld-1, -3, -4, -5, -8, and -18, provide high transepithelial electrical resistance (TEER), resulting in low solute permeability in the intestine. In contrast, the pore-forming proteins form channels that enable solute flow and include Cld-2 and Cld-10.[6]

Experimental assays demonstrate a wide array of interweaving pathways through which stress regulates commensal microbiota[7] and TJPs.[8] Chronic stress had a downmodulatory effect on the commensal bifidobacterial density in the colon of rats under restraint stress for 2 h a day over 4 days,[9] while the abundance in metagenomic terms of *Bifidobacterium pseudolongum* increased in the colon of mice subjected to chronic unpredictable mild stress.[10] Moreover, the interactions between chronic stress and microbiota through the brain–gut–microbiota axis impact behavior and TJP remodeling through pro-inflammatory cytokines.[11,12] Indeed, stress regulation of TJPs is partly mediated by TNF-α-induced expression of the myosin light chain kinase protein and the simultaneous opening of the gut barrier, as documented using in vitro Caco2-intestinal cultures.[13]

Water avoidance stress in male rats for 1 h a day over 10 days reduced the tissue location of Cld-1, occludin and zonular occludin-1 (ZO-1) in the colon but not in the jejunum. Interestingly, injecting corticosterone mimicked these effects, and they were blocked by the corticoid receptor antagonist RU-486, suggesting a regionalized stress-induced regulation of TJPs via corticosteroid receptor signaling.[14] In experimental settings of chronic isolation stress in female rats, treatment with spironolactone decreased Cld-15 mRNA levels only in small intestine regardless the stress conditions indicanting a role for MR in the Cld-15 expression[15] Moreover, rats subjected to subacute stress through isolation and limited movement for 24 h combined with chronic crowding stress for 14 days experienced a jejunal-specific decrease in Cld-2 messenger ribonucleic acid (mRNA) expression, with no colonic changes. However, exposure to subacute stress alone increased jejunal mRNA levels of Cld-1, Cld-5, Cld-8, occludin, and ZO-1, but did not affect the colon.[16] Other studies show that chronic restraint stress for 10 h a day over 3 days in mice decreased the expression of Cld-1 and occludin in the duodenum, jejunum and ileum.[17] Furthermore, chronic stress by tail suspension in rats for 14 or 21 days decreased the tissue expression and mRNA expression of occludin and ZO-1 in the jejunum.[18] Previous work also described the effects of immobilization stress on TJPs in the colon[19] and the contribution of stress to the progression and aggravation of pathologies affecting the small intestine.[20]

Objectives

This study aimed, for the first time, to assess the impact of chronic immobilization-induced stress on commensal bifidobacteria and epithelial TJPs of the duodenum and ileum by measuring pro-inflammatory and anti-inflammatory cytokines, and using albumin as a marker of permeability by assessing its extravasation from the blood to the intestinal lumen.[21]

Materials and methods

Animals

Twelve 6-week-old female BALB/c mice were housed at the Laboratory Animal Experimentation and Production Unit of the Metropolitan Autonomous University of Xochimilico (Mexico City, Mexico), in 2 groups of 6 mice each, under a 12-hour light/dark cycle (light on at 7:00 AM, light off at 7:00 PM), at room temperature (~20°C) and relative humidity of 55%. Mice were allowed free access to Laboratory Rodent Diet 5001 (LabDiet, St. Louis, USA) and water, and were housed for 2 weeks to adapt to the conditions. The same trained handler performed animal manipulations between 8:00 AM and 11:00 AM to reduce the influence of the circadian cycle on fluctuations in the levels of corticosterone and adrenocorticotropic hormones. The Internal Committee for the Use and Care of Laboratory Animals of the Metropolitan Autonomous University of Xochimilico approved the experimental interventions (protocol No. 176). Animals were maintained and handled following the Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999; Ministry of Agriculture, Mexico City, Mexico), and in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[22]

Body weight and energy intake

Body weight and food intake were determined on the 6th day for 7-week-old mice and on the 5th day for 8-week-old mice. The energy intake was calculated by multiplying the total food intake [g] and caloric content [kcal/g] (Laboratory Rodent Diet 5001 contains 4.675 kcal/g), and the values for each group from the 2 experiments were expressed as mean ± standard deviation (M ±SD).

Stress protocol

Eight-week-old mice were subjected to board immobilization stress for 2 h per day between the 2nd and 5th days using a previously described protocol.[19] Briefly, each mouse was placed on a polystyrene board in the prone position, with all 4 limbs and the middle part of the tail gently stretched, and then attached by placing a very low-adhesive tape directly on the skin or the rear central pads, overlaid with a high-adhesive tape. Curved strips of paperboard-adhesive tape reels were placed over the tape for the mouse to chew on, preventing self-inflicted injuries of the forelimb skin. The head was allowed to move freely, and limb twisting and whisker tearing were avoided. The tape was carefully removed after 2 h of immobilization, and the mouse was released. When the experimental group underwent the stress protocol, the control mice had no access to food or water.

Sampling

Upon completion of the stress protocol, mice were euthanized via exposure to isoflurane and exsanguination by cardiac puncture. The pyloric (proximal) and cecal (distal) segments of the small intestine were dissected into 7-centimeter-long sections, and fecal content was gently removed before flushing with 1.5 mL of sterile phosphate-buffered saline (PBS) (pH 7.2), containing a cOmplete Mini™ protease inhibitor cocktail (cat. No. 11836153001; Roche Diagnostics, Mannheim, Germany). Flushing was repeated 5 times using the same liquid and the samples were collected in a Petri dish to enrich the protein content before storing the samples at –20°C for the albumin enzyme-linked immunosorbent assay (ELISA). Then, 1-centimeter intestinal segments were opened longitudinally and deposited in preweighed sterile microcentrifuge tubes containing 0.5 mL of thioglycolate broth (cat. No. 211651; BD Biosciences, Sparks, USA) for total count of bifidobacteria. In addition, 1-centimeter-long segments were dissected, opened longitudinally and scraped using a glass microscope slide to collect whole mucosa samples that were immediately frozen at –70°C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assays.

Total count of bifidobacteria

Intestinal segments collected in sterile tubes containing thioglycolate broth (0.5 mL) were weighed and then fully homogenized. Intestinal homogenates were serially diluted (×10) in thioglycolate broth and 10 µL of the serial dilutions were plated on propionate agar (cat. No. 1.000430.0500) containing lithium mupirocin (cat. No. 1000.45.0010) (both from Merck, Darmstadt, Germany). After the incubation for 72 h at 37°C under anaerobic conditions using GasPack™ anaerobic sachets (cat. No. 260678) and GasPack™ dry anaerobic indicator strips (cat. No. 271051) (both from BD Biosciences), the number of colonies was counted to determine the colony-forming units per gram (CFU/g) of tissue.

Immuno-enzymatic assay

The Bradford assay quantified total protein in intestine samples using a commercial dye reagent (cat. No. 500-0006; Bio-Rad, Hercules, USA). Albumin quantitation was undertaken using sandwich ELISA in 96-well polystyrene microplates (cat. No. 8590; Costar Corning, Corning, USA) coated with goat IgG anti-mouse albumin (0.01 µg/mL) (cat. No. ab19194; Abcam, Waltham, USA) diluted in carbonate–bicarbonate buffer (pH 9.6). After the incubation overnight at 4°C, plates were washed with PBS containing 0.05% Tween 20 (PBST) (200 µL) and blocked with 5% w/v blotto (cat. No. 37530; Thermo Fisher Scientific, Waltham, USA) diluted in carbonate–bicarbonate buffer (pH 9.6), incubated for 2 h at 37°C, and then washed. The intestinal fluid samples were diluted in 5% w/v blotto in PBST, with the dilution adjusted for total protein concentration. After the incubation overnight at 4°C the wells were washed, horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse albumin antibody (cat. No. ab19195; Abcam) diluted in 5% w/v blotto–PBST was added, and the plates were incubated for 1 h at 37°C. The plates were washed, a substrate solution (hydrogen peroxide and orthophenylenediamine in citrate phosphate buffer (pH: 5.0)) was added, and the samples were incubated for 20 min in the dark at room temperature. The enzymatic reaction was stopped with 2.5 M sulfuric acid (100 µL/well), and the absorbance was measured at 492 nm using an Epoch™ microplate reader (BioTek, Winooski, USA). An ELISA assay using purified mouse albumin (cat. No. A3139; MilliporeSigma, St. Louis, USA) was used to prepare an albumin [µg/mL] standard curve, and albumin concentration was expressed per 100 mg of total protein in the intestinal samples. All reactants and samples were tested in triplicate.

Reverse transcription-quantitative polymerase chain reaction

The RNA was isolated from proximal and distal mucosa using TRI Reagent® (cat. No. TR 118; Molecular Research

Center, Inc., Cincinnati, USA), and total RNA (0.2 µg) was used as a template. The determination of mRNA expression of pro-inflammatory cytokines (TNF-α, interferon gamma (IFN-γ) and interleukin (IL)-6), an anti-inflammatory cytokine (IL-10), and TJPs (occludin, and Cld-2, -4, -7, -12, and -15) was performed using the $2^{-\Delta\Delta CQ}$ method[23] described in detail by Machorro-Rojas et al.[19]

Western blot assay

Protein levels of Cld-2, -4, -7, -12 and -15, and occludin were assayed with western blot, according to the method described in detail by Machorro-Rojas et al.,[19] using whole mucosa extracts from the proximal and distal segments of the small intestine.

Statistical analyses

All data analyses employed GraphPad Prism v. 8.0.1 software (GraphPad Software Inc., San Diego, USA), except for the energy intake and z-scores between the control and stress groups, which used a Social Science Statistics website (https://www.socscistatistics.com/tests/mannwhitney/default.aspx). Experimental assays were repeated twice, with representative data from 1 assay presented (n = 12). Data are expressed as the median with interquartile range (IQR) (Q1–Q3) (n = 6/group), and were compared using the nonparametric Mann–Whitney U test. A value of $p < 0.05$ indicated a statistically significant difference.

Results

Stress reduced distal bifidobacteria count, body weight and energy intake

Compared to the control group, stressed mice had a lower bifidobacteria count in the proximal segment (p = 0.522, z-score = 0.6405; Fig. 1A) and a significantly lower count in the distal region (p = 0.030, z-score = 2.1617; Fig. 1B). Also, body weight (p = 0.020, z-score = –2.321; Fig. 1C) and energy intake (p = 0.049; Fig. 1D) were significantly lower in the stress group than in the control group.

Stress displayed divergent effects on albumin and cytokine responses in each region

Albumin concentration was lower in the proximal region (p = 0.092, z-score = 1.6814; Fig. 2A) and higher in the distal region (p = 0.013 z-score = –2.4819; Fig. 2B) in the stress group compared to the control group. Meanwhile, relative mRNA expression of TNF-α (p = 0.008 z-score = 2.6473), IFN-γ (p = 0.005 z-score = –2.8022) and IL-6 (p = 0.008, z-score = 2.6473) were higher (Fig. 2C), and the expression of IL-10 was lower (p = 0.008, z-score = –2.6473; Fig. 2E) in the proximal region of the stress group. However, only mRNA expression of IL-6 significantly increased (p = 0.005, z-score = –2.8022) in the distal region of the stress group compared to the same region in the control group (Fig. 2D,F).

Stress increased the mRNA expression of ambiguous claudins in the proximal small intestine

The analysis of mRNA in the proximal region (Fig. 3A) demonstrated higher expression of the ambiguous Clds, Cld-7, Cld-12 and Cld-15 in the stress group than in the control group (all p = 0.005, z-score = –2.8022). Meanwhile, the mRNA expression of Cld-4 was lower (p = 0.005, z-score = 2.8022) in the stress group, and the expression of occludin (p = 0.065, z-score = –1.8414) and Cld-2 (p = 0.378, z-score = 0.8807) was nonsignificant. Western blot analysis (Fig. 3B) showed increased protein levels of Cld-2 (p = 0.005, z-score = –2.8022) and lower levels of Cld-15 (p = 0.005, z-score = 2.8022) in the stress group.

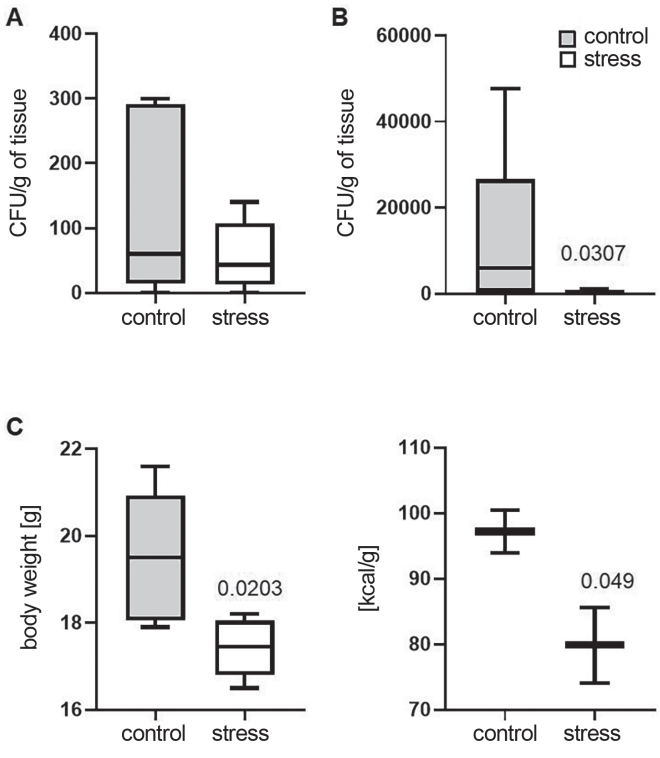

Fig. 1. Colony forming units (CFU) per gram of total bifidobacteria count in proximal (A) and distal (B) regions of the small intestine from the control and stress groups. Body weight [g] (C) and energy intake [kcal/g] (D) in the control and stress groups. Data (n = 6 per group) are presented as the median (midline), 1st quartile (Q1) (bottom line), 3rd quartile (Q3) (top line), and the minimum and maximum values (whiskers). The p-values in the stress column indicate significant differences in the stress group as compared to the control group, and were calculated using the Mann–Whitney U test

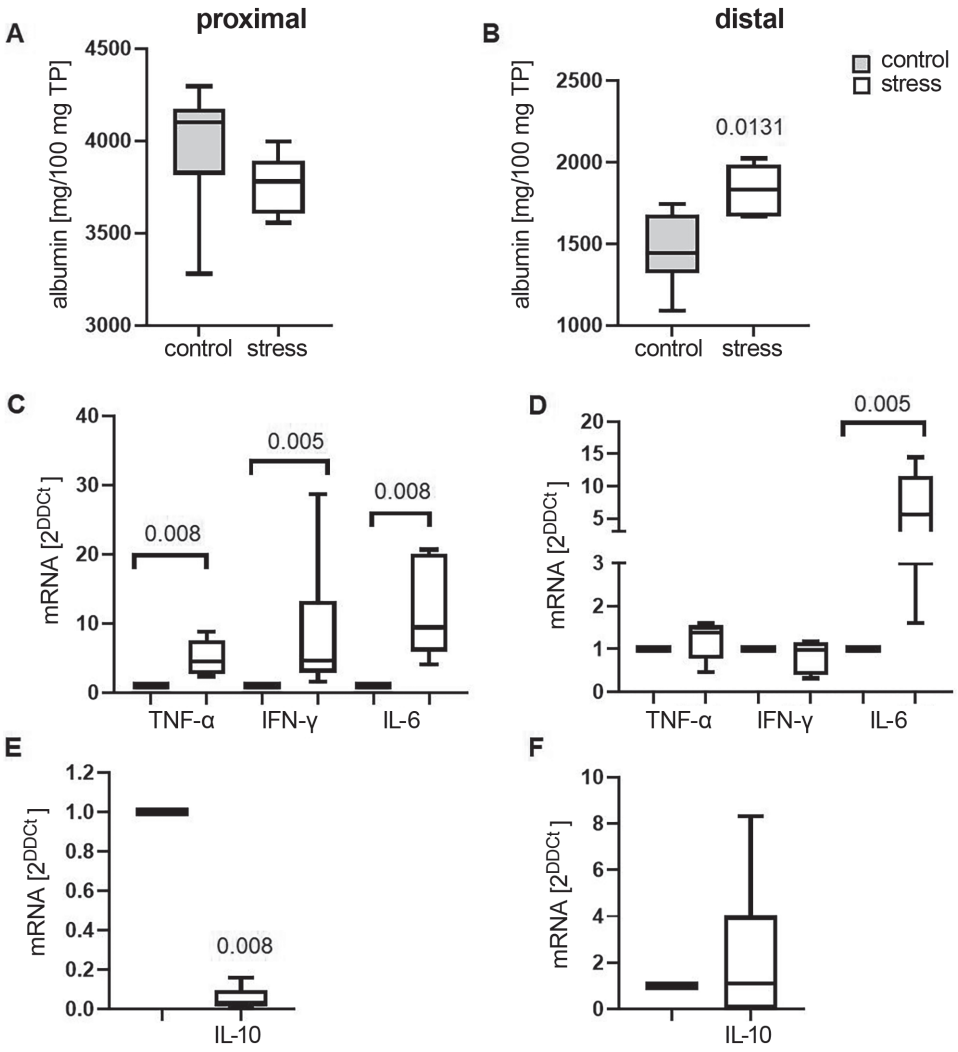

Fig. 2. Albumin concentration (µg/100 mg of total protein) in the proximal (A) and distal (B) small intestine segments from the control and stress groups. Relative messenger ribonucleic acid (mRNA) expression of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-6, and IL-10 in the proximal (C and E) and distal (D and F) regions of the small intestine from control and stress groups. Data (n = 6 per group) are presented as the median (midline), 1st quartile (Q1) (bottom line), 3rd quartile (Q3) (top line), and the minimum and maximum values (whiskers). The p-values in the stress column indicate significant differences in the stress group compared to the control group, and were calculated using Mann–Whitney U test

Stress decreased the mRNA expression of claudins in the distal small intestine

The analysis of mRNA in the distal region of the small intestine (Fig. 4A) showed lower expression of Cld-2, Cld-4 (both p = 0.005, z-score = 2.8022) and Cld-12 (p = 0.030, z-score = 2.1721) in the stress group. However, Cld-12 protein levels (Fig. 4B) were lower in the stress group compared to the controls (p = 0.032, z-score = 2.1529).

Discussion

Previous research described the effects of immobilization stress on TJPs in the colon,[19] so the present study focused on assessing, for the first time, the impact of chronic immobilization stress on selected markers of intestinal health and dysfunction in small intestine regions. In this regard, the study evaluated bifidobacteria, as they were found to be a reproducible and sensitive marker under the experimental conditions of stress used. Moreover, the approach is concordant with other studies in which bifidobacteria count was analyzed when monitoring the impact of stress on the intestine using conventional bacterial counts with selective agar plates.[24] Under the experimental conditions of this study, stress caused a significant decrease in the amount of bifidobacteria in the distal segment of the small intestine. This finding is in line with the reduced bifidobacteria load found in the colon and feces of rats and mice that underwent chronic restraint stress.[9,11] In addition, the apparent bifidobacteria amount decrease in the proximal region may be due to the bacterial load being below the limit of detection, as this area is scarcely populated with microbiota compared to the distal segment.[3]

Similar to restraint and crowding stress, the stress induced in the current study promoted the loss of body weight and reduced caloric intake.[11,16,25] Microbiota abundance is modulated by food intake and can be disrupted by increased energy expenditure under stressful conditions.[7,26] Moreover, stress-induced decreases in bifidobacteria load and body weight might contribute to anxiety and depression-like behaviors.[11] Mice under chronic restraint stress had decreased acetate levels in the cecum but not in the ileum,[12] and bifidobacteria are known to release acetate into the bloodstream.[27] The acetate can cross

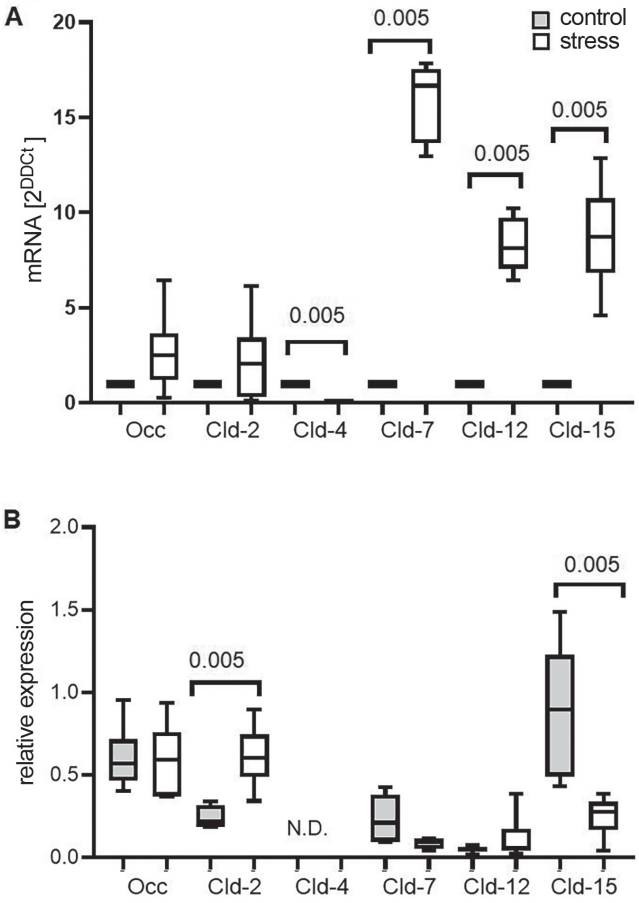

Fig. 3. Relative messenger ribonucleic acid (mRNA) expression (A) and protein levels (B) of occluding (Occ), claudin (Cld)-2, Cld-4, Cld-7, Cld-12, and Cld-15, in the proximal region of the small intestine from the control and stress groups. Data (n = 6 mice per group) are presented as the median (midline), 1st quartile (Q1) (bottom line), 3rd quartile (Q3) (top line), and the minimum and maximum values (whiskers). The p-values in the stress column indicate significant differences between the stress and control groups calculated using Mann–Whitney U test

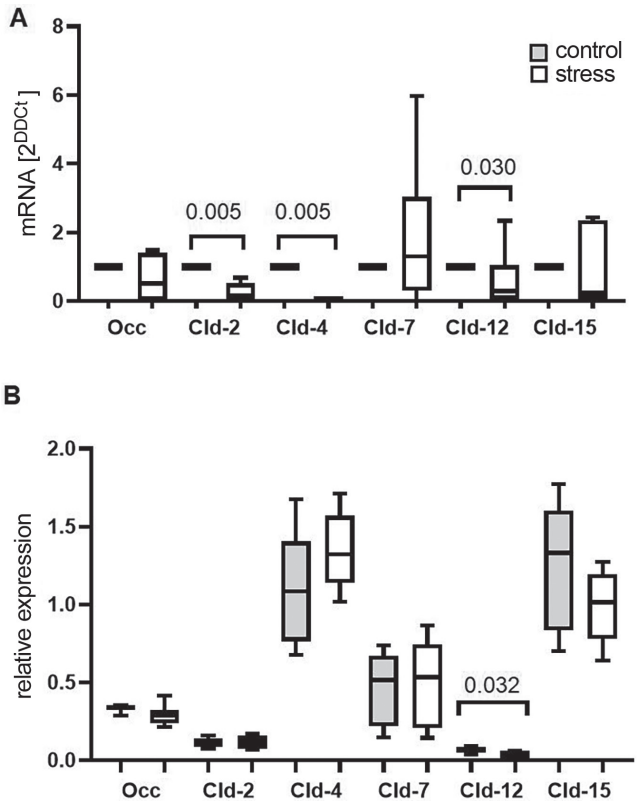

Fig. 4. Relative messenger ribonucleic acid (mRNA) expression (A) and protein levels (B) of occluding (Occ), claudin (Cld)-2, Cld-4, Cld-7, Cld-12, and Cld-15 in the distal segment of the small intestine from the control and stress groups. Data (n = 6 mice per group) are presented as the median (midline), 1st quartile (Q1) (bottom line), 3rd quartile (Q3) (top line), and the minimum and maximum values (whiskers). Calculated p-values displayed in the stress column indicate significant differences between the control and stress groups, and were calculated using Mann–Whitney U test

the blood–brain barrier and provide neuronal inputs that impact food intake,[28] while it acts as a ketogenic substrate for lipogenesis in peripheral tissues.[29]

The transport of mouse serum albumin from capillaries to the intestinal lumen requires crossing endothelial and epithelial barriers[30] as well as selective passage through the endothelial cell membrane, followed by passive intercellular transport as serum moves into the interstitial spaces during enterocyte disintegration.[31] In this study, albumin (66.5 kDa molecular mass) concentration increased in the distal region, suggesting that it is a target for stress-increased permeability. Meanwhile, chronic water avoidance stress in rats demonstrated increased polyethylene glycol-400 (400 Da molecular mass) permeability in the colon but not in the jejunum.[14] Evans blue (960.83 Da molecular mass) permeability increased in the duodenum but not in the ileum or colon, of mice under acute restraint stress.[32] These findings may be a result of, in part, the stress protocol used, the method of assessing permeability and the intestinal region analyzed.

The extravasation of luminal albumin was not stringently related to the mRNA expression of pro-inflammatory cytokines and TJPs in the proximal and distal small intestine regions. However, stress significantly increased mRNA levels of the mRNA of pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ) and reduced the anti-inflammatory cytokine IL-10 mRNA levels in the proximal region, whereas it only triggered increased IL-6 mRNA expression in the distal segment. Although the mRNA expression of pro-inflammatory cytokines has been shown in the colon of mice after board immobilization stress, it is unclear if these parameters have been documented in the duodenum using this model.[19] Nonetheless, previous studies demonstrated increased TNF-α and IFN-γ mRNA levels and decreased IL-10 mRNA levels in the ileum after chronic restraint stress.[33]

Pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6, among others, contribute to enhanced gut permeability and TJP remodeling in the colon[14,34] and ileum of rats after chronic water avoidance stress[35] and the ileum of mice under restraint stress.[33] Moreover, pro-inflammatory cytokines play a critical role in TJP expression.[36] In this study, no clear pattern was found between

cytokine mRNA expression and TJPs in the proximal and distal segments. Under stress conditions, mRNA expression of Cld-4 (barrier enhancer) decreased, and of Cld-7, Cld-12 and Cld-15 (ambiguous) increased in the proximal region, whereas mRNA expression of Cld-2 (pore-forming), Cld-4 and Cld-12 was reduced in the distal region. In addition, protein levels of Cld-4 were negligible in the proximal region in both groups, while Cld-15 was detected in both regions. These findings may result from the regionalized expression of Cld-4, which is predominantly found in the distal small intestine and colon, whereas Cld-15 is distributed along the entire gastrointestinal tract.[37]

The findings of this study may be due to the divergent effects of stress-induced hormones on TJP expression in each small intestine region. As documented previously, TJPs in the jejunum may be regulated by transcriptional factors other than glucocorticoid receptors (GRs). Indeed, GR protein expression was lower, and TJP expression was more stable in the jejunum than colon under stress conditions, which suggests that the stress-associated decrease in TJP expression in the colon resulted from proteasome degradation.[14] Moreover, a number of pathways control Cld transcription via several factors, including Snail, grainyhead-like 2 (GRHL2) and nuclear factor-kappa B (NF-κB). Furthermore, post-translational Cld modifications, including phosphorylation, ubiquitination and palmitoylation, may occur during endocytosis, exportation to subcellular compartments and proteasome degradation, among others.[38]

Limitations

The expression of GRs in the proximal and distal segments was not evaluated, and such data would have provided insight into the mechanisms driving the stress-induced effects.

Conclusions

The findings of this study indicate divergent outcomes of stress in the small intestine through increased pro-inflammatory and decreased anti-inflammatory cytokine mRNA expression in the duodenum. Furthermore, stress increased albumin extravasation and reduced bifidobacterial growth in the distal region of the small intestine.

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Effects of heat shock protein 90 on complement activation in myocardial ischemia/reperfusion injury after pioglitazone preconditioning

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Conflict of interest
None declared

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Abstract

Background. Heat shock protein 90 (HSP90) appears to have a pivotal function in ischemic preconditioning. Pioglitazone preconditioning (PioC) attenuates myocardial ischemia/reperfusion (I/R) injuries.

Objectives. The current study aims to investigate the role of HSP90, complement C3 and C5a, and nuclear factor kappa-B (NF-κB) in PioC-induced cardioprotection.

Materials and methods. A total of 80 rats were randomly categorized into 4 groups, as follows: sham, I/R, PioC, and PioC+HSP90 inhibitor geldanamycin (PioC+GA). The sham group rats had a thoracotomy, in which the ligature was passed by the heart with no ligation (150 min). The other 3 groups were exposed to ischemia (30 min) followed by reperfusion (2 h). In the PioC group, pioglitazone (3 mg/kg) was administered intravenously 24 h before ischemia. In the PioC+GA group, after being pretreated with pioglitazone, GA was administered (intraperitoneally, 1 mg/kg) 30 min before ischemia. Myocardial infarct sizes (ISs), apoptosis rates, creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and cardiac troponin I (cTnI) serum levels were determined. The HSP90, C3, NF-κB, C5a, B-cell lymphoma-2 (Bcl-2), and Bax expression levels, as well as interleukin (IL)-1β, IL-6, intercellular cell adhesion molecule-1 (ICAM-1), and tumor necrosis factor alpha (TNF-α) mRNA levels were measured.

Results. The myocardial ISs, serum CK-MB, cTnI and LDH levels, apoptosis rates, IL-1β, IL-6, TNF-α, ICAM-1 release, as well as Bax, C5a, C3, and NF-κB protein expression were considerably lower in the PioC group than in the I/R group ($p < 0.05$). The Bcl-2 and HSP90 expression was higher in the PioC group than in the I/R group ($p < 0.05$). Geldanamycin inhibited the effects of PioC. These data strongly demonstrate that the PioC-induced is dependent upon HSP90 activity.

Conclusions. The HSP90 is indispensable for PioC-mediated cardioprotection. The HSP90 attenuates I/R-induced ISs, apoptosis of cardiomyocytes and myocardial inflammation through C3, C5a and NF-κB activation inhibition.

Key words: NF-κB, C3, HSP90, C5a, pioglitazone preconditioning

Background

Myocardial ischemia/reperfusion (I/R) injury is a serious acute myocardial infarction (AMI) complication after revascularization. Research has focused on methods and strategies to reduce myocardial I/R injury.[1] The cardioprotective impact of peroxisome proliferator-activated receptor gamma (PPAR-γ) has gained increasing interest in this area.[2,3] Pioglitazone belongs to the thiazolidinedione class and is a second-line drug in the treatment of type 2 diabetes acting by activating PPAR-γ to increase insulin sensitivity and improve glucose and lipid metabolism.[4,5] Pioglitazone exerts beneficial cardiovascular outcomes influencing cardiac function, metabolism, cardiac remodeling, etc.[6,7] In a previous clinical trial, I/R injury was reduced in diabetic patients after AMI who were pretreated with pioglitazone.[8] Previous observations have shown that pioglitazone-induced cardioprotection may activate extracellular signal-regulated kinase (ERK1/2) signaling pathways using cyclooxygenase-2 (COX-2) as the downstream target, depend on endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS), activate Akt, and increase the expression of cytosolic phospholipase A2 (cPLA2).[5,9,10] Nevertheless, the cardioprotective mechanism underlying pioglitazone in I/R injuries has not been fully elucidated.

The complement system, a key player in innate immunity, causes inflammation by recruiting immune cells and inducing the release of inflammatory factors during myocardial injury. Activation of the complement cascade in I/R injury causes myocardial tissue damage.[11] Emerging evidence indicates that complement components C3 and C5a promote the release of inflammatory factors by inducing nuclear factor kappa-B (NF-κB) signaling, which further aggravates myocardial I/R injury.[12,13] According to the literature, ischemic preconditioning impedes the upregulation of complement C3 and C5a induced by I/R injury in vivo.[14] There is a paucity of information regarding pioglitazone preconditioning (PioC)-induced cardioprotection in terms of its inhibitory effects on C3, C5a and NF-κB.

Heat shock protein 90 (HSP90) is a highly conserved molecular chaperone and a vital effector molecule for cardioprotection during ischemic preconditioning.[15] The inhibition of HSP90 aggravates complement-mediated cell lysis.[16] The HSP90 can bind to molecular chaperones, which are resistant to complement-induced cell death.[17] In our recent study, we demonstrated a correlation between HSP90, C5a, C3, and NF-κB signaling in ischemic postconditioning.[18,19] However, whether HSP90 contributes to the protective effects of PioC in myocardial I/R injuries by suppressing C3, C5a and NF-κB remains unclear. Therefore, in the present study, we investigated the roles of HSP90, C3, C5a, and NF-κB in PioC-induced cardioprotection.

An I/R injury rat model was constructed to study the anti-inflammatory impact of PioC. We assessed interleukin (IL)-1β, IL-6, intercellular cell adhesion molecule 1 (ICAM-1), and tumor necrosis factor alpha (TNF-α) messenger RNA (mRNA) levels. Western blot analysis was conducted to determine HSP90, C3, C5a, B-cell lymphoma-2 (Bcl-2), NF-κB, and Bax proteins levels in the sham, I/R, PioC, and PioC+geldanamycin (GA) groups.

Objectives

The current study aims to investigate the role of HSP90, complement C3 and C5a, and NF-κB in PioC-induced cardioprotection.

Materials and methods

Animals

Guangxi Medical University Experimental Animal Center (certificate No. SYXK (Gui) 2020-0001; Nanning, China) provided 80 adult male Sprague Dawley rats (8 weeks old) weighing between 250–280 g and kept in a 12:12-hour light/dark cycle, 50 ±15% humidity and 25 ±2°C temperature conditions with ad libitum access to food and water. The National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals was followed. The Guangxi Medical University Animal Protection and Use Committee approved this study (approval No. 201909028).

Experimental model

Anesthesia was provided using intraperitoneal sodium pentobarbital injection (50 mg/kg). A small animal ventilator (ALC-V8; Alcott Biotech Co., Shanghai, China) was used to ventilate the animals. The heart was exposed by opening the chest through the left 5th intercostal area. Suture ligation of the left anterior descending (LAD) coronary artery was used for 30 min to create myocardial ischemia. The suture was removed, and reperfusion was performed for 2 h. An ST-segment elevation on electrocardiogram (ECG) and myocardium whitening in the LAD blood supply area indicated the successful construction of the model. The rats were euthanized immediately after the reperfusion. Blood samples were drawn from the left ventricular anterior wall close to the ventricular myocardium apex for further research.

Experimental groups

The rats were randomly divided into 4 groups (n = 20 per group): 1) sham group, where a ligature was passed around the LAD with no ligation (150 min); 2) I/R group, where

the rats had 30 min of ischemia and then 120 min of reperfusion; 3) PioC group, where pioglitazone (3 mg/kg[20]) was administered intravenously 24 h before ischemia, followed by 30 min of ischemia and then 120 min of reperfusion; 4) PioC+GA group, where pioglitazone (3 mg/kg[20]) was administered intravenously 24 h before ischemia, and 30 min before ischemia, an intraperitoneal injection of GA (1 mg/kg[21]) was administered, followed by 30 min of ischemia and 120 min of reperfusion.

Myocardial infarct size

Each group was tested separately to calculate myocardial infarct size (IS) (n = 5). After reperfusion, we tightened the LAD and stained the inferior vena cava with 2% Evans blue dye. The IS was determined after the stained heart was frozen, sectioned into 2-millimeter pieces, and treated for 15 min with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, USA) at 37°C. Using digital imaging software, the IS as a percentage of the left ventricle (LV) was calculated quantitatively.

Lactate dehydrogenase, creatine kinase-MB and cardiac troponin I plasma levels

The serum was obtained by collecting 5-milliliter blood samples and centrifuging them at 3000 × g. The cardiac troponin I (cTnI), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) serum concentrations were determined utilizing respective enzyme-linked immunosorbent assay (ELISA) kits (cTnI: CSB-E08594r, CK-MB: CSB-E14403r, LDH: CSB-E11324r; all acquired from CUSABIO, Wuhan, China) according to the manufacturer's instructions.

TUNEL staining

Cardiomyocyte apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) detection kit. Stained samples were visualized under a light microscope (model CKX41SF; Olympus Corp., Tokyo, Japan). A minimum of 5 randomly selected fields containing apoptotic cells were scored and recorded. Apoptosis was indicated by brown-stained nuclei (TUNEL-positive cells) and its index was computed using this formula: the number of TUNEL-positive cells / total number of myocytes × 100%.

mRNA expression quantification

Trizol reagent (Invitrogen, Carlsbad, USA) was used to extract 1 μg of total RNA from the myocardial tissue in the ischemic areas, and it was purified. The PrimeScript™ RT reagent Kit (Takara, Kusatsu, Japan) synthesized complement DNA (cDNA) and quantitative polymerase chain reaction (qPCR) was performed using the SYBR standard qPCR mix (Takara) on an ABI Prism 7500 System (Thermo Fisher Scientific, Waltham, USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *β-actin* served as the housekeeping genes. Statistics for the *β-actin* are presented in the Supplementary material (https://doi.org/10.5281/zenodo.7766218). The $2^{-\Delta\Delta CT}$ method was utilized for analyzing relative mRNA expression. The following primers were used:

IL-1β gene:
forward: 5'-TAGCAGCTTTCGACAGTGAGG-3',
reverse: 5'-CCACAGCCACAATGAGTGAC-3';
IL-6 gene:
forward: 5'-CTGGTCTTCTGGAGTTCCGTT-3',
reverse: 5'-GCATTGGAAGTTGGGGTAGGA-3';
TNF-α gene:
forward: 5'-ATGGGCTCCCTCTCATCAGT-3',
reverse: 5'-GCTTGGTGGTTTGCTACGAC-3';
and *ICAM-1* gene:
forward: 5'-TGTCGGTGCTCAGGTATCCATCC-3',
reverse: 5'-TTCGCAAGAGGAAGAGCAGTTCAC-3'.

Western blot

After reperfusion, myocardial tissue from the ischemic areas was lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio, Beijing, China), treated with an ultrasonic tissue homogenizer (Servicebio, Wuhan, China), and then centrifuged for 15 min at 4°C and 12,000 × g. The enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China) was used to determine protein concentrations. Equal protein amounts were subjected to gel electrophoresis before being moved to the polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Bedford, USA), which were blocked at room temperature using 5% bovine serum albumin for 1 h before being treated with the primary antibodies against HSP90 (1:5000; Proteintech, Chicago, USA), Bcl-2 (1:500, Proteintech), Bax (1:1000, Cell Signaling Technology, Danvers, USA), β-actin (1:5000; Proteintech), C5a (1:5000; Invitrogen), NF-κB p65 (1:1000; Invitrogen), and C3 (1:1000; Abcam, Cambridge, UK) for 1 full day at 4°C, and then washed by Tween 20 and treated with the horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin (1:12000) for 1 h. For the detection of protein bands, the FluorChemFC3 imaging system (ProteinSimple, Santa Clara, USA) and ImageJ v. 1.8.0 software (National Institutes of Health, Bethesda, USA) were used.

Statistical analyses

The IBM SPSS v. 23.0 software (IBM Corp., Armonk, USA) was utilized to analyze the data, which were presented as mean ± standard deviation (M ±SD) for normally distributed variables. The Shapiro–Wilk test was

used for the normal distribution of continuous variables. A one-way variance analysis (ANOVA) was utilized to compare several groups. The Tukey's honestly significant difference (HSD) test was performed as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Two animals died due to ventricular fibrillation and machine malfunction – 1 in the I/R group and 1 in the PioC+GA group. The final results correspond to the data obtained on 78 rats.

PioC upregulated HSP90

To explore the potential link between HSP90 and PioC, we identified the HSP90 expression in myocardial tissue for each group. The PioC group had a higher HSP90 protein level than the I/R group (84.60 ±3.31% compared to 69.13 ±5.58%, $p < 0.001$, ANOVA, $F = 57.2$; Fig. 1 and Table 1,2). Notably, treatment with the HSP90 inhibitor, GA, counteracted the effects of PioC.

PioC alleviated I/R-induced myocardial IS via HSP90

The PioC group had lower IS/LV rate than the I/R group (21.12 ±3.12% compared to 43.00 ±3.40%, $p < 0.001$, ANOVA, $F = 65.6$; Fig. 2 and Table 3,4). Geldanamycin reversed the PioC protective effects (21.12 ±3.12% compared to 42.68 ±3.83%, $p < 0.001$, ANOVA, $F = 65.6$). Neither the I/R group nor the PioC+GA group had a statistically significant difference in IS/LV ($p = 0.988$, ANOVA, $F = 65.6$).

PioC alleviated I/R-induced cardiomyocytes apoptosis via HSP90

The cardiomyocyte apoptotic index was examined to determine whether HSP90 has a role in PioC anti-apoptosis. The PioC group had lower apoptosis rates than the I/R group (23.71 ±2.08% compared to 45.98 ±1.70%, $p < 0.001$, ANOVA, $F = 866.0$; Fig. 3A and Table 5,6). The PioC group had higher Bcl-2 and lower Bax expression levels than the I/R group (66.69 ±3.83% compared to 22.46 ±1.71%, $p < 0.001$, ANOVA, $F = 332.9$; 54.09 ±5.26% compared to 71.97 ±3.75%, $p < 0.001$, ANOVA, $F = 141.0$; Fig. 3B and

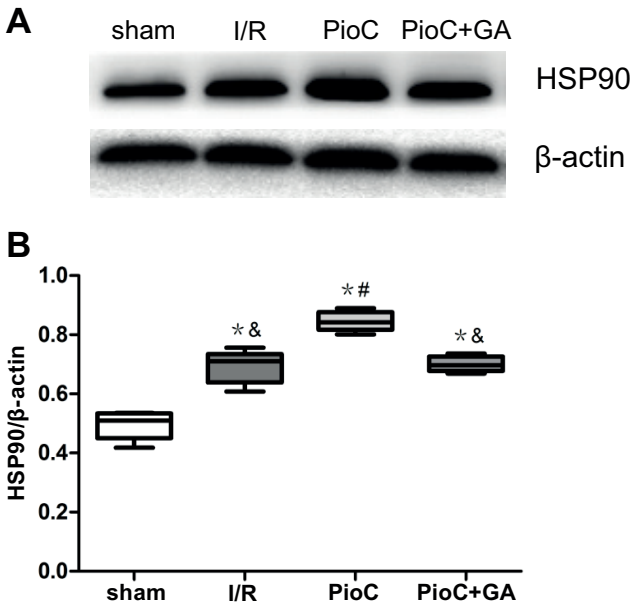

Fig. 1. Pioglitazone preconditioning (PioC) and geldanamycin (GA) effects on heat shock protein 90 (HSP90) expression following myocardial ischemia/reperfusion (I/R) injury. A. Western blots demonstrating HSP90 expression; B. Relative HSP90 expression; $p < 0.05$ compared to the *sham, #I/R and &PioC groups (Tukey's honestly significant difference (HSD) test following analysis of variance (ANOVA) testing); n = 5 in each group

Table 1. Results of ANOVA as presented in Fig. 1A,B

Variables		Sum of squares	df	Mean square	F	p-value
HSP90	between groups	0.310	3	0.103	57.219	<0.001
	within a group	0.029	16	0.002		
	total	0.339	19	N/A		

ANOVA – analysis of variance; HSP90 – heat shock protein 90; df – degrees of freedom; N/A – not applicable.

Table 2. Statistics for Fig. 1B

Variable		Min	Q1	Median	Q3	Max
HSP90	sham	0.4182547	0.4504220	0.5096487	0.5339405	0.5355048
	I/R	0.6075359	0.6388622	0.7102011	0.7343151	0.7561627
	PioC	0.8011262	0.8174776	0.8418486	0.8765956	0.8892547
	PioC+GA	0.6684666	0.6775302	0.6968863	0.7266420	0.7361755

Min – minimal value; Max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; HSP90 – heat shock protein 90; I/R – ischemia/reperfusion; PioC – pioglitazone preconditioning; GA – geldanamycin.

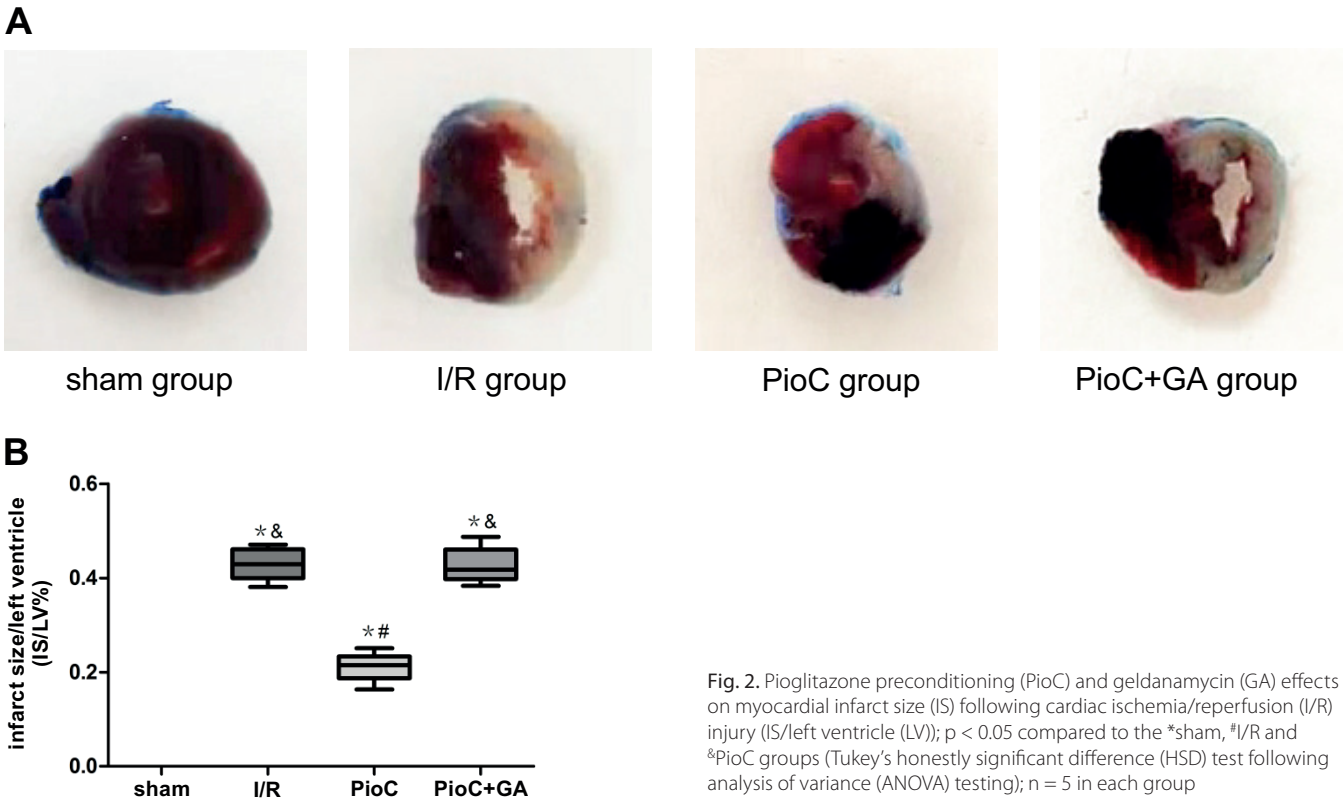

Fig. 2. Pioglitazone preconditioning (PioC) and geldanamycin (GA) effects on myocardial infarct size (IS) following cardiac ischemia/reperfusion (I/R) injury (IS/left ventricle (LV)); $p < 0.05$ compared to the *sham, $^{\#}$I/R and $^{\&}$PioC groups (Tukey's honestly significant difference (HSD) test following analysis of variance (ANOVA) testing); n = 5 in each group

Table 3. Results of ANOVA as presented in Fig. 2A,B

Variables		Sum of squares	df	Mean square	F	p-value
Infarct size	between groups	0.157	2	0.079	65.614	<0.001
	within a group	0.014	12	0.001		
	total	0.172	14	N/A		

ANOVA – analysis of variance; df – degrees of freedom; N/A – not applicable.

Table 4. Statistics for Fig. 2B

Variable		Min	Q1	Median	Q3	Max
Infarct size	sham	0	0	0	0	0
	I/R	0.3808338	0.3998195	0.4293745	0.4605114	0.4708008
	PioC	0.1631902	0.1873210	0.2146678	0.2332605	0.2505541
	PioC+GA	0.3834830	0.3977524	0.4178273	0.4602490	0.4873300

Min – minimal value; Max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; HSP90 – heat shock protein 90; I/R – ischemia/reperfusion; PioC – pioglitazone preconditioning; GA – geldanamycin.

Table 5,6), respectively. Geldanamycin inhibited the PioC anti-apoptotic effect on cardiomyocytes. These data suggest that HSP90 could effectively alleviate cardiomyocyte apoptosis and play a critical role in the anti-apoptotic effect of PioC.

PioC alleviated myocardial injury after I/R via HSP90

In comparison with the I/R group, CK-MB (1271.2 ±89.66 U/L compared to 828.6 ±53.13 U/L), LDH (1608.6 ±101.34 U/L compared to 824.0 ±22.36 U/L) and cTnI (325.44 ±34.67 ng/mL compared to 138.79 ±7.40 ng/mL) levels were considerably lower after I/R in the PioC group ($p < 0.001$, ANOVA, $F = 168.9$; $p < 0.001$, ANOVA, $F = 104.0$; $p < 0.001$, ANOVA, $F = 160.8$, respectively (Table 7,8). Geldanamycin reversed the beneficial effect of PioC in mitigating myocardial injury.

PioC alleviated I/R-induced activation of complement and NF-κB via HSP90

To explore whether PioC modulated the complement system and NF-κB signaling pathways through

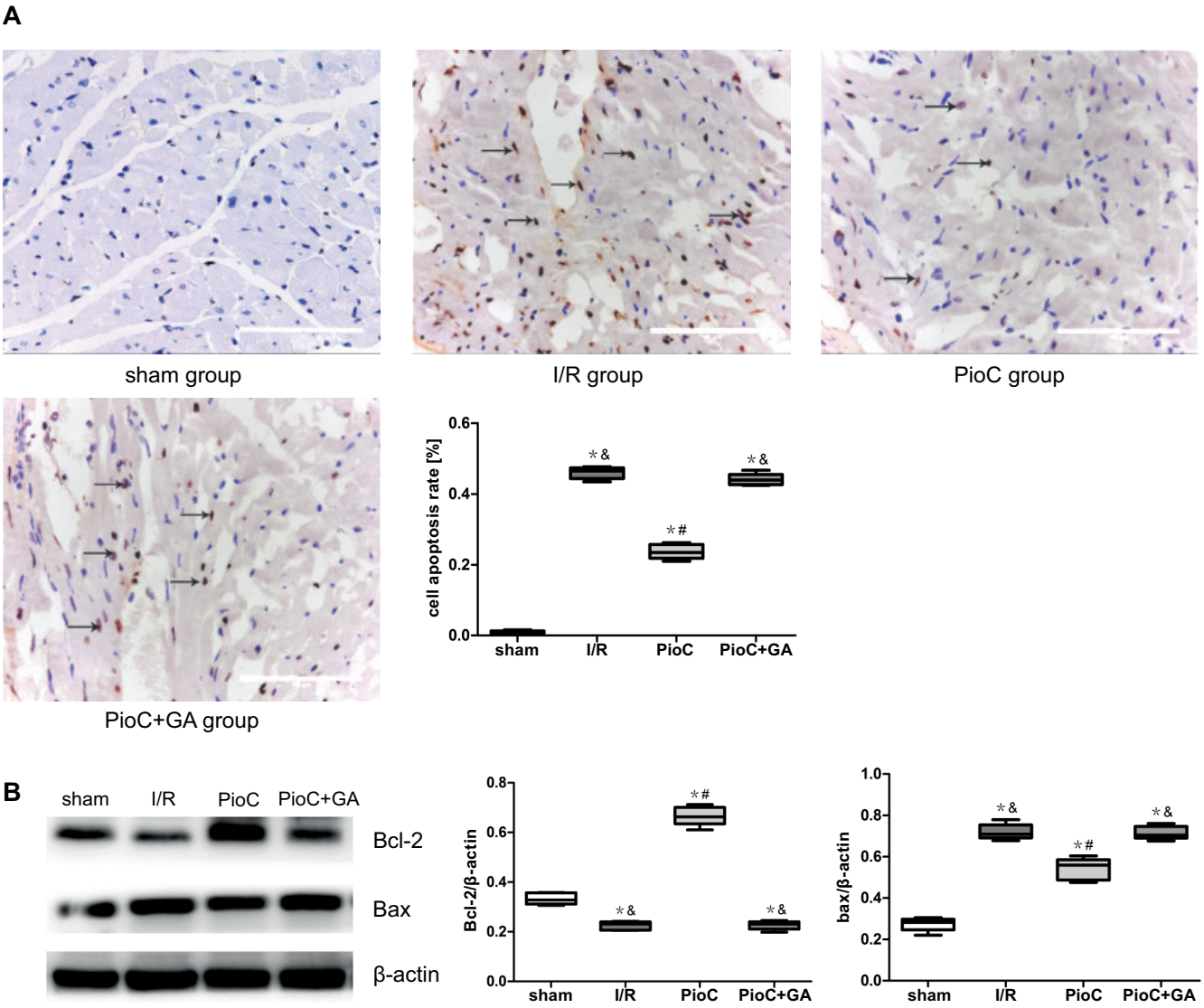

Fig. 3. A. Pioglitazone preconditioning (PioC) and geldanamycin (GA) effects on apoptosis following myocardial ischemia/reperfusion (I/R) injury (×400 magnification, bar = 100 μm). Normal cardiomyocytes are presented in blue, and an apoptotic nucleus is shown in brown (terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL)-positive cells). The arrow indicates TUNEL-positive cells; B. PioC and GA effects on B-cell lymphoma-2 (Bcl-2) and Bax protein expression; $p < 0.05$ compared to the *sham group, #I/R group and &PioC groups (Tukey's honestly significant difference (HSD) test following analysis of variance (ANOVA) testing); n = 5 in each group

Table 5. Results of ANOVA as presented in Fig. 3A,B

Variables		Sum of squares	df	Mean square	F	p-value
Apoptosis rate	between groups	0.663	3	0.221	866.049	<0.001
	within a group	0.004	16	<0.001		
	total	0.667	19	N/A		
Bcl-2	between groups	0.655	3	0.218	332.884	<0.001
	within a group	0.010	16	0.001		
	total	0.666	19	N/A		
Bax	between groups	0.660	3	0.220	141.028	<0.001
	within a group	0.025	16	0.002		
	total	0.685	19	N/A		

ANOVA – analysis of variance; df – degrees of freedom; N/A – not applicable; Bcl-2 – B-cell lymphoma-2.

Table 6. Statistics for Fig. 3A,B

Variable		Min	Q1	Median	Q3	Max
Apoptosis rate	sham	0.0066225	0.0066783	0.0098361	0.0129706	0.0159744
	I/R	0.4340836	0.4431144	0.4648829	0.4738945	0.4768212
	PioC	0.2100000	0.2181757	0.2343234	0.2574919	0.2624585
	PioC+GA	0.4244373	0.42602335	0.4391026	0.4547610	0.4668989
Bcl-2	sham	0.3060012	0.31137375	0.3274096	0.3573080	0.3580377
	I/R	0.2057575	0.2064249	0.2306815	0.2397666	0.242422
	PioC	0.6103339	0.6349405	0.6629329	0.7009239	0.7126300
	PioC+GA	0.1981251	0.2110812	0.2303862	0.2400198	0.2458526
Bax	sham	0.2200826	0.2461535	0.2823904	0.2969734	0.3052954
	I/R	0.6777682	0.6915758	0.7084501	0.7534922	0.778393
	PioC	0.4755463	0.4869095	0.5591846	0.5857422	0.6034487
	PioC+GA	0.6760882	0.6900726	0.7048742	0.7459117	0.760784

Min – minimal value; Max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; HSP90 – heat shock protein 90; Bcl-2 – B-cell lymphoma-2; I/R – ischemia/reperfusion; PioC – pioglitazone preconditioning; GA – geldanamycin.

Table 7. CK-MB, cTnI and LDH serum levels (M ±SD)

Groups	CK-MB [U/L]	cTnI [ng/mL]	LDH [U/L]
Sham	425.4 ±47.21$^{\#\&}$	7.96 ±1.51$^{\#\&}$	535.8 ±61.35$^{\#\&}$
I/R	1271.2 ±89.66$^{*\&}$	325.44 ±34.67$^{*\&}$	1608.0 ±101.34$^{*\&}$
PioC	828.6 ±53.13$^{*\#}$	138.79 ±7.40$^{*\#}$	824.0 ±22.36$^{*\#}$
PioC+GA	1193.4 ±68.57$^{*\&}$	310.33 ±39.73$^{*\&}$	1539.8 ±198.76$^{*\&}$

All results are shown as mean ± standard deviation (M ±SD). The Tukey's honestly significant difference (HSD) test was conducted following analysis of variance (ANOVA) testing. CK-MB – creatine kinase-MB; cTnI – cardiac troponin I; LDH – lactate dehydrogenase; I/R – ischemia/reperfusion; PioC – pioglitazone preconditioning; GA – geldanamycin. $p < 0.05$ compared to the *sham, $^{\#}$I/R and $^{\&}$PioC groups (Tukey's honestly significant difference (HSD) test following analysis of variance (ANOVA) testing); n = 5 in each group.

Table 8. Results of ANOVA as presented in Table 7

Variables		Sum of squares	df	Mean square	F	p-value
CK-MB	between groups	2253498.150	3	751166.050	168.876	<0.001
	within a group	71168.400	16	4448.025		
	total	2324666.550	19	N/A		
LDH	between groups	4215456.200	3	1405152.067	104.008	<0.001
	within a group	216161.600	16	13510.100		
	total	4431617.800	19	N/A		
cTnI	between groups	342268.241	3	114089.414	160.831	<0.001
	within a group	11349.980	16	709.374		
	total	353618.221	19	N/A		

ANOVA – analysis of variance; df – degrees of freedom; N/A – not applicable; CK-MB – creatine kinase-MB; LDH – lactate dehydrogenase; cTnI – cardiac troponin I.

HSP90, the complement components C3 and C5a and NF-κB expression were assessed in the presence of GA. The complement components C3 and C5a and NF-κB expression were assessed. The C3, C5a and NF-κB protein levels in the PioC group were lower than in the I/R group (65.03 ±3.32% compared to 78.62 ±3.04%, $p = 0.001$, ANOVA, $F = 105.2$; 57.31 ±4.71% compared to 75.35 ±5.49%, $p < 0.001$, ANOVA, $F = 190.8$; and 47.98 ±9.34% compared to 69.35 ±2.75%, $p < 0.001$, ANOVA, $F = 72.4$, respectively, Fig. 4 and Table 9,10). The mRNA levels of TNF-α, IL-1β, IL-6, and ICAM-1 in the PioC group were significantly lower than in the I/R group ($p < 0.001$, ANOVA, $F = 108.1$; $p < 0.001$, ANOVA, $F = 38.1$; $p < 0.001$, ANOVA, $F = 163.9$; $p = 0.017$, ANOVA, $F = 13.5$, respectively; Fig. 4B and Table 9,10). Geldanamycin negated the anti-inflammatory effects for PioC. These data demonstrate that PioC protects the heart from I/R-induced inflammatory responses by suppressing the activation of the complement system and NF-κB. Furthermore, the effects of PioC were closely linked to HSP90 activity.

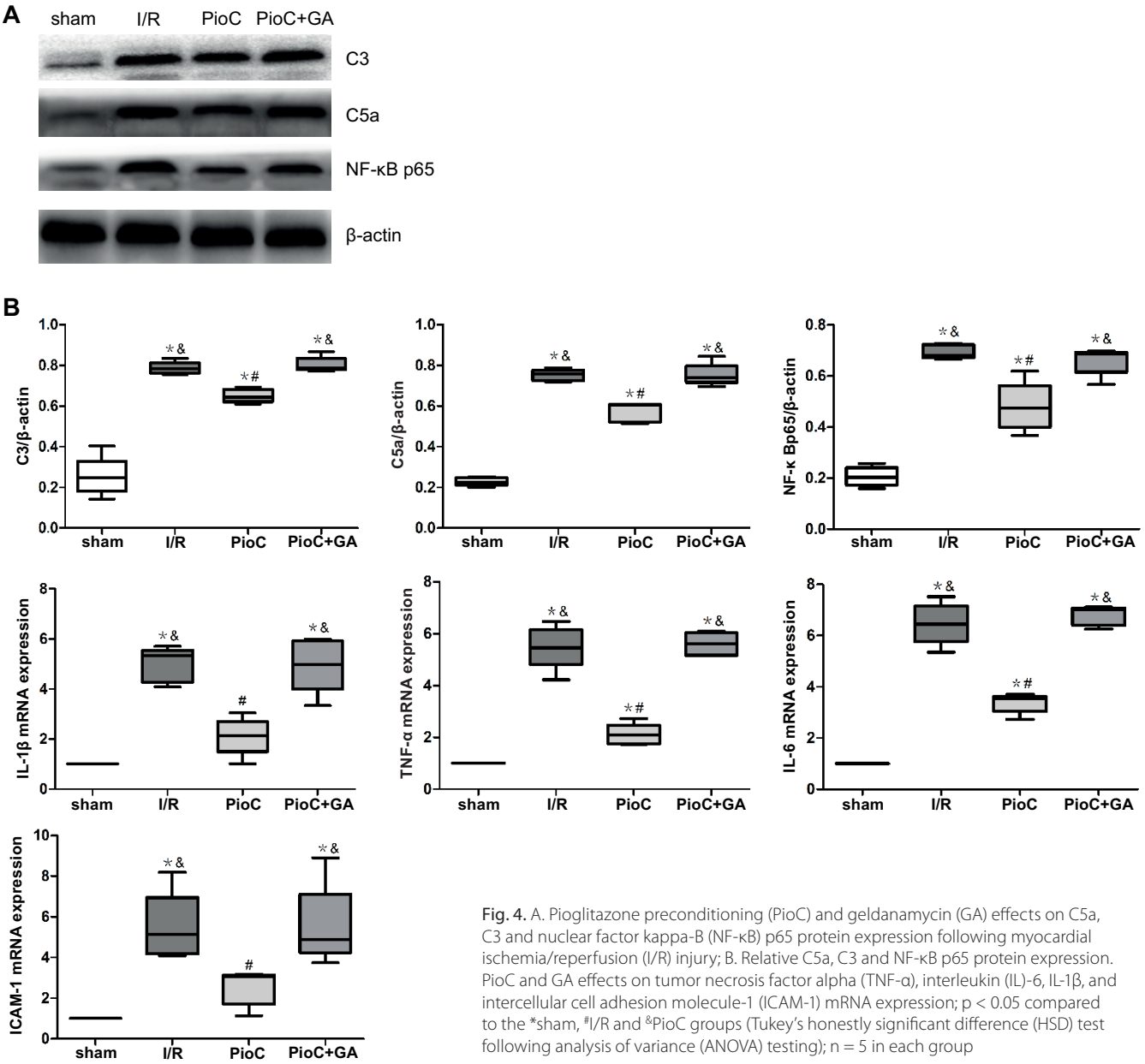

Fig. 4. A. Pioglitazone preconditioning (PioC) and geldanamycin (GA) effects on C5a, C3 and nuclear factor kappa-B (NF-κB) p65 protein expression following myocardial ischemia/reperfusion (I/R) injury; B. Relative C5a, C3 and NF-κB p65 protein expression. PioC and GA effects on tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, and intercellular cell adhesion molecule-1 (ICAM-1) mRNA expression; $p < 0.05$ compared to the *sham, #I/R and &PioC groups (Tukey's honestly significant difference (HSD) test following analysis of variance (ANOVA) testing); n = 5 in each group

Discussion

The major finding of this study was that PioC significantly decreased the I/R-induced activation of complement components C5a and C3 and NF-κB through HSP90. The PioC significantly alleviated I/R-induced myocardial injury, increased Bcl-2 expression and increased HSP90 expression. In rat hearts treated with GA, the cardioprotection was prevented as demonstrated by an increase in IS and apoptosis, CK-MB, cTnI, and LDH serum levels, C3, C5a, Bax, and NF-κB levels, and the inflammatory mediator expression of IL-6, TNF-α, ICAM-1, and IL-1β. These data highlight the pivotal function of HSP90 in inhibiting C3, C5a and NF-κB in PioC.

Pioglitazone, a PPAR-γ agonist, is widely used to manage type 2 diabetes. Based on previously published evidence, it can be stated that PPAR-γ has the potential as a new therapeutic target in I/R injury.[22,23] Clinical studies have confirmed the benefits of pioglitazone in alleviating reperfusion injury in diabetic patients with AMI.[8] Previous studies showed that PioC prevents I/R injury by increasing the expression of HSP32 (also called heme oxygenase 1) and HSP72.[24,25] Thus far, no studies have investigated whether PioC exerts a regulatory effect on HSP90, so herein, we constructed an I/R rat model, and found that PioC significantly upregulated the expression of HSP90 to prevent I/R injury, and the treatment with the HSP90 inhibitor GA reversed this effect.

The C3 is a vital component in the activation of the complement system, as demonstrated in reperfused myocardium, where C3 levels are markedly increased. Activation of C3 induces the production of C3a and C5a, which act as inflammatory mediators promoting the release of inflammatory cytokines (TNF-α, IL-1β and IL-6), which leads to an inflammatory response, and eventually the cleavage

Table 9. Results of ANOVA as presented in Fig. 4A,B

Variables		Sum of squares	df	Mean square	F	p-value
C3	between groups	0.982	3	0.327	105.242	<0.001
	within a group	0.050	16	0.003		
	total	1.031	19	N/A		
C5a	between groups	0.914	3	0.305	190.835	<0.001
	within a group	0.026	16	0.002		
	total	0.940	19	N/A		
NF-κB p65	between groups	0.746	3	0.249	72.394	<0.001
	within a group	0.055	16	0.003		
	total	0.801	19	N/A		
IL-1β	between groups	61.743	3	20.581	38.100	<0.001
	within a group	8.643	16	0.540		
	total	70.386	19	N/A		
TNF-α	between groups	82.487	3	27.496	108.121	<0.001
	within a group	4.069	16	0.254		
	total	66.556	19	N/A		
IL-6	between groups	112.913	3	37.638	163.904	<0.001
	within a group	3.674	16	0.230		
	total	116.587	19	N/A		
ICAM-1	between groups	75.110	3	25.037	13.496	0.001
	within a group	29.681	16	1.855		
	total	104.791	19	N/A		

ANOVA – analysis of variance; df – degrees of freedom; N/A – not applicable; NF-κB – nuclear factor kappa-B; IL – interleukin; TNF-α – tumor necrosis factor alpha; ICAM-1 – intercellular cell adhesion molecule-1.

of fragment C5b. The C5b binds with the other components (C6, C7, C8, and C9) to form C5b-9, also known as the membrane attack complex, which causes the subsequent cardiomyocyte damage and myocardium destruction.[11,26,27] Ischemic preconditioning and postconditioning inhibit C3 and C5a.[14,18] The PPAR-γ activation inhibits the inflammatory response by suppressing the production of inflammatory factors.[3,28] To date, it was unclear whether PioC had potent inhibitory effects on C3, C5a, TNF-α, IL-1β, IL-6, and ICAM-1. Our data revealed that the PioC group had lower C3, C5a, IL-1β, TNF-α, IL-6, and ICAM-1 levels than the I/R group. These data highlight the potential role of PioC in the suppression of C3, IL-1β, C5a, TNF-α, IL-6, and ICAM-1, anti-inflammatory induction, and cardioprotective effects.

The NF-κB is the key player in the inflammatory response after myocardial I/R injury.[29] It has previously been shown that NF-κB regulates complement component C3 to induce complement system activation and contribute to the complement-mediated immune–inflammatory response.[30] Other studies have revealed that NF-κB promotes the expression of pro-inflammatory cytokines in response to complement activation, which aggravates myocardial damage,[31,32] and that pioglitazone inhibits inflammation via suppression of NF-κB signaling.[33,34] In addition, pioglitazone was shown to combat myocardial I/R injury by inhibiting the overexpression of NF-κB activation.[20] Nevertheless, a correlation between complement components and NF-κB signaling in PioC-mediated cardioprotection remained unclear. Our results revealed that PioC lowers the protein expression levels of NF-κB, C3 and C5a. These data show that the PioC inhibitory effects on NF-κB signaling could be related to the regulation of the complement system.

The HSP90 is upregulated under stress and protects the heart from I/R injury during pharmacological conditioning and ischemic postconditioning.[21,35] Relevant investigations revealed a direct link between HSP90 and complement-mediated cell death events.[16,17] The C3 and C5a can be suppressed by preconditioning.[14] Previously, we found that ischemic postconditioning inhibited C3, C5a and NF-κB through HSP90.[18,19] These findings supported the view that PioC inhibits C3, C5a and NF-κB by upregulating HSP90. In the current study, the PioC group had higher HSP90 levels and lower C5a, C3 and NF-κB expression levels than the I/R group. Furthermore, GA reversed the PioC-mediated C3, C5a and NF-κB downregulation.

Cardiomyocyte apoptosis induced by I/R contributes to myocardial I/R injury dependent on the ratio of anti-apoptotic (Bcl-2) to proapoptotic (Bax) proteins.[36] Previous evidence demonstrated that pioglitazone reduced the levels of proapoptotic Bax and enhanced those of anti-apoptotic Bcl-2 proteins to attenuate myocardial apoptosis

Table 10. Statistics for Fig. 4B

Variable		Min	Q1	Median	Q3	Max
C3	sham	0.1420695	0.18196745	0.2468044	0.3278244	0.4038138
	I/R	0.7555192	0.7609642	0.7846901	0.8122560	0.8347841
	PioC	0.6096564	0.6210794	0.642889	0.6831024	0.693934
	PioC+GA	0.7715948	0.7791537	0.7878101	0.833518	0.8669052
C5a	sham	0.2001509	0.21247254	0.2270116	0.2463871	0.2520177
	I/R	0.7199085	0.72452212	0.7575603	0.7760201	0.7890712
	PioC	0.5146355	0.5217963	0.6039161	0.6089467	0.6099353
	PioC+GA	0.6962696	0.7165747	0.7407046	0.7967124	0.8447190
NF-κB p65	sham	0.1596326	0.1738399	0.20306	0.2413086	0.2568572
	I/R	0.6655821	0.6708702	0.6798548	0.7229049	0.7272208
	PioC	0.3669741	0.39992025	0.4747395	0.5623306	0.6184039
	PioC+GA	0.5667597	0.61475525	0.6869347	0.6925321	0.6974357
IL-1β	sham	1	1	1	1	1
	I/R	4.084700	4.2604795	5.350519	5.5542125	5.707974
	PioC	1.014345	1.5003725	2.143116	2.7007005	3.050886
	PioC+GA	3.343787	4.0029275	4.979283	5.916889	5.989528
TNF-α	sham	1	1	1	1	1
	I/R	4.225249	4.816789	5.457398	6.1509425	6.468019
	PioC	1.733721	1.756148	2.088649	2.471499	2.715366
	PioC+GA	5.157640	5.164975	5.604520	6.030263	6.086619
IL-6	sham	1	1	1	1	1
	I/R	5.351822	5.7666665	6.450896	7.1533675	7.520586
	PioC	2.729687	3.0352235	3.530458	3.632126	3.711900
	PioC+GA	6.248047	6.4101325	7.002700	7.073849	7.112963
ICAM-1	sham	1	1	1	1	1
	I/R	4.085224	4.1667785	5.120028	6.9497815	8.190071
	PioC	1.135953	1.7026610	3.050863	3.1361680	3.180996
	PioC+GA	3.736607	4.2366070	4.871034	7.1075915	8.898696

Min – minimal value; Max – maximal value; Q1 – 1st quartile; Q3 – 3rd quartile; HSP90 – heat shock protein 90; I/R – ischemia/reperfusion; PioC – pioglitazone preconditioning; GA – geldanamycin; NF-κB – nuclear factor kappa-B; IL – interleukin-1β; TNF-α – tumor necrosis factor alpha; ICAM-1 – intercellular cell adhesion molecule-1.

during I/R.[20] We reported that the Bax and Bcl-2 expression in postconditioning depended on HSP90.[21] In mast cells, the inhibition of HSP90 by GA inhibited the interaction of HSP90 with Bcl-2 and resulted in apoptosis.[37] In the present study, pioglitazone combined with GA reversed PioC-induced upregulation of Bcl-2, suggesting that the PioC anti-apoptotic effect is linked with HSP90.

These data support the evidence that HSP90 is a central player in the C3, C5a, NF-κB, and Bax inhibition during PioC. To the best of our knowledge, our observation is a pioneer in reporting the relationship between HSP90, NF-κB, C5a, C3, Bax, and Bcl-2 in PioC, which indicates that cardioprotection induced by PioC is closely related to HSP90 and the complement system. However, the detailed mechanism of the direct effect of PioC on HSP90 and the complement system needs to be clarified.

Limitations

There are some limitations to this investigation. First, we only applied the HSP90-specific modulator GA. The application of over- or underexpression of HSP90 using viral vectors to explore its role in PioC could be carried out in future studies. Second, our current work only showed the effect of HSP90 on complement activation in PioC; the application of complement inhibitors could further explore its effects on HSP90 and downstream signal transduction mechanisms of complement. Third, further research is needed to explore the detailed mechanism of the direct effect of PioC on HSP90 and the complement system. Fourth, because the sample size was very small, the Shapiro–Wilk test had low test power; thus, larger sample sizes should be applied in future studies.

Conclusions

The HSP90 plays a pivotal role in PioC-mediated cardioprotection, likely through the inhibition of C3, C5a and NF-κB activation, leading to a decrease in I/R-induced IS, myocardial inflammation and cardiomyocyte apoptosis. The current findings highlight the potential of HSP90 in treating I/R injury.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.7871084. The package contains the following files:

Supplementary Tables 1–4. Statistical analysis results of Fig. 1–4.

Supplementary Table 5. Data points of the study.

Supplementary Tables 6–12. Statistical analysis results of Supplementary Fig. 1.

Supplementary Fig. 1. The mRNA expression of inflammatory factors with *β-actin* as a housekeeping gene.

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Upregulation of ITGB6 in primary palmar hyperhidrosis

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Conflict of interest
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Abstract

Background. The regulatory effect of integrin β6 (ITGB6) on sweat gland cells in primary palmar hyperhidrosis (PPH) remains unclear.

Objectives. This study investigated the involvement of ITGB6 in the pathogenesis of PPH.

Materials and methods. Sweat gland tissues were collected from PPH patients and healthy volunteers. The expression levels of ITGB6 in sweat gland tissues were detected with quantitative polymerase chain reaction (qPCR), western blot and immunohistochemical staining. Sweat gland cells were extracted from PPH patients, and identified with immunofluorescence staining of CEA and CK7. The expression of aquaporin 5 (AQP5) and Na-K-Cl cotransporter 1 (NKCC1) in primary sweat gland cells that overexpress ITGB6 were also detected. Through a series of bioinformatic methods, differentially expressed genes in sweat gland tissues were examined and validated via comparing PPH samples and controls. The key proteins and biological functions enriched in PPH were determined using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

Results. The ITGB6 was upregulated in sweat gland tissues of PPH patients compared to that of healthy volunteers. The CEA and CK7 were positively expressed in sweat gland cells extracted from PPH patients. The overexpression of ITGB6 upregulated AQP5 and NKCC1 protein expression in the sweat gland cells of PPH patients. A total of 562 differentially expressed mRNAs were identified using high-throughput sequencing (394 upregulated, 168 downregulated), which were mainly active in the chemokine and Wnt signaling pathways. After verification with qPCR and western blot, the overexpression of ITGB6 significantly upregulated CXCL3, CXCL5, CXCL10, and CXCL11, and downregulated Wnt2 mRNA and protein expression in sweat gland cells.

Conclusions. The ITGB6 is upregulated in PPH patients. It may be involved in the pathogenesis of PPH by upregulating AQP5, NKCC1, CXCL3, CXCL5, CXCL10, and CXCL11, and downregulating Wnt2 expression in sweat glands.

Key words: chemokines, ITGB6, AQP5, primary palmar hyperhidrosis, human sweat gland cells

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Background

Sweat glands are important in adjusting body temperature in mammals.[1] Hyperhidrosis is characterized by abnormally excessive sweating that far exceeds physiological needs. Palmar hyperhidrosis is the most common form of hyperhidrosis, which can be exacerbated by stress, heat, taste, and olfactory stimuli.[2] It can seriously affect both the quality of life and work efficiency, and even cause severe psychological disorders if left untreated.[3] At present, the main treatment of primary palmar hyperhidrosis (PPH) includes drug therapy, Traditional Chinese Medicine (TCM), direct current stimulation, and computed tomography (CT)-guided percutaneous puncture thoracic sympathetic blockade.[4] However, most therapeutic strategies are limited to the relief of symptoms rather than a cure. Therefore, clarifying the molecular mechanism of PPH and developing target drugs are crucial.

As a cell surface receptor, integrins are transmembrane heterodimers composed of an α subunit and a β subunit that mediate signal transduction between the cells and the extracellular matrix.[5] Integrin β6 (ITGB6) belongs to the integrin family and is only expressed in epithelial tissues, having important roles in regulating wound healing, inflammatory responses and tumorigenesis. Wu et al. reported impaired wound repair in ITGB6$^{-/-}$ mice with corneal debridement and keratectomy wounds.[6] Moreover, Chen et al. demonstrated that the overexpression of ITGB6 in intestinal epithelial cells promotes dextran sulfate sodium (DSS)-induced colitis in mice.[7] Zheng et al. found that ITGB6 silencing can inhibit the progression of cervical cancer by modulating the JAK/STAT3 signaling pathway.[8] To date, the regulatory effect of ITGB6 on sweat gland cells in PPH remains uncertain.

Objectives

The aims of this study were to analyze the expression of ITGB6 in sweat gland tissue obtained from patients with PPH at the clinical level, explore the possible mechanism of ITGB6 in regulating the function of human sweat gland cells through the isolation of primary human sweat gland cells and the construction of ITGB6 overexpression vector, and analyze the underlying mechanism of ITGB6 participation in the pathogenesis of PPH. The findings of this study may serve as guidance for the treatment of PPH.

Materials and methods

Ethics approval

The Ethics Committee of The First Affiliated Hospital of Fujian Medical University (Fuzhou, China) approved this study (approval No. [2019]261). Written informed consent was obtained from all examined patients before sample collection.

Sample collection

Axillary all-layer skin samples (2 × 5 mm) of 8 patients with PPH treated with single-port surgery at the natural folds of the axilla in The First Affiliated Hospital of Fujian Medical University from January to September 2019 were collected as the experimental group. The samples were obtained from the right axilla by incision. Patients with a history of secondary hyperhidrosis, such as hyperthyroidism and tuberculosis, were excluded. All-layer skin samples (2 × 5 mm) from the right axilla of patients without hyperhidrosis (n = 11) who underwent axillary plastic surgery were surgically collected as the control group. The samples were obtained by incision. The patients had no history or family history of palmar and axillary hyperhidrosis and axillary osmidrosis.

Reagents and instruments

We used the following primary antibodies in our study: rabbit anti-ITGB6 antibody (19695-1-AP) was obtained from Proteintech (Wuhan, China), while rabbit anti-aquaporin 5 (AQP5) antibody (AF5169), rabbit anti-Na-K-Cl cotransporter 1 (NKCC1) antibody (DF2245), rabbit anti-CEA antibody (BF0092), rabbit anti-CK7 antibody (AF0195), rabbit anti-CXCL3 antibody (DF8554), rabbit anti-CXCL5 antibody (DF9919), rabbit anti-CXCL10 antibody (DF6417), rabbit anti-CXCL11 antibody (DF9917), and rabbit anti-WNT2 antibody (DF8067) were all purchased from Affinity Biosciences (Cincinnati, USA). The mouse monoclonal anti-GAPDH antibody (TA-08) and horseradish-labeled goat anti-rabbit IgG (H+L) antibody (ZB-2301) were both obtained from ZSGB-Bio (Beijing, China). The DAB Chromogenic Substrate Kit (CW0125), UltraSYBR Mixture (CW0957M), neutral resin (CW0136), BCA Protein Assay Kit (CW0014S), TRIzol reagent (CW0580S), UltraPure mRNA Purification Kit (CW0581M), and HiFiScript cDNA Synthesis Kit (CW2569M) were all purchased from CWBIO (Beijing, China). The hematoxylin and eosin (H&E) staining kit (AR1180-1) was purchased from Boster Bio (Pleasanton, USA), Opti-MEM (31985-062) from Gibco (Grand Island, USA), and radioimmunoprecipitation assay (RIPA) lysis buffer (C1053) from Applygen (Beijing, China).

Cell culture incubator (BPN-80CW) was purchased from Shanghai Yiheng Technology (Shanghai, China), and the bench-top low-temperature high-speed centrifuge (5424R) from Eppendorf (Hamburg, Germany). The CFX Connect™ real-time fluorescence PCR instrument was manufactured by Bio-Rad (Shanghai, China), and the vertical electrophoresis apparatus (DYY-6C) and microplate reader (WD-2102B) by Beijing Liuyi Biotechnology Co., Ltd. (Beijing, China). The automatic high-speed grinding device (Tiss-12) was obtained from Shanghai Jingxin Biological Pharmaceutical Co. Ltd. (Shanghai, China) and the ChemiDoc™ XRS+ System from Bio-Rad. Fluorescence

microscopes (model CX41 and model CKX53) were purchased from Olympus Corp. (Tokyo, Japan), the multifunctional enzyme labeling analyzer (Safire II) from Tecan (Männedorf, Switzerland), and the manual rotary microtome (2235) from Leica Biosystems (Wetzlar, Germany). The Nanodrop 2000 was obtained from Thermo Fisher Scientific (Waltham, USA), Agilent 2100 from Agilent Technologies (Santa Clara, USA), and the HiSeq4000 sequencing platform from Illumina (San Diego, USA).

Extraction of sweat gland cells

The human sweat gland tissues were collected, immersed in 75% ethanol for 10 s, and washed in phosphate-buffered saline (PBS) containing 1% penicillin and streptomycin. The apocrine sweat glands were isolated under a microscope and then incubated in PBS. The outer membrane of the apocrine sweat glands was peeled off, the remaining tissues were cut off and centrifuged for 4 min at 1000 rpm. The mixture was digested in 2 g/L collagenase II overnight, and for 10 min at 37°C the following day. After the termination of the enzymatic digestion, the mixture was centrifuged for 4 min at 1000 rpm, the supernatant was discarded, and freshly prepared Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) + 20% fetal bovine serum (FBS) + 1% penicillin and streptomycin were added, inoculated in a culture flask and placed in 37°C with 5% CO_2 for culture.

The cell culture supernatant was discarded, and cells were washed twice in 1× PBS. Then, 0.25% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA) was added. Cell digestion was terminated by adding the culture medium when the cell morphology became round. Then, the cell suspension was collected into a 10-milliliter centrifuge tube and centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the culture medium was added and resuspended evenly. The cell suspension was harvested into the culture plate and cultured in a 37°C, 5% CO_2 incubator.

Immunohistochemical staining

Paraffin-embedded tissue sections of sweat glands were dewaxed, incubated in citrate buffer for antigen retrieval, heated for 2 min in a pressure cooker, and then naturally cooled to room temperature. After washing with PBS, the sections were put in a wet box at room temperature, where newly prepared 3% H_2O_2 was added to quench endogenous peroxidase activity. Ten minutes later, the sections were washed in PBS for 5 min (3 times), and any excess reagent was wiped away. After blocking with 5% bovine serum albumin (BSA) at 37°C for 30 min, samples were incubated with the anti-ITGB6 antibody at 4°C overnight, followed by secondary antibodies at 37°C for 30 min. Then, the sections were stained with 3,3'-diaminobenzidine (DAB) solution, counterstained with hematoxylin, washed in tap water, mounted, and observed under a microscope (model CX41; Olympus Corp.).

Immunofluorescence

Cells cultured in dishes were washed in PBS 3 times for 3 min each, fixed in 4% paraformaldehyde for 15 min and rewashed in PBS 3 times for 3 min each. After immersion in PBS containing 0.5% Triton X-100 for 20 min at room temperature, the cells were washed in PBS 3 times for 5 min, and the medium was cleared. Then, the cells were blocked in 5% BSA for 30 min at 37°C and incubated with anti-CEA and anti-CK7 antibodies for 3 h at 37°C. Following 3 washes with PBS for 3 min each, the samples were incubated with the appropriate secondary antibody at 37°C in the dark for 45 min and washed 3 times in PBS for 3 min each. Then, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5 min for nuclear counterstaining and washed in PBS. Slides were sealed with 50% glycerol, followed by examination under a fluorescence microscope (model CKX53; Olympus Corp.) in the dark.

Cell transfection

The cells cultured to 70% density were prepared for transfection by replacing the media with serum-free medium. Two sterilized Eppendorf tubes were prepared with equal volumes of Opti-MEM. Then, 5 µL of Lipofectamine 3000 were added to 1 tube, while 2.5 µg of plasmid and 5 µL of P3000 were added to another tube, mixed well and incubated for 5 min at room temperature. Then, the 2 tubes were evenly mixed and incubated for 15 min at room temperature. The mixed solution was applied to a 6-well plate and cultured in the incubator. Four hours after the transfection, 1 mL of complete medium containing 20% serum was added, and verification was performed after 48 h.

High-throughput sequencing

After preparation of the cDNA library, total RNA was extracted using TRIzol reagent, and the concentration and purity were detected using a Nanodrop 2000. The RNA integrity was determined with gel electrophoresis. The RNA integrity number (RIN) value was measured using Agilent 2100. The total amount of RNA required for single library construction was >5 µg, the concentration was ≥200 ng/µL, and optical density (OD) 260/280 was between 1.8 and 2.2. The Ribo-Zero Magnetic kit (EpiCentre, Madison, USA) was used to remove rRNA, and RNase R (EpiCentre) was used to remove linear RNA. The TruSeq™ Stranded Total RNA Library Prep Kit (Illumina) was used to construct the paired-end sequencing library. The HiSeq4000 sequencing platform was used for sequencing and data analysis, and SepPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software were used to determine the quality of data. Qualified data were compared and analyzed with the reference genome data using the bowtie method.

Western blot

The samples from each group were added to the corresponding lysis buffer, lysed on ice for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was carefully aspirated to obtain the total protein, and the protein concentration analysis was performed using a bicinchoninic acid (BCA) kit. The protein was denatured, loaded and subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 1–2 h, followed by wet transfer to a polyvinylidene fluoride (PVDF) membrane for 30–50 min. The incubation with the primary antibody solution was conducted at 4°C overnight, followed by the secondary antibody at room temperature for 1–2 h. Then, the enhanced chemiluminescence (ECL) exposure solution was dropped on the membrane and imaged in a gel imaging system. The grey value of any bands that were present was analyzed with Quantity One software (Bio-Rad).

Quantitative polymerase chain reaction

The samples were collected, and TRIzol lysis buffer was added and mixed thoroughly with a pipette gun to ensure that the adherent cell suspension was in full contact with the lysis buffer. Then, the cell suspension was collected to extract the total RNA. The RNA was synthesized using reverse transcription HiFiScript first-strand cDNA synthesis kit. Fluorescence quantitative polymerase chain reaction (qPCR) was performed. The reaction system, steps and primer sequences for qPCR are presented in Table 1. Primers were synthesized by General Biol (Anhui) Corp. Ltd. (Chuzhou, China). The β-actin was used as an internal reference, and the relative expression was calculated according to the $2^{-\Delta\Delta CT}$ method.

Statistical analyses

The IBM SPSS v. 20.0 software (IBM Corp., Armonk, USA) was used for statistical analysis. All experiments were performed 3 times, and the quantitative results were expressed as mean ± 95% confidence interval (95% CI). Quantitative comparison between the 2 groups was performed using independent samples t-test (Student's t-test). When comparing multiple groups, a quantitative numerical comparison was performed with one-way analysis of variance (ANOVA) and post hoc Tukey's honest significant difference (HSD) test. The p-value of the test level was $\alpha = 0.05$, and $p < 0.05$ indicated a significant difference.

Principal component analysis

Principal component analysis (PCA) is often used to reduce the dimensionality of a dataset while maintaining the features that contribute the most to the variance, thereby effectively finding the main elements and structures in the data, and removing noise and redundancy. In addition, the original complex data are dimensionally reduced to reveal the simple structure hidden behind the complex data. By analyzing the components of different sample species and functions, the differences and distances between samples can be discovered. Principal component analysis uses variance decomposition to reflect the differences between multiple sets of data on the coordinate axis. For example, the more similar the sample composition, the closer the distance is reflected in the PCA diagram. Samples between different environments may show dispersive and aggregated distributions. The closer the samples are to different environments, the more likely they are to show a dispersive and agglomerated distribution, and the 2 or 3 components with the highest explanatory power for sample differences in PCA results can be used to verify hypothetical factors.

Table 1. Reaction system, steps and primer sequences for quantitative polymerase chain reaction (qPCR)

Reaction system	RNase-Free dH_2O	9.5 µL
	cDNA	1 µL
	upstream primer	1 µL
	downstream primer	1 µL
	2×SYBR Green PCR Master Mix	12.5 µL
Reaction steps	pre-denaturation	95°C, 10 min
	denaturation	95°C, 10 s
	annealing	58°C, 30 s
	extension	72°C, 30 s
	cycle, n	40
Primer sequence	ITGB6 forward primer	5′-CAGGACCAACCTGTGAACGA-3′
	ITGB6 reverse primer	5′-ACAGAACCATCCTTTGAGAAATC-3′
	β-actin forward primer	5′-TGGCACCCAGCACAATGAA-3′
	β-actin reverse primer	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

ITGB6 – integrin β6.

Volcano plot

A volcano plot is a type of scatterplot that shows statistical significance (p-value) compared to magnitude of change (fold change), and enables quick visual identification of genes with large fold changes that are also statistically significant. These may be the most biologically significant genes.

Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

Gene Ontology (GO) is an international standardized gene function classification system that provides a set of dynamically updated standard vocabulary (controlled vocabulary) to comprehensively describe the attributes of genes and gene products in organisms. Gene Ontology has 3 ontologies in total, which describe the molecular function, the cellular component and the biological process involved in the gene. The basic unit of GO is a term, and each term has a unique identifier (composed of "GO" plus 7 numbers, such as GO: 0072669). The terms of each type of ontology form a directed acyclic topology through their connections (is_a, part_of, regulate). The horizontal axis is the functional classification, and the vertical axis is the number of genes in the classification (right) and its percentage in the total number of annotated genes (left). Different colors represent different classifications. Light color represents target genes, and dark color represents all genes.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive database of biological systems, integrating genomic, chemical and system functional information. Among them, KEGG GENES collects the gene protein sequences of all known complete genomes, including the minimum information on each gene. The KEGG ORTHOLOGY (KO) system links various KEGG annotation systems together. Kyoto Encyclopedia of Genes and Genomes has established a complete KO annotation system, which can complete the functional annotation of the genomes or transcriptome of newly sequenced species.

Results

Demographic and clinical data

The control group included 3 males and 8 females, with a mean age of 21.8 ±5.0 years. The experimental group consisted of 2 males and 6 females, with a mean age of 21.4 ±5.3 years. All patients were of Han ethnicity with no previous history of surgical treatments. Both groups were comparable in terms of gender, mean age, ethnic background, and history of surgical treatments.

ITGB6 is upregulated in sweat gland tissue of patients with primary palmar hyperhidrosis

Compared with the healthy volunteers, both mRNA and protein expressions of ITGB6 in the sweat gland tissues of patients with PPH were significantly higher (Fig. 1A,B). Immunohistochemical staining consistently demonstrated a significantly increased staining of ITGB6 in the sweat gland tissue of patients with PPH compared to the controls (Fig. 1C).

Identification of human sweat gland cells and verification of ITGB6 overexpression transfection efficiency

The CEA and CK7 are generally considered markers of sweat gland cells,[9] and both CEA and CK7 proteins were expressed in primary isolated human sweat gland cells (Fig. 2A). The mRNA and protein expression of ITGB6 in cells transfected with ITGB6 overexpression (OE) were significantly upregulated compared to those transfected with OE negative control (NC), suggesting high transfection efficacy (Fig. 2B–D).

Regulatory effects of ITGB6 on aquaporin 5 and Na-K-Cl cotransporter 1 protein expression levels in sweat gland cells

Aquaporin 5 is a water channel protein that participates in the generation of saliva, tears and pulmonary secretions. Moreover, NKCC1 is vital for organs that secrete fluids. Therefore, we explored the influence of ITGB6 on AQP5 and NKCC1 protein expression levels in sweat gland cells extracted from patients with PPH using western blot. Interestingly, the overexpression of ITGB6 upregulated the AQP5 and NKCC1 protein expression levels (Fig. 3A,B).

mRNA high-throughput sequencing analysis

We found large differences in gene expression levels between the ITGB6 OE group and the control group (Fig. 4A–C). In Fig. 4D, the horizontal axis is the fold change (log (B/A)) value of the difference of transcript expression between samples of different groups, and the vertical axis is the p-value that represents the statistical significance of the expression changes. Compared with the OE NC group, a total of 562 differentially expressed mRNAs in the ITGB6 OE group were determined with high-throughput sequencing, involving 394 upregulated and 168 downregulated mRNAs. They were mainly enriched in the chemokine and Wnt signaling pathways. The results of GO and KEGG pathway enrichment analyses are presented in Fig. 5A and Fig. 5B, respectively.

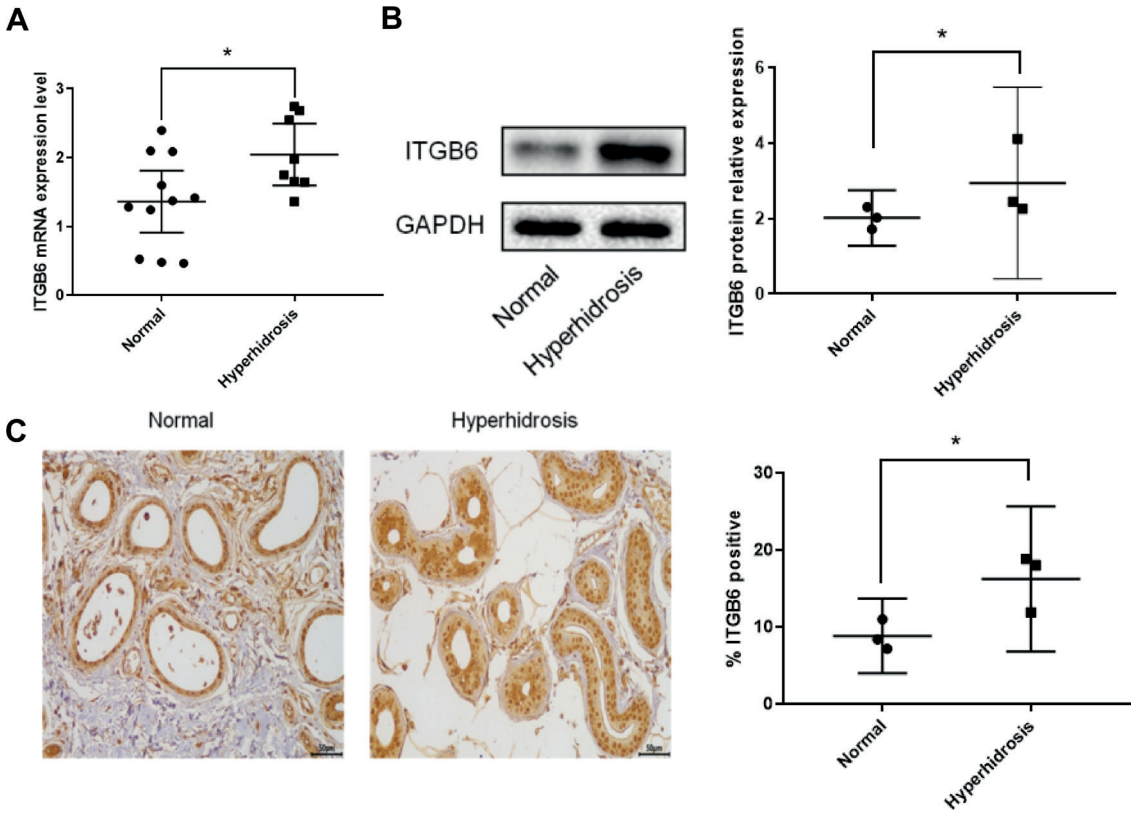

Fig. 1. A. The mRNA expression level (hyperhidrosis compared to healthy volunteers, Student's t-test, p = 0.030); B. Protein expression level (western blot images and quantitative analyses) (hyperhidrosis compared to healthy volunteers, Student's t-test, p = 0.027); C. Positive expression of integrin β6 (ITGB6) in the sweat gland tissues of patients with primary palmar hyperhidrosis and healthy volunteers (immunohistochemical staining and quantitative analyses; hyperhidrosis compared to healthy volunteers, Student's t-test, p = 0.040). The data are presented as mean ± 95% confidence interval (95% CI)

*p < 0.05, compared with the normal group.

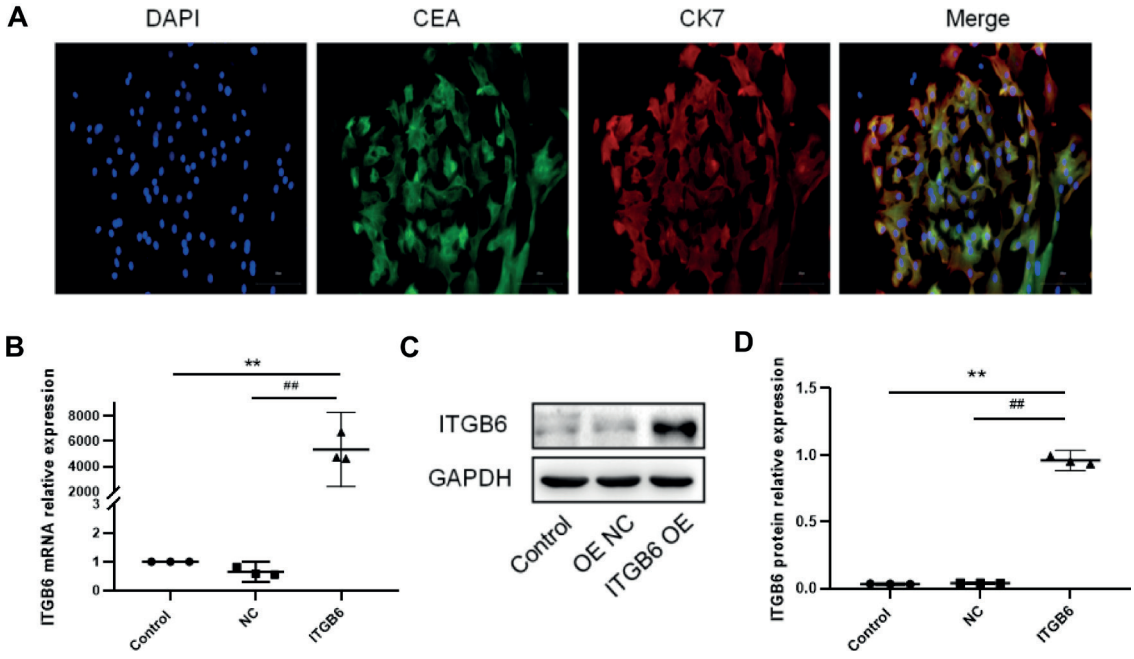

Fig. 2. Identification of human sweat gland cells and verification of integrin β6 (ITGB6) overexpression transfection efficiency. A. Immunofluorescence staining of CEA (green) and CK7 (red) in sweat gland cells extracted from patients with primary palmar hyperhidrosis. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue); B. Quantitative polymerase chain reaction (ITGB6 compared to control, Tukey's honest significant difference (HSD), p < 0.001; ITGB6 compared to negative control (NC), Tukey's HSD, p < 0.001); C,D. Western blot detection of the transfection efficiency of ITGB6 overexpression analysis results (ITGB6 compared to control, Tukey's HSD, p < 0.001; ITGB6 compared to NC, Tukey's HSD, p < 0.001). The data are presented as mean ± 95% confidence interval (95% CI)

**p < 0.01, compared with the control group; ##p < 0.01, compared with the NC group.

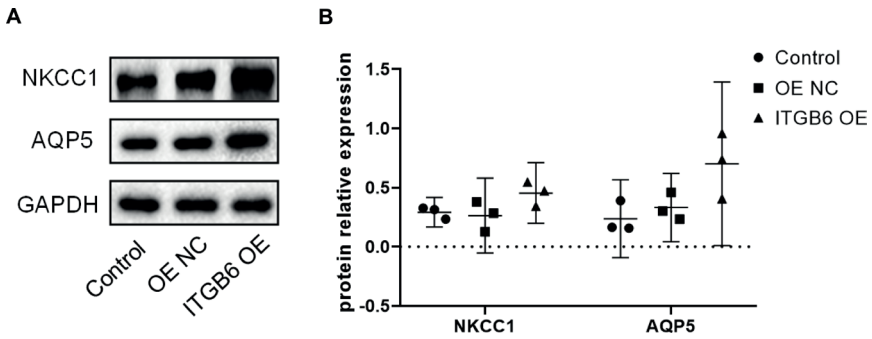

Fig. 3. Aquaporin 5 (AQP5) and Na-K-Cl cotransporter 1 (NKCC1) protein expression levels in sweat gland cells extracted from patients with primary palmar hyperhidrosis treated with blank control or transfected with overexpression negative control (OE NC) or integrin β6 (ITGB6) OE. Western blot images (A) and quantitative analyses (B) (ITGB6 OE compared to control, AQP5: Tukey's honest significant difference (HSD), p = 0.055; NKCC1: Tukey's HSD, p = 0.190). The data are presented as mean ± 95% confidence interval (95% CI)

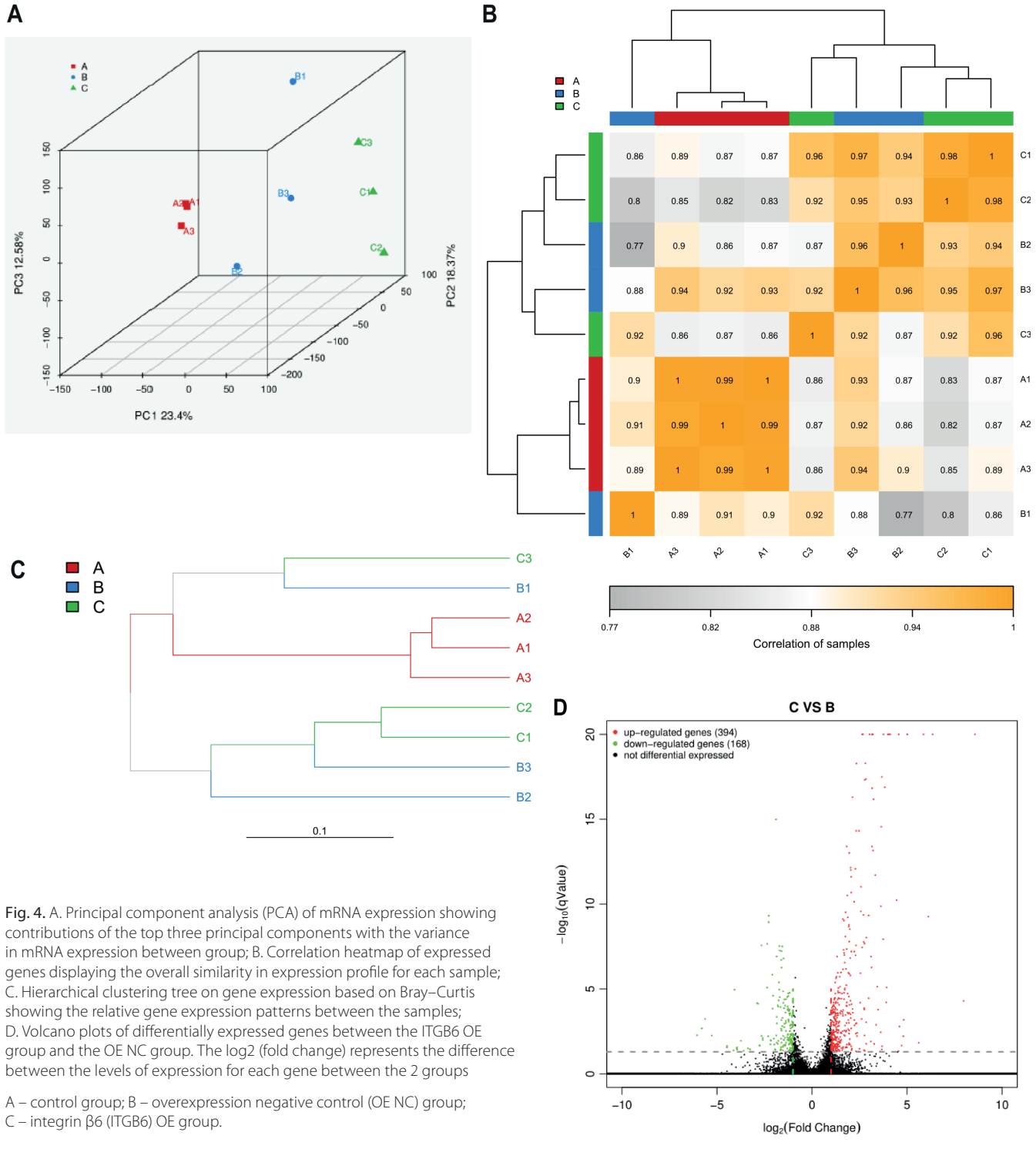

Fig. 4. A. Principal component analysis (PCA) of mRNA expression showing contributions of the top three principal components with the variance in mRNA expression between group; B. Correlation heatmap of expressed genes displaying the overall similarity in expression profile for each sample; C. Hierarchical clustering tree on gene expression based on Bray–Curtis showing the relative gene expression patterns between the samples; D. Volcano plots of differentially expressed genes between the ITGB6 OE group and the OE NC group. The log2 (fold change) represents the difference between the levels of expression for each gene between the 2 groups

A – control group; B – overexpression negative control (OE NC) group; C – integrin β6 (ITGB6) OE group.

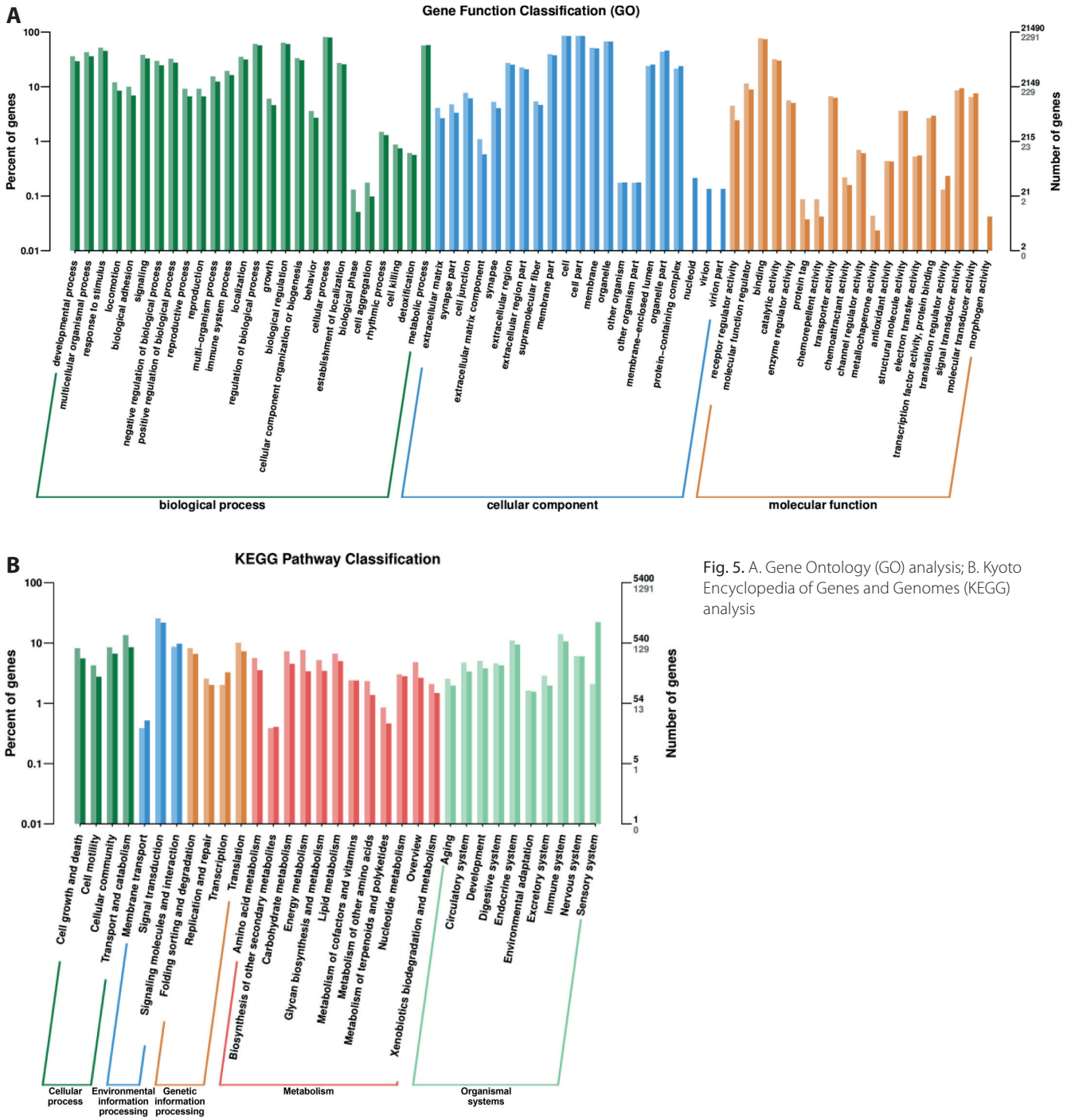

Fig. 5. A. Gene Ontology (GO) analysis; B. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Regulatory effects of ITGB6 on expression levels of CXCL3, CXCL5, CXCL10, CXCL11, and WNT2 in sweat gland cells

We further examined the expression of CXCL3, CXCL5, CXCL10, CXCL11, and WNT2 in sweat gland cells extracted from patients with PPH and transfected with OE NC or ITGB6 OE. We found that CXCL3, CXCL5, CXCL10, and CXCL11 mRNA and protein expression levels were significantly upregulated, whereas WNT2 was significantly downregulated in sweat gland cells overexpressing ITGB6 (Fig. 6A–C).

Discussion

Primary palmar hyperhidrosis refers to abnormal hypersecretion of sweat glands in the hands, which is not related to the temperature of the external environment. It manifests as wet palms in mild cases, while those severely affected may drip sweat from their hands, posing great psychological pressure and disrupting their social life.[10,11] At present, treatment measures can only alleviate symptoms rather than cure the disease,[12] and thus, it is of great importance to explore the pathogenesis of PPH and develop targeted interventions. In the present study, ITGB6 was

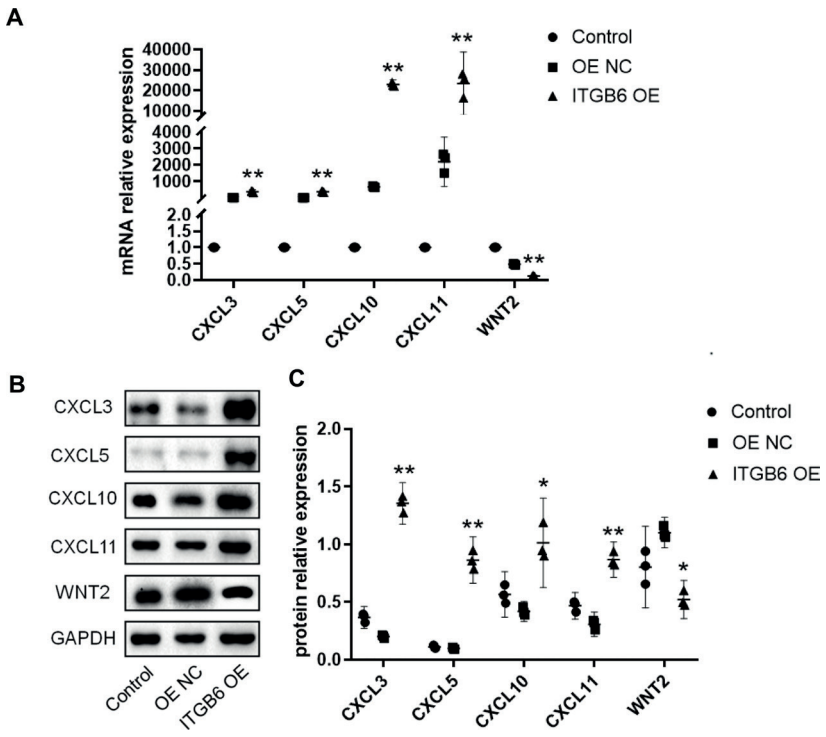

Fig. 6. Regulatory effects of integrin β6 (ITGB6) on CXCL3, CXCL5, CXCL10, CXCL11, and WNT2 expression levels in sweat gland cells. Sweat gland cells extracted from patients with primary palmar hyperhidrosis were treated with blank control or transfected with overexpression negative control (OE NC) or ITGB6 OE. The CXCL3, CXCL5, CXCL10, CXCL11, and WNT2 (A) mRNA (ITGB6 OE compared to control, Tukey's honest significant difference (HSD), $p < 0.001$, all), and (B,C) protein expression (ITGB6 OE compared to control, CXCL3, CXCL5, and CXCL11: Tukey's HSD, all $p < 0.001$; CXCL10: Tukey's HSD, $p = 0.004$; WNT2: Tukey's HSD, $p = 0.027$). The data are presented as mean ± 95% confidence interval (95% CI)

*$p < 0.05$, compared with the control group; **$p < 0.01$, compared with the control group.

found to be upregulated in sweat gland tissues of patients with PPH compared to healthy volunteers. Furthermore, it was shown that ITGB6 OE may upregulate AQP5 and NKCC1 in sweat gland cells extracted from patients with PPH. Combined with mRNA high-throughput sequencing and verification experiments, it was found that ITGB6 OE can significantly increase the CXCL3, CXCL5, CXCL10, and CXCL11 mRNA and protein levels while inhibiting WNT2 mRNA and protein expression.

As the main component of sweat, water molecules are regulated by relevant proteins expressed on the cell membrane of sweat glands. The AQP5 is a water channel protein that specifically transports water across the membrane. It is involved in the secretion and absorption of water and balances intra- and extracellular water through increasing water permeability of the cell membrane.[13] In recent years, studies have reported that AQP5 participates in various physiological functions of human water regulation. Wang et al. found that the inhibition of HMGB1 could alleviate the symptoms of Sjögren's syndrome by inhibiting the HMGB1/TLR4/NF-κB signaling pathway and upregulating the expression of AQP5.[14] Furthermore, Du et al. reported that AQP5 is highly expressed in the axillary sweat glands of patients with primary focal hyperhidrosis.[15] Our previous study demonstrated that ACVR1 can promote human sweat gland cell proliferation by upregulating AQP5 expression.[16] Finally, the downregulation of CHRNA1 can alleviate the symptoms of PPH by inhibiting AQP5 expression.[17] In the present study, ITGB6 OE may upregulate the expression of the AQP5 protein in sweat gland cells extracted from patients with PPH, suggesting that ITGB6 may aggravate the progression of PPH by regulating the expression of aquaporin. We found a similar result for NKCC1, whose main function is to transport Na, K and Cl ions into sweat gland cells in the ratio of 1:1:2. It plays an important role in cell ion transport, stabilizing ion gradient and regulating cell volume.[18,19] Our previous study found that ACVR1 can promote sweat gland cell proliferation by upregulating NKCC1 expression, and its main mechanism of action may be achieved by regulating the transport of Na, K and Cl ions.[16]

Chemokines contribute to the fight against and clearance of foreign bodies like invading pathogens through the directional chemotaxis of immune cells.[20–22] According to the differences in cysteine in the chemokines, they can be divided into 4 main subfamilies: CXC, CC, CX3C, and XC. Among them, the CXC subfamily, as a research hotspot, plays an important role in various diseases.[23] Pro-inflammatory factors, CXCL3, CXCL5, CXCL10, and CXCL11 have been reported to play key regulatory roles in various diseases such as acute lung injury,[24] atherosclerosis[25] and ovarian cancer.[26] However, their potential involvement in PPH is unclear. Our bioinformatic analysis revealed that CXCL3, CXCL5, CXCL10, and CXCL11 were upregulated in PPH with ITGB6 OE. As a result, we speculate that ITGB6 exacerbates the progression of PPH by promoting the accumulation of neuroinflammatory factors during sweat gland secretion.

The Wnt signaling pathway is highly conserved across species and exists widely in multicellular organisms.[27] It is capable of regulating cell proliferation, differentiation, apoptosis, migration, and invasion, and plays an important role in the occurrence and development of various diseases.[28] Cui et al. reported that the Wnt signaling pathway participates in the regulation of the induction and development of sweat glands.[29] Therefore, we speculated that ITGB6

promoted the progression of PPH by inhibiting the activation of Wnt signaling in the process of sweat gland secretion. Our results showed that ITGB6 OE downregulated WNT2 in sweat gland cells extracted from patients with PPH.

Limitations

This study mainly explored the potential mechanism of ITGB6 in the pathogenesis of PPH. Further studies may be needed to investigate the involved mechanism more deeply.

Conclusions

The ITGB6 was upregulated in patients with PPH, and the overexpression of ITGB6 may promote AQP5 and NKCC1 protein expression. Moreover, ITGB6 OE significantly increased CXCL3, CXCL5, CXCL10, and CXCL11 mRNA and protein expression, while inhibiting WNT2 mRNA and protein expression.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.7750959. The package contains the following files:

Supplementary Table 1. Data analysis for Fig. 1A–C, Fig. 2B,D, Fig. 3B, Fig. 4A–C, and Fig. 5A,C.

Supplementary Table 2. Data analysis for PCA rotation (Fig. 4A).

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Development and validation of metabolic models for predicting survival and immune status of hepatocellular carcinoma patients

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Conflict of interest
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Abstract

Background. Metabolic reprogramming is associated with the carcinogenesis of hepatocellular carcinoma (HCC). The effects of metabolism-related genes on predicting survival and immune status in HCC remain unclear.

Objectives. To develop and validate metabolic models for predicting the survival and immune status of HCC patients.

Materials and methods. The metabolic core genes for overall survival (OS) and disease-free survival (DFS) were retrieved. Then, glycolysis and fatty acid metabolism prognostic models were constructed and validated using The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) data. Decision trees based on machine learning were developed for classifying the prognostic risks of HCC patients. The associations between the metabolic signatures, immunotherapy and immune cell infiltration were investigated. Experimental validations were performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC).

Results. We identified 30 prognostic core genes for glycolysis metabolism and 12 prognostic core genes for fatty acid metabolism. Subsequently, 2 glycolysis models and 2 fatty acid metabolism models were developed to predict the OS and DFS of HCC patients, respectively. Two decision trees were constructed to classify the low-, intermediate- and high-risk groups of HCC patients for OS and DFS. Moreover, the patients in the high-risk groups of glycolysis and fatty acid metabolic models tended to have higher expression of programmed cell death ligand-1 (PD-L1 or CD274), programmed cell death 1 (PDCD1), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), and lymphocyte activating 3 (LAG3). Most of the metabolic core genes were significantly associated with immune cell infiltration. In addition, ATP-binding cassette subfamily B member 6 (ABCB6), peptidylprolyl isomerase A (PPIA), uroporphyrinogen decarboxylase (UROD), and non-SMC condensin II complex subunit H2 (NCAPH2) were positively correlated with both tumor mutational burden (TMB) and microsatellite instability (MSI) scores. The expression of ABCB6, PPIA, UROD, and NCAPH2 was validated using RT-qPCR and IHC.

Conclusions. We established novel prognostic models based on metabolism-related genes to better predict the outcome and immune status of HCC patients.

Key words: hepatocellular carcinoma, glycolysis, fatty acid metabolism, prognostic models, immune status

Background

Hepatocellular carcinoma (HCC) is characterized by a high malignancy and poor prognosis.[1,2] In China, the number of patients with hepatitis B virus-related HCC has been decreasing, while the incidence of fatty liver-related HCC is increasing due to accelerated aging and changes in diet composition.[3,4] Hepatocellular carcinoma remains a serious public health threat and increases the social and economic burdens on the government.[5]

The appropriate treatment depends on the physician's assessment of the patient's prognosis. For patients with a poor prognosis, doctors tend to use additional treatment methods, such as transcatheter arterial chemoembolization (TACE), targeted therapy and immunotherapy.[6] In the past, the prognostic evaluation of HCC mainly depended on the pathological stage. Several studies have shown that in addition to the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system, there are other clinicopathological features and specific gene expressions that can be used to predict the prognosis of HCC patients.[7,8]

Cancer development depends on the metabolic reprogramming of tumor cells, which is a hallmark of cancer and a direct or indirect result of oncogene mutations.[9] The abnormal expression of metabolic genes is closely related to the prognosis of cancer patients.[10] A prognostic model based on metabolic genes can be used to predict the prognosis of cancer patient.[11] The liver is the primary organ involved in metabolizing glucose and lipids. Abnormal gene regulation in the liver leads to abnormal glucose and lipid metabolism, thus inducing tumor progression.[12] In addition, abnormal metabolic regulation is closely associated with changes in the tumor microenvironment and immune cell infiltration.[13,14]

In recent years, there has been a breakthrough in the treatment of advanced HCC patients with immune checkpoint inhibitors (ICIs).[15] However, many patients do not respond to immunotherapy. Previous studies suggest that immune markers, such as programmed cell death ligand-1 (PD-L1 or CD274), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), tumor mutational burden (TMB), and microsatellite instability (MSI), can be used to predict the response to immunotherapy.[16,17] The expression of core metabolic genes is also considered a potential marker for predicting the effectiveness of immunotherapy.[18,19]

Objectives

This study aims to screen for prognostic core genes of glycolysis and fatty acid metabolism and build a prediction model combining TNM features with gene risk scores to better predict overall survival (OS), disease-free survival (DFS) and the immune status of HCC patients.

Materials and methods

Data collection

RNA-sequencing expression profiles and their corresponding clinical data were obtained from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov) and the International Cancer Genome Consortium (ICGC; https://icgc.org/). Using the bioinformatic analysis website Assistant for Clinical Bioinformatics (ACBI; https://www.aclbi.com/), RNA sequencing and survival data were obtained for 371 HCC patients from TCGA and 240 patients from the ICGC. Gene expression patterns varied significantly between histological subtypes; thus, intrahepatic cholangiocarcinoma and mixed HCC were excluded and only patients with HCC were selected. Clinical parameters, including age, gender, pathological stage (T, N and M stages), tumor grade, vital status, recurrence status, and follow-up time [years] were collected. The RNA count data were converted to transcripts per million (TPM). Hepatocellular carcinoma patients were divided into high- and low-expression groups using the median RNA expression as the cutoff value.

Acquisition of metabolic hallmark gene sets

The core metabolic genes were retrieved from the Molecular Signatures Database v. 7.5.1 (MSigDB, http://www.gsea-msigdb.org/gsea/msigdb). For further analysis, 200 glycolysis-related genes were collected from "HALLMARK_GLYCOLYSIS" and 158 fatty acid metabolism-related genes were collected from "HALLMARK_FATTY_ACID_METABOLISM" (Supplementary Table 1).

Identification of prognostic genes for OS and DFS

The univariate Cox regression analysis was used to assess the prognostic value of the glycolysis-related and fatty acid metabolism-related genes. Assistant for Clinical Bioinformatics was used for survival analyses of batches (https://www.aclbi.com/static/index.html#/). Hazard ratios (HRs), OS and DFS were calculated for each selected gene individually with 95% confidence intervals (95% CIs). Genes significantly associated with both OS and DFS were considered core metabolic genes and entered the next stage of model building. Differences in the expression levels of core metabolic genes in tumors and normal tissues were compared using the Wilcoxon test.

Construction of metabolic risk score prediction models

With 10-fold cross-validation, the Least Absolute Shrinkage and Selection Operator (LASSO) regression

Table 1. Risk factors for overall survival (OS) and disease-free survival (DFS) according to Cox proportional hazards regression model

Factors	Subgroup	OS set (n = 346)						DFS set (n = 298)					
		univariate analysis			multivariate analysis			univariate analysis			multivariate analysis		
		HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Age	≤60	1	–	0.172	–	–	–	1	–	0.897	1	–	–
	>60	1.87	0.89–1.87					1.02	0.73–1.44				
Gender	female	1	–	0.201	–	–	–	1	–	0.487	–	–	–
	male	0.78	0.54–1.14					1.14	0.79–1.65				
pT	T1	1	–	**0.000**	1	–	**0.002**	1	–	**0.000**	1	–	**0.000**
	T2	1.38	0.85–2.25		1.05	0.64–1.74		1.64	1.08–2.50		1.26	0.82–1.95	
	T3	2.56	1.66–3.94		2.02	1.29–3.16		2.73	1.80–4.16		2.37	1.55–3.62	
	T4	4.99	2.22–11.21		3.52	1.33–9.33		5.48	1.96–15.30		4.76	1.69–13.40	
pN	N0	1	–	0.348	1	–	–	1	–	0.499	1	–	
	N1	1.96	0.48–7.95		–			1.62	0.40–6.56				
pM	M0	1	–	**0.024**	1	–	0.214	–	–	–	–	–	
	M1	3.78	1.20–11.93		2.47	0.59–10.24							
Grade	G1	1	–	0.744	–	–	–	1	–	0.462	–	–	
	G2	1.15	0.64–2.08					1.27	0.71–2.28				
	G3	1.24	0.67–2.27					1.55	0.86–2.80				
	G4	1.74	0.62–4.86					1.18	0.39–3.58				
Glycolysis model	low risk	1	–	**0.000**	1	–	**0.032**	1	–	**0.000**	1	–	**0.001**
	high risk	2.46	1.68–3.59		1.70	1.05–2.75		2.50	1.76–3.57		2.01	1.35–2.99	
Fatty acid metabolism model	low risk	1	–	**0.000**	1	–	**0.019**	1	–	**0.000**	1	–	0.107
	high risk	2.40	1.64–3.51		1.75	1.09–2.78		1.90	1.34–2.69		1.37	0.93–2.01	

pT – pathological T stage; pN – pathological N stage; pM – pathological M stage; HR – hazard ratio; 95% CI – 95% confidence interval. Values in bold are statistically significant.

algorithm was used to further filter core metabolic genes to develop the prognostic glycolysis and fatty acid metabolism signatures using the "glmnet" R package (R Foundation for Statistical Computing, Vienna, Austria).[20] Risk scores for HCC patients were calculated based on the normalized gene expression levels (log2(TPM+1)) and corresponding regression coefficients in our models. The LASSO regression analysis was performed using the ACBI website. Hepatocellular carcinoma patients were then divided into high-risk and low-risk groups based on the median value of the risk scores. The Kaplan–Meier survival curves and log-rank tests were used to compare the OS and DFS between the 2 groups. To evaluate the efficacy of the glycolysis and fatty acid metabolic signatures, receiver operating characteristic (ROC) curves for 1, 3 and 5 years with the area under the curve (AUC) values were plotted using the "survivalROC" R package.

Integrated analysis of clinical parameters and metabolic risk score prediction models

Clinical information (age, gender, pathological stage: T, N and M, tumor grade) and glycolysis and fatty acid metabolism risk scores of HCC patients were integrated. To further build nomograms based on the pathological TNM data, patients with missing data were excluded. The univariate and multivariate Cox regression analyses were then performed to identify independent prognostic factors.

Development and validation of the nomograms for patients with HCC

To predict OS and DFS at 3 and 5 years in individual HCC patients, nomograms were developed by combining metabolic risks and clinical information based on the training groups, according to the results of the multivariate Cox regression (metabolic models). Corresponding models based on the pathologic TNM stage (TNM models) were constructed for head-to-head comparison using the metabolic models.

The discrimination of a predictive model was validated using concordance statistics. The concordance index (C-index) was analogous to the AUC value. In addition, the time-dependent ROC curve was used to summarize the predictive capacity of the models according to the continuous change over time.[21,22] Model calibration was validated using the calibration plot. We also developed a decision curve analysis (DCA) graph to test the clinical value of the metabolic models through visualizing the potential net benefits at each threshold probability between the metabolic and TNM models.[23]

Establishment and optimization of decision tree models

The Classification and Regression Trees (CART) algorithm is a non-parametric machine learning method,[24,25] and is not dependent on any type of distribution. The CART algorithm generates a top-down decision tree until the stopping criteria are met. The decision tree model has the advantage of being flexible and powerful, and can be presented in graphical form. We used the CART algorithm to establish decision tree models to identify low-, intermediate- and high-risk patient groups for OS and DFS, respectively. The glycolysis model (low risk, high risk), fatty acid metabolism (low risk, high risk) and pathological T (T1, T2, T3, and T4) were used as the covariates. The complexity parameter was used for tree pruning to optimize the decision tree. We used cross-validation to evaluate the relative error at different complexity parameter values. The "rpart" R package was used to generate the decision tree,[25] and the R codes are presented in the Supplementary material.

Conjoint analysis of TMB, MSI, immune cell infiltration, and metabolic core genes

The ACBI website was used to calculate TMB and MSI scores. Spearman's correlation analysis was then used to determine the relationship between TMB, MSI and the metabolic core genes. In addition, the relationship between immune cell infiltration and metabolic core gene expression was evaluated using the Tumor Immune Estimation Resource (TIMER) algorithm and Spearman's correlation analysis.

Experimental validation

Ten pairs of specimens were collected from postoperative patients with HCC from the Ningbo Medical Center Lihuili Hospital, China, between in 2021 and 2022. Total RNA was isolated using FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech, Nanjing, China) and cDNA was synthesized with HiScript II Q RT SuperMix for qPCR (Vazyme Biotech). The method of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was described in our previous study.[2] All primers were purchased from Tsingke Biotechnology (Beijing, China) and are listed in Supplementary Table 2. The RNA expression of the identified genes in different HCC tumor cells was extracted from the Cancer Cell Line Encyclopedia (CCLE, https://sites.broadinstitute.org/ccle),[26] and the results were visualized using Expression Atlas (https://www.ebi.ac.uk/gxa/experiments). Immunohistochemistry (IHC) results of prognostic genes were identified from The Human Protein Atlas (https://www.proteinatlas.org/). The Institutional Ethics Committee of Ningbo Medical Center Lihuili Hospital approved the study (approval No. KY2020PJ186), and written informed consent was acquired from the patients in accordance with the Declaration of Helsinki.

Table 2. The association between glycolysis core genes and tumor mutational burden (TMB) and microsatellite instability (MSI)

Gene	TMB		MSI	
	Spearman	p-value	Spearman	p-value
CENPA	0.07	0.211	0.15	**0.005**
B3GAT3	0.18	**0.001**	0.09	0.073
G6PD	0.10	0.087	0.09	0.077
HMMR	0.09	0.100	0.09	0.089
ABCB6	0.20	**0.000**	0.11	**0.041**
SAP30	0.05	0.354	0.02	0.644
KIF20A	0.05	0.355	0.13	0.011
SRD5A3	0.09	0.107	0.18	**0.000**
TXN	0.23	**0.000**	0.05	0.312
UGP2	–0.11	0.054	–0.11	**0.035**
PPIA	0.16	**0.004**	0.18	**0.001**
PHKA2	–0.02	0.728	0.00	0.944
VEGFA	0.02	0.748	0.09	0.097
NDST3	–0.06	0.382	–0.25	**0.000**
GLRX	0.08	0.163	–0.09	0.086

Values in bold are statistically significant.

Statistical analyses

The differences in gene expression between different groups were compared using the Wilcoxon test. Spearman's correlation analysis was performed to analyze the correlations. The Kaplan–Meier survival analysis was used to compare the survival differences with the log-rank test. The univariate and multivariate Cox regression analyses were used to estimate the HR for each potential risk factor. The two-tailed Student's t-test was used for quantitative data.

The Cox regression model met 2 assumptions: 1) the proportional hazards assumption and 2) that the relationship between each log's hazard and continuous covariate is linear. The proportional hazards assumption was tested graphically for categorical variables and with the scaled Schoenfeld residual test for continuous covariates. The Martingale residual plot was used to test the linear relationship between continuous covariates. If the data followed a normal distribution, the parametric t-test was used, and Welch's correction for variance heterogeneity was applied. Otherwise, the Mann–Whitney method for nonparametric t-tests was used (the results of assumption testing are shown in the Supplementary material).

A two-tailed p-value ≤0.05 was considered statistically significant. Statistical analyses were performed using R software v. 4.0.3 (R Foundation for Statistical Computing) and STATA v. 16.0 (StataCorp LLC, College Station, USA).

Results

Screening prognostic genes for OS and DFS

The study procedure is presented in the flowchart (Fig. 1). RNA sequencing and survival data for the 371 HCC patients from TCGA and 240 patients from the ICGC were acquired.

For the glycolic metabolism hallmark gene set, 85 genes were significantly associated with OS (Supplementary Fig. 1) and 35 genes were significantly associated with DFS (Supplementary Fig. 2). A Venn diagram was generated, and 30 genes were found to be statistically significant for both OS and DFS (Fig. 2A). The prognostic values of these 30 genes are shown using forest plots in Fig. 2B,C. Twenty-nine prognostic genes were significantly differentially expressed between tumors and normal tissues in HCC patients, except for the lactase (*LCT*) gene (Supplementary Fig. 3).

For the fatty acid metabolism hallmark gene set, 44 genes were significantly associated with OS (Supplementary Fig. 4), and 19 genes were significantly associated with DFS (Supplementary Fig. 5). The Venn diagram showed that there were 12 genes exhibiting statistical significance in both OS and DFS (Fig. 2D). The forest plots in Fig. 2E,F show the prognostic values of these 12 genes. Furthermore, these 12 prognostic genes were significantly differentially expressed between tumors and normal tissues in HCC patients (Supplementary Fig. 6).

Construction of prognostic models based on selected glycolysis-related genes

The 30 filtered genes were included in the LASSO regression algorithm to screen for glycolic prognostic signatures for OS and DFS (Fig. 3A–L), and cross-validation was performed.

We selected 8 prognostic signatures for predicting an individual's prognostic risk for OS (Fig. 3B). Figure 3C shows

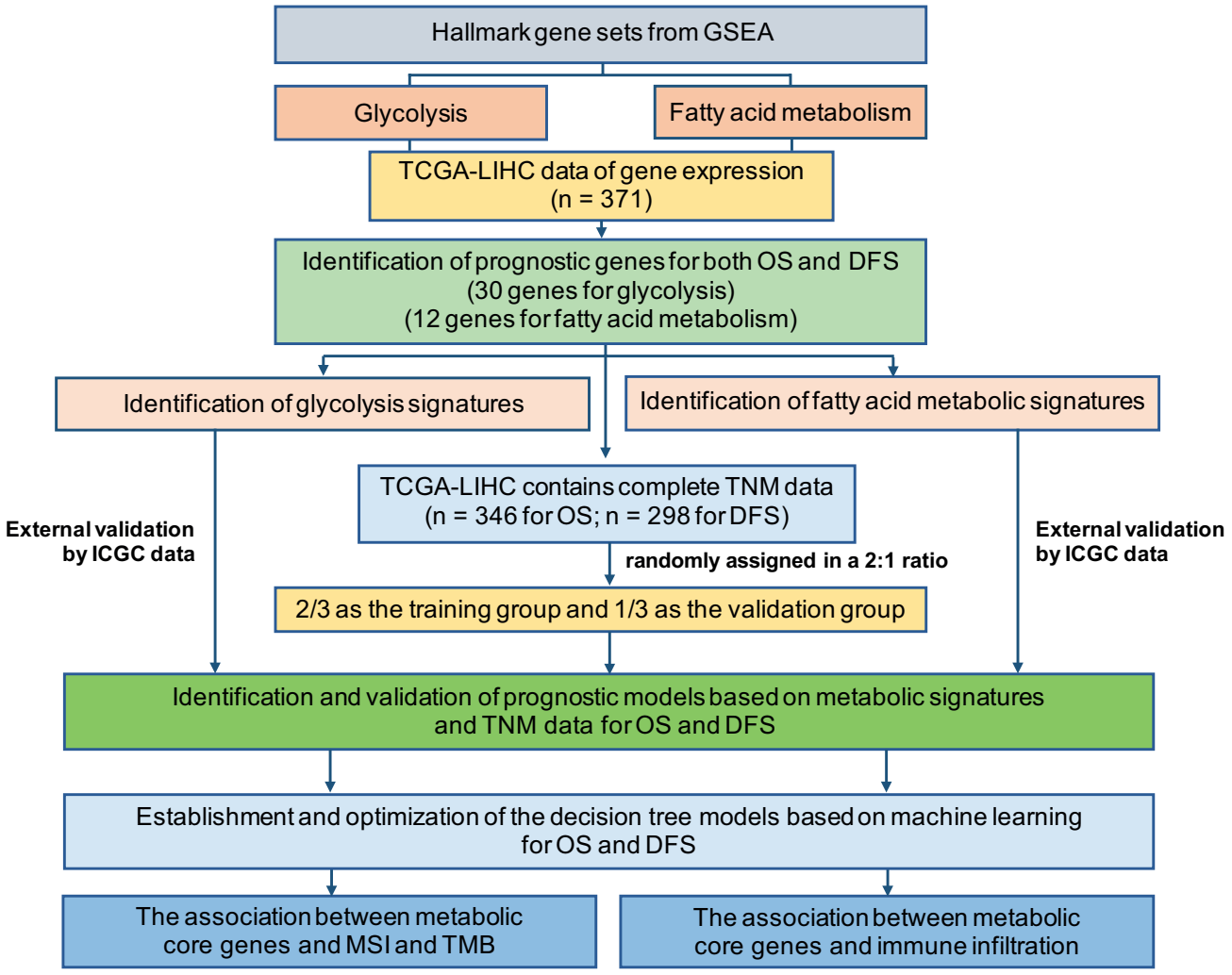

Fig. 1. Flowchart of the study

GSEA – Gene Set Enrichment Analysis; TCGA-LIHC – The Cancer Genome Atlas Liver Hepatocellular Carcinoma; OS – overall survival; DFS – disease-free survival; TNM – tumor-node-metastasis ; ICGC – International Cancer Genome Consortium; MSI – microsatellite instability; TMB – tumor mutational burden.

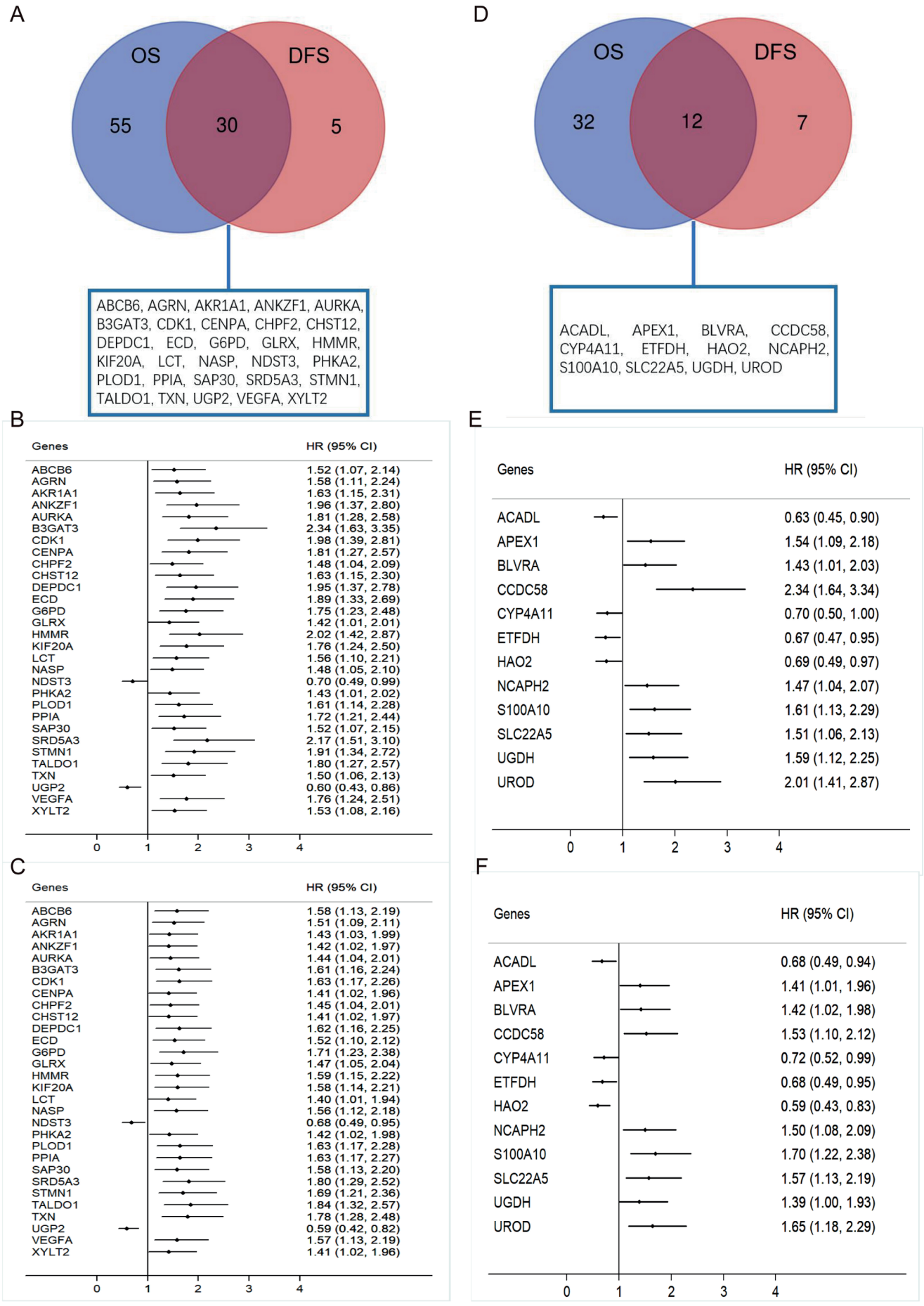

Fig. 2. Identification of the prognostic genes. A. Venn diagram of prognostic genes for glycolysis; B. Forest plot of glycolytic prognostic genes for overall survival (OS); C. Forest plot of glycolytic prognostic genes for disease-free survival (DFS); D. Venn diagram of prognostic genes for fatty acid metabolism; E. Forest plot of fatty acid metabolism prognostic genes for OS; F. Forest plot of fatty acid metabolism prognostic genes for DFS

HR – hazard ratio; 95% CI – 95% confidence interval.

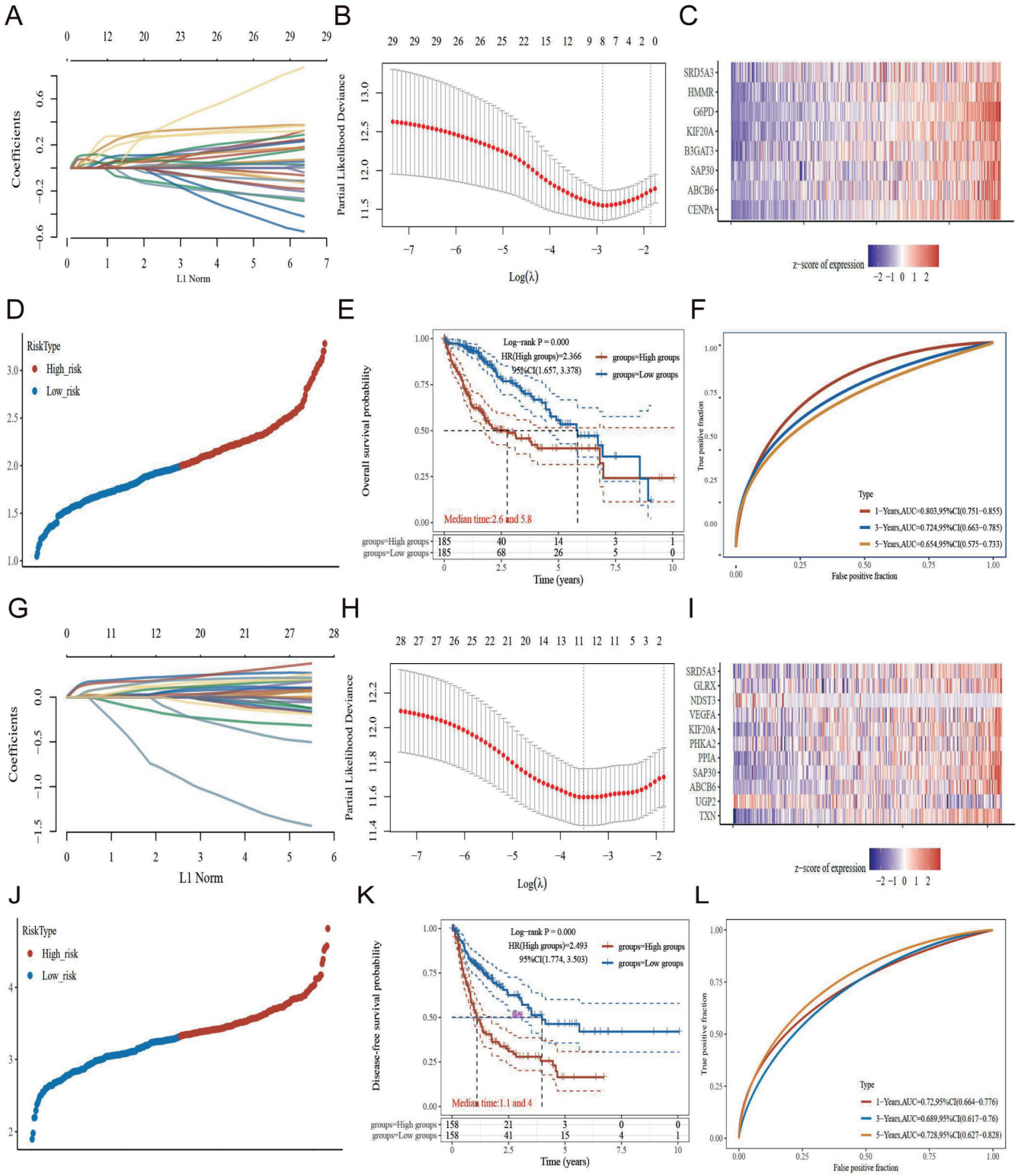

Fig. 3. Construction of the glycolysis-based signature for hepatocellular carcinoma (HCC) patients. A. The Least Absolute Shrinkage and Selection Operator (LASSO) coefficient of the glycolysis-based signature for overall survival (OS); B. The optimal parameter (λ) is selected in the LASSO regression model based on the minimum criteria for OS; C. The heatmap of the identified genes for the glycolysis-based signature for OS; D. Patients were divided into high-risk and low-risk groups based on the risk scores; E. The Kaplan–Meier curve of the high-risk and low-risk groups for OS; F. The receiver operating characteristic (ROC) curves of the glycolysis-based signature for OS; G. The LASSO coefficient of the glycolysis-based signature for disease-free survival (DFS); H. The optimal parameter (λ) is selected in the LASSO regression model based on the minimum criteria for DFS; I. The heatmap of the identified genes for the glycolysis-based signature for DFS; J. Patients were divided into high-risk and low-risk groups based on risk scores; K. The Kaplan–Meier curve of high-risk and low-risk groups for DFS; L. The ROC curves for the glycolysis-based signature for DFS

HR – hazard ratio; 95% CI – 95% confidence interval; AUC – area under the curve.

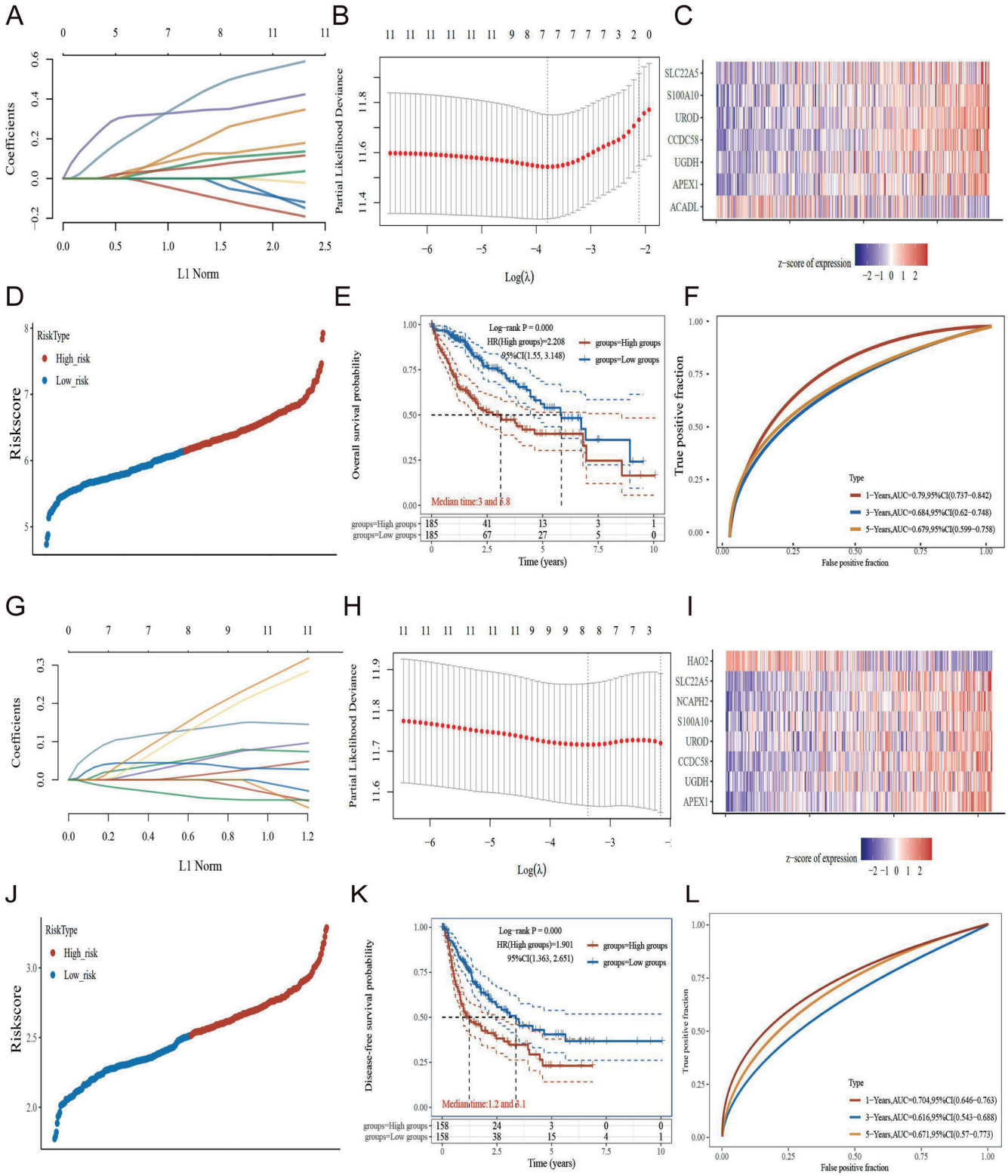

Fig. 4. Construction of the fatty acid metabolism-based signature for hepatocellular carcinoma (HCC) patients. A. The Least Absolute Shrinkage and Selection Operator (LASSO) coefficient for the fatty acid metabolism-based signature for overall survival (OS); B. The optimal parameter (λ) is selected in the LASSO regression model based on the minimum criteria for OS; C. The heatmap of the identified genes for the fatty acid metabolism-based signature for OS; D. Patients were divided into high-risk and low-risk groups based on risk scores; E. The Kaplan–Meier curve for the high-risk and low-risk groups for OS; F. The receiver operating characteristic (ROC) curves for the fatty acid metabolism-based signatures for OS; G. The LASSO coefficient of the fatty acid metabolism-based signature for disease-free survival (DFS); H. The optimal parameter (λ) is selected in the LASSO regression model based on the minimum criteria for DFS; I. The heatmap of the identified genes for the fatty acid metabolism-based signature for DFS; J. Patients were divided into high-risk and low-risk groups based on risk scores; K. The Kaplan–Meier curve of the high-risk and low-risk groups for DFS; L. The ROC curves of the fatty acid metabolism-based signatures for DFS

HR – hazard ratio; 95% CI – 95% confidence interval; AUC – area under the curve.

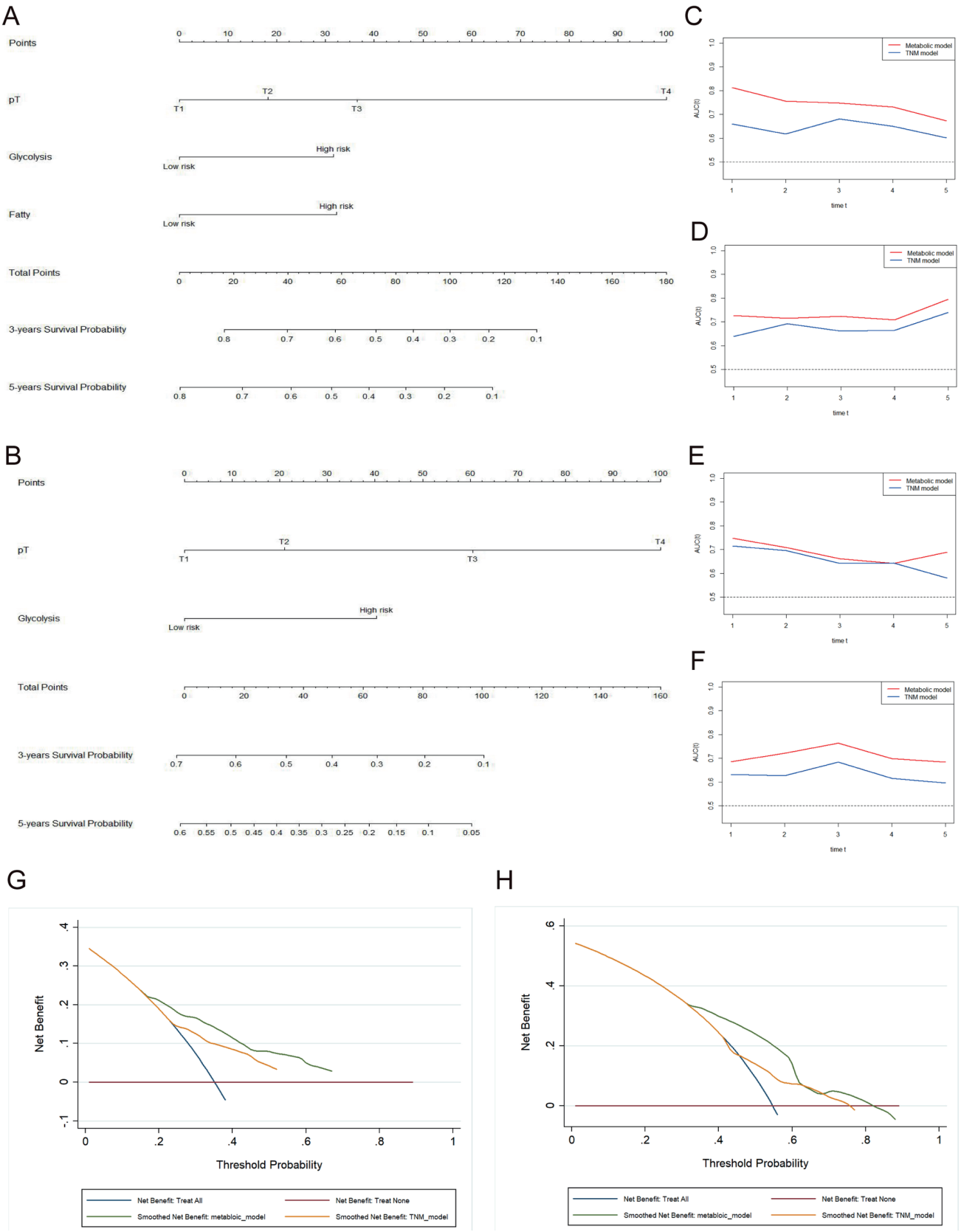

Fig. 5. Construction of the metabolic gene-based nomogram for predicting overall survival (OS) and disease-free survival (DFS). A. Nomogram for predicting OS; B. Nomogram for predicting DFS; C. Time-dependent receiver operating characteristic (ROC) curves of the training groups comparing the metabolic model with the tumor node metastasis (TNM) model for predicting OS; D. The time-dependent ROC curves of the validation groups comparing the metabolic model with the TNM model for predicting OS; E. The time-dependent ROC curves of the training groups comparing the metabolic model with the TNM model for predicting DFS; F. The time-dependent ROC curves of the validation groups comparing the metabolic model with the TNM model for predicting DFS; G. The decision curve analysis (DCA) curves represent the clinical value of the models for predicting 3-year OS; H. The DCA curves represent the clinical value of the models for predicting 3-year DFS

the heatmap of the 8 genes. The risk score for OS was calculated using the following formula (the gene names in the formula indicate the normalized gene expression levels (log2(TPM+1))): risk score = (0.054 × *CENPA*) + (0.2044 × *ABCB6*) + (0.0038 × *SAP30*) + (0.1457 × *B3GAT3*) + (0.0383 × *KIF20A*) + (0.1171 × *G6PD*) + (0.0465 × *HMMR*) + (0.0972 × *SRD5A3*).

The HCC patients were divided into high-risk and low-risk groups according to the median risk score for OS (Fig. 3D). The OS of the high-risk group was significantly worse than that of the low-risk group ($p < 0.001$, Fig. 3E). The ROC curves were constructed, and the AUCs at 1, 3 and 5 years were 0.803, 0.724 and 0.654, respectively (Fig. 3F). This model was validated using the independent data from the ICGC, where the AUCs at 1, 3 and 5 years were 0.706, 0.702 and 0.615, respectively.

We selected 11 prognostic signatures to predict an individual's prognostic risk for DFS (Fig. 3H). Figure 3I shows the heatmap of the 11 genes. The risk score for DFS was calculated using the following formula: risk score = (0.2161 × *TXN*) + (0.1311 × *UGP2*) + (0.1189 × *ABCB6*) + (0.1781 × *SAP30*) + (0.0303 × *PPIA*) + (0.0784 × *PHKA2*) + (0.0214 × *KIF20A*) + (0.022 × *VEGFA*) + (−0.6217 × *NDST3*) + (0.1059 × *GLRX*) + (0.157 × *SRD5A3*).

The HCC patients were again divided into high-risk and low-risk groups according to median risk scores for DFS (Fig. 3J). The high-risk group had a significantly worse DFS ($p < 0.001$, Fig. 3K). The ROC curves were constructed, and the AUCs at 1, 3 and 5 years were 0.720, 0.689 and 0.728, respectively (Fig. 3L). As there were no follow-up data for DFS in the ICGC, we did not perform the external validation for the DFS model.

Construction of prognostic models based on selected fatty acid metabolism-related genes

The 12 filtered genes were included in the LASSO regression algorithm to screen for prognostic signatures of fatty acid metabolism for OS and DFS (Fig. 4A–L), and cross-validation was performed.

We selected 7 genes to predict an individual's prognostic risk for OS (Fig. 4B). Figure 4C shows the heatmap of the 7 genes. The risk score for OS was calculated using the following formula: risk score = (−0.0793 × *ACADL*) + (0.1202 × *APEX1*) + (0.0852 × *UGDH*) + (0.342 × *CCDC58*) + (0.4259 × *UROD*) + (0.0578 × *S100A10*) + (0.1847 × *SLC22A5*).

The HCC patients were separated into high-risk and low-risk groups according to the median risk scores for OS (Fig. 4D). The Kaplan–Meier survival analysis showed that the OS of the high-risk group was significantly worse than that of the low-risk group ($p < 0.001$, Fig. 4E). The ROC curves were constructed, and the AUCs at 1, 3 and 5 years were 0.790, 0.684 and 0.679, respectively (Fig. 4F). This model was validated using independent data from the ICGC, and the AUCs at 1, 3 and 5 years were 0.607, 0.632 and 0.671, respectively.

We selected 8 genes to predict an individual's prognostic risk for DFS (Fig. 4H). Figure 4I shows the heatmap of the 8 genes. The risk score for DFS was calculated using the following formula: risk score = (0.0554 × *APEX1*) + (0.0481 × *UGDH*) + (0.1326 × *CCDC58*) + (0.1365 × *UROD*) + (0.0106 × *S100A10*) + (0.0422 × *NCAPH2*) + (0.1631 × *SLC22A5*) + (−0.0451 × *HAO2*).

The HCC patients were again divided into high-risk and low-risk groups according to the median risk scores for DFS (Fig. 4J). The high-risk group had a significantly worse DFS ($p < 0.001$, Fig. 4K). The ROC curves were constructed, and the AUCs at 1, 3 and 5 years were 0.704, 0.616 and 0.671, respectively (Fig. 4L). As there was no follow-up data for DFS in the ICGC, we did not perform the external validation for the DFS model.

Integrated analysis of clinical parameters and metabolic risk scores

After excluding patients with missing data for pathological T, N and M stages and follow-up time, 346 HCC patients with clinical data and risk scores were selected for the integrated analysis of OS, and 298 HCC patients were identified for the integrated analysis of DFS.

First, the univariate Cox regression analysis showed that T-stage, M-stage, glycolysis risk score (HR = 2.46; 95% CI: 1.68–3.59), and fatty acid metabolism risk score (HR = 2.40; 95% CI: 1.64–3.51) were significantly associated with the OS of HCC patients (Table 1). In addition, T-stage glycolysis risk score (HR = 2.50; 95% CI: 1.76–3.57) and fatty acid metabolism risk score (HR = 1.90; 95% CI: 1.34–2.69) were significantly correlated with the DFS of HCC patients (Table 1).

The multivariate Cox regression analysis was then performed, which showed that T stage, glycolysis risk score (HR = 1.70; 95% CI: 1.05–2.75) and fatty acid metabolism risk score (HR = 1.75; 95% CI: 1.09–2.78) were independent prognostic factors for OS. The T stage and glycolysis risk score (HR = 2.01; 95% CI: 1.35–2.99) were independent prognostic factors for DFS (Table 1).

Development and validation of the nomograms

The HCC patients were randomly assigned in a 2:1 ratio, considering 2/3 of the patients as the training group and the remaining patients as the validation group. Based on these results, 2 prognostic (metabolic) models were developed through combining clinical parameters and metabolic risk scores. In addition, graphical nomograms were generated to predict the probability of 3-year and 5-year survival for OS and DFS (Fig. 5A,B). The calibration plots showed good agreement between the observed outcome and the predicted probability (Supplementary

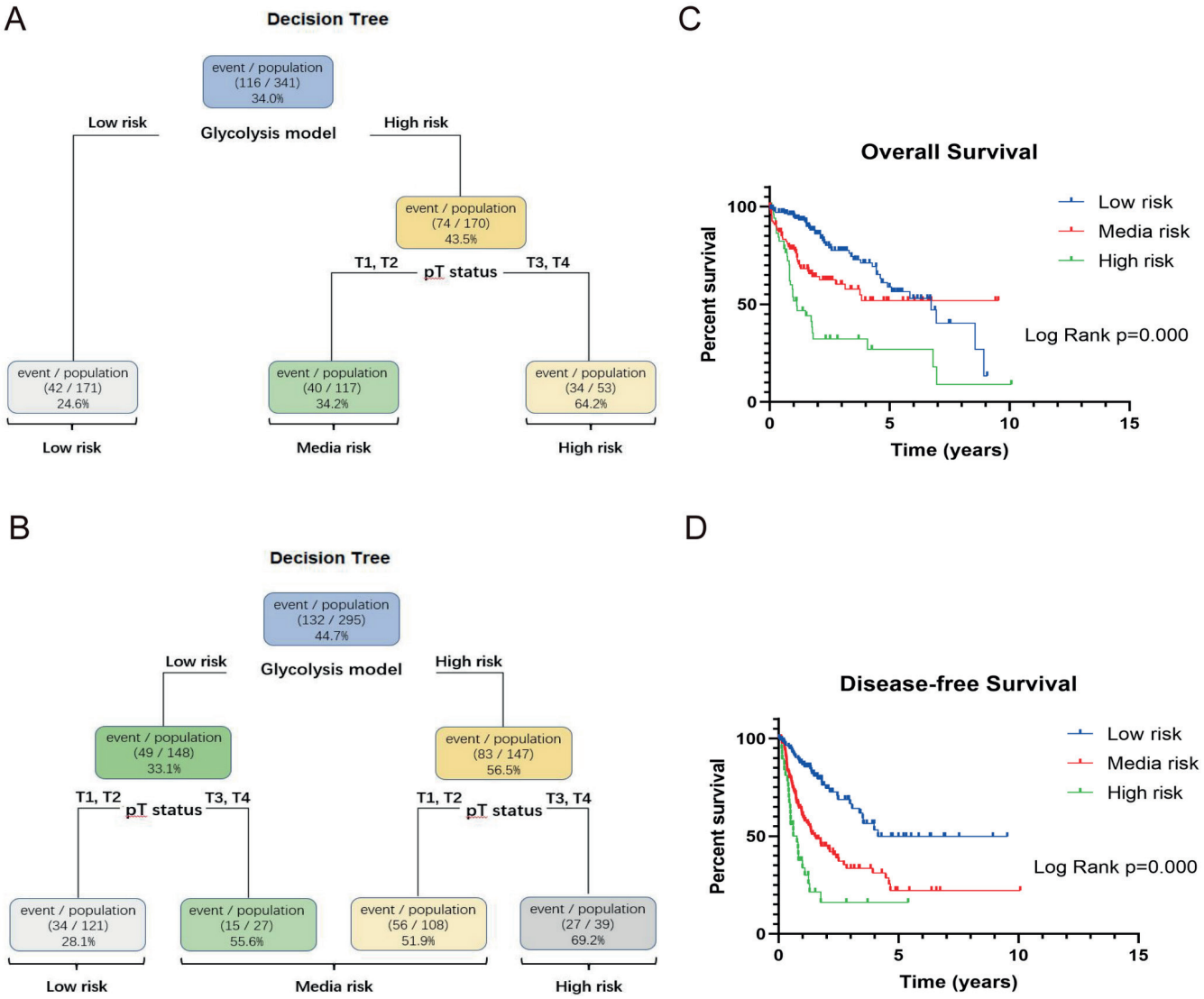

Fig. 6. Decision tree models for risk stratification. A. Decision tree model for overall survival (OS); B. Decision-tree model for disease-free survival (DFS); C. Kaplan–Meier curve of the high-risk, intermediate-risk and low-risk groups for OS; D. Kaplan–Meier curve of the high-risk, intermediate-risk and low-risk groups for DFS

Fig. 7A–F). In addition, 2 prediction models (TNM models) were constructed based on the TNM status of HCC patients for head-to-head comparison (Supplementary Fig. 8).

The predictive accuracy of the metabolic nomogram calculated using AUC was 0.773 for 3-year OS and 0.701 for 5-year OS. Furthermore, the AUC of the TNM model was 0.686 for 3-year OS and 0.663 for 5-year OS (Supplementary Fig. 7C,D). The time-dependent ROC curves revealed that the predictive accuracy of the metabolic model was significantly higher than that of the TNM model in both the training and validation groups (Fig. 5C,D).

The AUC of the metabolic nomogram was 0.680 for 3-year DFS and 0.676 for 5-year DFS. In addition, the AUC of the TNM model was 0.645 for 3-year DFS and 0.634 for 5-year DFS (Supplementary Fig. 7E,F). The time-dependent ROC curves showed that the predictive accuracy of the metabolic model was also significantly higher than that of the TNM model in both the training and validation groups (Fig. 5E,F).

In addition, the clinical value of the metabolic models was evaluated and compared with the TNM models using DCA curves. For almost all threshold probabilities, the metabolic models outperformed the TNM models in terms of net benefit for OS and DFS prediction (Fig. 5G,H).

Development and optimization of the decision tree

We generated 2 decision trees without pruning for OS or DFS. Then, the results of cross-validation showed that when the complexity parameter value was adjusted to 0.018 for OS and 0.014 for DFS, the models had the lowest relative error and minimum tree size (Supplementary Fig. 9).

Finally, 2 variables (glycolysis model and pathological T) were used in the converged optimal models (Fig. 6A,B).

Using the decision trees, HCC patients were classified into the low-, intermediate- and high-risk groups for OS and DFS. We found that the prognosis of the 3 groups was statistically different (Fig. 6C,D). The results indicated that the decision trees were useful for the risk stratification of HCC patients.

The association between the metabolic signatures, immunotherapy and immune cell infiltration

To assess the role of the metabolic signatures in immunotherapy, we calculated the association between ICI-related genes (*CD274* (*PD-L1*), *PDCD1* (*PD-1*), *CTLA-4*, and *LAG3*) and metabolic risk scores. The expression of *CD274*, *PDCD1*, *CTLA-4*, and *LAG3* was significantly higher in the high-risk groups for both glycolysis (Fig. 7A–D) and fatty acid metabolic (Fig. 7E–H) models for OS.

The *CD274*, *CTLA-4* and *LAG3* showed significantly higher expression levels in the high-risk group in the glycolysis model for DFS, whereas there was no difference in *PDCD1* expression between high- and low-risk groups (Fig. 7I–L). The high-risk group in the fatty acid metabolism model for DFS also had a higher expression of *CD274*, *PDCD1*, *CTLA-4*, and *LAG3* (Fig. 7M–P). These results suggest that high-risk patients may have a better response to immunotherapy.

We also analyzed the association between core metabolic genes and immune cell infiltration. As expected, most of the core metabolic genes were significantly associated with immune cell infiltration, including B cells, macrophages, myeloid dendritic cells, neutrophils, and $CD4^+$ and $CD8^+$ cells (Fig. 8). These results demonstrate the potential impact of the core metabolic genes on the immune microenvironment and immune response in HCC tumors.

We analyzed the association between the core metabolic genes (listed in Fig. 8) and TMB and MSI scores. The results of Spearman's correlation analyses revealed that *ABCB6* and *PPIA* from glycolysis models and *UROD* and *NCAPH2* from fatty acid metabolism models were positively correlated with both TMB and MSI (Table 2,3).

The prognostic values of *ABCB6*, *PPIA*, *UROD*, and *NCAPH2* were validated using the ICGC data (Fig. 9A). The RNA expression of *ABCB6*, *PPIA*, *UROD*, and *NCAPH2* in different HCC tumor cells was visualized in Fig. 9B. The RNA expression of *ABCB6*, *PPIA*, *UROD*, and *NCAPH2* in HCC was validated using RT-qPCR (Fig. 9C). The protein expression of ABCB6, PPIA, UROD, and NCAPH2 in HCC was validated with IHC (Fig. 9D–G).

These results suggest that these genes are potential biomarkers for immunotherapy prediction.

Table 3. The association between fatty acid metabolism core genes and tumor mutational burden (TMB) and microsatellite instability (MSI)

Gene	TMB		MSI	
	Spearman	p-value	Spearman	p-value
ACADL	0.07	0.232	−0.07	0.213
APEX1	0.03	0.597	0.12	**0.020**
UGDH	0.19	**0.001**	−0.01	0.797
UROD	0.25	**0.000**	0.13	**0.011**
S100A10	0.10	0.064	0.08	0.103
SLC22A5	−0.10	0.078	0.04	0.422
NCAPH2	0.13	**0.018**	0.14	**0.008**
HAO2	−0.01	0.890	−0.16	**0.002**

Values in bold are statistically significant.

Discussion

In this study, the core genes of glycolysis and fatty acid metabolism were screened from the hallmark gene sets in the MSigDB. The prognostic genes associated with OS and DFS were further identified based on the TCGA data. Then, 2 glycolysis metabolism models and 2 fatty acid metabolism models were constructed to predict the OS and DFS. Next, patients were divided into high-risk and low-risk groups according to the risk scores. The multivariate Cox regression analysis was performed using clinicopathological factors to analyze the independent risk factors. Then, metabolic models combining metabolic risk scores with pathological features were constructed based on the obtained independent risk factors, and nomograms were drawn to predict OS and DFS of HCC patients at 3 and 5 years. Compared with the TNM models, the metabolic models were found to improve the predictive accuracy of the TNM system. We also developed 2 decision trees based on machine learning to classify the prognostic risks of HCC patients. Finally, to provide ideas for basic research and clinical application of immunotherapy, the relationship between metabolic core genes and ICI-related markers, as well as the effects on immune cell infiltration, were analyzed.

Several studies presented prediction models related to glucose metabolism or lipid metabolism to predict the OS of HCC patients. For example, Chen et al. built a good prognostic signature with 4 glycolysis-related genes to predict OS in HCC patients.[11] Zhang et al. constructed a glycolysis-related gene prognostic model with internal and external validation.[27] Xu et al. reported a novel glycolysis-related gene signature to predict the survival and immune status of HCC patients.[28] Weng et al. developed a prognostic model based on 10 metabolic genes to reflect the prognosis of HCC patients and the metabolic characteristics of tumors.[10] He et al. and Hu et al. built lipid metabolism-related prognostic signatures to improve OS predictions in HCC patients.[29,30] However, few studies have focused on the prediction of DFS. Overall survival is affected by many confounding factors, such as the patient's

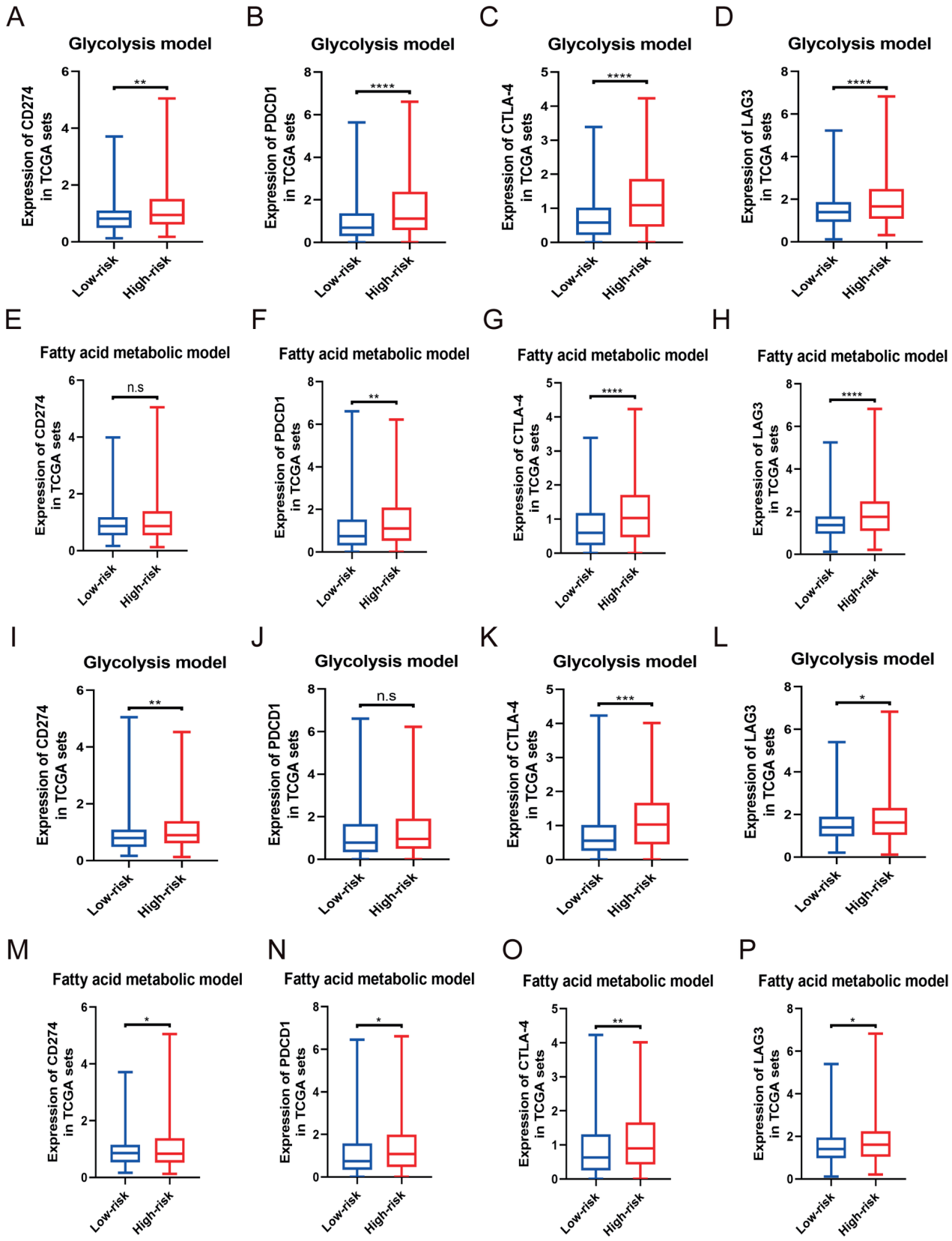

Fig. 7. Immunotherapy prediction of metabolic prognostic models for hepatocellular carcinoma (HCC). A–D. Immune checkpoint inhibitor (ICI)-related genes (*CD274*, *PDCD1*, *CTLA-4*, and *LAG3*) expression between high- and low-risk patients in the glycolysis-based prognostic model for overall survival (OS); E–H. ICI-related gene expression between high- and low-risk patients in the fatty acid metabolism-based prognostic model for OS; I–L. ICI-related gene expression between high- and low-risk patients in the glycolysis-based prognostic model for disease-free survival (DFS); M–P. ICI-related gene expression between high- and low-risk patients in the fatty acid metabolism-based prognostic model for DFS. The box and whiskers present median and ranges from minimum to maximum (Mann–Whitney test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

TCGA – The Cancer Genome Atlas.

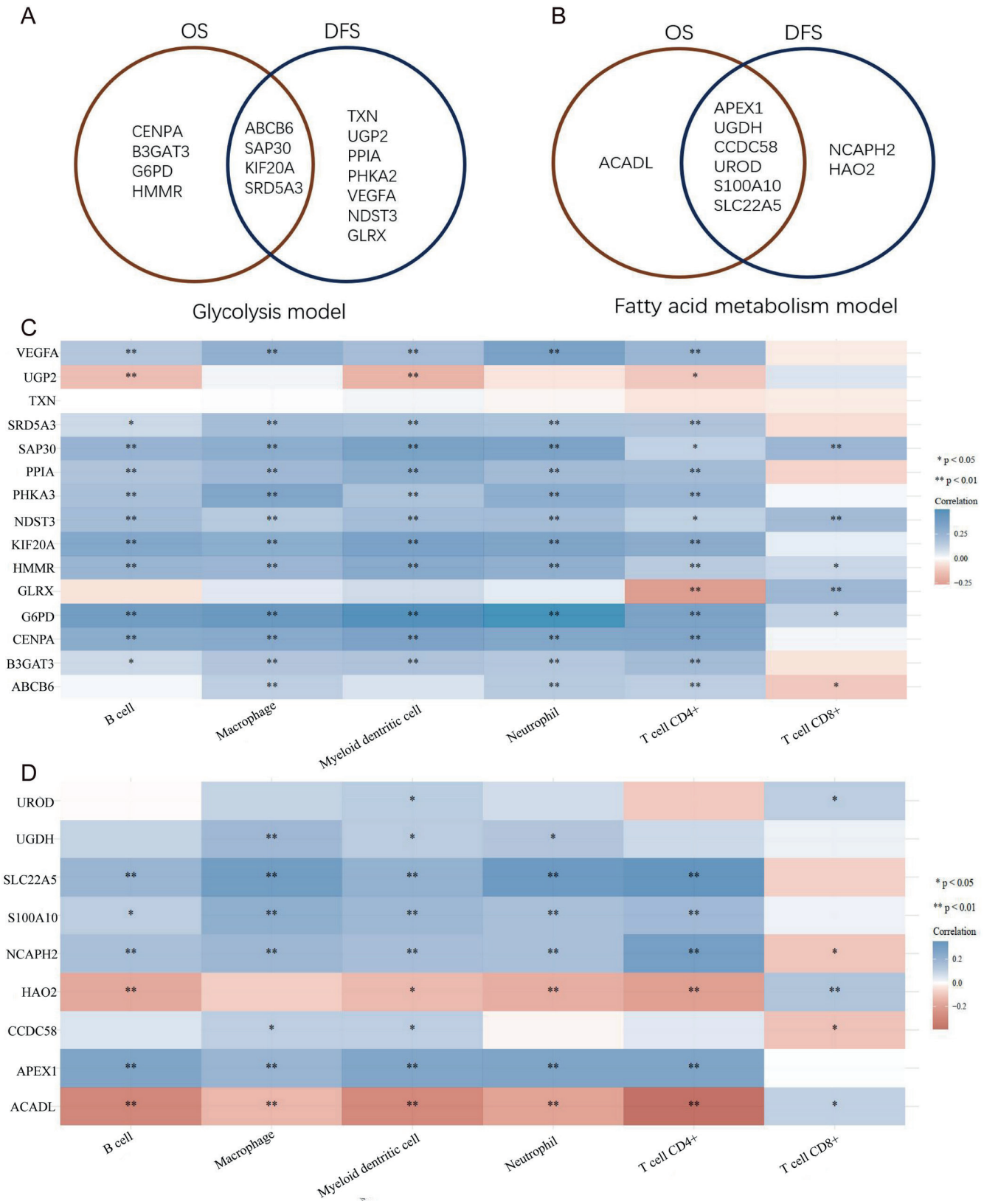

Fig. 8. The association between core metabolic genes and immune cell infiltration. A. Venn diagram of glycolytic core genes; B. Venn diagram of fatty acid metabolic core genes; C. Glycolytic core genes and immune cell infiltration; D. Fatty acid metabolic core genes and immune cell infiltration

age, underlying diseases and social factors. Disease-free survival can better describe the malignant grade of the carcinoma in terms of the biological behavior of the tumor. The present study is a valuable addition to previous studies because it focused on the effects of glycolysis and fatty acid metabolic genes on DFS predictions.

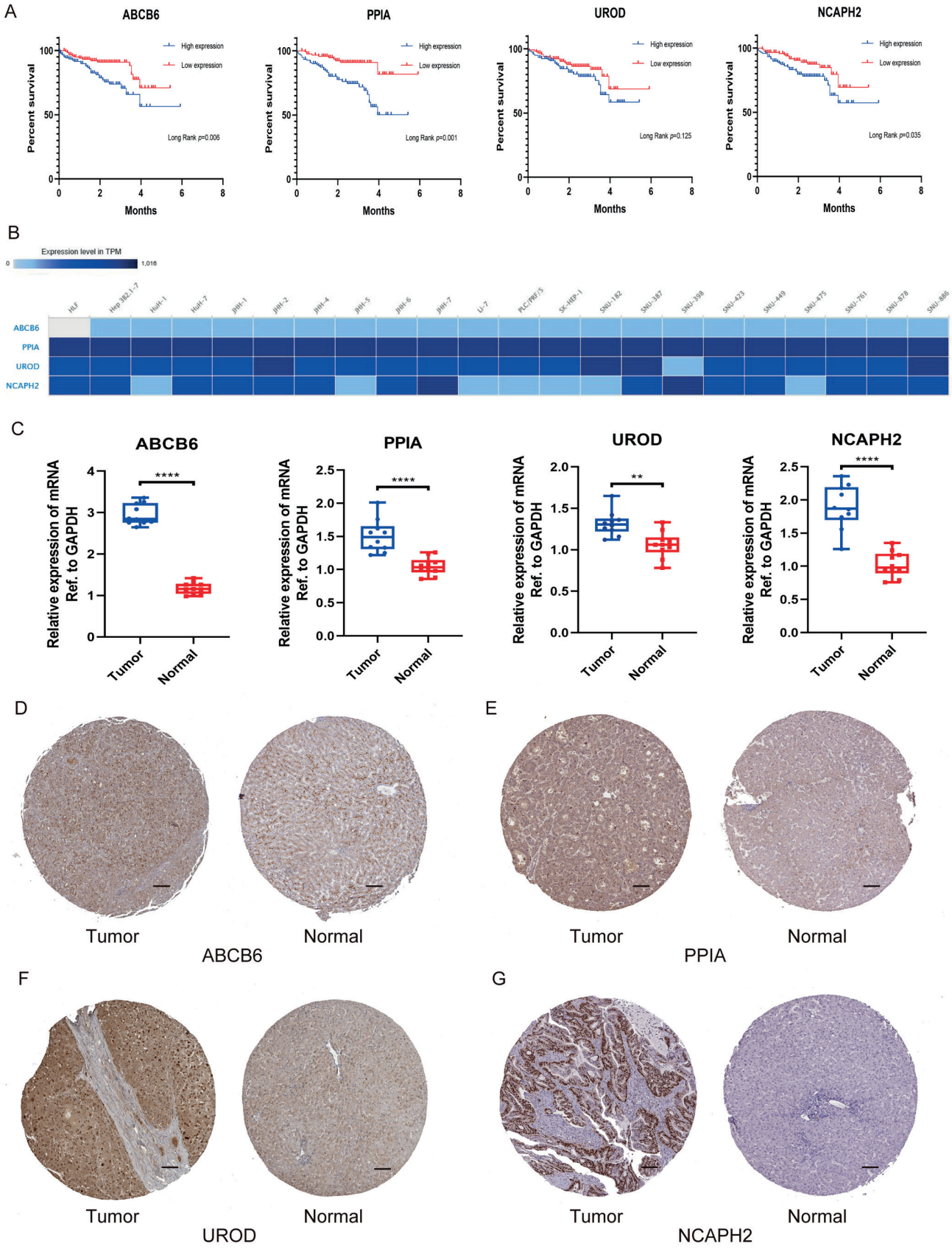

Fig. 9. Experimental validation of metabolic core genes. A. Kaplan–Meier curve for *ABCB6*, *PPIA*, *UROD*, and *NCAPH2* for overall survival (OS); B. RNA expression for *ABCB6*, *PPIA*, *UROD*, and *NCAPH2* in different hepatocellular carcinoma (HCC) tumor cells; C. Results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for ABCB6, PPIA, UROD, and NCAPH2. The box and whiskers present medians and ranges from minimum to maximum (**p < 0.01, ****p < 0.001; Student's t-test); D. Representative immunohistochemistry (IHC) images of *ABCB6* in tumor and normal tissues (scale bar, 100 µm); E. Representative IHC images of *PPIA* in tumor and normal tissues (scale bar, 100 µm); F. Representative IHC images of *UROD* in tumor and normal tissues (scale bar, 100 µm); G. Representative IHC images of *NCAPH2* in tumor and normal tissues (scale bar, 100 µm)

Most of the previous studies have focused on glucose or lipid metabolism.[11,28–30] However, these are 2 interdependent processes. They communicate with and influence each other through the tricarboxylic acid cycle. Therefore, this study focused on the effects of glycolysis and fatty acid metabolism together on predicting the prognosis. The results indicate that glycolysis and fatty acid metabolism are independent risk factors for OS. Glycolysis alone was an independent risk factor for DFS in HCC patients, and fatty acid metabolism did not statistically correlate with DFS. The above results suggest that the glycolysis core genes may play a more important role in the progression of HCC.

Some studies mainly focused on gene expressions but ignored the effects of clinicopathological features on the prognosis.[20,31] In this study, we tried to eliminate these confounding factors by combining gene expressions with clinicopathological features. The TNM staging system is the most widely used prognostic evaluation system in the clinic. However, it still has some limitations and deficiencies, such as an inability to consider gene expressions of individual patients.[32,33] Gene expression differences are one of the important factors causing tumor heterogeneity. In this study, TNM staging and metabolic risk scores were innovatively combined to predict the OS and DFS of HCC patients, taking clinicopathological features and gene expression differences into account. In addition, the head-to-head comparison with the TNM models showed that metabolic models could improve the predictive accuracy of the TNM models, which increases the clinical application value of this study.

Immunotherapy is a recent breakthrough in the treatment of advanced HCC.[34,35] However, due to tumor heterogeneity and immune escape, many patients do not respond to immunotherapy. Microsatellite instability status, TMB and PD-L1 expression can be used to screen for beneficiaries of immunotherapy.[36] However, the positive rate of these indicators was too low to meet the patients' needs. In this study, we observed that the expression of ICI-related markers was significantly increased in patients from the high metabolic risk groups, and the expression of metabolic core genes was correlated with immune cell infiltration. These results suggest that metabolic-related risk scores have potential feasibility as biomarkers for predicting the effects of immunotherapy. Furthermore, the abnormal expression of metabolic core genes may accelerate the development of HCC by affecting the components of the tumor immune microenvironment.

Limitations

This study has several limitations. First, external validation of independent data played a large role in model evaluation. However, because the follow-up data for DFS was missing in the ICGC and we did not find Gene Expression Omnibus (GEO) data with sufficient RNA-seq and DFS data, we could not perform the external validation for the DFS models. Second, only PCR and IHC were used for experimental validation. It is necessary to further explore the biological function and mechanism of core metabolic genes in HCC, especially their effects on the immune microenvironment and immunotherapy. Finally, we mainly focused on genes related to glycolysis and fatty acid metabolism, but not amino acid metabolism. Previous studies have found that glutamine metabolism is also closely involved in the progression of HCC.[12,37]

Conclusions

The novel prognostic models based on glycolysis- and fatty acid metabolic-related genes were developed to better predict the outcome and response of HCC patients to immunotherapy. The new metabolic models were found to improve the predictive accuracy of the TNM system. Further biological experiments are required to explore the mechanisms core metabolic genes play in HCC, especially their effects on the immune microenvironment and immunotherapy.

Supplementary material

The supplementary materials are available at https://doi.org/10.5281/zenodo.7863602. The package contains the following files:

Supplementary Fig. 1. Forest plots of the prognostic genes for OS based on glycolic hallmark gene set.

Supplementary Fig. 2. Forest plots of the prognostic genes for DFS based on glycolic hallmark gene set.

Supplementary Fig. 3. Expression of overlapping genes between tumor and normal tissues in HCC for glycolysis ($p < 0.001$).

Supplementary Fig. 4. Forest plots of the prognostic genes for OS based on fatty acid metabolic hallmark gene set.

Supplementary Fig. 5. Forest plots of the prognostic genes for DFS based on fatty acid metabolic hallmark gene set.

Supplementary Fig. 6. Expression of overlapping genes between tumor and normal tissues in HCC for fatty acid metabolism ($p < 0.001$).

Supplementary Fig. 7. A. Calibration plot of the metabolic prognostic model for OS; B. Calibration plot of the metabolic prognostic model for DFS; C. ROC curves of the metabolic and TNM models for predicting 3-year OS; D. ROC curves of the metabolic and TNM model for predicting 5-year OS; E. ROC curves of the metabolic and TNM models for predicting 3-year DFS; F. ROC curves of the metabolic and TNM model for predicting 5-year DFS.

Supplementary Fig. 8. Construction of the TNM nomograms to predict OS and DFS. A. TNM model for OS; B. TNM model for DFS.

Supplementary Fig. 9. Cost-complexity plot for the decision-tree models. A. Plot for OS; B. Plot for DFS.

Supplementary Table 1. Hallmark gene sets of glycolysis and fatty acid metabolism.

Supplementary Table 2. Primers used in this study.

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Possible effect of natural light on emotion recognition and the prefrontal cortex: A scoping review of near-infrared (NIR) spectroscopy

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Abstract

Near-infrared (NIR) spectroscopy, also known as functional NIR spectroscopy (fNIRS), is a tool for measuring the hemodynamic response of the prefrontal cortex (PFC) by using NIR light, enabling a noninvasive indirect neural activity assessment. The application of fNIRS in emotion recognition or the differential diagnosis of psychiatric disorders of depressive patients, including major depressive disorder, bipolar disorder and schizophrenia, has previously been reported. Although the use of fNIRS has gradually expanded in cognitive neuroscience studies, few researchers have focused on the effects of light exposure in fNIRS studies. In addition, to the best of our knowledge, there are no scoping reviews of fNIRS studies on light exposure. Because light is an important topic in cognitive neuroscience and psychiatry, we evaluated fNIRS studies on light exposure in humans. We reviewed 10 papers in their entirety. Bright light (BR) modulates fear, and the color differences showed no significance in 1 study, whereas other studies delved extensively into the effects of colored light, finding some individual hemodynamic responses. In our study, we highlighted that the effects of natural light have not been studied using fNIRS. Light is becoming a critical topic in cognitive neuroscience and psychiatry, and fNIRS is critical for improving public health and managing psychiatric disorders.

Key words: prefrontal cortex, emotion recognition, light, NIR spectroscopy

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Introduction

Functional near-infrared spectroscopy (fNIRS) is a tool for measuring cerebral hemodynamic responses, enabling a noninvasive assessment of indirect neural activity. This technique has developed rapidly over the 30 years since its first application.[1] The fNIRS tolerates moderate movements, which is an advantage over other tools, such as functional magnetic resonance imaging (fMRI).

The first demonstration of measuring cerebral oxygen level was performed by Jöbsis in 1977,[2] while the first commercial near-infrared (NIR) spectroscopy with a single channel was developed by Hamamatsu Photonics K.K. (Hamamatsu, Japan) in 1989. The original fNIRS system was expensive and cumbersome, and the many wires required rendered subjects immobile, making its application in clinical settings challenging. In 1998, a 10-channel system developed by Hitachi (Tokyo, Japan) was used on intractable epilepsy patients in a clinical setting.[3] Recently, researchers have elaborated on developing more miniaturized, wireless and low-cost fNIRS systems.[4–6]

The principles of the mainstream continuous wave (CW) fNIRS technique are outlined in Fig. 1. When NIR light travels through cerebral capillaries, the light is absorbed, scattered and transmitted. This light absorption can be calculated using the Beer–Lambert law. However, it is inappropriate to apply the original Beer–Lambert law. First, scattering light should be considered because the human brain includes blood vessels and other highly scattering tissues, such as the skin and skull. Second, the trajectory of light is not linear but banana-shaped (Fig. 1) when applying NIRS to the human brain. Therefore, the law needed to be modified.[7] When measuring light absorption of 2 different wavelengths, the common factor of scattering is negligible. Because the molar extinction coefficient for oxygenated hemoglobin (oxy-Hb) and deoxygenated hemoglobin (H-Hb) can be obtained experimentally, the relative concentrations of both oxy-Hb and H-Hb result from solving the 2 equations simultaneously.

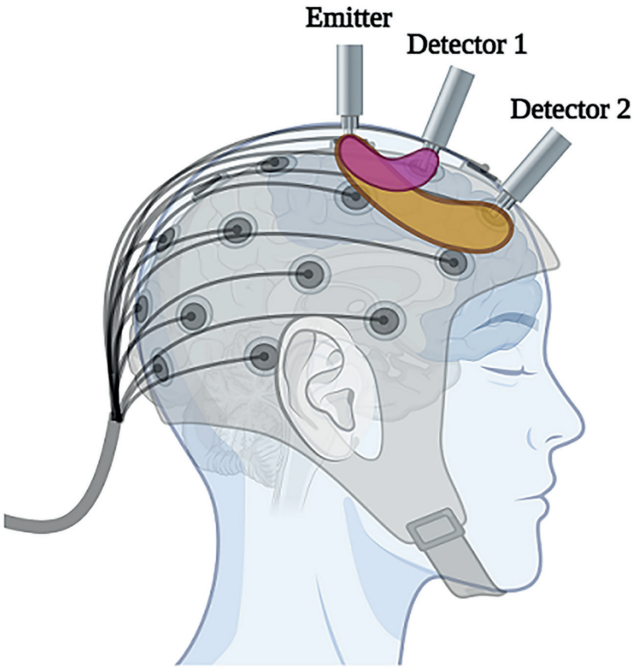

Fig. 1. The principle of the continuous wave (CW) functional near-infrared spectroscopy (fNIRS) system. Two different wavelengths of light are emitted from the emitter and then detected with 2 detectors. Detection is performed under the principles of the modified Beer–Lambert law; therefore, 2 equations are formulated. Finally, these equations are solved to calculate the oxygenated hemoglobin (oxy-Hb) concentration

Functional near-infrared spectroscopy can be a useful tool for measuring brain activity in various settings besides limited laboratory situations. However, there are critical disadvantages of fNIRS to consider. Although fNIRS measures hemodynamic response noninvasively,[8–10] the spatial resolution is not as high compared to an electroencephalogram. In addition, the deep brain of adults cannot be measured since light penetration does not reach it. Therefore, fNIRS can only target the lateral surface of the brain hemispheres, mainly the cortex.[11] The forehead is easy to approach regions of interest in fNIRS. These regions includes motor functionality (the primary sensorimotor cortex and the premotor cortex[12]) and higher-order functionality (the frontopolar cortex and the prefrontal cortex (PFC)[13]).

The PFC contributes to both fear acquisition and extinction, as PFC has the ability to learn from a precedent fear.[14] Recent studies have suggested that the medial PFC is impaired by the basolateral complex of the amygdala (BLA), which is activated by the locus coeruleus (LC). In addition, the posterior ventromedial PFC (BA11) activates powerfully during late fear conditioning.[15] In contrast, the LC activates the medial PFC, and the medial PFC inhibits the BLA.[16] Therefore, the LC, medial PFC and BLA are vital for noradrenergic fear modulation, but the mechanisms differ between fear acquisition and fear extinction. In functional brain imaging studies, the fear and anxiety regions of the frontal cortex are not only in the medial prefrontal but also the dorsal, ventrolateral and prefrontal orbitofrontal cortex.[17] In patients with post-traumatic stress disorder (PTSD), impairment of recalling fear extinction from the hypoactivity of the hippocampus was reported, whereas patients were conditioned to both fear acquisition and extinction normally.[14] Also, the hippocampus projection to the PFC is aberrant in patients with PTSD, depression and schizophrenia,[18] suggesting that hippocampal function affects fear, anxiety and cognition.

The fNIRS application in the differential diagnosis of depressive patients, including major depressive disorder, bipolar disorder and schizophrenia, has been reported.[19] In Japan, fNIRS, as an optical topography test, has been covered by the healthcare insurance system for the differential diagnosis of the depressive state. However, clinical applications in neuroimaging are still controversial. In 2018, the American Psychiatric Association (APA)

concluded that neuroimaging should remain a research tool only. There may be 2 main reasons for this. Firstly, most studies have a small number of participants and identify a relatively tiny effect size. Moreover, although neuroimaging is noninvasive, it is challenging to draw an identical assessment between clinicians, which makes it difficult to support its application for clinical diagnosis.

The use of fNIRS has expanded gradually in cognitive neuroscience studies. Cognitive neuroscience is a broad multidisciplinary field focusing mainly on the neural mechanisms behind cognitive function by linking together disciplines such as physiology, anatomy, psychology, psychiatry, and computational biology.[20] Table 1 shows the main subjects of cognitive neuroscience with reported topics for fNIRS application. It should be noted that the mentioned studies were almost but not entirely separate, and each field will overlap with other fields. For example, dyslexia is in the reading field but can also be categorized in the decision or language fields. In terms of the attention field, attention deficit hyperactivity disorder (ADHD) during the developmental ages is a critical topic in fNIRS studies.[21] Studies regarding traumatic brain injury,[22,23] autism spectrum disorders,[24,25] drowsy driving,[26] and meditation[27] are also recent clinical applications of fNIRS. Studies on consciousness, anesthesia management,[28] disorders of consciousness,[29] and locked-in syndrome[30] have been reported. Decision-making is one of the essential applications of cognitive neuroscience. The application of the brain–computer interface[31] to examine group decision,[32] purchase decision[33,34] and risk-taking[35,36] yielded some pieces of evidence. In terms of executive control, obesity,[37] exercise[38] and older adults[39] have been researched. In the intelligence field, problem-solving[40] and work performance[41] have been studied. The language field[42,43] is the most extensively studied topic in cognitive neuroscience, and the verbal fluency task (VFT) has recently demonstrated its ability to measure depressive disorder.[44] We perceive almost all stimuli under any circumstances; therefore, it is challenging to aggregate perception studies. To the best of our knowledge, perception of depth,[45] emotion,[46–48] face,[49] reward,[50,51] self-agency,[52] and time[53] have been reported. Finally, reading studies include dyslexia[54] and overt reading[55] (reading aloud).

Table 1. Major topics of cognitive neuroscience and reported application of functional near-infrared spectroscopy (fNIRS)

Topic	Application
Attention	attention deficit hyperactivity disorder (ADHD)
	impairments after traumatic brain injury
	autism spectrum disorder
	drowsy driving
	meditation practice
Consciousness	anesthesia monitoring
	disorders of consciousness
	locked-in syndrome
Decision	brain–computer interface
	group decision
	purchasing decisions
	risk behavior in financial decisions
	risky decision-making in social interactions
Executive control	central obesity
	exercise
	older adults
Intelligence	problem solving
	work performance
Language	aphasia
	bilinguality
	developments
	presurgical assessment
	second language
	unknown language
	verbal fluency
Perception	depth
	emotion
	light
	face
	reward
	self-agency
	time
Reading	dyslexia
	overt reading

Objectives

Light is essential not only for visualization but also for sleep, mood and cognition. Meta-analyses and systematic reviews support the impact of light on alertness.[56] In addition, light therapy is an effective treatment for depression in Parkinson's disease[57] and premenstrual dysphoric disorder,[58] and the effects of light therapy on sleep disturbance have also been reported.[57,59–61] In cognitive neuroscience and psychiatry, light is an important topic. However, few researchers have focused on the effects of light exposure measured using fNIRS. In addition, to the best of our knowledge, there is no scoping review of fNIRS studies on light exposure. Therefore, in the present review, we evaluate fNIRS studies on light exposure in humans.

Review protocol

We delineated 4 inclusion criteria. First, studies must be performed on humans. Second, studies should focus on light exposure. Therefore, papers that only mentioned light exposure for the technical principles of fNIRS or light effect on fNIRS experiments itself[62] (ambient light) were

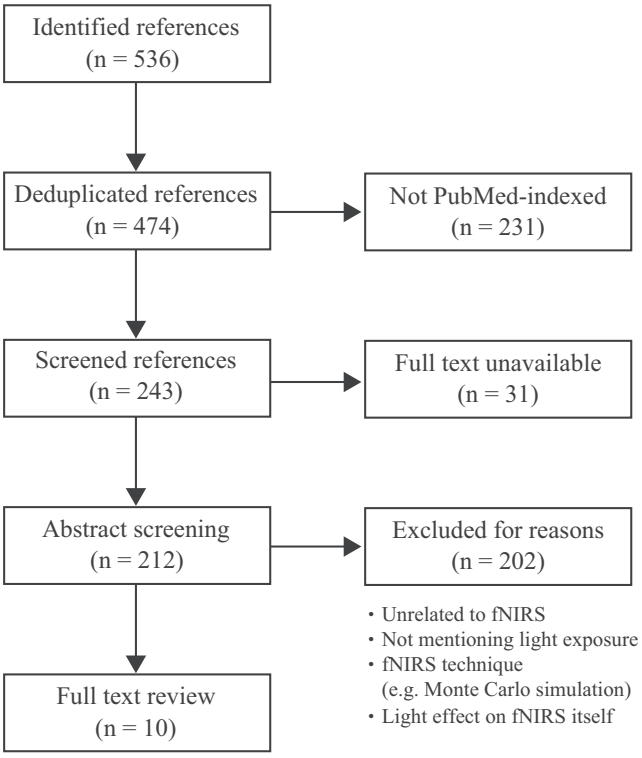

Fig. 2. Selection of target literature

fNIRS – functional near-infrared spectroscopy.

excluded from our review. Third, not only direct but also indirect light exposure measurements were included, as was smartphone use[63] (effect of blue light (BL)). However, studies were excluded if there were other non-negligible effects, such as the natural light effect on garden therapy[64] with green plants. Fourth, since the first fNIRS study was published in 1991, studies published only after that year were included in the analysis.[1] Following an initial Internet search, we concluded the literature on light exposure encompassed various fields such as architecture, education, religion, and complementary medicine. Therefore, from the standpoint of scientific rigor, we required papers to have a PubMed Identifier (PMID) in PubMed and did not take into account information available from other websites, dissertations and books. Our review presents the whole PubMed-indexed research on the topic.

We started the literature search on November 15, 2022, and finished it on January 20, 2023. The process of selecting literature is shown in Fig. 2. We used Google Scholar, Scopus and PubMed databases to identify relevant studies, and carefully selected queries in order to identify fNIRS research on light exposure. The queries that were used are listed in Table 2. Google Scholar facilitates literature identification,[65] but many irrelevant results appear because of its low precision.[66] In addition, search results are not reproducible.[67] Therefore, using Google Scholar solely is challenging in identifying literature[68]; thus, Scopus and PubMed were used to improve coverage and maintain search consistency. On January 20, 2023, 2 authors (RN and TF) independently searched the databases and found that all results were consistent. In addition, we confirmed that the results had not been updated since the previous search. After literature identification, we deduplicated the references using Zotero v. 6.0.18 (https://www.zotero.org/). After deduplication, articles with unavailable full-text articles were excluded, abstracts were screened and 10 papers remained for full-text review. The main highlight of the fNIRS studies was the oxy-Hb concentration.

The bias risk assessment was performed based on the characteristics and number of participants and study protocols. Moreover, the study protocol was assessed. We used robvis tool to create risk-of-bias plots.[69]

Results

A brief overview of the 10 reviewed studies is presented in Table 3. In summary, bright light (BR) modulates fear. Regarding the color differences, no significance was indicated in 1 study, whereas other studies suggested that colored light produced some individual hemodynamic responses.

Bias risk assessment

Figure 3 summarizes the risk of bias. All studies included both male and female participants. Although the number of participants ranged from 10 to 34 in most studies, 1 study had 757 participants. Due to the inhomogeneous signal processing methods, comparing oxy-Hb concentrations between studies would be inappropriate. In 4 studies,[70–73] the reported number, male/female proportion, standard deviation (SD), mean (M), and age ranges of participants were identical. In 1 study, a machine learning technique was used.[71] However, although the machine learning technique is overused in various fields, artificial intelligence (AI) does not apply to small biased datasets.[74]

Table 2. Queries used for database search

Database	Queries	Matches
Google Scholar	"functional near-infrared spectroscopy" AND "light exposure"	212
	"functional near-infrared spectroscopy" in title: "light"	231
PubMed	"functional near-infrared spectroscopy (title)" AND "light (title/abstract)"	83
Scopus	TITLE-ABS-KEY ("functional near-infrared spectroscopy" OR "functional near infrared spectroscopy" OR "fNIRS") AND TITLE-ABS-KEY ("light W/3 exposure" AND (LIMIT-TO (DOCTYPE, "ar") OR LIMIT-TO (DOCTYPE, "re")	10

Table 3. Summary of fully reviewed references

Year	Author and reference	Types of light	Participants, n (male/female)	Age [years]			fNIRS findings
				range	M	SD	
2011	Weinzirl et al.[78]	C	13 (7/6)	18–44	30.0	N/A	indifferent to color sequence
2012	Weinzirl et al.[77]	C	10 (5/5)	23–44	27	N/A	BL decreases oxygen demand
2017	Hori et al.[75]	C	fNIRS measurement: 757 (294/463) questionnaire: 299 (118/181)	6–78	32.0	14.0	light itself does not change an emotion
	Metz et al.[82]	C	17 (11/6)	25–65	N/A*	N/A	yellow light elevates oxy-Hb concentration in the left PFC
	Scholkmann et al.[83]	C	14 (9/5)	24–57	33.4	10.5	only BL elicits PFC oxy-Hb concentration elevation
2018	Yoshiike et al.[76]	BR	29 (10/19)	20–25	21.7	N/A	BR decreases oxy-Hb concentration in the PFC
2021	Zohdi et al.[72,73]	C	32 (17/15)	N/A	25.5	4.3	indicates inter-participant hemodynamic variability
		C	32 (17/15)	19–45	25.5	4.3	various deviations in cerebral hemodynamics
2022	Honma et al.[63]	BL	34 (14/20)	N/A	20.4	0.8	BL overly activates the PFC
	Zohdi et al.[71]	C	32 (17/15)	N/A	25.5	4.3	–
	Scholkmann et al.[70]	C	32 (17/15)	19–45	25.5	4.3	oxy-Hb and deoxy-Hb concentration change is color-independent; BL rather than red light response is stronger in the VC

BL – blue light; BR – bright light; C – colored light; N/A – not available; oxy-Hb – oxygenated hemoglobin; PFC – prefrontal cortex; fNIRS – functional near-infrared spectroscopy; M – mean; SD – standard deviation; VC – visual cortex; * median age was 29 years.

Therefore, even if machine learning methods successfully find plausible clustering, these models are not always suitable for all datasets, and sometimes the clustering is a result of sheer luck.

Detailed description of the reviewed literature

Hori et al. stimulated 757 subjects with various colored lights and waterfall sounds using different frequencies to study the emotional effects on the PFC.[75] Their feedback system enabled them to alter the stimuli in response to increased oxy-Hb concentration in the PFC, resulting from pleasantness or unpleasantness. Pleasantness or unpleasantness was determined according to a previous study.[11] After the stimulation, 298 participants answered a questionnaire to assess their comfort with the stimuli, ranging from –5 (unpleasant) to +5 (pleasant). Results indicated that sound frequency rather than light color affects how pleasant or unpleasant the feelings of the participants were. High-frequency sounds accounted for approx. 43% of both pleasantness and unpleasantness responses.[75]

Yoshiike et al. performed an fNIRS study to show the effect of BR on PFC activity during human fear conditioning and fear extinction. They conducted a single-blinded study by exposing 29 participants to either bright (9000 lx) or regular (<500 lx) light, and presented visual conditioned stimuli (CS) randomly, followed by unconditioned stimuli (US) using an unpleasant but not painful electric shock. For CS, each sign represented a learning process using triangles, squares and circles to represent fear extinction, fear acquisition and a safety state, respectively. In addition to the PFC activity on fNIRS, skin conductance was recorded with each response to stimuli.[76] The results were intriguing. Bright light decreases skin conductance during the recall session of any learning process. However, fNIRS showed that BR modulates fear immediately and differently, promoting fear extinction and safe learning while inhibiting fear acquisition. Moreover, BR modulates fear in any type of learning, which generally lasts at least 24 h.

Wolf's research group extensively focused on the color of light and conducted 8 fNIRS studies in the years 2011–2022.[70–73,77,78,82,83] In 2011, they first investigated the effect of red and blue light on oxygen consumption in the brain and muscles of 10 participants.[77] Then, they exposed the 12 volunteers to these 2-colored lights sequentially to examine the hypothesis stating that red light enhances the effects of BL.[78] The source of light was not a light-emitting diode (LED) but a white light bulb with colored filters. The constant hemoglobin concentration and high tissue oxygen saturation indicated that BL, but not red light, exposure decreases oxygen demand in the brain.[77,78] However, this contradicts the activation of the PFC in both long (30 min) and short (1 min) BL

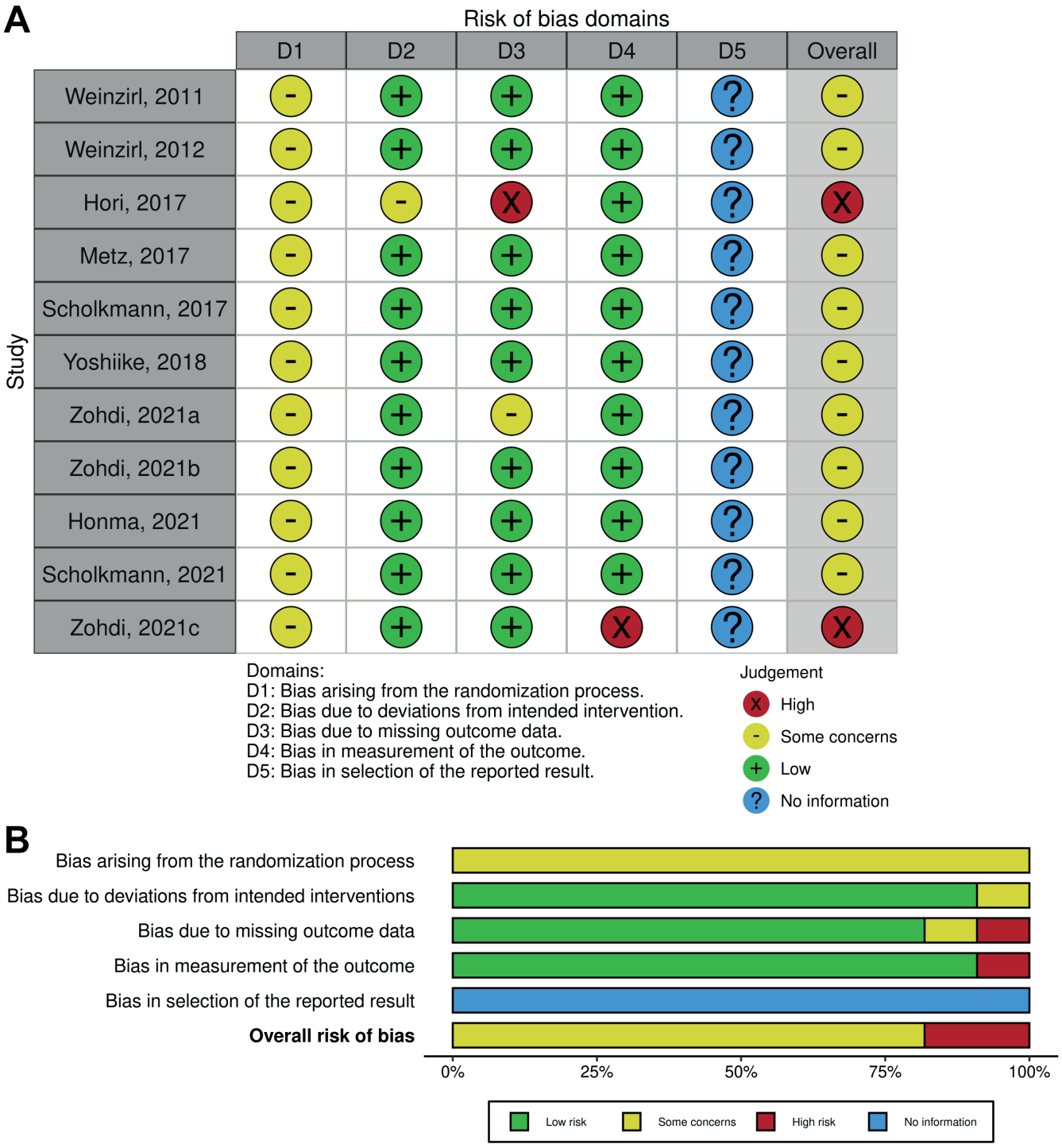

Fig. 3. Summary of bias risk assessment. A. Risk of bias for each study and domain; B. Summarized bias risk assessment for all 10 studies

exposures.[79–81] Interestingly, blood oxygenation was independent of the sequence, and the oxygenation in both sequences was equivalent to a single red light exposure, suggesting that red light is preponderant to BL.[78]

Metz et al. in 2017 proposed physiology-augmented fNIRS (SPA-fNIRS) and conducted the first 2 SPA-fNIRS studies. As the name suggests, SPA-fNIRS associates fNIRS with physiological parameters, including skin conductance, end-tidal carbon dioxide ($P_{ET}CO_2$) and heart rate (HR).[82,83] Both studies exposed the participants to colored light, but the major difference was whether the exposure was continuous[82] or intermittent.[83]

Metz et al. continuously exposed the 12 participants to blue, green, red, and yellow LED lights for 10 min while recording their physiological and neurological activities using SPA-fNIRS. Skin conductance increased for all colors except green, and only yellow light significantly elevated oxy-Hb concentration in the left but not the right PFC.

Additionally, as in their previous study, oxygen demand decreases were only observed when exposed to blue, but not red light. Except for the green light, HR increased while the partial pressure of CO_2 (P_aCO_2, equivalent to $P_{ET}CO_2$[84]) decreased. The HR elevation and $P_{ET}CO_2$ reduction indicate that participants hyperventilated during fNIRS. They described that this hyperventilation occurred due to weak pain in the recovery phase, but not from the light itself.

Scholkmann et al. used blue, green and red LEDs to beam light for 10 min, on and off every 20 s, but did not use yellow LEDs. In addition to PFC activity, visual cortex (VC) activity was measured using the SPA-fNIRS. The results indicated that BL and red light significantly increased respiratory rate (RR), whereas green light decreased it. Regarding HR and $P_{ET}CO_2$, only BL elevated HR, and $P_{ET}CO_2$ showed no significant alteration. In addition, only BL elicited an elevation in PFC oxy-Hb, while VC activity-related oxy-Hb elevation was observed with any color.[83]

In 2021, followed by another SPA-fNIRS study to investigate the colored light effect (CLE) during a VFT,[73] Wolf's research group delved into the interindividual differences of hemodynamic responses and consecutively published 4 studies in January 2021, April 2021, May 2022, and October 2022.[70–73] Briefly, followed by performing a VFT under CLE, they patterned or clustered hemodynamic responses using various techniques, including unsupervised machine learning,[71] namely k-means.

In 2022, Honma et al. enrolled 34 healthy individuals to compare reading comprehension on a smartphone compared to paper by recording the number of sighs the subjects made while reading and bilateral PFC activity obtained using fNIRS. The 10-point comprehension test used 10 multiple-choice questions. This crossover study revealed that reading on paper achieves at least 1 point higher score than reading on a smartphone. In addition, sighs decreased by approx. 70% while reading on a smartphone, and the left PFC was simultaneously overactivated. These findings indicated that BL decreases sighs and increases left PFC overactivation, resulting in poor comprehension due to excessive cognitive loads.[63]

Discussion

Results show that the CLEs on verbal fluency have been extensively researched, and the hemodynamic responses may differ individually. In addition, BL overly activates the PFC. Moreover, BR modulates fear immediately and differently between learning processes, promoting fear extinction and safe learning, as well as inhibiting fear acquisition.

Although our review provides some evidence of the effects of colored light, some concerns should be mentioned. First, according to previously mentioned findings,[75] light color does not affect emotions. On the other hand, Battaglia et al. suggested that both positive and negative emotions equally control our behaviors.[85] Therefore, light color differences may not affect our behaviors. However, it is not surprising that numerous studies suggest that color underlies mood, quality of life (QOL) and cognition. In 2010, Carruthers et al. developed the Manchester Color Wheel to assess patients by the color of light they were exposed to using 38 colors representing positive, neutral or negative conditions that were rigorously reproduced on the participants.[86] Color-using art therapy can increase purpose in the life of stroke patients and their caregivers, resulting in QOL improvements.[87] In addition, as Berndt et al. indicated in 2020, color plays a vital role in language comprehension, facilitating anagram solving by presenting the referent color of the solution words.[88]

Second, as previously mentioned, Metz et al. reported that exposure to green light did not increase HR and decrease $PaCO_2$,[82] suggesting that green light did not induce hyperventilation (in turn caused by weak pain) during fNIRS. However, the light color should not be the only difference studied, and hyperventilation during fNIRS needs to be elucidated more complex. Although previously mentioned, it should be noted again that noninvasiveness is a core feature of fNIRS. Moreover, hyperventilation suggesting HR elevation and concurrent $P_{ET}CO_2$ reduction were not observed with intermittent light exposure.[83] Research on the effect of green light is scarce because exposure to green light in the natural environment is rare; nonetheless, the effects of green light need to be elucidated.

Finally, there are some discrepancies to be noted when comparing continuous light[82] and intermittent light[83] exposure. Regarding the BL effect, continuous light does not induce activation,[77,82] whereas intermittent light induces an oxy-Hb concentration increase in the PFC.[83] However, interindividual differences can be the main plausible cause of varying results, as mentioned.[70–72]

Blue light activates intrinsically photosensitive retinal ganglion cells (ipRGCs), and the signal is projected to several brain regions, including the ventral tegmental area, raphe and LC via the suprachiasmatic nucleus, to modulate circadian rhythms.[89] The ipRGCs do not form a vision, suggesting that even in blind people exposure to colored light may result in circadian modulation.[80,90,91] A LED display on smartphones is an example of BL emitter. Blue light harms photoreceptors and pigmented epithelium cells,[92] and the reviewed fNIRS study suggests the negative impact of BL on reading comprehension.[63]

In the reviewed studies, BL increased total hemoglobin concentrations when the participants were exposed to light emitted by a color-filtered white lightbulb, but it remained constant under LED illumination. However, the difference between incandescent light and LED can be applied to this result. Recently, Farghly et al. reared rabbits under various light conditions and found that rearing animals under LED conditions resulted in the highest glucose concentrations.[93] In addition, Niemierzycka et al. showed that LED light with a color temperature of 4200 K achieves higher speeds with

maintained accuracy on the Kraepelin test.[94] As the Kraepelin test induces PFC activity[95] and glucose is primarily present in the brain, it can be inferred that LED light plays a different role than incandescent light on the PFC.

The present review indicated that there are only 10 studies on the effects of light exposure measured using fNIRS measurements in PubMed. In addition, we should carefully interpret these results. For example, since colored light effects have been studied only by a single research group, there are some non-negligible biases, such as confirmation bias. Thereby, the present review identified the sparsity of light exposure studies with fNIRS usage.

The effect of natural light has not been studied using fNIRS. As previously mentioned, garden therapy was studied,[64] but the effect of green plants is not negligible. However, studying solely natural light is challenging because natural light is a component of the environment. For example, when downtown, there can be additional stimuli like noise from cars or rising temperatures caused by the heat island effect.

We hypothesize that fNIRS can yield critical findings in combination with other biomarkers. For example, because fNIRS measures the oxidation of hemoglobin in the cerebral capillaries, cerebral oxidative stress can be noninvasively measured in combination with other oxidative indicators, such as the kynurenine-3-monooxygenase, which is activated by oxygen.[96–101] Moreover, fNIRS might facilitate the understanding of fear learning, in combination with cardiac autonomic dynamics of heart rate variability.[102]

Another important hypothesis is that fNIRS can bring critical findings for psychiatric disorders in terms of light effects. For example, since light therapy was reported to be effective in seasonal affective disorder (SAD) in 1984,[103] only the atypical pattern of rod electroretinogram has been reported as a possible biomarker.[104] Due to a much stronger invasiveness of electroretinogram than fNIRS, the present review suggests the need for developing a biomarker in patients with SAD. The fNIRS can also be used in combination with inflammatory cytokines in dementia[105,106] or the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in various gut microbiota-related psychiatric illnesses.[107] Although MRI is essential for diseases such as cerebral small vessel disease,[108] there might be a possibility of using fNIRS to predict the critical need for an MRI, which would significantly spare the clinician's time and effort.

As previously mentioned, fNIRS is an effective tool for cognitive neuroscience due to its non-invasiveness, tolerance of movements and high resolution. On the other hand, most fNIRS studies corroborate PFC activity by associating oxy-Hb concentration increase with brain activity.[109–113] The idea of such association is derived from the neurovascular coupling theory. Although the mechanism by which neural tissue activation leads to increased cerebral blood flow remains unclear,[114,115] in terms of neurovascular coupling, the integration of fNIRS and functional MRI can be helpful in understanding brain hemodynamics.[116]

Limitations

Although this scoping review presents the whole PubMed-indexed research on fNIRS and the effects of light exposure, it has some limitations. We focused only on PubMed-indexed literature, which excludes information available from websites, dissertations and books. In addition, fNIRS-related fields, such as engineering, computer science and physics, were not incorporated because PubMed does not include biomedical journals. Finally, we did not include studies published after January 2023.

Conclusions

The fNIRS techniques enable real-time monitoring of human brain activity with tolerance to motion artifacts to some extent. Humans live with light, but its quality changes from natural to artificial light exposure. Notwithstanding that natural light is still essential, its effect on the brain's hemodynamic response has not been thoroughly researched. Delving into light effects under commonplace environments using fNIRS is a reasonable approach. In conclusion, light will be a more critical topic in cognitive neuroscience and psychiatry, and fNIRS can be a critical tool for improving public health and managing psychiatric disorders.

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Central nervous system nocardiosis diagnosed by metagenomic next-generation sequencing: A case series and literature review

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Conflict of interest
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Abstract

Background. Central nervous system (CNS) nocardiosis is a rare suppurative disease caused by the genus *Nocardia*. It is found most frequently in immunocompromised individuals.

Objectives. In this study, we retrospectively reviewed the clinical presentations, laboratory examination, therapy and outcomes of 9 patients with CNS nocardiosis diagnosed using metagenomic next-generation sequencing (mNGS) in our hospital.

Materials and methods. We reviewed 9 patients with confirmed diagnosis of CNS *Nocardia* infection from January 2017 to December 2021 in the Department of Neurology at The Third Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). In addition, we searched literature related to CNS *Nocardia* infection on PubMed and included all case reports with proven CNS nocardiosis since 2016.

Results. The metagenomic next-generation sequencing (mNGS) of CSF can be used for the rapid diagnosis of nocardiosis in CNS and *N. farcinica* are the most commonly isolated species. Underlying autoimmune diseases, immunosuppressive agents including corticosteroids and organ transplantation are predisposing factors of developing CNS nocardiosis. Single or multiple hyper-enhanced ring lesions indicative of cerebral abscesses are commonly presented in brain imaging. Trimethoprim-sulfamethoxazole (TMP-SMX) is used as the primary agent for the antibacterial therapy and in combination with other antibacterial agents.

Conclusions. Our study demonstrated that mNGS of CSF can be conducted for definitive and rapid diagnosis for CNS nocardiosis.

Key words: infection, central nervous system, brain abscess, metagenomic next-generation sequencing, nocardia

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Background

Nocardiosis is a rare suppurative disease caused by gram-positive aerobic bacteria in the genus *Nocardia*. As an opportunistic infection, it is found most frequently in immunocompromised individuals, such as organ transplant recipients, patients with human immunodeficiency virus (HIV) infection, and those with hematologic malignancies.[1,2] *Nocardia* infection causes at least 6 forms of disease in humans including pulmonary, systemic, central nervous system (CNS), extra-pulmonary, cutaneous nocardiosis, and actinomycetoma.[3] The incidence of CNS nocardiosis is often secondary to lung or systemic nocardiosis,[3] and its clinical symptoms are variable, even without any external neurologic signs.[4–6] A multicentric cohort study in Japan reported 57 patients (56/317, 17.7%) had CNS nocardiosis, secondary to pulmonary nocardiosis (207/317, 65.3%).[7] *Nocardia farcinica* was found to be the most prevalent species in solid organ transplant recipients.[8] *Nocardia asteroides, N. nova* and *N. abscessus* also were the majority of invasive infection reported in the literature to date.[9,10]

Objectives

In this study, we retrospectively reviewed the clinical presentations, laboratory examination, implemented therapy, and outcomes of 9 patients with CNS nocardiosis diagnosed using metagenomic next-generation sequencing (mNGS) in our hospital.

Materials and methods

Study design

We reviewed 9 patients with confirmed diagnosis of CNS *Nocardia* infection from January 2017 to December 2021 in the Department of Neurology at The Third Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Demographic information, underlying comorbidities, clinical features, and radiological examinations were recorded. In addition, the presence of underlying disease conditions and the use of immunosuppressants comprising corticosteroids were identified. Diagnosis was made based on clinical characteristics, computer tomography (CT) or magnetic resonance imaging (MRI), as well as mNGS of the cerebrospinal fluid (CSF). Furthermore, we analyzed treatment approaches including medication and surgery.

mNGS

The general workflow of mNGS includes cerebrospinal fluid specimen collection, nucleic acid extraction, library generation, and sequencing, and aligning against published microbial genome databases. Cerebrospinal fluid DNA was extracted and purified following the standard procedures of QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). Qubit 4.0 (Thermo Fisher Scientific, Waltham, USA) was used for DNA concentration and quality control. Libraries were constructed using QIAseq Ultralow Input Library Kit (Qiagen). Once qualified, the libraries were sequenced on the Nextseq 550 platform (Illumina, San Diego, USA). The raw reads generated after mNGS sequencing were filtered by removing adapters, low quality and short reads (<35 bp) to obtain clean data. Bowtie2 was used for excluding human sequences by mapping the clean data to the human reference genome (hg38). For microorganism identification, the remaining reads were then aligned against published microbial genome databases downloaded from the National Center for Biotechnology Information (fttp://ftp.ncbi.nlm.nih.gov/genomes/).

The study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (approval No. [2021]02-121-01). Written informed consent was obtained from all participants.

Literature review

We searched the existing English-language literature related to CNS *Nocardia* infection on PubMed. Key words included "nocardia", "nocardiosis", "central nervous system", "meningitis", "encephalitis", and "brain abscess". We included all case reports with proven CNS nocardiosis between 2016 and 2021.

Results

Underlying conditions

The clinical and microbiological features of patients were summarized in Table 1. The mean age of these CNS nocardiosis cases was 45 years, ranging from 26 to 67 years. Female patients accounted for 44% (4 of 9). Most cases (7 of 9, 77.8%) presented autoimmune diseases, including SLE (n = 4), pemphigus (n = 1), nephrotic syndrome (membranous nephropathy, n = 1), and myasthenia gravis (n = 1). Other underlying conditions consisted of renal transplantation (n = 1) and malignancy (nasopharyngeal carcinoma treated with radiotherapy; n = 1). Of these, 8 individuals were receiving immunosuppressants such as corticosteroids (1 with prednisone and 6 with methylprednisolone), mycophenolate mofetil (n = 2), cyclophosphamide (n = 1), methotrexate (n = 1), azathioprine (n = 1), and tacrolimus (n = 1). None of the patients had HIV infection, tuberculosis, cerebral vascular diseases, chronic lung disease, or hematologic malignancies.

Clinical characteristics at admission

The clinical manifestations varied in this cohort of patients. Headache was the most common symptom present in all cases. Other symptoms included fever in 7 cases and weakness in 4 cases. Two patients manifested abnormal vision: 1 with blurred vision and the other with metamorphopsia. There were 2 cases of pulmonary symptoms, of which 2 cases had cough and 1 case had expectoration. Seizure, dizziness, muscle soreness, disorientation, confusion, and diplopia were also recorded (Table 1). Patients with brain abscess (case 5, 6 and 8) presented signs of neurological deficits. Case 5 developed hemiplegia on the left side of the body, while case 6 developed abduction dysfunction of the right eye movement. Cases 5, 6 and 8 showed decreased muscle strength and abnormal muscle tone. Case 7 with meningitis showed neck rigidity and positive meningeal signs including Kernig's sign and Brudzinski's sign, but no neurological deficits were detected. For the remaining patients, no positive or specific signs were found in the neurological examination.

Laboratory test and brain imaging

On the day following admission to our hospital, all patients underwent CSF examinations. Only 3 of these patients manifested an increased intracerebral pressure (ICP). White blood cell (WBC) count and total protein were elevated in 8 and 9 patients, respectively. The levels of chloride and glucose decreased in 6 patients. The mNGS of CSF was conducted in all cases. The confidence level and specific reads were recorded in Table 1. Figure 1 shows

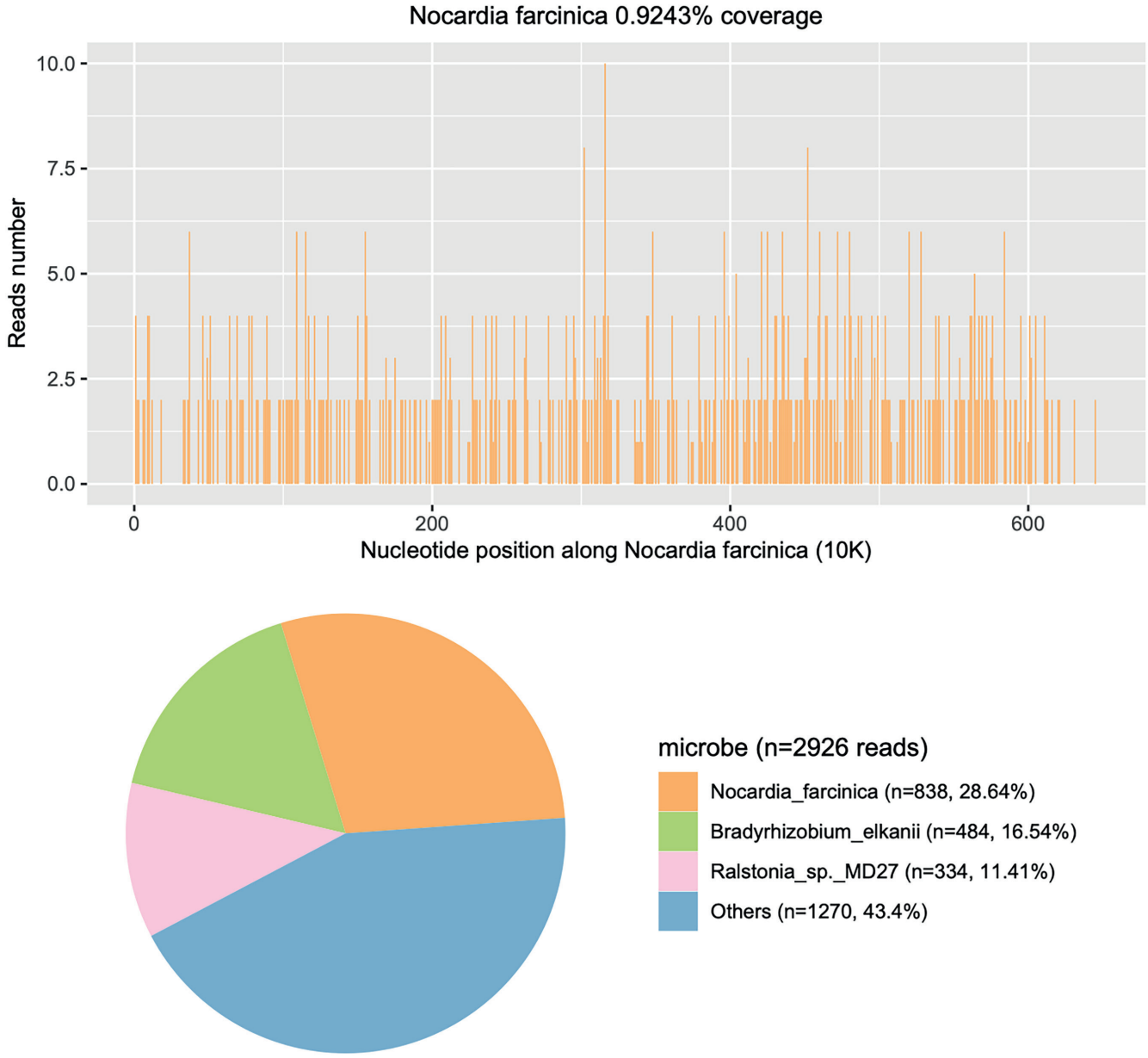

Fig. 1. Metagenomic next-generation sequencing (mNGS) results of case 6

Table 1. Nine cases of central nervous system (CNS) nocardiosis in The Third Affiliated Hospital, Sun Yat-sen University (Guangzhou, China)

Characteristic		Patient								
		1	2	3	4	5	6	7	8	9
Age/sex		26/M	47/F	44/F	28/M	56/M	26/F	67/M	55/F	53/M
Comorbidities		SLE	nasopharyngeal carcinoma	SLE	renal transplantation	pemphigus	SLE	myasthenia gravis	SLE	nephrotic syndrome (membranous nephropathy)
Immunosuppressants		prednisone, cyclophosphamide, methotrexate	none	methylprednisolone, mycophenolate mofetil	methylprednisolone, mycophenolate mofetil	methylprednisolone	methylprednisolone	azathioprine	methylprednisolone	methylprednisolone, tacrolimus
Species		*N. abscessus*	*N. farcinica*	*N. terpenica*	*N. farcinica*	*N. farcinica*	*N. farcinica*	*N. farcinica*	*N. farcinica*	*N. farcinica*
mNGS	yes/no	yes	yes	yes	yes	yes	yes	yes	yes	yes
	confidence degree	medium	none	high	high	high	high	high	high	high
	specific reads	12	none	34	29559	35	838	110	1043	71
Type of infection		meningoencephalitis, brain abscess	meningoencephalitis	meningitis	brain abscess	brain abscess	brain abscess, meningoencephalitis	meningitis	brain abscess	brain abscess
Clinical manifestations		headache, fever, blurred vision	headache, fever, weakness	fever, dizziness, headache, muscle soreness	headache, fever, seizure, metamorphopsia	fever, headache, weakness, disorientation, hemiplegia on the left side of the body, decreased muscle strength and abnormal muscle tone	cough, fever, headache, weakness, confusion, abduction dysfunction on right eye movement, decreased muscle strength and abnormal muscle tone	fever, headache, cough, expectoration, neck rigidity, Kernig's sign and Brudzinski's sign	headache, weakness, diplopia, seizure, decreased muscle strength and abnormal muscle tone	lumbosacral pain, fever, dizziness, slow response
First in-hospital CSF examination	ICP [mm H_2O]	240	150	315	170	150	160	none	200	330
	WBC counts [*10^6/L]	146	160	420	42	178	510	3680	2	2600
	chloride [mmol/L]	94.2	117.9	113.8	125.9	125	112	110.2	123.8	99.4
	glucose [mmol/L]	0.91	2.89	2.28	2.63	2.08	1.21	1.53	3.35	0.04
	total protein [mmol/L]	6.13	0.81	1.34	0.85	1.26	1.13	1.88	0.93	2.14

Table 1. Nine cases of central nervous system (CNS) nocardiosis in The Third Affiliated Hospital, Sun Yat-sen University – cont.

Characteristic		Patient								
		1	2	3	4	5	6	7	8	9
CNS imaging	CT	multiple ring-enhancing lesions in bilateral frontal and left parietal lobes	multiple ring-enhancing lesions in bilateral frontal and left temporal lobes	white matter lesion not excluded	multiple ring-enhancing lesions in left frontal and bilateral temporal occipital lobes	multiple ring-enhancing lesions in the right cerebral hemisphere, left frontal lobe, corona radiata, basal ganglia, and insula	right basal ganglia lesion	none	multiple ring-enhancing lesions in left cerebral peduncle, pons, right cerebellar hemisphere, right frontal, parietal, and occipital lobes	left basal ganglia lesion
	MRI	meningoencephalitis, multiple abscesses in bilateral frontal lobe and left posterior horn of lateral ventricle, surrounding brain edema, ventriculitis and left choroid plexus inflammation	meningoencephalitis in the left temporal lobe, right part of pons, anterior pontine cistern, cerebellopontine angle cistern and internal auditory canal	none	multiple lesions in bilateral cerebral hemisphere and cerebellum	multiple lesions with surrounding edema in the right cerebral hemisphere, left basal ganglia, insular lobe and paraventricular posterior horn	meningoencephalitis, multiple lesions in bilateral frontal, parietal lobes, corona radiata, right lateral ventricle, right basal ganglia and right para-hippocampal gyrus	meningitis, supratentorial ventricular system moderately dilated with paraventricular white matter edema (hydrocephalus)	multiple lesions with surrounding edema in right frontal lobe, parietal lobe, occipital lobe, left cerebral peduncle, pons, and right cerebellar hemisphere	multiple lesions in left radial crown-basal ganglia and posterior horn of lateral ventricle
Antibiotics		TMP-SMX, linezolid, meropenem	TMP-SMX, linezolid, moxifloxacin, ceftriaxone, vancomycin	TMP-SMX, linezolid, meropenem	TMP-SMX, linezolid, meropenem, moxifloxacin, minocycline	TMP-SMX, linezolid, meropenem, moxifloxacin	TMP-SMX, linezolid, meropenem, minocycline, amoxicillin clavulanate	TMP-SMX, linezolid, amoxicillin clavulanate, doxycycline	TMP-SMX, linezolid, meropenem, moxifloxacin, amikacin, teicoplanin	TMP-SMX, linezolid, meropenem, moxifloxacin
Surgery		EVD	none	none	none	EVD, decompressive craniectomy	none	none	EVD	none
Outcomes		death	recovery	recovery	recovery	recovery	recovery	recovery	recovery	recovery

SLE – systemic lupus erythematosus; mNGS – metagenomic next-generation sequencing; CSF – cerebrospinal fluid; CT – computed tomography; MRI – magnetic resonance imaging; ICP – intracranial pressure; WBC – white blood cells; TMP-SMX – trimethoprim-sulfamethoxazole; EVD – external ventricular drainage. In case #2, the confidence dgree and specific reads of mNGS was lost, while *N. farcinica* was detected in mNGS of CSF indeed and this patient was comfirmed as CNS nocardia infection.

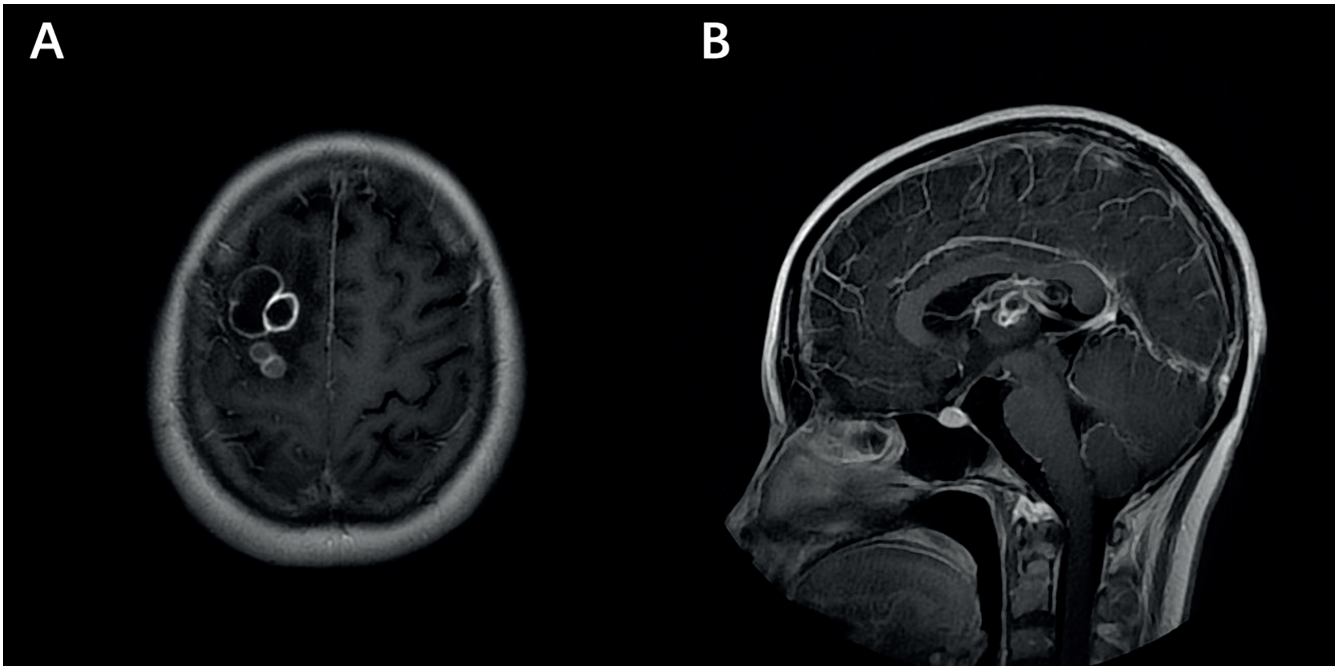

Fig. 2. Representative magnetic resonance images (MRI) images of the patients. A. The axial gadolinium-enhanced MRI image of case 8 shows multiple ring-enhancing lesions of varying sizes in the right frontal and parietal lobes; B. Sagittal T1 gadolinium-enhanced MRI image of case 6 shows ring-enhancing lesions in the right basal ganglia, with meningeal enhancement

representative mNGS results of case 6. The coverage and specific reads of *N. farcinica* detected with mNGS were 0.92% and 838, respectively. Most patients (7 of 9) were identified as *N. farcinica*-infected, and the other 2 patients had *N. abscessus* and *N. terpenica*, respectively.

The most common type of *Nocardia* infection of the CNS was brain abscess, which occurred in 6 cases according to the brain CT or MRI scan. Other types were meningoencephalitis (n = 3) and meningitis (n = 2). Brain CT scans were performed in 8 patients. Multiple ring-enhancing lesions occurred in 6 patients and single lesions in 3 patients. The frontal lobe was the most common brain region involved, occurring in 5 cases. Brain MRI was performed in 8 patients. One patient showed moderate expansion of supratentorial ventricular system with paraventricular white matter edema, suggesting hydrocephalus. Peripheral edema surrounding the lesion was seen on CT and MRI in 3 patients. Figure 2 showed representative MRI images of the patients presented.

Treatment and outcome

Both medical and surgical interventions were included in the treatment strategy. Three patients received a combination of medical and surgical treatment (patients 1, 5 and 8; Table 1) and underwent external ventricular drainage (EVD). One patient had a combined surgical procedure of EVD and decompressive craniectomy due to high ICP (patient 5). The remaining patients received antimicrobial therapy only. Both trimethoprim-sulfamethoxazole (TMP-SMX) and linezolid were prescribed in the antimicrobial therapy of all patients. In addition, 7 patients received combined therapy with meropenem, and 5 patients received moxifloxacin as part of the combined regimen. Two patients were treated with amoxicillin-clavulanate or minocycline, and 1 each with vancomycin, doxycycline, amikacin, and teicoplanin, respectively. Most patients (8/9) recovered after antimicrobial therapy with or without surgery. Only 1 patient died from a *Nocardia* complication.

Literature review

We included 57 reported cases of CNS nocardiosis published on PubMed from 2016 to 2021.[11–65] The clinical and microbiological characteristics of patients in literature review are summarized in Table 2 and the Supplementary data (https://doi.org/10.5281/zenodo.10153255). The mean age of patients was 60 years. Overall, 79.6% (43/54) of the patients were male and 20.3% (11/54) were female. A significant number of cases (14/57, 24.6%) had no comorbidities. The most common comorbid condition was diabetic mellitus, which was reported in 22.8% of the patients. Malignancy and autoimmune disease were the 2nd and 3rd most common comorbidities, reported in 21.1% and 19.3% of patients, respectively. Five patients (8.7%) received bone marrow or solid organ transplant. The most common organ transplanted was kidney (n = 3), followed by bone marrow (n = 1) and heart (n = 1). Five patients (8.7%) had a history of alcoholism. Other comorbidities included chronic lung disease (4/57, 7.0%), chronic kidney disease (3/57, 5.3%) and HIV infection (2/57, 3.5%).

Table 2. Clinical and microbiological characteristics of literature patients (n = 57)

Mean age	Gender	Comorbidities	Species	Sequencing	Type of infection	Medication	Surgery	Outcome
60	male (43/54, 79.6%) female (11/54, 20.3%)	diabetes mellitus (13/57, 22.8%) malignancy (12/57, 21.1%) autoimmune disease (11/57, 19.3%) transplant (5/57, 8.7%) alcohol abuse (5/57, 8.7%) chronic lung disease (4/57, 7.0%) chronic kidney disease (3/57, 5.3%) HIV infection (2/57, 3.5%) none (14/57, 24.6%)	*Nocardia farcinica* (20/52, 38.5%) *Nocardia cyriacigeorgica* (5/52, 9.6%) *Nocardia asiatica* (4/52, 7.7%) *Nocardia beijingensis* (4/52, 7.7%) *Nocardia paucivorans* (3/52, 5.8%) *Nocardia otitidiscaviarum* (3/52, 5.8%) *Nocardia nova* (2/52, 3.9%) *Nocardia brasiliensis* (2/52, 3.9%) *Nocardia araoensis* (2/52, 3.9%) *Nocardia abscessus* (2/52, 3.9%) *Nocardia asteroids* (1/52, 1.9%) *Nocardia kroppenstedtii* sp. *nov* (1/52, 1.9%) *Nocardia elegans/aobensis/africana complex* (1/52, 1.9%) *Nocardia mexicana* (1/52, 1.9%) *Nocardia thailandica* (1/52, 1.9%)	16S rRNA (27/57, 47.4%)	brain abscesses (46/57, 80.7%)	empiric treatment (33/56, 58.9%)	34/57, 59.6%	recovery (45/55, 81.8%)
				mNGS (3/57, 5.3%)	meningitis (5/57, 8.8%)	targeted treatment (23/56, 41.1%)	–	death (10/55, 18.2%)
				DNA sequencing (2/57, 3.5%)	disseminated infection (5/57, 8.8%)	–	–	–
				no (25/57, 43.8%)	spinal abscesses (3/57, 5.3%)	–	–	–
				–	endogenous endophthalmitis (2/57, 3.5%)	–	–	–
				–	endocarditis (1/57, 1.7%)	–	–	–
				–	ventriculitis (1/57, 1.7%)	–	–	–
				–	infectious intracranial aneurysm (1/57, 1.7%)	–	–	–
				–	–	–	–	–

HIV – human immunodeficiency virus; mNGS – metagenomic next-generation sequencing.

Of these 57 patients, 46 (80.7%) had brain abscesses on brain imaging, 5 had meningitis and 3 had spinal abscesses. Two patients with brain abscesses also had endogenous endophthalmitis. Patients with brain abscesses had endocarditis (n = 1), ventriculitis (n = 1) and infectious intracranial aneurysm (n = 1). Five patients had disseminated *Nocardia* infection in addition to CNS involvement. Other sites of infection included the lungs, skin and back. The most frequently reported species was *N. farcinica* (20/52, 38.5%), followed by *N. cyriacigeorgica* (5/52, 9.6%), *N. asiatica* (4/52, 7.7%), *N. beijingensis* (4/52, 7.7%), *N. otitidiscaviarum* (3/52, 5.8%), and *N. paucivorans* (3/52, 5.8%). Other less common species included *N. brasiliensis* (n = 2), *N. nova* (n = 2), *N. araoensis* (n = 2), *N. asteroids* (n = 1), *N. kroppenstedtii* sp. *nov.* (n = 1), *N. elegans/aobensis/africana complex* (n = 1), *N. Mexicana* (n = 1), and *N. thailandica* (n = 1). A large proportion of the patients were diagnosed using 16S rRNA sequencing (27/57, 47.4%) or mNGS (3/57, 5.3%). However, other patients were confirmed to have *Nocardia* infection using DNA sequencing, acid-fast staining and culture, etc.

In this group of patients, 41.1% (23/56) were reported to have received targeted treatment according to antimicrobial susceptibility testing and 58.9% (33/56) received antibiotics empirically. Most patients (48/56, 85.7%) underwent combined anti-microbial therapy consisting of at least 2 antibiotics. Overall, 41.1% (23/56) of the patients were administered TMP-SMX alone or with other antibiotics. A variety of other antimicrobials were used in the treatment, including ceftriaxone (17/56, 30.4%), imipenem (13/56, 23.2%), meropenem (13/56, 23.2%), linezolid (11/56, 19.6%), or amikacin (9/56, 16.1%). A large proportion of patients (34/57, 59.6%) underwent surgical intervention with combination antimicrobial therapy.

Results were reported for 55 patients. Overall, 45 patients (81.8%) recovered completely or with complications, and 10 patients (18.2%) died.

Discussion

To date, only limited case reports described using mNGS to diagnose CNS nocardiosis. In this study, we retrospectively reported 9 cases of CNS *Nocardia* infection confirmed with mNGS, and analyzed the clinical and microbiological features, treatment strategies, and outcomes. To the best of our knowledge, this is the largest case series of CNS nocardiosis diagnosed using mNGS.

It is of great importance to isolate and culture *Nocardia* spp. for microbiological diagnosis and use of sensitive antimicrobial agents. Nevertheless, *Nocardia* spp. grow relatively slowly, which makes them difficult to culture in the laboratory. In recent years, sequencing technologies including 16S rRNA sequencing and mNGS were developed for definitive and rapid diagnosis of clinical infectious diseases.[28] The mNGS allows direct detection of the microbial community in the natural state, without isolation and culture, and therefore could be applied to diagnosing bacterial infection caused by those more difficult to culture, such as *Nocardia* spp.[45,66] In the literature review, nearly half of patients underwent 16S rRNA sequencing, but only 3 patients used mNGS for diagnosis.

Predisposing factors for the development of CNS nocardiosis include age, gender and immunosuppressive status.[67,68] The mean age of CNS nocardiosis patients in our hospital and in the literature was 45 and 60 years, respectively. The higher incidence of CNS nocardiosis in the elderly may be result of immunosenescence, which refers to the decline of the immune system associated with aging and may lead to excessive accumulation of pro-inflammatory cytokines and inflammaging.[69,70] However, the mean age of our case series was much younger than the literature review and previous studies, which may be due to the presence of comorbidities and immunosuppressive conditions in our cohort.

It is well known that systemic *Nocardia* infection occurs mainly in immunocompromised patients. Accordingly, most of the patients (8/9, 88.9%) in our study had received immunosuppressive agents, with 7 patients receiving corticosteroid treatment (prednisone and methylprednisolone). In a previous study, high-dose and long-term corticosteroid treatment was considered a risk factor for the development of nocardiosis.[71,72]

It is noteworthy that 7 patients in our group had autoimmune diseases, with systemic lupus erythematosus (SLE) being the most common (n = 4). Cell-mediated immunodeficiency was implied as one of the major risk factors for the development of *Nocardia* infection.[73] In our case series, patients with autoimmune diseases were using steroids and other immunosuppressive agents such as mycophenolate mofetil, azathioprine and methotrexate, which inhibit cell-mediated immunity.

The incidence of *Nocardia* infection in organ transplant patients has been reported to range from <1% to 3.5%.[74–76] In our case series and literature review, we found that kidney transplant recipients ranked the 1st place, accounting for 66.6% (4/6) of the transplant patients. However, in a matched case-control study comprising of 5,126 organ transplant recipients, the highest rate (3.5%) of *Nocardia* infection was found in lung transplant recipients, followed heart (2.5%), intestine (1.3%), kidney (0.2%), and liver (0.1%) transplants.[74] Risk factors for post-transplant patients developing this disease include the use of high-dose corticosteroids, anti-lymphocyte globulin, higher levels of calcineurin inhibitors, and previous cytomegalovirus disease.[8,77]

As seen in our case series, most patients presented with fever and headache, typical of those with CNS infection. Neurological complaints including weakness, seizures, dizziness, disorientation, and confusion were also present in this case series. However, in a Queensland (Australia) case series of 20 cases, few patients presented with classic infectious symptoms.[78] Clinical presentation varies from patient to patient and there are no specific symptoms to guide clinicians in making the diagnosis. Patients with CNS nocardiosis may have single or multiple hyperenhancing ring lesions suggestive of cerebral abscesses on CT or MRI. The frontal lobe was the most common site of infection according to our study and a recent report,[68] due to inhalation or direct spread from paranasal sinuses.[79]

Brain abscess and/or meningitis caused by other opportunistic infectious pathogens such as *Klebsiella pneumoniae*[80] and *Escherichia coli*[81] should be considered in differential diagnosis of CNS nocardiosis. Central nervous system nocardiosis masquerading brain metastasis is not uncommon and has been reported.[47,82] For example, in a case report by Voide et al.,[83] a patient initially diagnosed with non-small cell lung cancer presented delirium during chemotherapy treatment, and subsequently underwent CT and MRI. The brain lesions were initially interpreted as CNS metastasis, but later stereotactic biopsy and Ziehl–Neelsen staining of lesion showed *N. farcinica* infection. In addition, CNS nocardiosis should be differentiated from ischemic stroke. Lavalard et al.[84] reported an immunocompromised female initially treated as cerebral stroke but later confirmed to have CNS *Nocardia* infection. Despite treatment with cotrimoxazole and rifampicin, she did not improve and died 3 months after treatment concluded.

Nocardia farcinica was the most common species found in both our patients and patients in the literature. This is consistent with previous reports that *N. farcinica* was the most commonly isolated species in patients.[68,85] Compared with other species, *N. farcinica* is more virulent and more likely to cause CNS nocardiosis or disseminated disease.[78,86] However, *N. abscessus* and *N. asteroids* are quite rare in both our case series and literature patients, whereas these 2 species were common in previously published studies.[78,85] To date, the current study reports the 2nd case of CNS nocardiosis infected with *N. terpenica*. The first reported patient with CNS *N. terpenica* infection was from Nanchang, China.[87]

Due to its low rate of resistance and good penetration into the CNS,[88] TMP-SMX is considered as the fundamental treatment in CNS *Nocardia* infection. All patients in our cohort and the majority of patients in the literature were taking TMP-SMX. In the literature, patients receiving antibiotic regimens that included eTMP-SMX had higher survival rates and lower relapse rates.[78,89] Although there

are no definitive clinical guidelines, it is recommended that patients with CNS nocardia infection should receive TMP-SMX at a dose of 25–50 mg/kg per day for at least 12 months to prevent relapse.[71]

In addition to TMP-SMX, other effective antimicrobials against *Nocardia* spp. include linezolid, meropenem, moxifloxacin, imipenem, and ceftriaxone in our series or in patients from the literature. Although targeted antimicrobial treatment based on antimicrobial susceptibility testing is widely recognized as the optimal therapy, patients in our hospital or in a large proportion of case reports receive only empirical treatment. However, mNGS cannot detect the resistance of *Nocardia* spp. Because CNS nocardiosis is rare in the world, there is a lack of definitive and effective antibiotic regimens, and larger, multicenter randomized controlled trial is needed to determine the optimal treatment for this disease.

As is shown in our case series, $^1/_3$ of the patients in our study and 59.6% (34/57) of the literature patients underwent neurosurgery including EVD and decompressive craniectomy. In the previous studies, patients treated with a combination therapy of medicine and surgery had higher survival rates than those who received antibiotics only.[68,78,86] However, the prognosis of patients with CNS nocardiosis relies on multiple factors such as age, immune status of patients, time from disease onset to diagnosis, different *Nocardia* spp., antimicrobial regimens, and the duration of treatment, among others. Therefore, the favorable prognosis of surgery may be due to the bias of selecting patients with better status for surgery.

Limitations

This study has several limitations. Our case series is a single center study and only 9 patients were included. In a recent study involving 24 patients with *Nocardial* brain abscess, lung and skin were the most common primary infectious sources (37.5% and 12.5%, respectively).[68] Unfortunately, the primary infectious sites of this case series were not recorded in our electronic medical records. Therefore, we were unable to identify the primary source of the CNS *Nocardia* infection. In addition, all patients in our cohort were treated empirically, as no antimicrobial susceptibility testing was performed.

Conclusions

Our study demonstrated that CSF mNGS can be conducted for definitive and rapid diagnosis of CNS nocardiosis. Elderly and immunocompromised individuals, especially those receiving immunosuppressive drugs, have a higher incidence of CNS *nocardia* infection. Antimicrobial therapy including TMP-SMX in combination with neurosurgery may reduce mortality and recurrence rates.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10153255. The package includes the following files:

Supplementary Table 1. Clinical and microbiological characteristics of literature patients.

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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Immature platelet fraction and immature platelet count as novel biomarkers of elevated platelet reactivity in NSTE-ACS patients receiving dual antiplatelet therapy

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Abstract

Background. Antiplatelet therapy is the cornerstone of treatment for patients presenting with acute coronary syndrome (ACS) treated with percutaneous coronary intervention (PCI). Some patients may not respond to such therapy adequately, which is associated with a greater risk of ischemic events. Reticulated platelets are the youngest, largest, and most active platelet subtype. They have been initially shown to be associated with an increased risk of cardiovascular (CV) events and increased platelet activity.

Objectives. The aim of the presented study was to evaluate whether the immature platelet fraction (IPF) reflects the response to antiplatelet treatment in invasively managed ACS patients.

Materials and methods. This prospective study enrolled ACS patients treated with PCI and dual antiplatelet therapy (DAPT) comprising acetylsalicylic acid (ASA) and clopidogrel or ticagrelor. In all patients, venous blood was collected within 24 h after the procedure. Platelet parameters were measured, including IPF using the Sysmex hematological analyzer and adenosine diphosphate (ADP)-induced platelet reactivity using the Multiplate® Analyzer.

Results. A total of 108 patients were enrolled, including 62 with ST-segment elevation ACS (STE-ACS) and 46 with non-ST-segment elevation ACS (NSTE-ACS). Of them, 20.4% had diabetes mellitus, 26.9% had a history of MI and 59.2% of smoking. Spearman's correlation analysis demonstrated that higher IPF and immature platelet count (IPC) values are associated with increased ADP-induced platelet reactivity (respectively: rho = 0.387, 95% confidence interval (95% CI): 0.101–0.615, p = 0.008; and rho = 0.458, 95% CI: 0.185–0.666, p = 0.001) in NSTE-ACS but not in STE-ACS patients.

Conclusions. Immature platelet count and IPF may be valuable markers of platelet activity in patients with NSTE-ACS treated invasively and receiving DAPT (ClinicalTrials.gov No. NCT06177587).

Key words: platelet reactivity, acute coronary syndrome, dual anti-platelet therapy, immature platelet fraction

Background

Platelets have a significant role in the pathophysiology of cardiovascular (CV) events, including acute coronary syndrome (ACS), especially concerning patients treated invasively.[1,2] Therefore, therapy aimed at inhibiting platelet activity is an essential part of treatment to prevent, i.a., stent thrombosis (ST) or recurrent myocardial infarction (MI). As standard, such patients receive 2 antiplatelet drugs that act by different mechanisms: acetylsalicylic acid (ASA) and a $P2Y_{12}$ inhibitor, for 1 year, according to guidelines.[3,4] However, the response to treatment varies significantly depending on individual patients' characteristics, which may require adjusting the intensity or duration of treatment.[5,6] To date, there are no simple and accessible methods to effectively predict, and therefore prevent, high on-treatment platelet reactivity (HTPR).

Immature, newly released to the circulation reticulated platelets (RPs) are more reactive than mature ones.[7] Studies have shown that their levels represented as a percentage of RPs among all platelets, named immature platelet fraction (IPF), may have a predictive value for the occurrence of CV events in patients treated with dual antiplatelet therapy (DAPT).[8–11] However, their exact significance in assessing response to treatment is not fully understood.

Objectives

The aim of the presented study was to evaluate whether IPF could be a valuable parameter for determining on-treatment platelet reactivity and predicting response to antiplatelet therapy in ACS patients treated with percutaneous coronary intervention (PCI).

Materials and methods

This was a prospective, single-center study conducted in a tertiary cardiology clinical center. Written informed consent was obtained from each participant. This study was conducted according to the principles outlined in the Declaration of Helsinki and by the Bioethics Committee of Medical University of Warsaw under reference No. KB/242/2015. The clinical trial was registered with ClinicalTrials.gov under the identifier NCT06177587.

Patients

Consecutive patients presenting with ACS between July 2017 and May 2018 were enrolled. The inclusion criteria were: age >18 years, admission due to ACS, the need for immediate (<2 h) or early (<24 h) invasive treatment with stent implantation, treatment with DAPT, and ability to sign informed consent. The patients were excluded if they received any other medication that affects platelet activity or blood coagulation, had any contraindications to take ASA or $P2Y_{12}$ inhibitor, or had coagulation disorders. All patients received a loading dose of ASA (300 mg) and $P2Y_{12}$ inhibitor (300 mg of clopidogrel or 180 mg of ticagrelor) periprocedurally, and were treated thereafter with 75 mg of ASA daily and either clopidogrel (75 mg once a day) or ticagrelor (90 mg twice a day).

Laboratory tests

Blood sampling for all analyzed parameters was obtained from the peripheral vein in the first 24 h after PCI. Blood collection had taken place while the patients were still in the catheterization laboratory, before they were transported to the ward, so in 88% of cases, it was performed within the first 2 h after the PCI. Platelet count (PLT), hemoglobin, platelet distribution width, mean platelet volume (MPV), and IPF were assessed in whole blood anticoagulated with ethylenediaminetetraacetic (K3EDTA) using an automated hematological analyzer (Sysmex XN 2000; Sysmex, Kope, Japan). In the case of 2 IPF measurements, the average value was used for analyses. Immature platelet count (IPC) was calculated as a product of IPF and PLT. For platelet reactivity measurements, blood samples were drawn from the peripheral vein and collected in hirudin-containing tubes. Impedance aggregometry using Multiplate® Analyzer (Roche Diagnostics, Basel, Switzerland) with adenosine diphosphate (ADP) as agonist was performed 30–120 min after sampling. The test was carried out as instructed by the manufacturer. Maximum platelet aggregation and aggregation velocity are expressed in arbitrary units AUC (area under the curve of aggregation units (AU) over time (min)). Clinical data was collected from an electronic patients' database.

Statistical analyses

The statistical analysis was performed using IBM SPSS Statistics v. 28.0 (IBM Corp., Armonk, USA). The distribution of continuous data was assessed with Shapiro – Wilk test. Data were presented as mean and standard deviation (SD) and compared with Student's t-test, or as median with interquartile range (IQR) and compared with Mann–Whitney U test for parametric and nonparametric variables, respectively. Categorical data were presented as number and percentage. The Spearman's rank correlation coefficient was used to assess the relationship between platelet aggregation and RPs parameters. Two-sided p-values <0.05 were considered statistically significant.

Results

A total of 108 ACS patients were enrolled; 62 of them presented with ST-segment elevation ACS (STE-ACS) and 46 with non-ST-segment elevation ACS (NSTE-ACS). Baseline characteristics (Table 1) did not differ significantly

Table 1. Baseline characteristics (values in bold are statistically significant)

Variable		All (108)	NSTE-ACS (46)	STE-ACS (62)	p-value
Female gender, n (%)		28 (25.9)	12 (26.1)	16 (25.8)	0.974
Age [years], mean (SD)		66.7 (10.7)	69.0 (9.2)	65.8 (11.9)	0.084
HT, n (%)		70 (64.8)	35 (76.1)	35 (56.5)	**0.022**
DM, n (%)		22 (20.4)	11 (23.9)	11 (17.7)	0.364
HL, n (%)		73 (67.6)	31 (67.4)	42 (67.7)	0.903
HF, n (%)		36 (33.3)	15 (32.6)	21 (33.9)	0.954
CKD, n (%)		14 (13.0)	10 (21.7)	4 (6.5)	**0.017**
Current smoker, n (%)		39 (36.1)	16 (34.8)	23 (37.1)	0.908
Past smoker, n (%)		25 (23.1)	8 (17.4)	17 (27.4)	0.166
Previous MI, n (%)		29 (26.9)	15 (32.6)	14 (22.6)	0.245
Previous PCI, n (%)		19 (17.6)	11 (23.9)	8 (12.9)	0.110
MVD, n (%)		53 (49.1)	23 (50.0)	30 (48.4)	0.781
Clopidogrel, n (%)		82 (75.9)	36 (78.3)	46 (74.2)	0.625
Ticagrelor, n (%)		26 (24.1)	10 (21.7)	16 (25.8)	
Creatinine [mg/dL], median (IQR)		1.02 (0.34)	1.03 (0.38)	1.04 (0.34)	0.320
eGFR [mL/min/1.73m^2], median (IQR)		74.0 (28.0)	68.0 (34.5)	74.5 (25.3)	0.100
RBC [10^6/μL], median (IQR)		4.53 (0.69)	4.46 (0.64)	4.63 (0.70)	0.131
HGB [g/dL], median (IQR)		14.1 (2.2)	13.8 (2.1)	14.1 (1.9)	0.32
PLT [10^3/μL], median (IQR)		217 (63)	210 (77)	219 (60)	0.546
Cholesterol [mg/dL], mean (SD)		167 (43)	159 (33)	173 (48)	**0.010**
HDL [mg/dL], median (IQR)		41.5 (21.0)	42.5 (21.0)	41.0 (22.5)	0.540
LDL [mg/dL], mean (SD)		94.3 (37.6)	83.3 (30.4)	103.6 (39.0)	**0.008**
TG [mg/dL], median (IQR)		114 (58)	115 (54)	114 (53)	0.208
EF (%), median (IQR)		49.0 (12.8)	53.5 (9.5)	45.0 (13.5)	**<0.001**
Troponin [ng/mL], median (IQR)		10.3 (29.0)	6.0 (17.0)	17.1 (55.1)	**0.012**
Number of vessels	1	39 (36.1)	14 (30.4)	25 (40.3)	0.228
	2	25 (23.1)	10 (21.7)	15 (24.2)	
	3	24 (22.2)	13 (28.3)	11 (17.7)	
	4	14 (13.0)	5 (10.9)	9 (14.5)	
	5	6 (5.6)	4 (8.7)	2 (3.2)	
Final TIMI flow, mean (SD)		2.9 (0.5)	3.0 (0.0)	2.8 (0.6)	0.111
ASA prior to hospitalization, n (%)		23 (21.3)	11 (23.9)	12 (19.4)	0.567
Satin, n (%)		106 (98.1)	45 (97.8)	61 (98.4)	0.831
β-blocker, n (%)		97 (89.8)	45 (97.8)	52 (83.9)	**0.018**
ACEI/ARB, n (%)		103 (95.4)	44 (95.7)	59 (95.2)	0.904
CCB, n (%)		12 (11.1)	9 (19.6)	3 (4.8)	**0.016**
PPI, n (%)		97 (89.8)	40 (87.0)	57 (91.9)	0.398

ASA – acetylsalicylic acid; ACEI – angiotensin-converting enzyme inhibitor; ARB – angiotensin receptor blockers; CCB – calcium channel blocker; CKD – chronic kidney disease; DM – diabetes mellitus; EF – ejection fraction; eGFR – estimated glomerular filtration rate; HDL – high-density lipoprotein; HF – heart failure; HGB – hemoglobin; HL – hyperlipidemia; HT – hypertension; PPI – proton pump inhibitor; IQR – interquartile range; LDL – low-density lipoprotein; MI – myocardial infarction; MVD – multi-vessel disease; n – number; NSTE-ACS – non-ST-elevation acute coronary syndrome; PCI – percutaneous coronary intervention; PLT – platelets; RBC – red blood cells; SD – standard deviation; STE-ACS – ST-elevation acute coronary syndrome; TIMI – thrombolysis in myocardial infarction; TG – triglycerides.

between the groups, except for a higher prevalence of hypertension in STE-ACS patients and a greater incidence of chronic kidney disease in the NSTE-ACS group. Additionally, the NSTE-ACS group exhibited lower troponin and cholesterol levels, including LDL, as well as a higher ejection fraction compared to the STE-ACS group. Ticagrelor was received by 26 (24.1%) and clopidogrel by 82 (75.9%) patients.

The analysis revealed that the level of IPF correlates with ADP-induced platelet reactivity in NSTE-ACS patients (rho = 0.387, 95% confidence interval (95% CI): 0.101–0.615,

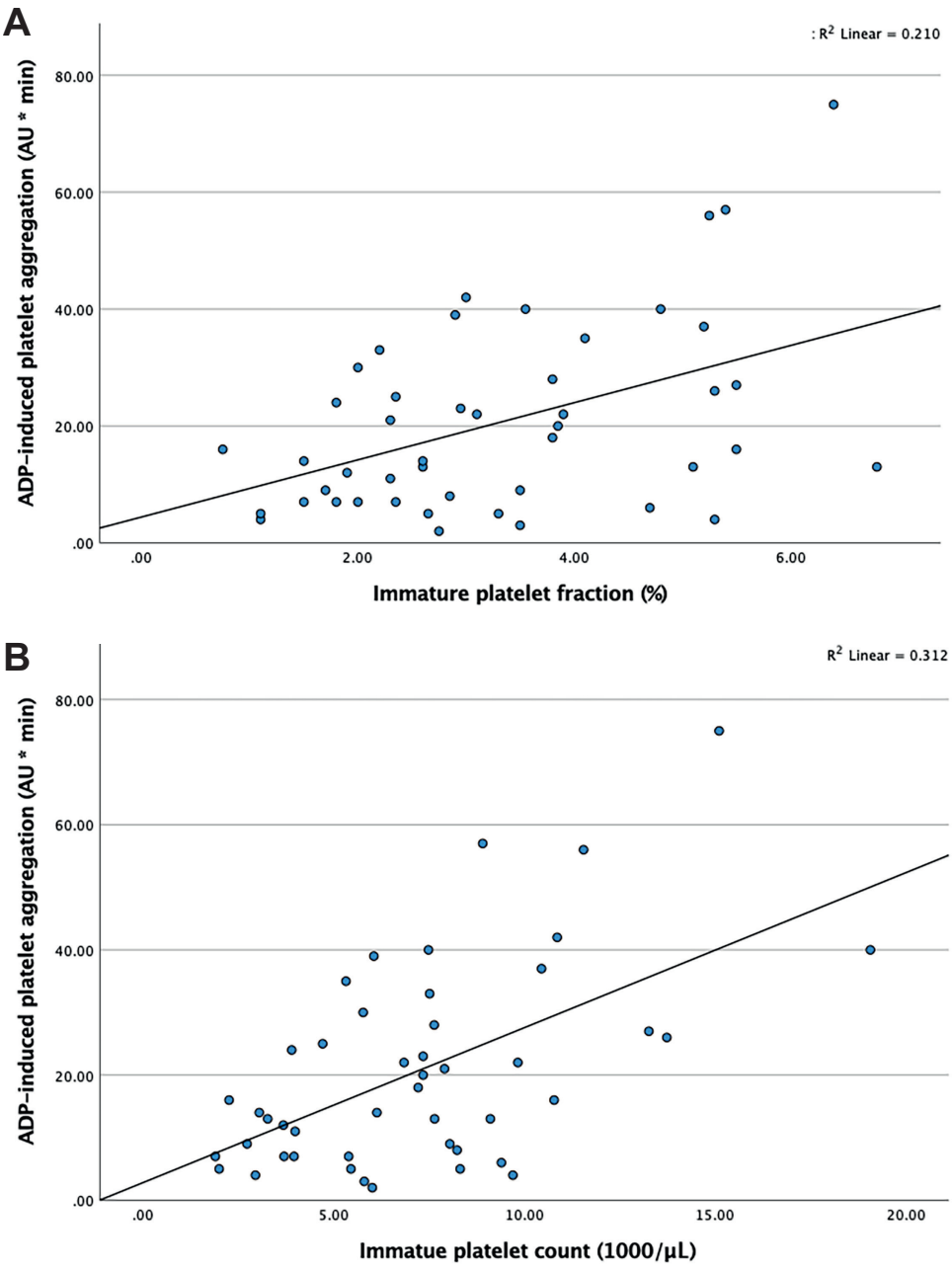

Fig. 1. Correlation between adenosine diphosphate (ADP)-induced platelet reactivity and (A) immature platelet fraction and (B) immature platelet count

p = 0.008); Fig. 1). However, this relationship was not observed in the STE-ACS group.

In the NSTE-ACS group, 36 patients were treated with clopidogrel and 10 with ticagrelor. We analyzed the relationship between ADP-induced platelet reactivity and IPF in both groups separately. For clopidogrel, the positive correlation was also present (rho = 0.346, 95% CI: 0.010–0.612, p = 0.039), whereas in the ticagrelor group, the observed correlation did not reach a statistically significant level (rho = 0.610, 95% CI: −0.054–0.900, p = 0.061).

Analysis concerning IPC revealed an even stronger correlation with ADP-induced platelet reactivity in NSTE-ACS patients (rho = 0.458, 95% CI: 0.185–0.666, p = 0.001). Moreover, this relationship was maintained in both clopidogrel and ticagrelor treated cohorts analyzed separately (rho = 0.378, 95% CI: 0.047–0.635, p = 0.023; and rho = 0.854, 95% CI: 0.467–0.966, p = 0.002, respectively). Again, the relationship was absent in STE-ACS patients.

Partial Spearman's correlation for potentially confounding variables including age, gender, diabetes mellitus, smoking status, and antiplatelet agent was also performed in the NSTE-ACS cohort. It revealed that the relationship between ADP and IPF as well as between ADP and IPC remained while controlling for all the variables mentioned above. The detailed results of the analysis are presented in Supplementary Table 1. Moreover, we showed that clinical presentation did not significantly impact the level of platelet reactivity, also after adjustment for potentially confounding variables (Supplementary Table 2), and that there were no differences in platelet parameters according to diabetes status, insulin treatment and the $P2Y_{12}$ inhibitor received (Supplementary Table 3).

Discussion

We demonstrated that the levels of both studied RP parameters, i.e., IPF and IPC, correlate with ADP-induced platelet aggregometry among patients with NSTE-ACS treated with PCI and DAPT. This relationship was not observed in STE-ACS patients.

Optimal platelet inhibition stands as a crucial factor influencing the prognosis of post-PCI patients.[12] Inadequate response to antiplatelet treatment remains an open problem related to serious consequences such as ST, MI, or CV death.[13] Despite numerous attempts and tests evaluated so far, routine identification of HTPR on a large scale was not found cost-effective and is currently not recommended in the society guidelines.[3,14]

Several studies have indicated the relationship between the level of RPs and antiplatelet therapy response, particularly notable in patients receiving thienopyridine therapy. However, it was not apparent in the ticagrelor-treated group.[15–18] Most of the patients in our study were treated with clopidogrel. Therefore, the issue of the relationship between IPF and platelet activity in ticagrelor-treated patients remains to be further elucidated. Despite the limited sample size, it is noteworthy that among ticagrelor-treated patients, there was a rising trend in IPF as ADP-induced platelet aggregation levels rose. Moreover, a statistically significant correlation was identified with regard to IPC. Based on the existing literature, the influence of clopidogrel treatment compared to ticagrelor appears to elicit varying effects on IPC levels in a long-term observation.[19] However, our findings, as presented, reveal that baseline platelet parameters and their correlation with platelet reactivity persist irrespective of the administered medication at a saturating dose.

Immature platelets, known for their heightened prothrombotic potential, can be reflected by IPF level – a reliable marker of platelet turnover. Elevated IPF is characteristic for specific patient groups including smokers, diabetics or the ones with ongoing inflammation,[20–23] as well as ACS patients.[24] Baseline IPF serves as a predictor of major adverse CV events (MACE) in patients with coronary artery disease (CAD) treated invasively and with DAPT.[8,9,11] Similar findings extend to IPC, which was also more strongly associated with antiplatelet response.[10,25] Patients with higher baseline levels of both parameters face a higher risk of ischemic events, indicating increased platelet turnover and reactivity despite adequate therapy. Regarding patients treated percutaneously with stent implantation, there is an additional risk of ST.

Interestingly, the correlation in our study did not exist for STE-ACS patients. Prior studies suggested that patients with STE-ACS have a higher IPF level than NSTE-ACS patients.[22] This was not observed in our population, where the distribution of IPF and IPC was similar in both groups. It can be due to the fact that blood parameters were obtained after an initial treatment including PCI and the loading doses of antiplatelet drugs. Perl et al. described the correlation between RPs level and platelet reactivity in STE-ACS patients, yet the measurements in that study were performed 2–4 days after the start of the treatment and later after 30 days.[26] The short interval between the onset of STE-ACS and the measurements in our study could be a factor contributing to this observation. Subsequent studies should focus on selecting the most optimal measurement time when IPF or IPC values reliably reflect platelet activity.

Immature platelet fraction can be easily, inexpensively measured using automatic hematology analyzers during a complete blood count test, providing the results quickly.[27,28] The same applies to IPC, which can be calculated from IPF and PLT. As such, RPs parameters may become useful markers for guiding antiplatelet therapy once the above findings are confirmed in further studies with larger cohorts.

Limitations

Our study predominantly included clopidogrel-treated NSTE-ACS patients, warranting further research specific to ticagrelor. The pharmacokinetics and pharmacodynamics differ among $P2Y_{12}$ inhibitors. It cannot be excluded that the relationship between platelet reactivity and the level of RPs depends on the drug received. Moreover, our focus on parameters shortly after the procedure prevents us from confirming whether this relationship persists in longer-term follow-up.

Conclusions

Immature platelet count and IPF may hold promise as potential markers of platelet reactivity in patients with NSTE-ACS undergoing invasive treatment and receiving DAPT. Given their accessibility, these markers could prove valuable for assessing an individual's responsiveness to antiplatelet therapy or aid in identifying individuals who are at higher risk of thrombotic events. Further research is needed to establish their effectiveness in this regard.

Supplementary data

Supplementary materials are available at https://zenodo.org/doi/10.5281/zenodo.10219636. The package contains the following files:

Supplementary Table 1. Partial Spearman's correlation analysis between ADP and IPF/IPC for potentially confounding variables.

Supplementary Table 2. Multivariate analysis showing the relation between ADP-induced platelet aggregation and the clinical presentation of ACS after adjustment for potential confounding variables.

Supplementary Table 3. The differences between platelet parameters (IPF, IPC and ADP-induced PA) in groups divided by diabetic status, insulin intake or P2Y12 inhibitor used.

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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