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Contents

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Monthly Volume 12 Number 12 December 15, 2021

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ABOUT COVER

Editorial Board Member of *World Journal of Diabetes*, Caterina Conte, MD, PhD, Associate Professor, Department of Human Sciences and Promotion of the Quality of Life, San Raffaele Roma Open University, Rome 0166, Italy. caterina.conte@uniroma5.it

AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64□Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

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EDITORIAL

Meeting report of the chief editorial board meeting for *World Journal of Diabetes* 2021

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Author contributions: Wang JL analyzed the data and drafted the manuscript; Yan JP, Li X, Ma N and Ma L contributed to organizing the meeting; Islam MS is the original contributor of Figure 9; Islam MS, Xiao JB, Cai L, and Ma LS proofread the manuscript.

Conflict-of-interest statement: None declared.

Country/Territory of origin: United States

Specialty type: Endocrinology and Metabolism

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0

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Abstract

The 2021 online editorial board meeting of the *World Journal of Diabetes* (*WJD*) was held on November 9, 2021. Jin-Lei Wang, General Manager on behalf of the Baishideng Publishing Group, and Professor Islam, one of the Editors-in-Chiefs (EiCs) of the *WJD*, organized the meeting. Three EiCs and 18 Baishideng Publishing Group staff attended the meeting. The meeting goal was to brief the EiCs on the journal's performance, discuss the issues of concern of the EiCs, and gather ideas for the journal's development in 2022. As of November 8, the *WJD* had received 287 manuscripts since the year's start, among which 122 met the criteria for publication. These numbers represent an increase of 117.4% for submissions and 110.3% for publications compared to those in 2020. However, how to effectively control the academic quality of manuscripts and attract high-quality original article submissions remain a challenge. The EiCs provided feedback and suggestions centered on three topics: (1) Who should and how to control the academic quality of the manuscripts; (2) How the EiCs perform their responsibilities; and (3) The distinctive and shared responsibilities of the publisher and the EiCs.

Key Words: *World Journal of Diabetes*; Baishideng; Editorial board meeting; Journal development; Bibliometrics; Scientific literature

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Grade E (Poor): 0

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P-Reviewer: El-Yazbi AF, Rydosz A
S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

Core Tip: The 2021 *World Journal of Diabetes* editorial board meeting was held on November 9, 2021. The meeting goal was to brief Editors-in-Chiefs (EiCs) on the journal's performance, discuss issues of concerns of the EiCs, and gather ideas for the journal's development in 2022. The discussion focused on: (1) Who should and how to control the academic quality of the manuscripts; (2) How the EiCs perform their responsibilities; and (3) The distinctive and shared responsibilities of the publisher and the EiCs.

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INTRODUCTION

Since new Editors-in-Chiefs (EiCs) were appointed to the *World Journal of Diabetes* (*WJD*) in August 2021, Professor Islam, one of the EiCs, suggested to organize a meeting from the editorial office in order to understand the editorial process of the journal so that the EiCs may serve their role in an optimized manner. According to the EiCs' suggestions, the *WJD* editorial office hosted the meeting on November 9, 2021 to review the journal's performance, discuss issues of concerns of the EiCs, and gather ideas for the journal's development in 2022. The meeting was moderated by Miss Na Ma, Company Vice-Editor-in-Chief. The first part of the meeting consisted of presentations on journal status review, and the second part consisted of open discussions with the EiCs to garner their feedback and suggestions.

ATTENDEES

This online meeting brought together three EiCs, namely Professor Jian-Bo Xiao, Professor Lu Cai, Professor Md Shahidul Islam, and 18 Editors (Na Ma, Jin-Lei Wang, Xiang Li, Ze-Mao Gong, Jia-Ping Yan, Ya-Juan Ma, Yun-XiaoJian Wu, Jia-Ru Fan, Chen-Chen Gao, Man Liu, Ji-Hong Liu, Yu-Jie Ma, Yan-Xia Xing, Yan-Liang Zhang, Kai-Le Chang, Jing-Jie Wang, Li-Li Wang, and Han Zhang) from Baishideng Publishing Group Inc (Figure 1).

REPORTS

Jin-Lei Wang began the meeting by offering an overview of the *WJD*'s journal statistics, status quo of the editorial board, publication process for manuscripts, *etc*.

Introduction of WJD

Overview of the journal: The *WJD* is a high-quality, monthly, online, open-access, single-blind peer-reviewed journal. The *WJD* publishes articles based upon their academic quality, academic norms, academic ethics, and academic integrity, with an aim of disseminating academic research results to the world and leading the healthy development of the academic community. The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Current Contents®/Clinical Medicine, Scopus, PubMed, and PubMed Central.

Journal metrics: The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 83 among 146 journals in endocrinology and metabolism; and Quartile category: Q3.

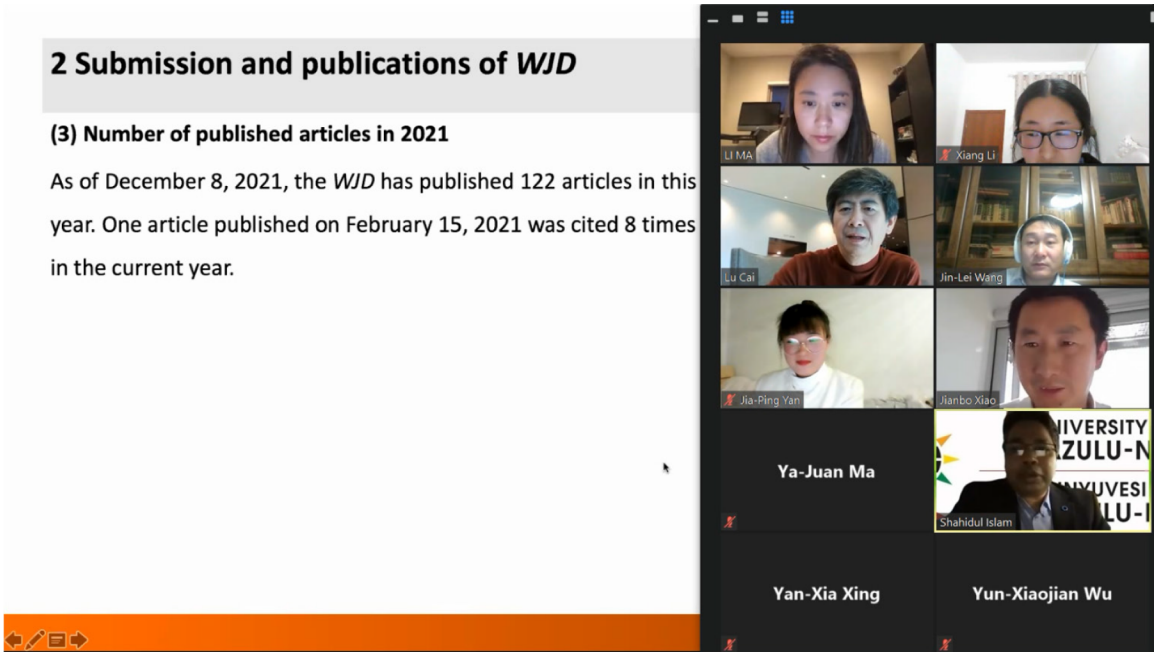

Figure 1 2021 *World Journal of Diabetes* chief editorial board meeting presenters.

The *WJD* was included in SCIE in 2019. The *WJD*'s first IF was 3.247 and the IF without journal self cites was 3.222 (in 2019). The IF in 2020 increased by 15.9% compared with the IF in 2019 (Figure 2).

Submission and publications of WJD

Number of submissions in recent years: From 2013 to 2021, the *WJD* received a total of 1476 manuscript submissions for consideration of publication, with an average number of submissions per year of 164 (Figure 3). As of November 8, 2021, the *WJD* had received 287 manuscript submissions since the beginning of the calendar year, whose authors were from 46 countries/territories.

Number of published manuscripts during 2010-2021: Based on our recently developed "Reference Citation Analysis" system (https://www.referencecitation-analysis.com/), the *WJD* published 752 articles since its launch in 2010 to 2021. Of note, the five most cited articles include: one article published in 2015[1] has been cited 438 times, its article influence (Calculation for Article Influence: Times cited/Number of years = Article influence™) is 73.0 (Figure 4); another article published in 2015[2] has been cited 353 times, its article influence is 58.8; one article published in 2014[3] has been cited 312 times, its article influence is 44.6; one article published in 2015[4] has been cited 312 times, its article influence is 52.0; and one article published in 2010[5] has been cited 269 times, its article influence is 24.5. The total 752 articles published in *WJD* during 2010-2021 includes 463 reviews, 207 original articles, 63 editorials, 7 case reports, and 12 articles of other types (Figure 5). As of November 8, 2021, 500 of the articles are indexed in the Web of Science, the authors of which are located in 51 countries/territories, including United States (102 articles, 20.4%), China (57 articles, 11.4%), India (42 articles, 8.4%), Japan (31 articles, 6.2%), Australia (26 articles, 5.2%), and United Kingdom (26 articles, 5.2%), *etc*. (data from Web of Science; Figure 6).

Number of published articles in 2021: As of November 8, 2021, the *WJD* had published 122 articles in the calendar year. One article[6] published on February 15, 2021 was cited 8 times in the current year, and other five articles[7-11] were cited 3 times in the current year.

Number of submission and publications during 2013-2021: Compared with the numbers of submissions and publications in 2020, *WJD* submissions and publications increased by 117.4% and 110.3%, respectively, in 2021 (Figure 7).

Publication process for manuscripts from now

The publication process for any manuscript submitted to the *WJD* is as follows: Manuscript reception and registration; Single-blind external peer review; First

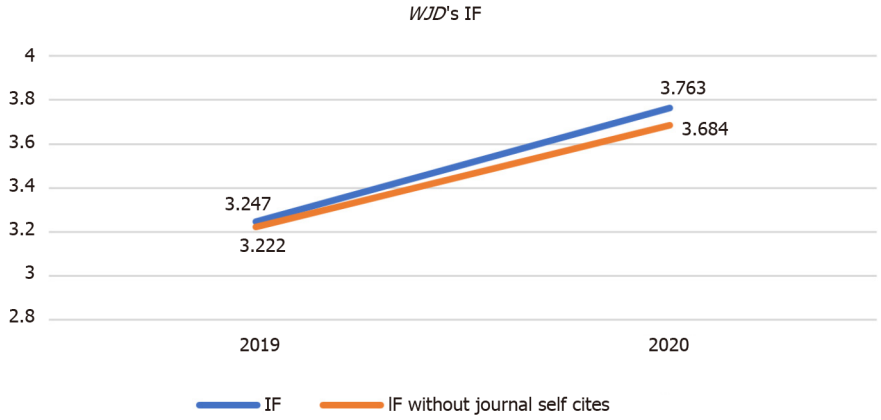

Figure 2 The impact factor in 2020 increased by 15.9% compared with the impact factor in 2019. IF: Impact factor; *WJD*: *World Journal of Diabetes*.

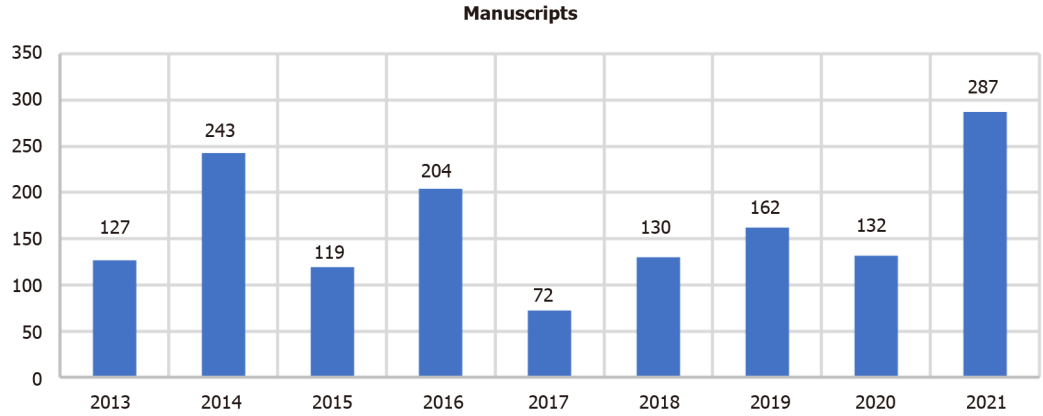

Figure 3 From 2013 to 2021, the *World Journal of Diabetes* received a total of 1476 manuscripts, with an average number of 164 submissions per year.

decision; Revision by the author(s); Second decision; Final review by the EiC; Final acceptance; Production; and Published online (Figure 8).

Single-blind external peer review: The peer review model is single-blinded; however, the reviewer can choose to hide or not hide his/her name to the author. Both invited and unsolicited manuscripts will be externally peer reviewed. Our artificial intelligence system automatically selects 20 external peer reviewers for each manuscript from our database of high-impact experts and scholars. At the same time, our science editor manually selects 10 Editorial Board Members in a single-blind manner to review the manuscript. Usually, the peer review process takes 7-14 d to complete. Before the formal peer review is initiated, peer reviewers should consider and answer the following two questions: (1) Does the manuscript have a conflict with your interests? And (2) Does the research content of the manuscript meet the requirements of ethical norms?

First decision by Academic Editor/Editorial Board/Science Editor: After the manuscript's peer review is completed by the peer reviewer(s) and/or editorial board member(s), we invite the Academic Editor/Editorial Board/Science Editor to make the first decision for the manuscript and write an evaluation report of this first decision. The main task of the first decision is to verify and evaluate the academic quality of manuscripts based on the peer-review report and key points for first decision of manuscripts. Academic editors will independently decide to accept or reject the manuscript or suggest to the authors a transfer of the manuscript to another of our journals. Currently, the *WJD* has 8 online Academic Editors.

Second decision: After the authors have revised the manuscript in accordance with the peer-review report and the criteria for manuscript revision, and responded to the issues raised in the peer-review report, the revised manuscript will be edited by the

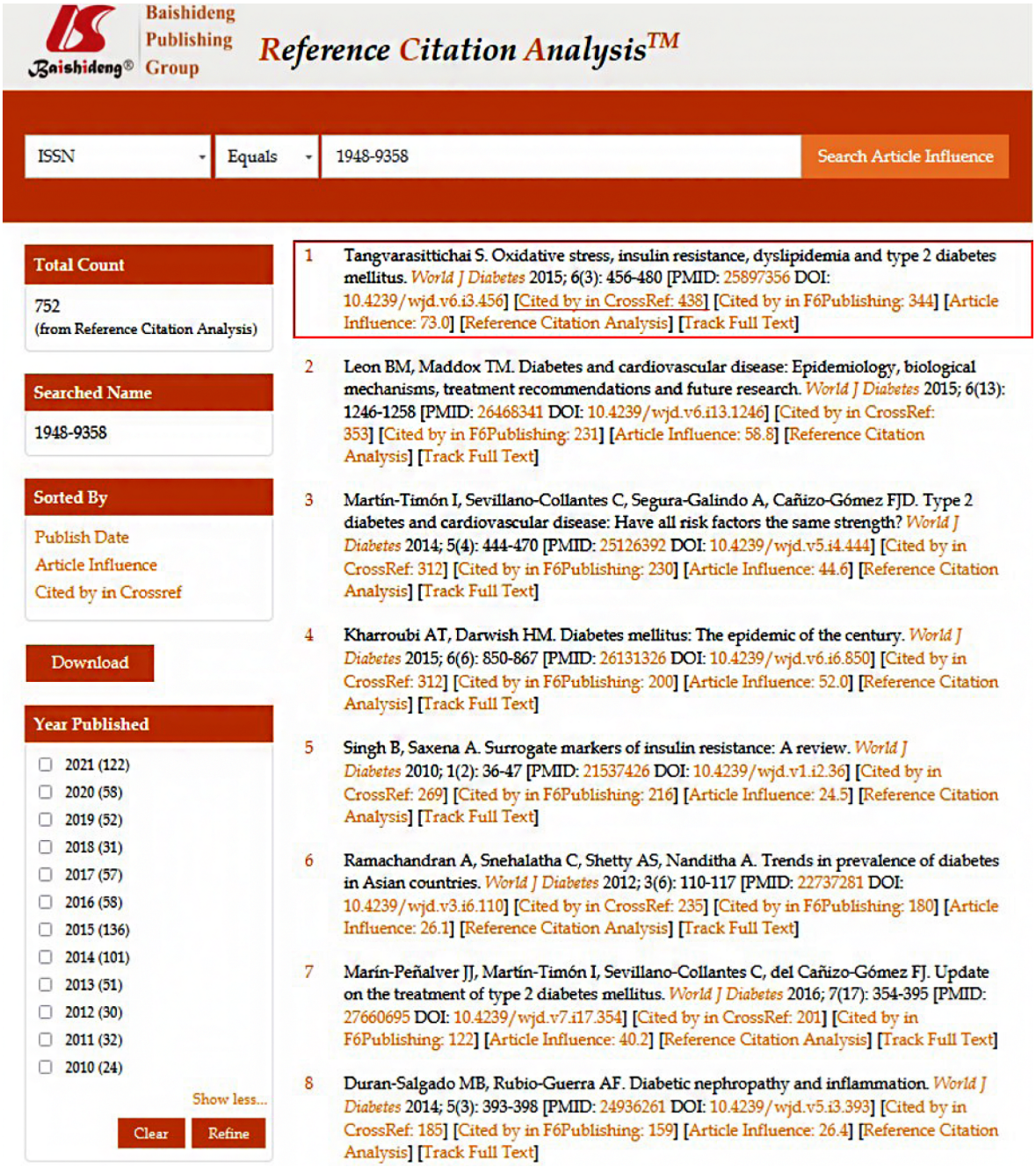

Figure 4 One *World Journal of Diabetes* article published in 2015 has been cited 438 times.

science editor according to the journal's guidelines. Then, the EiCs will make the second decision to reject or accept the manuscript based on all relevant documents submitted by science editor. And the company EiC (internal) will re-verify whether the manuscript conforms to academic ethics, rules, and norms and makes the final decision to accept or reject the manuscript.

WJD's editorial system - F6Publishing system

F6Publishing is an artificial intelligence-based system that integrates the functions of online manuscript submission, peer review, manuscript revision by authors, manuscript production, manuscript publishing and release, editing and publishing management, and statistics of publishing fees and costs (https://www.f6publishing.com).

Maximize article impact for authors: To enable more peers to read, share, and cite authors' published research results, to help enhance authors' global academic influence, and to promote the overall development of the field, Baishideng accurately

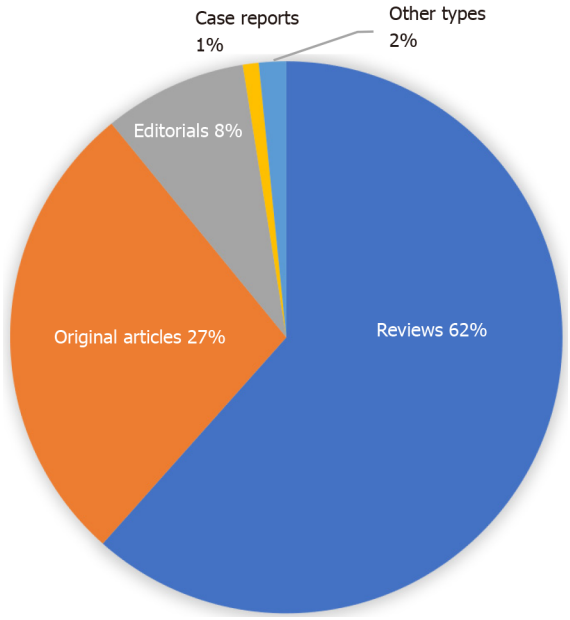

Figure 5 A total of 752 articles were published in *World Journal of Diabetes* during 2010-2021.

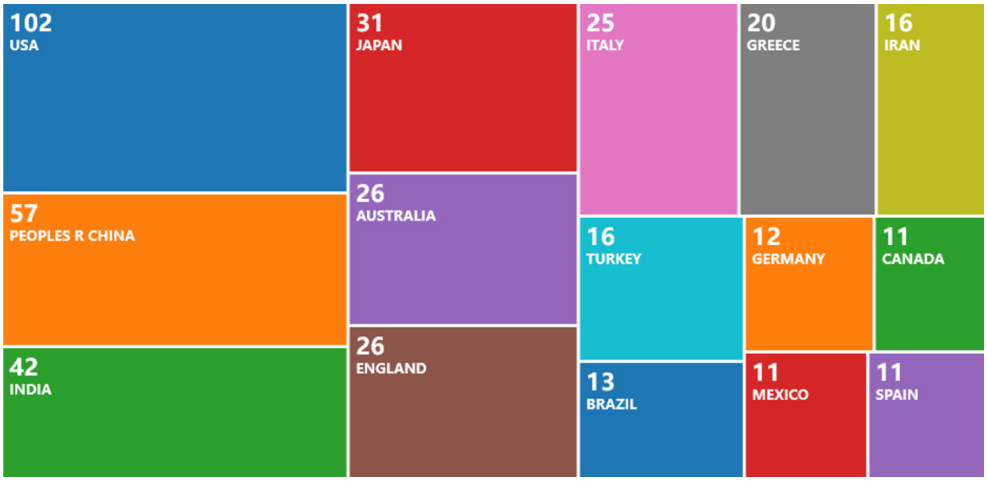

Figure 6 A total of 500 articles published in *World Journal of Diabetes* are indexed in the Web of Science, by authors located in 51 countries/territories. This figure was downloaded from Web of Science.

sends published articles to 1000-10000 highly influential experts. After completing this, Baishideng will formally notify authors of the number of experts to whom their manuscript was sent *via* email.

Editorial Board introduction

The 2021 Editorial Board of *WJD* is composed of 101 Editorial Board Members. They are from 35 countries or regions, including 20 (19.8%) in China, 10 (9.9%) in United States, 8 (7.9%) in Italy, 6 (5.9%) in India, 5 (5.0%) in Greece, and 52 (51.5%) in other countries or regions.

Responsibilities of EiCs: In addition to the responsibilities of the Editorial Board members, the EiCs of the *WJD* will be involved in the second decision making (final reviewing) of manuscripts.

Responsibilities of Editorial Board Members: (1) Conducting peer review of at least six manuscripts each year. Reviewing the academic and language quality of manuscripts to ensure the academic level of journals. Conducting the manuscript peer-review task timely and carefully. Making a fair and objective evaluation of manuscripts, and giving detailed reasons for acceptance/rejection of a manuscript and

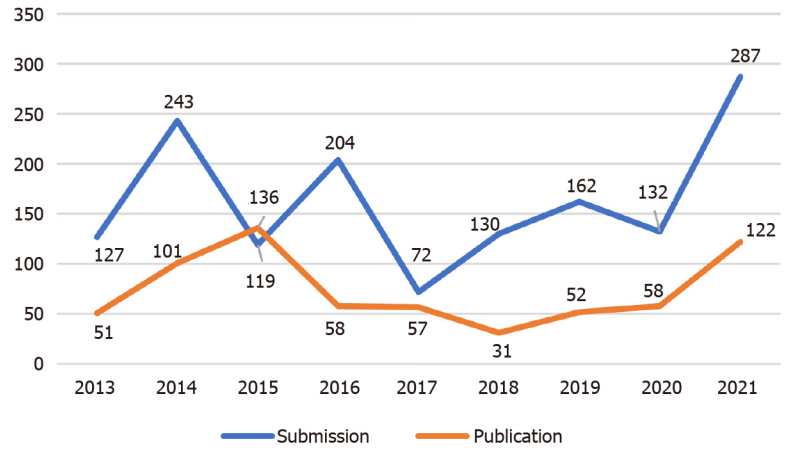

Figure 7 Number of submissions to and publications in *World Journal of Diabetes* during 2013-2021. As some of the manuscripts submitted in 2014 were peer-reviewed and published online in 2015, the publication number in 2015 is higher than the submission number.

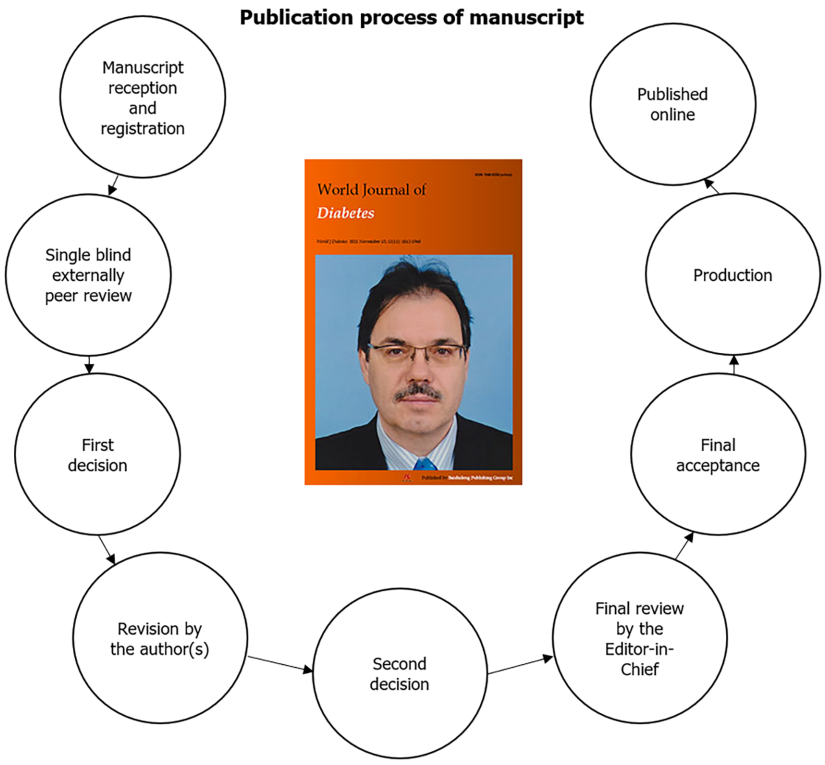

Figure 8 Publication process for manuscripts in *World Journal of Diabetes*.

detailed comments for its revision; (2) Willingness to track the post-publication evaluations on the manuscripts you have reviewed; (3) Willingness to organize a special topic, carried out by inviting 7-10 manuscripts from experts and scholars in the field to contribute manuscripts on a hot topic; (4) Recommending high-quality manuscripts for submission to the journal; (5) Promoting published articles, including thorough use of personal social media to forward the articles published in the journal and share them with more colleagues; and (6) Recommending distinguished experts and scholars in the field to join the Editorial Board as new members.

Privileges of Editorial Board Members: (1) Regular invitations to contribute editorials and review articles for the journal, for which the publication fee will be waived; (2) A 10% discount of the publishing fee for other articles submitted by the member; (3) A listing of personal professional information on the journal's homepage; (4) Public acknowledgement of related contributions to the journal, by posting of the member's name and number of manuscripts he/she reviewed on the journal's website. For

details, please visit: https://www.f6publishing.com/HighlyInfluentialPeerReviewers; and (5) Selection of outstanding members of the Editorial Board to appear as the cover figures published on the cover page of each issue.

Invitation for manuscripts for WJD in 2021

As of November 8, 2021, the *WJD* has received a total of 109 titles of invited manuscripts, including 43 (39.4%) for review articles, 56 (51.4%) for original articles, and 10 (9.2%) for other types of articles.

In December 2021, we plan to continue to invite global experts and scholars in the field of diabetes to write high-quality reviews, original articles, and special issue articles for the *WJD* in 2022.

OPEN DISCUSSION FROM EICS FOCUSED ON THREE MAIN TOPICS

Academic quality control of the manuscripts

Professor Islam cited concern about the publication process for manuscripts. He provided alternative opinions about the processes for manuscript peer-review, manuscript first decision, manuscript acceptance, *etc*. He suggested that the Associate Editors and the EiCs should be responsible for the academic quality of the manuscript, and the following editorial work flow was suggested: (1) The manuscripts should initially checked by the journal office and sent back to authors or sent to an Associate Editor in that area of research; (2) The Associate Editor will carry out scientific scrutinization and may reject or send the manuscript to multiple peer-reviewers for review; (3) That same Associate Editor will judge the peer-review report and send the manuscript back to the authors for revision or reject the manuscript; (4) If the manuscript is acceptable after revision, then a preliminary decision will be made by that same Associate Editor, who will send it to one of the EiCs to make a final decision; and (5) If the manuscript is accepted, the manuscript will go the production editor for production purposes and the journal office will assume handling from that point onwards.

Professor Cai also suggested that an Associate Editor should carry out the scientific scrutinization and thereafter decide to send the manuscript to peer-review or reject the manuscript immediately.

EiCs' responsibilities

Professor Islam pointed out that the EiCs' responsibilities should be more focused. The EiCs do not necessarily need to participate in the production of manuscripts and other editing processes of the manuscript. Professor Xiao pointed out that the EiCs can recommend Editorial Board Members and organize special issues on hot topics.

Responsibilities of the publisher

Professor Islam pointed out that the publisher should be more focused on the production of the manuscript, and not interfere with the academic quality control of the manuscript. The EiCs and/or the Associate Editor should control the academic quality of the manuscripts.

ACTIONS AND PLANS FROM THE EDITORIAL OFFICE

First, the editorial office clarified that (1) the academic quality of the manuscripts will be controlled by the EiCs through their conducting of the first decision for peer-reviewed manuscripts and final review of the revised manuscript, (2) to avoid conflicts of interest, the technical quality of the publications will remain controlled by the publisher, and (3) the copyrights of the published articles will continue to belong to the authors.

Second, the editorial office updated the responsibilities of EiCs and Associate Editors, as follows in detail: (1) Conduct of the first-decision of peer-reviewed manuscripts. The main task of this work is to verify and evaluate the academic quality of manuscripts submitted for consideration of publication, based on the peer-review report and key points for first decision. The purpose of this work is to ensure the academic quality of articles published in our journals, thereby leading academic innovation in general alongside the healthy development of Baishideng journals.

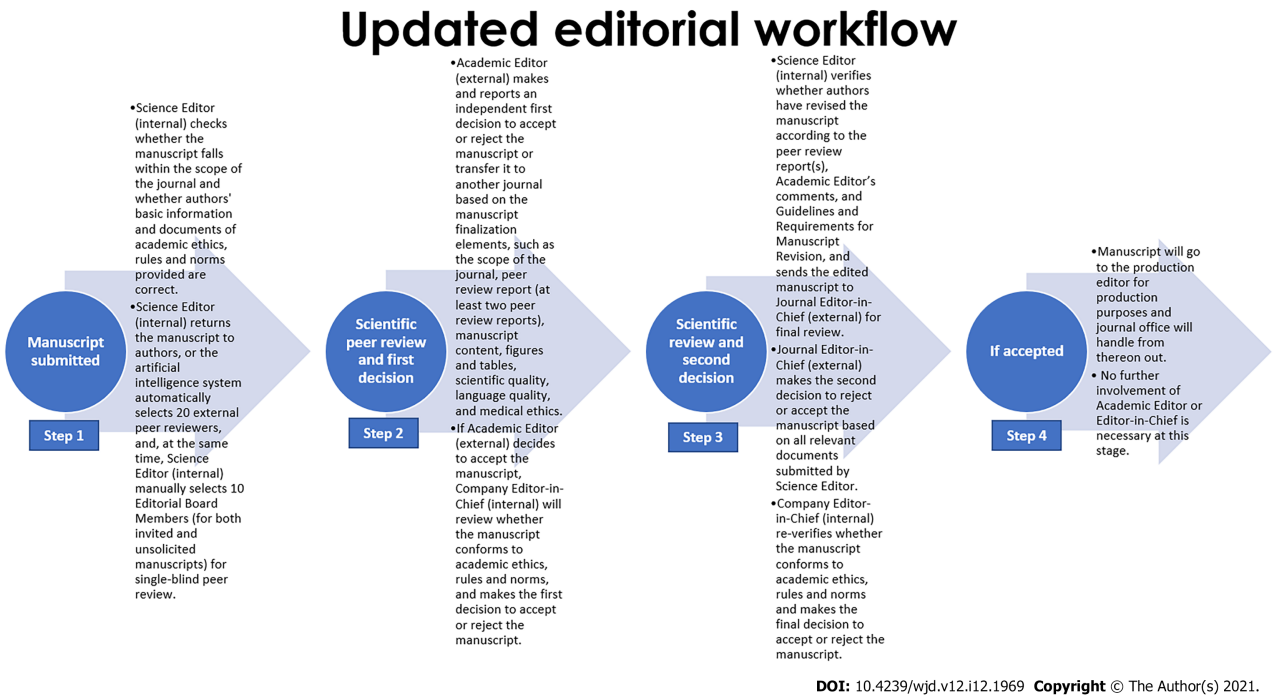

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Figure 9 Updated editorial workflow from 2022.

Relying on the key points for first decision of manuscripts (including manuscript theme, peer-review report, manuscript content, figures and tables, language quality, manuscript format, medical ethics, and statistical analysis), EiCs will independently decide to accept or reject the manuscript or suggest to the authors a transfer of the manuscript to another, more appropriate Baishideng journal. They will also provide a summary of this decision, in order to help the authors and the journal to further improve the academic quality of the manuscript under consideration; (2) Inviting manuscripts for the journal. This task involves organizing a special topic by inviting 7-10 manuscripts on a hot topic from experts and scholars in the field. These invited manuscripts will be published free-of-charge after successful completion of peer review; (3) Conduct of peer review of manuscripts. This task involves conducting peer review of at least six manuscripts each year. The focus of this review activity is the academic and language quality of the manuscripts, to ensure the academic level of the journals. The manuscript peer-review task should be conducted in both a timely and careful manner. The ultimate aim is to make a fair and objective evaluation of manuscripts, giving detailed reasons for acceptance/rejection of a manuscript and detailed comments for revision; (4) Final review of the authors' revised manuscript; and (5) Other responsibilities. This task relies on a willingness to track the post-publication evaluations of the manuscripts you have reviewed. The EiCs will also recommend high-quality manuscripts for submission to the journal, promote its published articles, including through use of personal social media to forward the articles published in the journal and share them with more colleagues, and recommend distinguished experts and scholars in the field to join the Editorial Board as new members.

Third, after discussion with our colleagues, the editorial office carefully updated the editorial workflow according to Professor Islam's suggestions, and more detailed clarifications were added to the editorial workflow (Figure 9).

CONCLUSION

All EiCs expressed commitment and enthusiasm to help the journal grow. Both the EiCs and the editorial office are on the same team and moving toward the same goal on an agreed upon path; therefore, we will work together to make the *WJD* better and promote it to be a top journal in the field. Future online editorial board meetings are expected to be set every 3 months. All editorial members are welcome to attend any and all future meetings. Stay tuned for more details please!

ACKNOWLEDGEMENTS

The *WJD* editorial office thanks all the EiCs for their enthusiasm, guidance and contributions to the journal's growth.

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REVIEW

Thioredoxin interacting protein, a key molecular switch between oxidative stress and sterile inflammation in cellular response

Islam N Mohamed, Luling Li, Saifudeen Ismael, Tauheed Ishrat, Azza B El-Remessy

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Abstract

Tissue and systemic inflammation have been the main culprit behind the cellular response to multiple insults and maintaining homeostasis. Obesity is an independent disease state that has been reported as a common risk factor for multiple metabolic and microvascular diseases including nonalcoholic fatty liver disease (NAFLD), retinopathy, critical limb ischemia, and impaired angiogenesis. Sterile inflammation driven by high-fat diet, increased formation of reactive oxygen species, alteration of intracellular calcium level and associated release of inflammatory mediators, are the main common underlying forces in the pathophysiology of NAFLD, ischemic retinopathy, stroke, and aging brain. This work aims to examine the contribution of the pro-oxidative and pro-inflammatory thioredoxin interacting protein (TXNIP) to the expression and activation of NLRP3-inflammasome resulting in initiation or exacerbation of sterile inflammation in these disease states. Finally, the potential for TXNIP as a therapeutic target and whether TXNIP expression can be modulated using natural antioxidants or repurposing other drugs will be discussed.

Key Words: Thioredoxin interacting protein; NOD-like receptor pyrin domain containing 3; Inflammasome; Interleukin 1b; Inflammation; Obesity; High-fat diet; Ischemia; Reperfusion; Oxidative stress

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Core Tip: Inflammation has been postulated as the central pathway involved in maintaining homeostasis and in cellular response to insults. High fat diet-induced inflammasome activation have been reported to predispose microvascular diseases including retinopathy, and nonalcoholic fatty liver disease. Inflammation can alter vascular recovery in response to ischemic insult including ischemic retinopathy, stroke and critical limb ischemia. Thioredoxin interacting protein (TXNIP) is required for the activation but not necessarily for expression of NOD-like receptor pyrin domain containing 3-inflammasome resulting in initiation or exacerbation of the disease state. A list of natural antioxidants or repurposed drugs is included to modulate TXNIP expression.

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INTRODUCTION

Sterile inflammation as a physiological and pathological response

Inflammation is the body's natural defense mechanism to recognize and react to harmful insults or stimuli in effort to eliminate or mitigate these damaging threats and maintain normal tissue and organ homeostasis[1,2]. Therefore, as illustrated in Figure 1, depending on the nature of these threatening insults or stimuli, inflammation can be generally classified into two major categories: (1) Microbial inflammation, resulting from the major group of microbial triggers, known as the pathogen associated molecular patterns (PAMPs). Examples of PAMPs include danger signals from invading microorganisms like; the whole microorganism (bacteria, virus or fungi), their byproducts (bacterial enzymes and/or toxins), or their subcellular components [bacterial lipopolysaccharide (LPS)]; and (2) Sterile inflammation, which is associated with non-microbial related insults, known as the damage associated molecular patterns (DAMPs). DAMPs include any chemical, biochemical triggers or metabolic by products or danger signals released as a result of tissue damage or cellular injury excluding microorganisms. Examples of DAMPs associated diseases include amyloid beta plaques in Alzheimer's disease (AD), cholesterol crystals in atherosclerosis, glucose in diabetes mellitus, glutamate in neurotoxicity and neurodegenerative diseases, monosodium urate crystals in gout, and saturated fatty acids (ex: Palmitate) in obesity (Reviewed in[2-4]). Therefore, sterile inflammation can be defined as: Inflammation that occurs in absence of or irrespective to invading microorganisms or their byproducts. Both PAMPs and DAMPs triggers are recognized by a large family of pattern recognition receptors (PRRs) which provoke the expression of pro-inflammatory cytokines to further instigate the activation and recruitment of the pro-inflammatory and immune response *via* immune and non-immune cells. As illustrated in Figure 2, PRRs are generally classified into five major classes according to their subcellular location, activating PAMPs or DAMPs and their corresponding pro-inflammatory signaling pathways. NOD-like receptor (NLR), present in the cytoplasm, is one of the five major receptor classes of PRR that has been directly linked to major metabolic, micro and macrovascular diseases[2]. Upon recognition by a stimulus, NLR pyrin domain containing 3 (NLRP3) inflammasome signaling is initiated. NLRP3-inflammasome consists of the sensor NLRP3, the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and the effector caspase-1. NLRP3 activation process occurs *via* two steps: Priming and activation. The priming step requires NFkB-mediated transcriptional expression of the component of NLRP3-inflammasome and pro-cytokine namely pro-interleukin (IL)-1b. In contrast, pro-IL-18 is constitutively expressed. The assembly and activation of inflammasome result in pro-caspase-1 activation, which could subsequently cleave pro-cytokines namely pro-IL-1b and pro-IL-18 into their active forms IL-1b and IL-18, respectively[2,5].

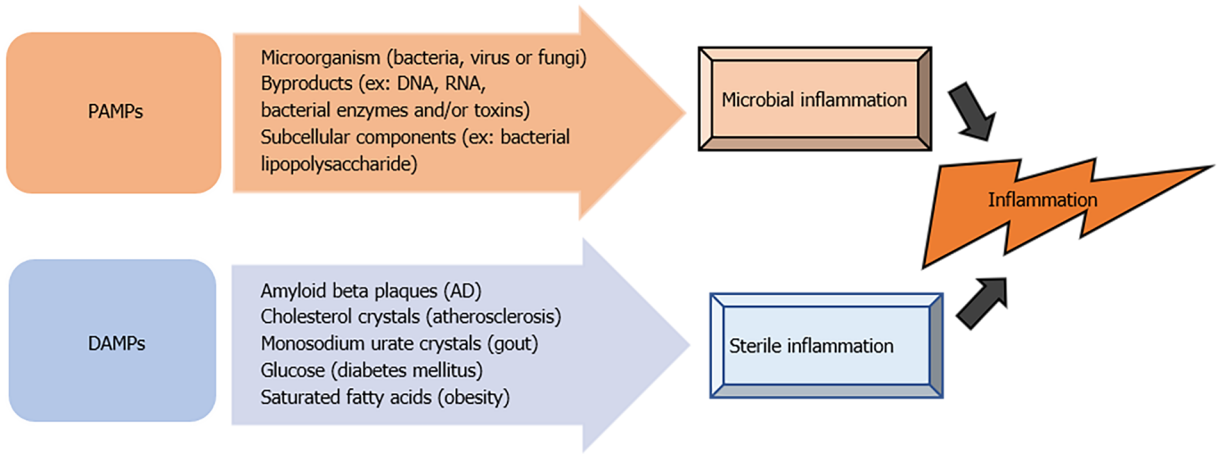

Figure 1 A diagram showing sterile inflammation and microbial inflammation. Inflammation results from activation of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include microorganisms, its byproducts or subcellular components. Examples of DAMPs include glucose, saturated fatty acids, uric acid, or amyloid beta plaques. DAMP: Damage-associated molecular pattern; PAMP: Pathogen-associated molecular pattern; AD: Alzheimer's disease.

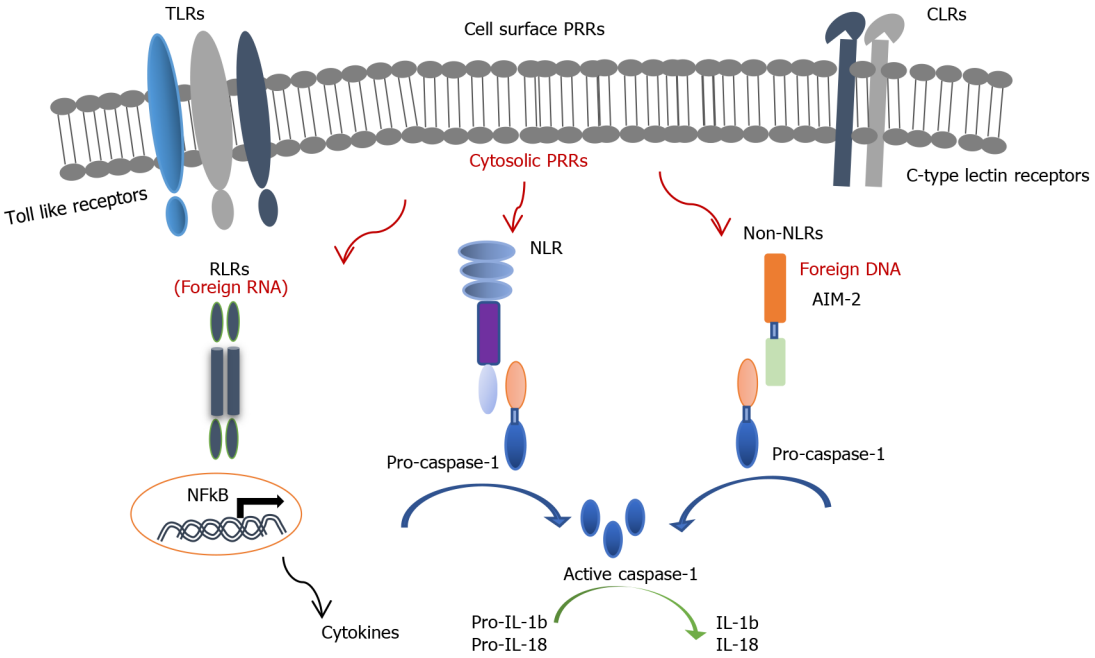

Figure 2 A diagram that depicts the five major classes of protein recognition receptors that are identified for sensing pathogen-associated molecular patterns and damage-associated molecular patterns and subsequent stimulation of proinflammatory responses. The cell surface pattern recognition receptors (PRRs) include Toll-like receptors and c-type lectin receptors. The cytoplasmic PRRs include NOD-like receptors (NLRs), retinoic acid-inducible gene-1-like receptors (RLRs), and the non-NLRs. RLRs recognize double-stranded RNA viruses and activate NFκB to increase the transcription of cytokines. The signaling of NLRs requires the initial expression of inflammasome and cytokine precursors such as pro-IL-1β or pro-IL-18. Assembly of the NLR-inflammasome results in caspase-1 activation and subsequently processing and secretion of cytokines IL-1β and IL-18. Non-NLRs, known also as AIM-2 can recognize double-stranded DNA viruses. Similarly, AIM-2 signals *via* activation of cleavage and release of active caspase-1 to process and mature IL-1β and IL-18. TLRs: Toll-like receptors; NLRs: NOD-like receptors; CLRs: c-type lectin receptors; PRR: Pattern recognition receptors; RLRs: Retinoic acid-inducible gene-1-like receptors.

TXNIP-NLRP3 inflammasome axis as a pivotal pathway for sterile inflammation

Thioredoxin-interacting protein (TXNIP), also known as vitamin D3 upregulated protein-1 or thioredoxin-binding protein-2, is an endogenous inhibitor of thioredoxin (TRX). TXNIP is a key regulator of oxidative stress and has been linked also to inflammation[2]. TRX serves as a multifunctional regulator that is necessary for the control of cellular survival, inflammation, glucose and lipid metabolism, angiogenesis, carcinogenesis, and oxidative stress[6]. Trx also acts on the apoptosis signal-regulating kinase 1 (ASK-1) to block the subsequent activation of the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways that eventually leads to apoptosis stimulated by tumor necrosis

factor alpha (TNF-α)[7,8]. In the reduction-oxidation (redox) dependent state, the oxidized TXNIP-cysteine-247 binds to the active site of reduced TRX-cysteine-32 *via* disulfide bonds[6].

In addition, TXNIP works in a redox-independent fashion by stimulating NLRP3 inflammasome axis resulting in further activation of pro-caspase-1 and subsequently pro-IL-1β to IL-1β. As such, TXNIP is postulated to play a central role as a pro-inflammatory switch of the TXNIP-NLRP3 inflammasome axis. Several instigating metabolic insults and/or pro-inflammatory DAMPs converge to promote TXNIP-sterile inflammation as TXNIP is the common denominator for these metabolic stressors. This review will summarize the published evidence of TXNIP contribution in stimulating NLRP3-inflammasome and mediating sterile inflammation in response to metabolic and ischemic cellular events. In particular, we will attempt to highlight the major cellular activities that can trigger TXNIP and NLRP3 inflammasome activation and understand how sterile inflammation associated with high fat diet (HFD)-associated obesity and its impact on retinopathy, steatohepatitis and delayed vascular recovery. Further, we examined the impact of ischemia-reperfusion and the associated sterile inflammation in various disease states including ischemic retinopathy, ischemic stroke, brain aging and critical limb ischemia.

CELLULAR ACTIVITIES THAT CAN TRIGGER TXNIP AND NLRP3 INFLAMMASOME ACTIVATION

At the cellular level, sterile inflammation and activation of NLRP3-inflammasome could be triggered by multiple activities including K^+ efflux and lysosomal disruption induced by particles such as silica, cholesterol, and uric acid crystals. Mitochondrial dysfunction and formation of reactive oxygen species (ROS) are also important upstream events of NLRP3 activation. As depicted in Figure 3, TXNIP expression can be triggered at the transcription level by saturated fatty acids[9], hyperglycemia[10], calcium influx[7]. TXNIP can be post-transcriptionally regulated by endoplasmic reticulum (ER) stress and microRNA (miRNA)[11,12]. Further, TXNIP is an established regulator of cellular oxidative stress where ROS dissociates TXNIP from TRX and increase its level. Together, increases in TXNIP facilitate its binding to NLRP3, resulting in NLRP3-inflammasome activation (reviewed in[2]).

ER stress regulates TXNIP expression

Unfolded protein response (UPR) is an adaptive response, which prevents the accumulation of misfolded proteins in the lumen of the ER. The UPR is transduced by three major ER-resident stress sensors, namely protein kinase RNA-like ER kinase, activating transcription factor 6, and inositol requiring enzyme 1 (IRE1). When protein misfolding exceeds the capacity of the UPR, an ER-stress state that can trigger programmed cell death[13]. We and others have shown that ER stress can enhance TXNIP expression and NLRP3-inflammation, suggesting TXNIP as critical signaling node that links ER stress and inflammation[11,14,15].

Several studies have established the role of the Toll-like receptors (TLR) as upstream signal to mediate ER-stress response to the saturated fatty acid palmitate in cultured hepatocytes through the IRE1 pathway[16-18]. A prior study showed that TLR2 deficiency protected against hepatic steatosis in a murine model of diet-induced metabolic syndrome[17]. Deletion of TLR4 resulted in protection against development of non-alcoholic steatohepatitis (NASH) in apolipoprotein E deficient mice fed with high-fat and high-cholesterol diet as a model of obesity[18]. Release of glutamate, one of the well-identified DAMPs, was shown to trigger TXNIP expression[7,19,20]. Increased glutamate also has been shown to induce ER stress and result in neuronal damage in a model of brain ischemia[20]. Inhibition of ER stress with tauroursodeoxycholic acid reduced TXNIP activation suggesting the role of ER stress in the induction of TXNIP expression[20]. Ding *et al*[21] demonstrated a temporal relationship between TXNIP, ER stress and neuroinflammation in rat model of cerebral venous sinus thrombosis[21]. They found that oxidative stress and ER stress contribute to the activation of TXNIP, which further induces NLRP3 inflammasome and neuronal pyroptosis. Similarly, supplementation of ketogenic diet improved ischemic tolerance in mice through inhibition of ER stress and associated TXNIP-NLRP3 inflammasome activation[22].

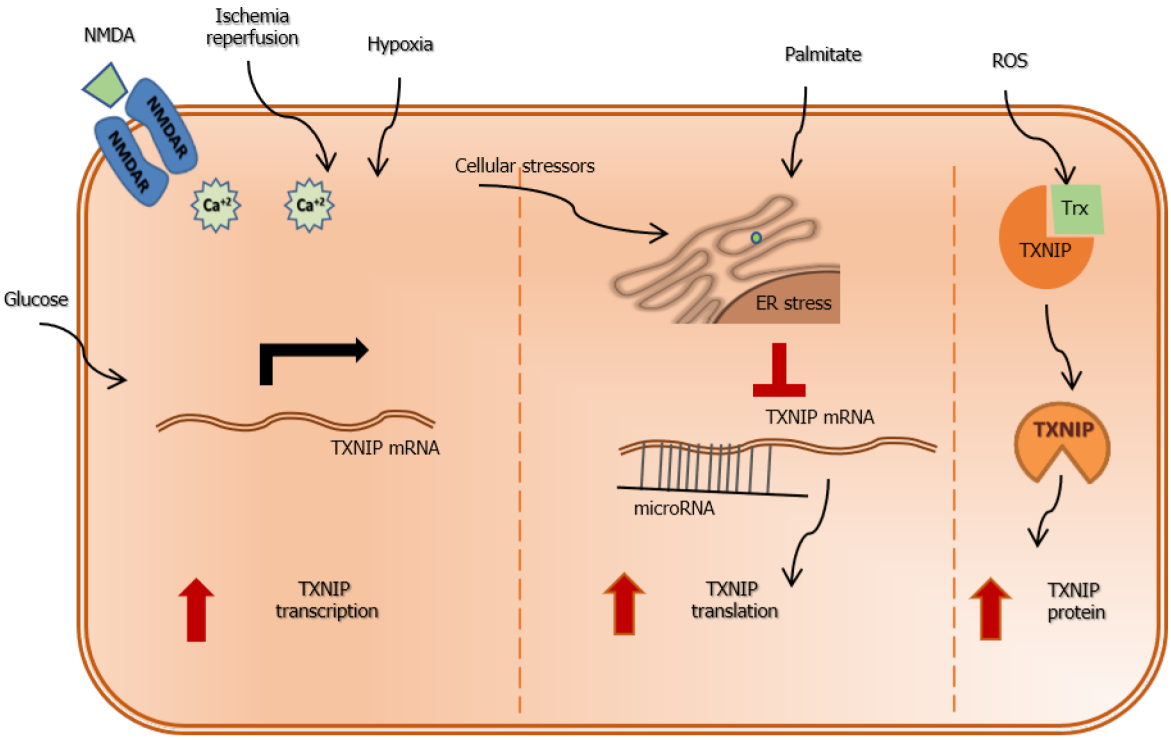

Figure 3 A diagram that depicts various ways of regulation of thioredoxin interacting protein expression. At the transcriptional level, thioredoxin interacting protein (TXNIP) expression can be triggered by hyperglycemia as it contains carbohydrate response element. Ischemia-reperfusion injury, hypoxia and activation of the n-methyl D-aspartate receptor result in significant increase in calcium influx that trigger TXNIP expression *via* activation of the Ca-response element. Further, TXNIP can be post-transcriptionally regulated by endoplasmic reticulum (ER) stress and microRNA (miRNA) that traditionally bind to the 3 UTR region of TXNIP mRNA and repress its translation. Under conditions of cellular stressors, saturated fatty acids such as palmitate result in increases in ER stress and degradation of miRNA resulting in increases in TXNIP expression. Finally, oxidative stress and formation of reactive oxygen species dissociates TXNIP from thioredoxin and increase its level that facilitate activation of NLRP3-inflammsome and release of inflammatory mediators. TXNIP: Thioredoxin interacting protein; NMDA: N-methyl D-aspartate; ER: Endoplasmic reticulum; ROS: Reactive oxygen species.

Role of miRNA and regulation of TXNIP expression

miRNAs are a class of highly conserved, endogenous non-coding RNAs containing 19 to 25 nucleotides. miRNA anneal to target genes and simultaneously control their translation and transcription. The stability of mRNA is governed by binding of specific miRNAs to complementary sequences in the 3′ untranslated region (UTR) of gene target, which is then degraded or silenced[23]. Bioinformatic analysis of the TXNIP 3′ UTR identified several conserved binding sites for miRNA that were implicated in the negative regulation of TXNIP expression (reviewed in[13]). Recent literature shows growing number of newly-identified miRNA that regulate TXNIP expression in various models. One of the examples include miR-20b-3p that has been demonstrated to negatively regulate TXNIP expression in models of diabetic retinopathy and cerebral ischemia[24,25]. Another example is miR-146a-5p, that has been shown to regulate TXNIP expression and subsequently the inflammatory and apoptotic response in human chondrocytes cell line[26]. TXNIP was also reported to be a potential target of miR-125b promoting metastasis and progression of pancreatic cancer *via* the HIF1α pathway[27,28]. In colorectal cancer tissues, miR-135b-5p was upregulated whereas TXNIP was downregulated, which promoted cell proliferation, migration and invasion, and suppressed apoptosis of cancer cells[29]. Overexpression of miR-148a reduced infarct size in vivo, and alleviated dysregulation of cardiac enzymes and Ca^{2+} overload in myocardial ischemia/reperfusion *via* down-regulating TXNIP and inactivating the TLR4/NF-κB/NLRP3 inflammasome signaling pathway [30]. Myocardial ischemia reperfusion induced TXNIP expression and lower miR-150-5p levels, along with increased cardiomyocyte apoptosis[31]. Injection of MSCs-derived exosomes containing miR-150-5p resulted in downregulation of TXNIP and showed a reduction in myocardial remodeling[31]. Similarly, myocardial I/R triggered TXNIP expression both mRNA and protein in diabetic mice and miR-135a expression level was reduced in diabetic mice regardless of I/R injury or not[32]. Of note, the regulation of TXNIP expression by miR-17-5p is well-studied in various models and will be discussed in the following section.

Role of miRNA miR-17-5p in regulation of TXNIP expression

Recent work showed that known DAMPs such as palmitate and hypoxia triggered the expression of ER-stress markers and TXNIP in retinal Müller cells[11,33]. Among the ER-stress markers, IRE1α, is an ER bifunctional kinase/RNase that has been shown to destabilize number of RNA and miRNA including miR-17-5p. The latter is a small non-coding RNAs that control the translation and transcription of TXNIP[12]. When levels of miR-17-5p decline under stress condition, TXNIP expression is enhanced. Our work demonstrated that treatment of retinal Müller cells with ER-stress inhibitor phenyl-butyric acid or with the pharmacological inhibitor of IRE1α, dramatically restored TXNIP expression back to normal level[11]. Similarly, exposure to strong DAMPs such as hypoxia *in vitro* and ischemic/reperfusion injury *in vivo* has been shown to trigger ER-stress and TXNIP expression[33,34]. Modulation of miR-17-5p activity or expression reduced the increased TXNIP-NLRP3-mediated inflammation [33,34]. These studies highlight the potential contribution of ER stress to fine-tune expression of TXNIP and regulate its associated inflammation. In consistence, several reports have shown that ER stress can enhance TXNIP expression and NLRP3-inflammation, suggesting TXNIP as critical signaling hub that links ER stress and inflammation[11,14,15].

Cross-talk of miR17-5p, PPAR-g and TXNIP expression

Umbelliferone, a natural antioxidant, has been proven effective against neurodegenerative and inflammatory processes[35]. Inhibition of TXNIP expression and NLRP3 inflammasome activation was also associated with upregulation of PPAR-γ. Interestingly, maintaining the redox state suggest a balance between activation of PPAR-γ and down-regulation of TXNIP, indicating that PPAR-γ is a negative regulator of TXNIP[36]. In particular, TXNIP expression has been demonstrated to be regulated by PPAR isoform PPAR-β/δ. Treatment with GW0742, PPAR-β/δ, agonist attenuated the expression of TXNIP-NLRP3, microglial activation and improved neurological outcome in rat pups following hypoxic ischemia[34]. The inhibitory effect of PPAR-β/δ on TXNIP is also mediated by upregulation of transcriptional regulator miR17-5p confirming the regulatory role of PPARs on TXNIP[37].

OBESITY AND HFD ARE MAJOR DRIVERS FOR STERILE INFLAMMATION

Obesity is characterized by excessive accumulation of fat in the body that impairs personal health and a body mass index of more than 30. Among all the detrimental causes of obesity, HFD and lack of exercise are the most compelling factors[1]. Because of the accumulation of macronutrients in the adipose tissue, inflammatory mediators, such as TNFα and IL-6, are stimulated, which lead to the activation of pro-inflammatory state and oxidative stress[1]. Further, chronic sterile inflammation is not only integral to the pathobiology of obesity, but it also causes development of insulin resistance resulting in a vicious cycle to sustain obesity[38]. obesity is a risk factor for development of type II diabetes, cardiovascular events and can cause microvascular dysfunctions[39]. Therefore, understanding the interaction between obesity and sterile inflammation in various disease states has become crucial. In the next section, we will examine the role of TXNIP in mediating the interaction between obesity and sterile inflammation in select disease state including retinopathy, steatohepatitis and delayed vascular recovery after critical limb ischemia.

TXNIP-NLRP3 inflammasome activation and HFD-systemic inflammation

Similar to humans, when rodents are fed with HFD, they present with an increase of body weight, total cholesterol, triglyceride, insulin resistance and high glucose level[9,40,41]. We and others showed that HFD is associated with increased systemic and local production of IL-1β detected in the circulation and across several tissues[40,42]. The contribution of TXNIP is best demonstrated by the findings that genetic deletion of TXNIP not only resulted in alleviated glucose intolerance in HFD, but it also mitigated HFD-induced systemic and tissue inflammation[9,40].

Direct role of TXNIP-NLRP3 Inflammasome activation in microvascular dysfunction

Microvascular dysfunction and subsequent cell death can be generally classified according to the root cause into: (1) Primary dysfunction; that occurs as a result of mechanical, metabolic and/or biochemical insults and associated sterile inflammation within the vascular cells themselves (ex: Endothelial cells, pericytes); and (2)

Secondary dysfunction; that occurs in response to the sterile inflammation driven by the same kind of insults in non-vascular cells (ex: Infiltrating and resident proinflammatory monocytes and macrophages and/or specialized cells like astrocytes and glial cells). In response to different types of DAMPs, several studies have established the direct role of TXNIP-NLRP3 Inflammasome activation in several microvascular beds. Ischemia reperfusion injury was shown to induce the specific TXNIP-mediated upregulation and activation of the NLRP3 inflammasome axis and associated oxidative stress, endothelial barrier dysfunction and pro-inflammatory response in animal models of cerebral and cardiac ischemia and corresponding cultured brain and cardiac microvascular endothelial cells rather than non-vascular cells[43,44]. Furthermore, similar findings were also reported showcasing the protective effect of genetic and pharmacological inhibition of the TXNIP-NLRP3 inflammasome axis and associated microvascular dysfunction, vascular permeability, apoptosis, and angiogenic response in hyperglycemic animal models of diabetic retinopathy and cultured retinal endothelial cells[24,45,46].

In effort to dissect the direct role of the TXNIP-NLRP3 inflammasome axis in microvascular *vs* non-vascular cells, cultured retinal endothelial cells treated with saturated fatty acid palmitate coupled to BSA to mimic HFD-obesity showed significant increase in TXNIP expression as well as NLRP3-inflammasome activation and IL-1β expression[9,11,41]. Knocking-down TXNIP expression mitigated expression and activation of NLRP3-inflammasome components evident by increased caspase-1, and IL-1β[9,11]. Furthermore, overexpression of TXNIP plasmid mimicked the HFD-induced sterile inflammation *in vitro* evidenced by the significant elevation of NLRP3, caspase-1, IL-1β, and TNF-α, which were attenuated upon treatment with IL-1 receptor antagonist. Hence, indicating the essential role of the TXNIP-NLRP3 inflammasome activation in driving sterile inflammation in autocrine fashion through IL1-b[41]. Similarly, isolated Muller cells from TXNIP knockout (TKO) mice showed a blunted NLRP3 inflammasome activation response to saturated fatty acid palmitate treatment compared to the primary cultures from WT mice[11]. Interestingly, our studies in cultured retinal endothelial cells showed that knocking down TXNIP expression specifically blunted palmitate-induced, but not peroxynitrite-induced release of IL-1b to the condition medium[9]. TXNIP, as a member of the alpha arrestin scaffolding proteins, plays an essential role in intracellular cargo trafficking. Hence, subcellular localization of TXNIP in response to different insults might enhance or inhibit release of mature IL-1β[9]. These findings confirmed the integral role of TXNIP in not only expression and activation of IL-1β, but also its release extracellularly and to the systemic circulation.

TXNIP-NLRP3 inflammasome activation and HFD-induced retinal microvascular dysfunction and degeneration

In addition to the central role of TXNIP in mediating HFD-induced metabolic response, TXNIP can directly connect HFD-induced metabolic stress and sterile inflammation. Clinical and preclinical studies have established obesity among other components of the metabolic syndrome as an independent risk factor risk for development of retinal microvascular dysfunction with or without diabetes[47-51]. Our group was the first to report that HFD can selectively result in inducing TXNIP expression and its direct association with NLRP3 inflammasome activation evidenced by increased cleaved caspase-1 and cleaved IL-1b levels in a rat model of HFD for 8-10 wk[9]. In this study, immunohistochemical analysis revealed strong TXNIP expression in the retinal ganglion cell layer and inner nuclear layer, which colocalized within Müller cell end-feet and retinal microvasculature that constitute two major components of the retinal neurovascular unit[9]. In subsequent series of studies, we further elucidated that HFD triggers the unfolded protein ER-stress response in retinas from 4 wk HFD fed mice *in vivo* and in cultured retinal Muller cells treated with saturated fatty acid palmitate. Inhibiting ER-stress significantly blunted the increase in HFD-induced TXNIP expression without altering its associated insulin resistance in the HFD treated group. In line with our findings, another report also showed that retinas from HFD exhibited neural inflammasome activation at 3-mo of HFD, before the development of systemic glucose intolerance, electroretinographic defects, or microvascular disease[52]. Moreover, HFD or Western diet was also shown to enhance TXNIP expression in the retina that resulted in TXNIP-dependent JNK activation and retinal cell death[53]. Retinal neurodegeneration and decreased retinal function were also observed in response to HFD combined with Streptozotocin injection as a model for type 2 diabetes, that was alleviated by over expression of TRX suggesting the involvement of the TXNIP-mediated activation of the mTOR pathway and associated

inhibition of autophagy[54]. Furthermore, we sought to examine the effect of TXNIP deletion on HFD-induced retinal microvascular inflammation and degeneration. In WT mice, short-term HFD feeding for 8 wk resulted in enhanced TXNIP expression along with increased levels of cleaved caspase-1, cleaved IL-1b as the classic markers for NLRP3 inflammasome activation. Increased levels of retinal adhesion molecules, ICAM-1 and VCAM-1, retinal vascular permeability and associated leukocytes-induced obstruction of the retinal vasculatures, were also elevated[9,41]. However, such effects were ameliorated in TXNIP knock out mice fed with HFD. In parallel, 18 wk of HFD also resulted in increased degeneration of retinal microvascular capillaries and morphological changes in WT mice fed; on the other hand, TXNIP deletion abrogated such presentations[9,41]. Together, these findings signify the direct causal role of TXNIP in mediating HFD-Induced NLRP3 inflammasome activation and retinal microvascular dysfunction.

TXNIP-NLRP3 inflammasome activation and HFD-induced steatohepatitis

The global prevalence of non-alcoholic fatty liver disease (NAFLD) is 25.2% with a 40.7% incidence rate of developing into cirrhosis, which is a sequala resulted from NASH, a serious form of NAFLD [systemic review by Younossi *et al*[55]. Among individuals with NAFLD, 51.94% of them have a comorbidity of obesity, 22.5% with type-II diabetes, 69.2% with hyperlipidemia, and 42.5% metabolic syndrome, which implicated the strong correlation between steatohepatitis and metabolic dysfunctions [55]. While the pathogenesis of disease progression from NAFLD to NASH is not fully understood, involvement of sterile inflammation, TLR signaling, and gut-liver axis are among suggested pathways[56].

Several studies established the role of the TLRs and downstream activation of the NLRP3 inflammasome in the pathophysiology of NAFLD using various models of diet-induced liver dysfunction[17,57-59]. HFD-induced hepatic steatosis was associated with statistically significant expression of TLR2, total-NFκB, NLRP3, cleaved caspase-1, cleaved IL-1β, and TNF-α[58]. Genetic deletion of TXNIP attenuated the expression of TLR2, T-NFkB NLRP3 and their downstream inflammatory markers [58]. In the same study, IL-1β expression coincided within the same areas of steatosis, inflammatory cell infiltration, collagen deposition and α-SMA expression in WT-HFD mice[58]. We and others have shown that genetic deletion of TXNIP resulted in the alleviation of hepatic steatosis, hepatocyte inflammation, and fibrogenesis[17,57,58]. In parallel, genetic modulation or pharmacological inhibition of caspase-1, as a central mediator, protected against HFD-induced hepatic steatosis, inflammation and early fibrogenesis[60-62]. In addition, both genetic deletion and pharmacological inhibition of NLRP3 reduced hepatic inflammation and the expression of hepatic caspase-1 and IL-1β to normal level in two murine models of steatohepatitis fed by methionine/choline deficient or atherogenic-HFD diet models[57,63]. Interestingly, knocking-down TXNIP augmented steatohepatitis and hepatic fibrosis in methionine choline-deficient diet-fed mice[64]. Such discrepancy could be attributed to the different nature of experimental model of steatohepatitis.

Multiple studies using the pharmacological inhibition of TXNIP and NLRP3-inflammasome showed reduction in the hepatic pro-inflammatory markers and lipid accumulation by using salidroside, verapamil, quercetin, allopurinol, dietary curcumin, salvianolic acid A, and berberine[21,65-67]. Mitigating oxidative stress and TXNIP expression in the liver resulted in suppression of TNF-α, NLRP3, caspase-1, and IL-1β in a mouse model of HFD-induced NAFLD or from the diabetic mouse model induced by either streptozotocin injection[66] or fructose-fed diet[68]. Kim *et al* [69] depicted that dietary curcumin could also downregulate TXNIP expression, which protected fatty liver in a high fat/high sugar induced mouse model. Furthermore, berberine also demonstrated its ameliorative effect on steatohepatitis in methionine-choline deficient-fed mice; and its inhibitory effect of NLRP3 inflammasome activation *via* the ROS/TXNIP axis *in vitro*[70].

ISCHEMIA-REPERFUSION AS A MAJOR DRIVER FOR STERILE INFLAMMATION

Post-ischemic event, the restoration of blood flow is usually the primary therapeutic approach as reperfusion is essential to restore oxygen and nutrients. However, reperfusion can induce further tissue damage in the ischemic organ and adjacent ones. Ischemia-reperfusion is associated with alteration of intracellular calcium level and release of ROS, known upstream triggers of cell injury. ROS disturb TRX system by

increasing TXNIP expression and inhibiting TRX from binding and inhibiting the activity of ASK-1, resulting in activation of apoptotic pathways. In parallel, displacement of TXNIP triggers NLRP3-inflammasome activation and subsequent inflammation signaling pathways. Further, TXNIP has been shown to modulate angiogenic response in addition to metabolic and inflammatory response[71]. Prior study showed that TXNIP is required for VEGF-mediated angiogenic signal and response in endothelial cells[72]. In the next section, we will review the evidence of how TXNIP contributed to NLRP-3 inflammasome activation in the pathogenesis of ischemic-reperfusion and secondary microvascular dysfunction and/or neurodegenerative events.

TXNIP-NLRP3 inflammasome axis activation in critical limb ischemia

Critical limb ischemia is a major symptomatic manifestation of peripheral arterial disease and failure to establish revascularization eventually leads to amputation. Traditionally, TRX stimulates ischemia-induced angiogenesis through two primary mechanisms; by regulating ROS that resulted in increased level of nitric oxide and nitrotyrosine formation; or by suppressing the ASK-1 that promotes endothelial cells apoptosis[73]. Recent evidence shows compromised functional recovery to ischemia in patients with frank diabetes and in non-diabetic patients with insulin resistance.[74] We and others have shown that metabolic disorders including obesity is associated with impaired vascular recovery post-ischemic events in experimental models[40,75,76]. Increases in oxidative stress and TXNIP expression have been implicated as major players in impaired vascular recovery. Indeed, deletion of TXNIP improved vascular recovery and protected mice from the reduction of blood flow due in HFD-obesity[40,77]. One of the possible mechanisms of impaired vascular recovery is the loss of ischemia-induced VEGF expression and/or activation of its receptor (VEGFR2) due to metabolic stress. Interestingly, the expression of VEGF and VEGFR2 activation were similar across mice lacking TXNIP expression (TKO) regardless of the diet choice (normal diet *vs* HFD) or ischemic condition (ischemic side *vs* sham)[40]. The other postulated mechanism of impaired vascular recovery is the activation of NLRP3-inflammasome in response to stress conditions such as ischemia or HFD, which led to a rise in inflammatory mediators such as IL-1β[40,77]. Deletion of TXNIP significantly reduced NLRP3-inflalmmasome activation evident by reduced tissue and circulatory level of IL-1b[40]. In agreement, genetic deletion of TL4 or NLRP3 demonstrated similar vascular protective effects *via* modulation of TXNIP-NLRP3-inflammasome activation[76,78]. These studies confirmed the integral role of TLR4-mediated TXNIP and NLRP3-inflammasome signaling interfering with perfusion recovery in muscle ischemia. These signaling molecules may represent a therapeutic target to improve vascular recovery and preserve limb salvage.

TXNIP-NLRP3 inflammasome activation in retinal ischemia-reperfusion model

Retinal neurodegeneration, an early characteristic of several blinding diseases, triggers glial activation, resulting in inflammation, secondary damage and visual impairment. Treatments that focused only at neuroprotection have failed clinically. Therefore, there is significant need for treatment strategy that target secondary damage. Exposure to transient ischemia alters TRX antioxidant defenses, resulting in significant increases in retinal oxidative stress and TXNIP expression. Indeed, exposure to transient ischemia resulted in early increase in TXNIP mRNA expression that persisted for 14 d in a model IR, compared to sham controls[33]. Colocalization studies showed that TXNIP localized within activated glial Müller cells in IR-retinas. Exposure of Müller cells to hypoxia-reoxygenation injury triggered ER stress markers and inflammasome activation in cells isolated from WT mice, but not in cells isolated from TKO mice. Secondary damage was triggered by TXNIP-NLRP3-inflammasome activation evident by increase in inflammatory mediators, sustained neurodegeneration. Furthermore, secondary damage was sustained 14-days post IR injury assessed by the significant increase in the number of occluded acellular capillaries and visual impairment in IR-WT mice, but not in IR-TKO. Intervention with TXNIP-antisense oligomers (ASO) prevented ischemia-induced glial activation and neuro-vascular degeneration, and improved visual function compared to untreated WT[33]. In a recent study, sulforaphane administration significantly inhibited IR-mediated changes in retinal thickness and prevented retinal ganglion cell death. Sulforaphane suppressed inflammatory cytokines production, microglia activation, and inflammasome activation. In parallel, knockdown of NLRP3 was performed, and the according changes of retinal ganglion cells assessed. In accordance, NLRP3 knockdown presented the similar inhibitory effect on IR rats[79]. The protective of the effect of TXNIP inhibition is partly mediated through inhibition of NLRP3-inflammasome components including cleaved

caspase-1 and IL-1b[80]. Therefore, targeting TXNIP expression may offer an effective approach in the prevention of secondary damage associated with retinal neurodegenerative diseases.

TXNIP-NLRP3 inflammasome activation in ischemic stroke

Ischemic stroke is a leading cause of death and long-term disability in the US with limited therapeutic window for reperfusion therapy. Thus, there is a great need to identify effective therapeutics that could be administered in a more practical window. Recent mRNA profiling analysis by Tian *et al*[81] demonstrated that TXNIP signaling is one of the major gene hubs differentially expressed in rat brain following middle cerebral artery occlusion. In agreement, our studies showed that ischemic injury-induced TXNIP expression was associated with significant increases in expression of NLRP3-inflammasome components and its activation[80]. Consistently, genetic deletion or pharmacological inhibition of TXNIP with resveratrol resulted in protection of mice from ischemic reperfusion injury and improved neurological outcome following embolic middle cerebral artery occlusion[80]. Elevated expression of TNF-α, and apoptotic markers including cleaved caspase-3 and PARP were attenuated by TXNIP deletion or resveratrol treatment. We and others demonstrated also that genetic deletion of TXNIP or overexpression of TRX have showed neuroprotective effects against ischemic brain damage[80,82]. TKO mice showed higher expression of TRX with reciprocal decrease in the makers of oxidative stress including nitrotyrosine along with inhibition of inflammation activation. In support, Hua *et al*[83] demonstrated in rat model with middle cerebral artery occlusion/ reperfusion, increased expression TXNIP, elevated level of markers of oxidative stress and reciprocal decrease in the expression TRX.

Diabetes is the leading co-morbidity, which increases the risk of hemorrhagic transformation and poor recovery in stroke patients. Recently, we reported that thrombolytic therapy with tissue plasminogen activator (tPA) worsened ischemic reperfusion injury under hyperglycemic condition along with activation TXNIP-NLRP3 axis[84]. Pharmacological modulation of TXNIP expression with verapamil attenuated NLRP3 inflammasome activation, hemorrhagic transformation, and blood brain barrier (BBB) damage. Treatment with verapamil attenuated activation of ASC, cleaved caspase-1 and IL-1β[84]. Although verapamil is not a specific activator for TXNIP, it can be considered as an adjunctive therapy to mitigate the detrimental effect of tPA in hyperglycemic condition[85]. Consistently, attenuation of TXNIP-NLRP3 activation with hyperbaric oxygen preconditioning also activation thereby ameliorated hyperglycemia associated hemorrhagic transformation and stroke outcome[3]. In support, Cao *et al*[43] demonstrated that ischemic stroke associated BBB damage along with activation of TXNIP and NLRP3 inflammasome and activation of MAPKs including p38 and JNK. Further, treatment with ruscogenin, an anti-inflammatory steroid sapogenin could inhibit the activation of MAPK, TXNIP/NLRP3 pathway and BBB damage in mouse model of ischemic stroke. Ruscogenin attenuated the ischemia associated activation of NLRP3-inflammasome and subsequently mitigated levels of caspase-1 and IL-1β.

TXNIP-NLRP3 INFLAMMASOME ACTIVATION IN NEURODEGENERATION

TXNIP-NLRP3 inflammasome and development of AD

Neurodegenerative diseases such as AD, Parkinson's, and Huntington's disease are characterized by the progressive loss of specific synapses and neurons[86]. Microglia are the principle innate immune cells in the CNS that express TLRs, and it has been shown that amyloid plaques, characteristic of AD can activate the innate immune response by interacting with TLRs[87]. Excessive inflammation can be linked to altered TLR4 signaling and increased possibility of developing AD[87,88]. HFD has been implicated in driving inflammation in regards to AD. It has been shown that a caloric deficit has the ability to possess anti-inflammatory effects and downregulate the TLR4/NF-$_{\kappa}$B signaling cascade[89]. Several studies have shown the protective effects of TXNIP inhibition against NLRP3-inflammasome activation in genetic models of cognitive dysfunction and AD. Examples of these models include the Swedish mutant form of APP (APPswe)/PSEN1dE9 transgenic mice[90], ibotenic acid-induced neurological disorder in rats and in cells[87].

TXNIP-NLRP3-inflammsome activation in brain aging

Recent study by Oberacker *et al*[91] demonstrated that TXNIP is candidate gene for aging as is found to be elevated in aged human primary T cells, hematopoietic progenitor cells and monocytes. Consistently, elevated mRNA expression of TXNIP was reported in aged human cortices (81-95 year) in comparison with young (25-37 year)[92]. Later, Zhou *et al*[67] found that aging is associated with elevated microglial activation and neuroinflammation along with increased TXNIP/NLRP3 expression in aged rat (24 mo) in comparison with young rats (12 mo). Further, modulating TXNIP-NLRP3 activation with a traditional anti-inflammatory Chinese medicine attenuated age-associated neurodegeneration in aging rats[93]. Consistently, we recently identified aged-dependent activation of TXNIP-NLRP3 inflammasome in the cortex and hippocampus of brain in both male and female mice[94]. Our studies showed that enhanced TXNIP expression in aged mice is associated with decreased TRX expression and oxidative damage. It is further evident from increased expression of ASC, cleaved caspase-1 and cleaved IL-1β. Genetic deletion of TXNIP attenuated activation NLRP3-inflammasome activation with parallel decrease in the expression of caspase-1 and IL-β. Further, pharmacological inhibition of TXNIP with verapamil significantly attenuated neuroinflammation and age associated-cognitive impairment confirming the contributory role of TXNIP in age associated neurodegeneration[94].

THE POTENTIAL FOR TXNIP AS A THERAPEUTIC TARGET

TXNIP is a multi-faceted protein that plays pro-inflammatory, pro-oxidative stress, and pro-apoptotic functions. As reviewed, TXNIP mediated NLRP3-inflammasome activation has been implicated in the detrimental micro- and macrovascular complications of various disease states. Although TXNIP has been widely suggested a promising therapeutic target, there is lack of specific pharmacological inhibitor. Therefore, other therapeutic tools for inhibiting TXNIP were developed that extend from natural compounds, phytochemicals to repurposing of drugs that are already approved for clinical use[71,95]. In the next section, we will review some of the potential therapeutic tools for targeting TXNIP expression.

Natural antioxidants inhibit TXNIP expression

Pharmacologically, there are number of natural antioxidants that have been reported to exert their protective effects *via* inhibition of TXNIP and NLRP3-inflammasome (see Table 1). Such list included the following; salidroside, quercetin, allopurinol, dietary curcumin, salvianolic acid A, and berberine[21,65,66]. The protective effects of these natural antioxidants were mainly mediated by mitigating oxidative stress, TXNIP and NLRP3 expression and lipid accumulation in the liver, which led to future suppression of TNF-α, NLRP3, caspase-1, and IL-1β in a mouse model of HFD-induced NAFLD.

Ischemic stroke models were used to demonstrate the protective effects of natural and herbal medicine *via* inhibitory effect on TXNIP expression. Taohong Siwu decoction (THSWD), a traditional Chinese medicine could inhibit the expression of TXNIP thereby improved neurobehavioral outcome, inhibited NLRP3-inflammasome activation and pyroptosis following ischemic reperfusion injury in rat[96]. Treatment with THSWD attenuated activation of NLRP3-inflammasome evident by lower level of caspase-1, IL-1β, TNFα, IL-6, HMGB1 and IL-18. Similarly Z-Guggulsterone, a herbal steroid has shown its protective effect in ischemic reperfusion injury by inhibiting TXNIP/NLRP3-inflammasome demonstrated by down regulation of IL-1β, IL-6, and IL-18[97]. Wang *et al*[98], demonstrated that Umbelliferone, a natural antioxidant belongs to coumarin derivative, could inhibit TXNIP expression in a rat model of focal cerebral ischemia. Pretreatment with Umbelliferone, 7-d before ischemic stroke ameliorated infarct size and brain edema with improved neurological outcome[98]. The beneficial effects of Umbelliferone is mediated by inhibition of TXNIP-NLRP3 inflammasome activation[35]. Treatment with GW0742, PPARβ/δ, agonist attenuated the expression of TXNIP-NLRP3, microglial activation and improved neurological outcome in rat pups following hypoxic ischemia[34].

Drug repurposing to inhibit TXNIP expression

Repurposing of clinically approved drugs in the market provide promising and safe therapeutic options (listed in Table 2). Verapamil, a calcium channel blocker, has been widely used to inhibit TXNIP expression. Verapamil-mediated TXNIP inhibition is conferred by reduction of intracellular calcium, inhibition of calcineurin signaling, and nuclear exclusion. Verapamil has been shown to exert antidiabetic effects and reduce

Table 1 Summary of studies on modulation of thioredoxin interacting protein using natural antioxidants animal models

Ref.	Treatment	Animal model	Main findings
[1]	Taohong Siwu decoction 18, 9 and 4.5mg/kg Intragastric administration for 7 d	Rat with middle cerebral artery occlusion	Improved neubehavioral function and inflammation and inhibited pyroptosis following ischemic stroke
[2]	Z-Guggulsterone, 12.5, 25, 50 mg/kg, (*ip*) Intraperitoneal administration for 6 d	Rat with middle cerebral artery occlusion	Z-Guggulsterone improved neurological deficit and, modulated redox imbalance and inflammation through inhibition of TXNIP/NLRP3 signaling
[11]	Curcumin 50 mg/kg, One hour before surgery, (*ip*)	Rat with cerebral artery occlusion	Attenuated ischemic brain injury. Modulation of TXNIP/NLRP3 inflammasome activation by suppression of ER stress.
[70] [69]	Curcumin	HFD/ High sugar diet	Prevented fatty liver *via* inhibition of TXNIP
[66]	Qurecetin	diabetes	Prevented inflammation, liver TXNIP, lipid accumulation
[6]	Ketogenic diet 3 wk	Mouse model of middle cerebral artery occlusion	Ketogenic diet improved ischemic tolerance, Attenuated ER stress and TXNIP/NLRP3 activation
[7]	Umbelliferone, 15and 30 mg /kg Pretreatment for 7 d (*ip*)	Rat with middle cerebral artery occlusion	Protected against cerebral ischemia reperfusion injury by suppressing TXNIP/NLRP3 inflammasome activation
[8]	Ruscogenin, 10 mg/kg One hour before surgery, (Intra gastic admin.	Mice with middle cerebral artery occlusion	Decreased brain infarction, edema, improved neurological outcome by suppressing a TXNIP/NLRP3 inflammasome activation and MAPK pathway
[9]	Resveratrol, 5 mg/Kg 3 h post-embolic occlusion. (*iv*)	WT mice with embolic middle cerebral artery occlusion	Protected from ischemic injury, improved neurological score suppressed TXNIP/NLRP3 inflammasome and apoptosis
[21]	Salvianolic acid	HFD- Rats	Prevented HFD-induced NAFLD
[65]	Salidroside		Prevented HFD-induced NAFLD
[12]	Compound 10b, 3 mg/kg At the onset of reperfusion	Rat with middle cerebral artery occlusion	Attenuated cerebral ischemia by upregulating endogenous antioxidant system and down regulation of oxidative stress.

TXNIP: Thioredoxin interacting protein; NLRP3: NOD-like receptor pyrin domain containing 3; HFD: High fat diet.

glucose toxicity *via* decreasing the binding of carbohydrate response element-binding protein to the E-box repeat in the TXNIP promoter[10]. Oral administration of verapamil prevented N-methyl D-aspartate (NMDA)-induced retinal neurotoxicity by three different mechanisms, inducing release of inflammatory mediators such as TNF-α and IL-1β, altering antioxidant status and disrupting the Trx-ASK-1 inhibitory complex leading to activation of the p38 MAPK/JNK apoptotic pathway[7]. Inhibiting TXNIP expression with verapamil attenuated NLRP3 inflammasome activation, hemorrhagic transformation, BBB damage[84]. Verapamil improves hepatic inflammation and improves metabolic homeostasis in NAFLD[67].

Other drugs such as metformin has been shown to reduce TXNIP expression *in vitro* using differentiated macrophages in response to high glucose[99]. In vivo, treatment of apoE$^{-/-}$ mice alleviated diabetes-induced metabolic disorders and atherosclerosis. The postulated protective mechanism of metformin involved inhibition of TXNIP-mediated NLRP3 inflammasome activation[99]. Earlier study using STZ-diabetes model showed that metformin and resveratrol can modulate ROS production and ER-stress *via* reducing TXNIP expression. Further, metformin mitigated inflammation and apoptosis *via* inhibition of TXNIP and NLRP3-inflammasome activation[100].

Finally, Ezetimibe, hypolipidemic drug has shown its beneficial effect in ischemic stroke by modulation of TXNIP-NLRP3 inflammasome activation through modulation of AMPK and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway [101]. Activation of Nrf2 with tert-butylhydroquinone inhibited the activation TXNIP/NLRP3-inflammasome in a rat model of MCAO[102]. Conversely, genetic deletion of Nrf2 abolished the activation of TXNIP following MCAO demonstrating the contribution of oxidative stress in the activation of TXNIP in ischemic stroke.

Table 2 Summary of studies on modulation of thioredoxin interacting protein expression using drug repurposing in animal models

Ref.	Treatment		Animal model	Main findings
[84]	Verapamil (0.15 mg/kg), intra venous	1 h	Hyperglycemic mouse model middle cerebral artery occlusion	Reduced infarct area, hemorrhagic transformation and blood brain barrier damage. Improved stroke outcome and neuro inflammation in response to hyperglycemic stroke
[7]	Verapamil po	1 h	NMDA- optic neuropathy	Improved retinal neurodegeneration by altering antioxidant status and disrupting the Trx-ASK-1 inhibitory complex
[67]	Verapamil, 25 mg/kg/d, IP	1 wk	high-fat diet-induced obesity- 10 wk	Improved hepatic inflammation, metabolic homeostasis in NAFLD *via* TXNIP-NLRP3 inflammasome activation
[104]	Verapamil		High-fat diet-prediabetic neuropathy	improved prediabetic neuropathy, inflammation *via* inhibition of TXNIP and NLRP3-inflammasome activation
[10], [105]	Verapamil, 100 mg/kg	Po daily	STZ- and HFD-obesity model	Inhibit TXNIP expression and restore beta-cell function, improve glucose level in STZ- and HFD-obesity model
[100]	Metformin		STZ-diabetes mouse	Suppressed TXNIP/NLRP3 inflammasome activation, reduced cell apoptosis in adipose tissue
[99]	Metformin		$ApoE^{-/-}$ + STZ mice	Inhibited TXNIP/NLRP3 inflammasome activation, and suppressed diabetes-accelerated atherosclerosis in apoE-/- mice
[101]	Ezetimibe (250 μg, 500 μg, 1 mg)	1 hIntra-nasal	Rat model middle cerebral artery occlusion	Improved infarct volume, neurological outcome Increased activation of AMPK, modulated oxidative stress, microglial activation and TXNIP/NLRP3 activation
[103]	SRI-37330	Po daily	STZ-mouse model and obesity-induced (db/db) diabetes	Inhibited glucagon secretion and function, reduced hepatic glucose production, and reversed hepatic steatosis
[105]	W2476, 200 mg/kg	Po daily	STZ- and HFD-obesity model	Inhibit TXNIP expression and restore beta-cell function, improve glucose level in STZ- and HFD-obesity model
[34]	GW0742 (25 μg/kg; intranasal)	1 h/ 24 h	Rat pups with hypoxic ischemia	GW0742 significantly reduced the activation of TXNIP/NLRP3 inflammasome, pro-inflammatory microglia

TXNIP: Thioredoxin interacting protein; NLRP3: NOD-like receptor pyrin domain containing 3; HFD: High fat diet; NMDA: N-methyl D-aspartate.

Since TXNIP modulates several of the essential metabolic and homeostatic pathways, there are challenges that can hinder the full development of TXNIP as a druggable target[71,95]. For instance, ablation of TXNIP at the systemic level carries a considerable risk for disrupting the physiological roles of TXNIP in regulating beta cell function, insulin release and regulating fatty acids metabolism[71,95]. Of note, targeting TXNIP may pose possible risks for sacrificing its proapoptotic action in the treatment of cancer[95], a disease state that is beyond the scope of this review. On the other hand, targeted delivery of specific TXNIP inhibitors in more confined organs (like the retina or the liver) might provide safer therapeutic opportunities. For example, intravitreal injection of TXNIP ASO prevented vision loss post-IR injury[33]. The protective effects of TXNIP ASO involved mitigation of TXNIP expression, inflammasome activation and secondary damage[33]. A novel oral form of small molecule SRI-37330 that shows benefit effects against the development of obesity and diabetes [103]. The drug has attenuated the formation of hepatic glucose and reversing steatohepatitis *via* the inhibition of TXNIP[103]. Finally, expanded pre-clinical studies for similar types of new drug molecules, followed by larger studies in subsequent stages of clinical development will remain key in ultimate evaluation of the efficacy *vs* the safety promise for TXNIP as a very attractive therapeutic target.

CONCLUSION

In summary, sterile inflammation is a central pathway that is involved in both physiological and pathological cellular processes to maintain homeostasis. TXNIP, a pro-oxidative, pro-inflammatory and pro-apoptotic protein that has been implicated in sterile inflammation. Evidence from literature showed that TXNIP is required for the activation but not necessarily expression of NLRP3-inflammasome in response to various stimuli as summarized in Table 3 for *in vivo* studies and Table 4 for *in vitro* studies. While there is no specific inhibitor for TXNIP, there is a long list of natural antioxidants and other drugs that could be repurposed are to modulate TXNIP expression (see Tables 1 and 2). Furthermore, potential specific small molecule

Table 3 Summary of the *in vivo* studies

Ref.	Duration of Studies	Insult	TXNIP	NLRP3	CASP-1	IL-1β	TNF-a	NFKB	Casp-3	NY	Other markers
Mohamed *et al*[2], 2015	Rat retina, 10 wk	HFD	+	+	+	+	+	+	+	+	Acellular capillaries
Coucha *et al*[11], 2017	Mouse retina, 8 wk	HFD	+ mRNA								ER-stress, miR17-5p
Mohamed *et al*[41], 2020	Mouse retina, 8 wk	HFD	+	-	-	+					Leukostasis, acellular capillaries
Mohamed *et al*[58], 2018	Mouse liver, 8 wk	HFD	+	+	+	+	Trend	+			TLR2 signal +, fibrosis
Elshaer *et al*[40], 2017	Mouse sk. Muscle, 8 wk	HFD	+	-	+	+				+	Systemic IL-1b, vascular recovery
Coucha *et al*[33], 2019	Mouse-retina, 1-3 d, 14 d	I/R	+ protein + mRNA	+	+	+	+				Acellular capillary, visual acuity
El-Azab *et al*[19], 2014	Mouse-retina, 1-d	NMDA	+			+	+		+	+	Acellular capillary, neurode -generation, ERG
Al-Gayyar *et al*[7], 2011	Rat-retina, 1-d	NMDA	+			+	+	+	+	+	Neurode -generation
Ishrat *et al* [80],2015	Mouse; Brain	Embolic -stroke	+	+	+	+	+		+	+	Neurological function, cerebral blood flow
Ismael *et al*[94], 2021	Mouse brain, 24 h	Stroke+ HG	+	=	+	+	+	+ trend			Hemorrhagic transformation
Wang *et al*[24], 2020	Rat brain, 7-d	Stroke	+	+	+	+		+			Pyroptosis, inflammation
Liu *et al* [97], 2020	Rat brain, 7 d	Stroke	+ mRNA + protein	+	-	+	+				Neurological deficit, inflamm
Gamdzyk *et al*[34], 2020	Rat pups brain, 24 h	Hyp-oxia	+	+	+	+					Microglial activation, TXNIP
Ding *et al* [21], 2016	Rat brain, 14 d	Throm-bosis	+	+	+	+				+	ER- stress neural pyroptosis
Yin *et al* [29], 2021	Rat brain, 72 h	Stroke	+	+	+	+					Microglial activation, ROS
Tian *et al* [81], 2012	Rat brain, 24 h	Stroke	+								MAPK activa -tion and Nrf2
Guo *et al* [3], 2018	Mice, 72 h	Stroke	+	+	+ active	+					Elevated ER stress, neurode -generation
Hou *et al* [102], 2018	Rat brain, 24 h	Stroke	+	+		+					Nrf2 and NL -RP3 through TXNIP

Cao *et al* [43], 2016	Mice brain, 24 h	Stroke	+	+		+					Neuro. deficit, BBB damage
Guo *et al* [3], 2016	Rat brain, 24 h	HG + stroke	+	+	+	+					Hemorrhagic transformation
Hua *et al* [83], 2015	Rat brain, 24 h	Stroke	+					+		+	Neurological deficit
Wang *et al*[98], 2015	Rat brain	Stroke	+	+	+	+					PPARγ, nega -tive regulator of TXNIP
Li *et al* [20], 2015	Rat brain, 24 h	Stroke	+	+	+	+					ER stress me -diates TXNIP activation

HFD: High fat diet; TXNIP: Thioredoxin interacting protein; NLRP3: NOD-like receptor pyrin domain containing 3; ROS: Reactive oxygen species; ER: Endoplasmic reticulum; BBB: Blood brain barrier.

Table 4 Summary of the *in vitro* Studies

Ref.	Cell type	Insult	TXNIP	NLRP3	CASP-1	IL-1β	TNF-a	NFKB	Casp-3	Other markers
Mohamed *et al* [2], 2015	EC	Palmitate	+	+	+	+			+	IL1-b in cell lysate and CM Adhesion Molecules
Mohamed *et al* [41], 2020	EC	TXNIP++		+	trend	+	+			Adhesion Molecules
Coucha *et al*[11], 2017	Muller	Palmitate	+ protein + mRNA	trend	trend	+				IL1-b in cell lysate
Coucha *et al*[33], 2019	Muller	Hypoxia	+ mRNA	trend	+	+				IL-1b in cell lysate
El-Azab *et al*[19], 2014		NMDA	+	+	+	+	+			IL1-b in CM
Gamdzyk *et al* [34], 2020	P12 cells	OGD	+			+	+			Cell death, miR-17-5p
Tian *et al*[81], 2012	Primary rat cortical neuron	OGD	+							Oxidative stress and activation of MAPK
Liu *et al*[97], 2020	Primary rat neurons	OGD	+	+	+	+				TXNIP NLRP3
Guo *et al*[3], 2018	SH-SY-5Y cells	OGD		+	+ activity	+				Activation of ER stress
Cao *et al*[43], 2016	bEnd.3	OGD	+	+	+					MAPK activation, EC-damage

TXNIP: Thioredoxin interacting protein; NLRP3: NOD-like receptor pyrin domain containing 3; ER: Endoplasmic reticulum; TNF-α: Tumor necrosis factor alpha; NMDA: N-methyl D-aspartate.

inhibitors are currently under development and will provide a much-needed treatment for TXNIP-associated disease states.

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MINIREVIEWS

Progress and prospect of stem cell therapy for diabetic erectile dysfunction

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Abstract

Diabetic erectile dysfunction (DED) is a common complication of diabetes mellitus, significantly impairing the quality of life of patients. The conventional clinical treatment still has limitations. Stem cells (SCs), as a type of cells with multidirectional or directional differentiation capability and sustainable self-renewal potential, are widely used in regenerative medicine and tissue engineering. With the continuous update of regenerative medicine theory and the success of animal experiments, SCs as a treatment for male erectile dysfunction, especially DED, have attracted widespread attention because of curable possibility. This review focus on the current progress in the clinical application of SC treatment for DED. Moreover, we summarize the development prospects of SCs in the field of DMED therapy.

Key Words: Stem cell; Diabetic erectile dysfunction; Extracellular vesicles; Gene editing

Core Tip: Diabetic erectile dysfunction (DED) is a common complication of diabetes. Conventional clinical treatments like phosphodiesterase-5 inhibitors, intracavernosal vasoactive drug injection, negative pressure suction device, and low-intensity shock wave therapy are the conventional methods of clinical treatment. However, none of the above therapies has the potential of curing. Stem cell therapy is currently the only possible cure for DED and can avoid the potential complications of conventional therapies. Here we discuss the current role and progress of stem cells in the treatment

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INTRODUCTION

Diabetes mellitus (DM) is a chronic, non-communicable disease caused by genetic, environmental, and other factors. Over the past three decades, the number of people with DM has more than doubled globally, making it one of the most important public health challenges worldwide[1]. Erectile dysfunction, defined as the inability to achieve and/or maintain an erection sufficient to permit satisfactory sexual intercourse [2], is a major complication of DM[3]. Diabetes is considered the main risk factor for the development of erectile dysfunction, and since the 1970s, the association between diabetes and the development of erectile dysfunction has been documented both in animal models and humans[4]. Increasing attention focuses on erectile dysfunction in men with diabetes due to its multifactorial pathophysiology and the concurrence of the same components as vasculopathy, neuropathy, and depression[5]. Although oral phosphodiesterase-5 inhibitors (PDE5Is) represent as a successful first-line therapy, a considerable proportion of men do not respond to oral PDE5Is[6-8]. Other conventional therapeutics for ED consist of oral medications, intracavernosal injections, vacuum erection devices, and penile implants. However, due to the lack of high quality evidence of efficacy and safety, poor patient compliance, and poor treatment outcomes, the above treatment regimens are still controversial in clinical application[9-13]. Recent 20 years have witnessed the progress of stem cell therapy (SCT), and it is expected to be an alternative option in the treatment of diabetic erectile dysfunctions (DED) and replace the current conventional treatment options.

STEM CELL THERAPEUTIC POTENTIAL AND CLASSIFICATION

The term ‘stem cells’ encompasses various cells with self-renewal and differentiation abilities, and many of them can potentially be used therapeutically[14,15]. In addition to differentiation into mature tissue cells, stem cells can also play a therapeutic role due to their chemotaxis, anti-inflammatory, regenerative, angiogenic, and anti-apoptotic properties[16-21]. A large number of regenerative medicine studies have proved the therapeutic potential of stem cells[22].

There are two major types of stem cells, embryonic stem cells (ESCs) and adult stem cells (ASCs)[14]. ESCs are derived from the inner cell mass of preimplantation embryos. Limited by biological and medical ethics, the clinical application of ESCs in the treatment of ED is restricted[23]. ASCs are ethical and easier to obtain from the host. Therefore, ASCs derived from patients themselves have been favored by researchers in recent years[24-31]. At present, many preclinical studies have used various types of ASCs to confirm the conclusion of improving erectile function, including bone marrow-derived mesenchymal stem cells (BMSCs)[32], adipose-derived stem cells (ADSCs)[33], neuro-derived stem cells[34], mesenchymal stem cell (MSCs)[35] , and urine-derived stem cells (USCs)[36,37]. They are characterized by the narrowest differentiation capabilities and a special property of dividing repeatedly, making them a promising candidate for therapeutic use in regenerative medicine.

In 2006, Takahashi and Yamanaka[38] reported a method to reprogram multipotent ASCs to the pluripotent state. Retrovirus-mediated transduction of mouse fibroblasts with four transcription factors (Oct-3/4, Sox2, KLF4, and c-Myc) could induce the fibroblasts to become pluripotent. This new form of stem cells is named induced pluripotent stem cells (iPSCs)[39]. One year later, the experiment also succeeded with human cells[40]. iPSCs opened a new field in stem cell research, because they can proliferate indefinitely and differentiate into any kind of cell with unlimited sources. iPSCs bypass the need for embryos in SCT, because they are obtained from the host patient’s own cells, and they are autologous and no longer generate any risk of

immune rejection[41,42] (Table 1).

STEM CELL THERAPY IN DIABETIC ERECTILE DYSFUNCTION MODELS

Stem cell-based therapies have been investigated in the field of DED. The overall aim is to repair the underlying corpus cavernosum cellular damage. Yang *et al*[43] injected ADSCs and endothelial progenitor cells (EPC) alone or in combination in DMED rat models and found that on the 28th day after injection, the mean arterial pressure (MAP) ratio were significantly higher in the ADSC group, EPC group, and ADSC combined with EPC group than in the DED group, and in the ADSC combined with EPC group than in the DED group, ADSC group, and EPC group ($P < 0.05$). It is revealed that both ADSCs and EPCS can improve erectile function, and the combined transplantation of ADSCs and EPC can synergistically improve the endothelial function in DMED rats. The therapeutic effect is better than the treatment with ADSCs or EPCs alone. Zhang *et al*[44] explored the therapeutic mechanism of USCs, using advanced glycation end products to treat rat cavernous endothelial cells to simulate a diabetic environment. Then, the two cells were co-cultured to evaluate the protective effect of USCs *in vitro*. Finally, they concluded that autophagy dysfunction is related to cavernous endothelial function and erectile dysfunction in DMED rats. In addition, USCs can up-regulate the autophagy activity of sponge endothelial cells, which improves endothelial sponge dysfunction and is ultimately expected to improve erectile dysfunction caused by diabetes. These stem cell-based preclinical studies have emphasized the improvement of the erectile function of DED animals after stem cell treatment and explained the underlying mechanism.

Some researchers also explored the combination of stem cells with conventional therapy. Liu *et al*[45] conducted related studies and used hepatocyte growth factor (HGF) combined with ADSCs to treat DMED rat models. One month later, treatment with ADSCs alone can significantly improve the erectile function of diabetic rats. However, the combined application with HGF has a more significant effect, making the erectile function even close to the normal level. It shows that HGF can significantly enhance the beneficial effects of ADSCs on the erectile function of diabetic rats, and its mechanism may be closely related to the down-regulation of the TGF-β1-Smad signaling pathway. Shan *et al*[46] combined BMSCs and low energy shock wave (LESWT) to treat a DMED rat model. On days 1, 3, and 28 after BMSC transplantation, they observed the number of surviving BMSCs in the cavernous body. The result showed that combined application of LESWT and BMSC transplantation maintained more BMSCs in cavernous body and improved the erectile function of diabetic rats more effectively than LESWT or BMSC transplantation alone. This may promote the expression of stromal cell-derived factor-1 in the diabetic cavernous tissue, thus promoting angiogenesis. In another study, Jung *et al*[47] used an oxygen-releasing hollow microparticle (HP) system with human BMSC to attempt to overcome certain limitations of SCT, including insufficient nutrient and oxygen supplies for transplanted stem cells. The results demonstrated that a stem cell/oxygen-releasing HP hybrid system could further improve erectile function, cyclic guanosine monophosphate level, and nitric oxide synthase (NOS) level in a bilateral cavernous nerve injury rat model through prolonged stem cell survival. Their data suggest that a stem cell/oxygen-releasing HP system is a promising clinical treatment option for postprostatectomy erectile dysfunction. Furthermore, this system may be relevant in different disease therapies and regenerative medicine.

Next-generation stem cells may serve as therapeutic agents by overexpressing neurotrophic factors, chemokines, anti-inflammatory molecules, angiogenic factors, and other molecules that aid in recovery and endogenous repair of tissues[48]. Modified stem cells may repair the damaged signaling pathway in DED that causes endothelial dysfunction, confirmed by various studies. We have prepared ADSCs expressing large amounts of vascular endothelial growth factor (VEGF) through virus transfection[49], and found that these ADSCs could stimulate endothelial function, increase the content of smooth muscle and pericytes, and significantly improve the erectile function of DED rats. Kizub *et al*[50] also conducted similar experiments in MSCs, and MSCs expressing VEGF also showed the same advantages. In addition, ADSC injection combined with insulin therapy can produce better therapeutic effects in a DMED rat model[51]. The above experiments show that stem cell injection can help restore the erection and other physiological functions of the penis.

The use of stem cell-derived extracellular vesicles (EVs) avoids the risks of any undesired stem cell growth or potential for tumor-promoting effects, which has been

Table 1 Characteristics of the articles published

Ref.	Treatment factor	Method/transfer/period	Evidence	Outcome
[42]	Rat ADSCs/EPCs/combined ADSCs and EPCs	Injection with ADSCs (1×10^6), EPCs (1×10^6), and ADSCs/EPCs ($0.5 \times 10^6/0.5 \times 10^6$)	ADSC/EPC group displayed more significantly enhanced ICP and ICP/MAP than the DED or ADSC or EPC group ($P < 0.05$). (After 28 d)	Combined transplantation of ADSCs and EPCs has a synergic effect in repairing the endothelial function of DED rats
[43]	Human USCs	Coculture of USCs and rat corpus (CCECs) treated with AGEs/injection with USCs (1×10^6) in rat DED model	USCs protected CCECs from AGE-induced autophagic dysfunction and cellular damage/DED rats showed lower ratio of ICP/MAP, reduced expression of endothelial markers, and fewer autophagic vacuoles in the cavernosal endothelium	Intracavernous injection of USCs up-regulates autophagic activity in the cavernosal endothelium
[44]	Combined HGF and ADSCs	Injection with ADSCs alone or combined with HGF	Significant down-regulation of TGFβ1-Smad signaling	HGF enhance the beneficial effects of ADSCs on DED through down-regulation of the TGFb1-Smad signaling pathway
[45]	Combined BMSCs and LESWT	1/3 d after BMSC transplantation, the number of surviving BMSCs in the cavernous body was counted	LESWT favored the survival of transplanted BMSCs in the cavernous body, increased stromal cell-derived factor-1, and enhanced angiogenesis	Combined LESWT and BMSC improve the erectile function of DED rats more effectively than either alone
[46]	HP and human BMSCs	hBMSC (1×10^6 cells/mL) seeded on oxygen-saturated HPs	cGMP and NOS levels rose through prolonged stem cell survival	Stem cell/oxygen-releasing HP hybrid system could further improve erectile function
[48]	ADSCs expressing VEGF	ADSCs expressing large amounts of VEGF through virus transfection	VEGF-ADSCs stimulated endothelial function, and increased the content of smooth muscle and pericytes	VEGF-ADSCs improve erectile function
[57]	ADSC EVs	ADSC EVs through ultracentrifugation and treatment of DED rat model through ICI	ADSC-derived EXOs and ADSCs increased the ratio of intracavernous pressure	ADSC-derived EXOs and ADSCs are able to rescue corpus cavernosum endothelial and smooth muscle cells by inhibiting apoptosis
	USC EVs	USC EVs through ultracentrifugation and treatment of DED rat model through ICI	USC-EVS enhanced the expression of endothelial cell markers in DED rats, reduced collagen deposition, and improved neurogenic erectile response through pro-angiogenic miRNAs	USC-EV transplantation can ameliorate DED in rats *via* mechanisms that may involve the delivery of proangiogenic miRNAs

ADSCs: Adipose tissue-derived stem cells; EPCs: Endothelial progenitor cells; ICP: Intracavernous pressure; MAP: Mean arterial pressure; DED: Diabetic erectile dysfunction; CCECs: Cavernosal vascular endothelial cells; AGEs: Glycation end products; HGF: Hepatocyte growth factor; BMSCs: Bone marrow mesenchymal stem cells; LESWT: Low energy shock wave; HPs: Oxygen-releasing hollow microparticles; EVs: Extracellular vesicles; USCs: Urine derived stem cells.

noted for MSCs in certain situations[52]. At present, stem cells are widely used as biomaterials for the regeneration of tissue defects. The treatment mechanism is based on the differentiation into specific target cells after implantation and the paracrine release effect of EVs[53,54]. EVs secreted by stem cells are believed to play a major role in the treatment of DED. The paracrine release effect of EVs means that the exosomes after injection can repair damaged tissues by delivering cytoprotective molecules, anti-inflammatory molecules, and anti-apoptotic molecules. Many studies support this view, proposing the direct use of stem cell EV therapy to restore the function of damaged organs and tissues and achieve the direction of cell-free SCT[55-57]. Chen *et al*[58] acquired ADSC EVs through ultracentrifugation and treated a DED rat model through intracavernous injection (ICI). The results show that, like ADSCs, exosomes derived from ADSCs can increase the number of sponge endothelial cells and smooth muscle cells by inhibiting cell apoptosis, thereby promoting the recovery of erectile function in type 2 diabetic rats. Ouyang *et al* used the same method to obtain the EVs of USCs[59]. Their results showed that USC-EVs enhance the expression of endothelial cell markers in DED rats, reduce collagen deposition, and improve neurogenic erectile response. The transport of pro-angiogenic microRNAs secreted by EVs may play an important role in endothelial repair. EVs secreted from engineered stem cells are considered an alternative cell-free next-generation approach for delivering vascular repair factors, anti-apoptosis agents, and other tissue-repairing agents.

HUMAN CLINICAL TRIALS OF SCT

Although SCT has been proved effective in multiple animal ED models, there are only a few human clinical trials of SCT, thus we include clinical trials of other types of ED. Bahk *et al*[60] recruited seven patients with type 2 DED in the study, all of whom were insensitive to drug treatment for more than 6 mo and needed to wait for penile prosthesis implantation. The researchers injected 1.5 × 1.7 human umbilical cord blood cells into the cavernous body and recorded their International Erectile Function Index-5, Sexual Encounter Profile, Global Assessment Question, erectile diary, blood glucose diary, and medication dosage. The results showed that three of the six patients recovered the morning erection reaction after 1 mo, and it rose to five after 3 mo. At the 6 mo follow-up, two of the patients completed their sexual life and reached orgasm under the premise of oral PDE5i. After 9 mo, all five patients felt an increase in libido. During the entire follow-up process, two patients quit to implant a penile prosthesis, and four had recovered erectile function. And no side effect of SCT was reported. Levy *et al*[61] injected placental matrix-MSCs to treat patients with ED into eight patients with ED who had failed to take oral medications, and MSC treatment was followed at 6 wk, 3 mo, and 6 mo to assess peak systolic velocity (PSV), end diastolic velocity, stretched penile length, penile width, and erectile function status based on the International Index of Erectile Function questionnaire. The results showed that at 6 wk, two patients developed spontaneous and maintained erections; at 3 mo, one patient can erect spontaneously. The average PSV values of patients at 3 and 6 mo were significantly higher than those before treatment. However, the measured end-diastolic speed changes, extended penis length, penis width, and erectile function international index score were not statistically significant. In a phase 1 clinical trial[62], 12 patients who had ED after radical resection of prostate cancer (drug treatment failed) were given ICI of BMSCs four times, and the injection dose of BMSCs was increased gradually (2×10^7, 2×10^8, 1×10^9, and 2×10^9). The results showed that 9 out of 12 patients exhibited significant improvement in erectile function under the premise of oral PDE5i. It was found that as the injection dose increased, the incidence of spontaneous erection increased, and the study did not report any serious adverse reactions after injection. In phase II clinical trial of the same study, six more patients were recruited, and the best dose (1×10^9) of BMSCs determined in phase 1 was injected. The results showed that the sexual satisfaction scores of 18 patients in the IIEF-5 index and the erectile function scores were significantly improved compared to those before treatment. However, the six patients who participated in the second stage had lower erectile function scores than the 12 patients who participated in the first stage. In the first phase, no prostate cancer recurrence was detected[63]. In the phase I clinical trial conducted by Demour *et al*[64] in 2018, human BMSCs were used for the first time to treat DED. They included four patients with DED who had failed medical treatment and injected the BMSCs extracted from the patients into the penile sponge twice. The patients' tolerability was evaluated immediately after injection and 24 h later. The effectiveness was evaluated by IIEF-5 and EHS scores 1 year later, and safety was evaluated 2 years later. The results showed that all patients tolerated the operation well, and there was no obvious adverse reaction. After 12 mo of follow-up, the patients' IIEF-15, EHS, erectile function, libido, sexual satisfaction, and overall satisfaction scores were significantly improved compared to those before treatment. Haahr *et al*[65] discussed the effectiveness and safety of ADRCs in the treatment of patients with ED after RP. The researchers injected 21 patients' own ADSCs into the cavernous body at one time, followed them for 1 year, and evaluated and recorded adverse events, IIEF-5 score, and EHS score. The results showed that no serious adverse events occurred during the follow-up process, but eight cases had reversible adverse reactions related to liposuction, including three cases of redness and swelling in the penis and five cases of mild hematoma in the abdomen, all of which occurred within a short period of time after treatment and subsided without any sequelae. Eight of the 21 participants (38%) recovered sufficient erectile function during the 12-mo observation period. These eight patients had poor erection assistance effects before receiving stem cell treatment. After treatment, three men could complete sexual intercourse without erectile assistance. Six of all participants had erectile dysfunction before RP, and these six patients did not recover their erectile function after receiving SCT.

DISSCUSSION

According to the current multiple preclinical studies and a small number of clinical studies for the treatment of organic ED with SCT, the effectiveness and safety of SCT are considerable, and it is also considered to be a very promising way to treat organic ED in the future. A meta-analysis included ten preclinical studies on SCT in ED rat models, with a total of 302 rats. The results showed that SCT improved erectile function in diabetic rats (standard mean difference = 4.03, 95% confidence interval: 3.22-4.84, $P < 0.001$)[66]. The contents of vascular smooth muscle and endothelial cells in the stem cell treatment group were significantly higher than those in the control group. The expression of endothelial NOS and neural NOS, smooth muscle/collagen ratio, and VEGF secretion were significantly increased. In addition, stem cell treatment can reduce apoptotic cells. Subpopulation analysis showed that modified stem cells were more effective than unmodified stem cells. These results suggested that SCT can significantly improve the erectile function of diabetic rats. Some specific modifications, especially the genetic modification of growth factors, can improve the effectiveness of SCT. SCT may become an effective strategy for the treatment of diabetic ED.

There are only nine clinical trials reporting the efficacy and safety of stem cell treatment in men with ED, and the trial sample size is less than 100 cases. Based on the results of each study, the overall penile hemodynamic and erectile function scores of patients are significantly improved. No major adverse reactions was observed.

After years of exploration in many preclinical trials, the current mechanism of SCT for organic ED has been clarified. There are two major hypotheses about SCT. First, SCT can repair and replace cavernous nerves. SC differentiate into cavernous nerve cells, smooth muscle cells, and endothelial cells[67]. Second, SCT provides penile tissue support through the paracrine effect of EVs, delivering proangiogenic, anti-inflammatory, anti-apoptotic, and anti-fibrotic properties[68]. Studies have shown that the release of paracrine repair cytokines is an endogenous mechanism independent of the differentiation of stem cells into different cell types[69].

Although SCT seems to have good prospects in the treatment of DED, there are still many unresolved challenges. First, there is still a lack of large-scale clinical studies to verify its effectiveness and safety. Although SCT has achieved great success in some animal experiments, due to the huge anatomy and physiology gap between animal DED model and human DED[70], it is difficult to clarify whether the successful results of animal experiments will still work in human DED due to the lack of high-quality and large-scale human trials of SCT. Most of existed clinical trials are phase I studies, with a small sample size and lack of blinding method, as well as ambiguous inclusion and exclusion criteria, and primary and secondary outcome indicators. The inclusion of negative studies makes these studies insufficient to provide convincing evidence.

Second, SCT may have safety issues. Although previous studies have not found serious complications after treatment, some studies reported that MSCs could penetrate into prostate cancer cells, including prostate tissue[71], and SCs may act as a tumor promoter to affect its occurrence and development[72].

Third, the cell source selection of SCT warrants further investigation. Existing studies have found that both autologous and foreign SCs can improve the erectile function in ED. As for the trade-off between the pros and cons of choosing autologous or allogeneic SCs, only one study has explored this issue, but it still does not directly compare the application of autologous cells and allogeneic cells[73]. It is believed that choosing autologous stem cells will bring obvious safety advantages and it is convenient to obtain materials because it can overcome the antigenic problem of allogeneic cell transplantation. However, recent studies have found that allogeneic ADSCs can induce a locally suppressed microenvironment by secreting intraoperative cytokines to regulate the function of natural killer cells and T cells, thereby avoiding allogeneic immune rejection[67]. Meanwhile, in another study on a rat model of acute myocardial infarction treated with SCs, it was found that allogeneic transplantation of MSCs only maintained their ability to overcome allogeneic immune rejection in a short period of time. As the treatment time prolonged, MSCs differentiated in myocardial tissue and lost their immune privilege status, thereby affecting the therapeutic effect [74]. Autologous stem cells are not suitable for all patients. For example, for elderly patients, the proliferation and differentiation ability of stem cells in the body decreases significantly as the body ages[75]. In addition, for cancer patients, the use of autologous stem cells for transplantation is prohibited[76].

Fourth, treatment methods have not yet been standardized. Multiple studies have demonstrated the presence of MSCs in the lung, immediately after injection[77-80]. However, the majority of cells are, however, cleared within the first days of treatment [81]. It is reported that less than 1% of stem cells injected intravenously in SCT actually

reached the target tissue, but the treatment effect was not affected[82]. Hence, there is a demand for retaining stem cells in the corpus cavernosum after ICI for ED therapy. ADSCs incubated with NanoShuttle were magnetic and maintained in the corpus cavernosum. This improved the adipose-derived SCT of ED in a CNI rat model[83]. Nevertheless, the cell type, the injection concentration, the course of treatment, and the evaluation endpoint of the treatment effect have not yet been determined.

Fifth, there are ethical issues. SCs constitute one of the most promising tools for regenerative medicine. Thus, it seems morally compelling to explore all the sources that might provide us with them. However, some of these sources, such as somatic cell nuclear transfer, embryo destruction, or even induced pluripotency obtained by reprogramming, have raised serious ethical issues[84]. For the use of cell, tissue, and stem-cell products in treating patients, the US Food and Drug Administration[85], the European Medicines Agency guidelines, and the International Society for Stem Cell Research have also developed or updated several specific guidelines for SCT with the help of stem cell experts from all around the world[86]. According to these guidelines, the most important topics of SCT related to ethical, legal, and social considerations of cell therapy include: (1) Manufacturing conditions and characterization of clinical-grade cells; (2) genetic material and confidential personal information; (3) informed consent; (4) genetic manipulation of the cells; and (5) intellectual property and patents, along with some other important issues[87].

CONCLUSION

SCT for DED has broad development prospects. However, many problems need to be solved to achieve an effective and safe clinical treatment plan. In addition to the need to further clarify its specific mechanism of action, effectiveness, and safety, it is also necessary to clarify the optimal treatment plan to solve the immunogenicity and heterogeneity of SCT, and improve its high cost and low efficiency in application. In the future, more phase II and phase III clinical trials are needed to make full preparations for the transformation of SC treatment of ED from preclinical to clinical trials and translate into clinical applications.

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MINIREVIEWS

Overcoming ischemia in the diabetic foot: Minimally invasive treatment options

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Abstract

As the global burden of diabetes is rapidly increasing, the incidence of diabetic foot ulcers is continuously increasing as the mean age of the world population increases and the obesity epidemic advances. A significant percentage of diabetic foot ulcers are caused by mixed micro and macro-vascular dysfunction leading to impaired perfusion of foot tissue. Left untreated, chronic limb-threatening ischemia has a poor prognosis and is correlated with limb loss and increased mortality; prompt treatment is required. In this review, the diagnostic challenges in diabetic foot disease are discussed and available data on minimally invasive treatment options such as endovascular revascularization, stem cells, and gene therapy are examined.

Key Words: Diabetic foot; Peripheral artery disease; Critical limb ischemia; Endovascular revascularization techniques; Gene and stem cells delivery; Hyperbaric oxygen treatment

Core Tip: Recognizing and promptly treating ischemia in patients with diabetic foot ulcers is essential for wound healing and limb salvage. A plethora of novel minimally invasive technologies and techniques are currently available, including dedicated peripheral angioplasty balloon catheters, drug-eluting stents, drug-coated balloons,

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angiosome-guided angioplasty, pedal arch angioplasty, and percutaneous deep vein arterialization, while research on gene and stem cell therapies is ongoing and initial data are deemed positive. Large, multicenter randomized trials specifically focused on optimizing endovascular treatment options for diabetic foot ulcers remain limited, and more high-quality, long-term, data are expected.

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INTRODUCTION

The global burden of diabetes mellitus (DM) has rapidly increased over the past decade, and many international scientific organizations now consider DM as the upcoming public health emergency of the 21st century, while health professionals and patients are becoming gradually aware of the gravity of diabetes-related complications [1]. Diabetes is the foremost cause of lower-limb loss worldwide. Every year, more than one million patients with DM suffer a lower limb amputation, and nearly every 20 s, an amputation is performed due to diabetic complications. Diabetic foot (DF) ulcers (DFU) are continuously becoming more frequent, and the incidence will further increase as the mean age of the world population increases and the obesity epidemic advances[1].

Moreover, diabetic patients are twice as likely to suffer from peripheral artery disease (PAD) in comparison with the non-diabetic population[2]. It has been also estimated that in middle and high-income countries, nearly half of patients with diabetes and foot ulceration suffer from underlying PAD and present with mixed neuroischemic type ulcers. On the contrary, neuropathic ulcers are less common and usually more frequent in lower-income countries[3,4]. Interestingly, in subjects with diabetes, PAD may remain undiagnosed before tissue loss, as patients may not experience any preceding clinical symptoms of PAD such as claudication or rest pain [5]. The pathophysiology of critical limb ischemia (CLI) involves chronic atheroma development, epithelial injury, and thrombus formation. This entity results in both lower limb micro and macro-vascular disease.

Established treatment options include open surgical and percutaneous endovascular revascularization techniques, while the experience gained from coronary interventions has also broadened peripheral endovascular capabilities with the use of drug-eluting stents (DES) and drug-coated balloons (DCB)[6]. For very small vessel disease, novel therapeutic options, at present under investigation, include gene and stem cell therapy aimed at local, targeted drug delivery triggering angiogenesis and vasculogenesis.

The purpose of this review is to present currently available minimally invasive interventions, for the management of ischemia in the diabetic foot.

DEFINITIONS AND PATHOPHYSIOLOGY

The first step in the treatment of DF-related ischemia is prompt differentiation between purely neuropathic and neuroischemic DFU. DFU is defined as a complicated pathology of infection, ulceration, or destruction of tissues of the foot linked to neuropathy and/or peripheral artery disease in the lower extremity of a patient with a history of DM[7]. The key components of diabetic foot pathophysiology are a triad: Neuropathy, angiopathy, and structural and/or gait abnormalities. Peripheral neuropathy is one of the major factors correlated to diabetic ulcerations. Due to the loss of this nociceptive mechanism, patients are incapable of appreciating local foot trauma. As a result, the foot is at high risk of trauma and ulceration, which could lead to amputation[9].

Types of ischemia in diabetic foot ulcers

Concomitant lower-limb arterial, atherosclerotic, steno-occlusive disease, PAD, is common in individuals with long-standing diabetes. Many mechanisms contribute to the development of PAD, particularly, endothelium dysfunction, arterial stiffness, thrombotic abnormalities, low-grade inflammation, advanced glycation end-products, and oxidative stress[10]. DFU are classified according to the underlying pathology in namely three categories: Neuropathic, ischemic, and neuroischemic. The ischemic component (PAD) is considered a form of macro-vascular complication and is positively associated with age, smoking, and other forms of macro-vascular complications, including hypertension and myocardial infarction, which increase the risk of cardiovascular death. On the other hand, peripheral neuropathy is a form of micro-vascular complication of diabetes. A mixed micro- and macro-vascular dysfunction results in neuroischemic disturbances where micro-vascular abnormalities impair perfusion of DF[3].

Diagnosis

Physical examination of the foot is essential. Meticulous inspection is advised for the identification of neuropathic changes such as dry skin, cracks, malformations, callus, foot structure abnormalities, ulceration, and nail lesions. Major ischemia can be suspected in the presence of hair loss on the foot's dorsal aspect and should be assessed by careful examination of peripheral pulses (common femoral, popliteal, distal foot arteries). Sensory neuropathy can be tested using monofilaments, biothesiometry or cotton wool, pinprick, and vibration sense for light touch[11]. A portable pocket doppler device can confirm the presence of pulses and quantify the arterial supply of the foot. The ankle-brachial index (ABI) should also be measured. However, ABI is not a reliable test for subjects with diabetes as they may present incompressible calf vessels due to significant Monckeberg medial calcific sclerosis, and therefore false-negative results are very common. The American Diabetes Association recommends that all people with diabetes and a foot wound should have pedal perfusion assessed by ABI and either toe-brachial index (TBI) or transcutaneous oxygen pressure ($TcPO_2$)[12]. The 2019 Global vascular guidelines on the management of chronic limb-threatening ischemia, endorsed by the Society for Vascular Surgery, European Society for Vascular Surgery, and World Federation of Vascular Societies, does not suggest computed tomography angiography for the detailed visualization of infrapopliteal disease and recommends that patients with suspected chronic limb-threatening ischemia who are suitable candidates for limb salvage should not be denied revascularization without first undergoing complete diagnostic angiography including the ankle and foot[13].

Neuroischemic wounds are more arduous to heal than nonischemic and are correlated with higher rates of amputation and mortality. Thus, prompt revascularization for the treatment of mixed-neuroischemic DFU is today considered a medical emergency and should be performed using surgical and/or endovascular techniques, following a multidisciplinary team, case-sensitive decision[13,14].

Classification of ischemia

The Fontaine and Rutherford-Becker classification systems have become obsolete for everyday clinical practice, as a wide spectrum of underlying factors such as degree of arterial disease, ulcer type, anatomical location and extent, presence, and severity of infection have been highly correlated to limb salvage in patients suffering from DFU. To address the need for a more accurate wound description, the Society for Vascular Surgery Lower Extremity Guidelines Committee recommended in 2014 a new classification system, based on three major factors: Wound, ischemia, and foot infection (WIfI). The WIfI classification system epitomizes a synthesis of many formerly published classification schemes and merges systems focused only on DFU or pure ischemia models. A brief description of the WIfI classification is presented in Table 1 [15].

MINIMALLY INVASIVE TREATMENT OPTIONS

Endovascular revascularization

Due to very limited level Ia evidence comparing open *vs* endovascular revascularization, in subjects with DM the decision to proceed with open surgical bypass or endovascular treatment is case-sensitive and should be discussed in the ambit of a multi-disciplinary team meeting involving vascular surgeons, interventional

Table 1 Wound Ischemia and foot Infection score

Score	Wound	Ischemia (Toe pressure $TcPO_2$)	Foot infection
0	No ulcer and no gangrene	60 mmHg	Uninfected
1	Small ulcer no gangrene	40-59 mmHg	Mild (< 2 cm cellulitis)
2	Deep ulcer and gangrene limited to toes	30-39 mmHg	Moderate (> 2 cm cellulitis/purulence)
3	Extensive ulcer or extensive gangrene	< 30 mmHg	Severe (systematic response/sepsis)

$TcPO_2$: Transcutaneous oxygen pressure.

radiologists, and diabetologists. Factors influencing treatment choice include patient's age, comorbidities, surgical risk, location, extent of arterial disease, DFU characteristics, the availability of healthy vein for distal bypass, and local expertise. Endovascular treatment is often preferred over surgery (debridement, lower limb amputation, skin drafting, incision-drainage, sequestrectomy) in the presence of severe comorbidities and disease of the pedal outflow vessels. On the other hand, endovascular treatment should be conducted in large-volume dedicated centers by experienced hands[16].

In subjects with DM, atherosclerosis is more prevalent in the infrapopliteal arteries; however, concomitant femoropopliteal arterial disease is also common, while iliac artery disease, especially isolated, is less frequent, but not rare[17]. Therefore, multilevel revascularization of various arterial segments, with variable lumen diameters and different histology, is commonly required.

Significant advantages of minimal invasive treatment options over open surgical repair include less hospital stay and decreased periprocedural morbidity and mortality, especially in critically ill patients and those at high surgical risk. In the vast majority of cases general anesthesia is not required, and revascularization can be obtained using local anesthesia and mild conscious sedation. Moreover, endovascular methods represent the only option in patients with significant pedal arch arterial disease in which surgery is not an option for technical reasons[13,16,17].

Plain balloon angioplasty

Plain balloon angioplasty (POBA) remains the first-line endovascular treatment option in long infrapopliteal lesions, typically noted in patients with DM, although studies investigating POBA in exclusively diabetic populations are scarce. Starting from 2000, several studies have documented a high immediate technical success rate ranging between 80%-100% and a satisfactory (up to 80%) limb salvage rate at the 2-year follow-up in mixed diabetic and non-diabetic populations. Since 2005, the BASIL randomized trial remains the only randomized comparison of open surgical bypass *vs* POBA. Of 452 patients (42% with DM) presenting to 27 United Kingdom hospitals, 228 were randomly assigned to a bypass surgery-first and 224 to a balloon angioplasty-first revascularization treatment. Follow-up period was set for at least 3 years. Patients with infrapopliteal disease (with or without femoropopliteal disease) demonstrated similar amputation-free and overall survival, following vein bypass surgery or endovascular treatment[18]. Notable to mention, Faglia *et al*[19] published a population based cohort study with 292 diabetic patients with CLI according to the TransAtlantic Inter-Society Consensus II recommendations. Researchers report that angioplasty for diabetic patients with type D and/or long infrapopliteal lesions without good run-off at the foot and/or high surgical risk achieved high revascularization rates as well as less amputation rates[19].

In 2008, Romiti *et al*[20] published a meta-analysis of 30 studies (2557 patients, 2.653 limbs) to assess the mid-term outcomes of infrapopliteal POBA in patients with CLI and compared results with a meta-analysis of popliteal-to-distal vein bypass. Although both treatment modalities resulted in similar limb salvage rates, significantly lower 1-year patency rates were noted for POBA (48.6% ± 8.0% *vs* 72.3% ± 2.7%). The 30-d mortality and complication rates were significantly higher for infrainguinal bypass[20]. However, it should be noted that only two studies included in this meta-analysis investigated only diabetic patients, while the conclusions may not be reliable due to the methodological limitations of this review.

According to the existing literature data, the main disadvantage of POBA remains the development of neointimal hyperplasia, resulting in short-term restenosis, low patency rates, and clinical relapse, requiring more reintervention to sustain clinical

outcomes. To overcome the limitation of restenosis following POBA, several new technologies were investigated. In 2010, Cryoplasty was compared with POBA in a single-center randomized trial that included 50 diabetic patients with femoropopliteal disease. The Cryoplasty balloon catheter (Boston Scientific, Boston, MA, United States) uses low-temperature angioplasty to induce smooth muscle cell apoptosis and reduce neointimal hyperplasia. However, at the 3-year follow-up there were no significant differences to patients' survival and lower limb salvage, while lower primary patency and more repeat procedures due to clinical relapse were observed in the Cryoplasty subgroup[21].

The latest comparative studies also suggest that endovascular treatment demonstrates a similar limb salvage rate to open bypass. Specifically, in 2016 Patel *et al*[22] reported outcomes of a large retrospective, controlled study using propensity score matching to compare POBA with distal bypass surgery for the treatment of CLI. The study included 243 patients (with DM: 48.8% in the surgical group and 55.2% in the endovascular group in the propensity score-matched groups), and similar limb salvage rates were noted at 1-year follow-up (94.2% endovascular *vs* 90.4% surgery). However, at 1-year, primary (54.4% *vs* 51.4%), assisted (77.5 *vs* 62.7%), and secondary (84.4% *vs* 65.8%) patency rates were significantly better following open surgery. On the other hand, overall complications and length of hospital stay were significantly lower following endovascular treatment. Interestingly, according to binary logistic regression analysis, DM was identified as a preoperative factor favoring bypass surgery as the treatment choice[22].

Drug-eluting stents and drug-coated balloons

Following the establishment of the long-term efficacy of DES in the treatment of coronary disease, the use of infrapopliteal DES has been investigated in retrospective analysis providing optimistic initial results that were further validated by multicenter randomized clinical trials (RCTs). Scheinert *et al*[23] published the "ACHILLES" multicenter RCT (200 patients; 64% with DM), which was the first-ever designed to investigate the efficacy and safety of a balloon-expandable, sirolimus-eluting stent compared to POBA in patients with symptomatic infrapopliteal arterial disease up to 90 mm in length. At 1-year follow-up, lower angiographic restenosis rates (22.4% *vs* 41.9%, $P = 0.019$), as well as superior vessel patency (75.0% *vs* 57.1%, $P = 0.025$), were noted in the sirolimus-eluting stent group, while similar death, repeat revascularization, and index-limb amputation rates were reported[23]. Additionally, two multicenter RCTs produced similar outcomes favoring DES over bare-metal stents in short- to medium-length infrapopliteal lesions, and one multicenter RCT demonstrated the long-term safety and efficacy of infrapopliteal paclitaxel-eluting stents over POBA[24,25].

Long-term, 10-year clinical results of infrapopliteal DES in an exclusively diabetic population are also available from a single-center retrospective study published in 2015. In total 214 patients (311 limbs, 562 arteries, 679 lesions) with DM and CLI were treated. At the 1-, 5-, and 10-year follow-up, survival and amputation-free rates were 90.8%, 55.5%, and 36.2%, and 94.9%,90.4%, and 90.4%, respectively, while target limb reintervention-free rates were 79.7%, 55.2%, and 49.7%, at 1, 5, and 10 years. Long-standing diabetes, concomitant coronary artery disease, and dialysis were identified as independent predictors of decreased survival[26]. However, limitations of infrapopliteal DES use include the presence of a continuous mechanical stimulus of the vessel wall eventually leading to restenosis, even in the long-term, the increased cost for the treatment of long lesions where multiple stents are required, and stent fractures occurring in specific various locations such as the distal below ankle arterial segments and the pedal arch[27].

To overcome such issues, DCB have emerged as a promising technology developed to overcome the limitations of standard balloon angioplasty and stenting. Specially designed paclitaxel-coatings have been developed to deliver a single dose of the cytotoxic agent paclitaxel to inhibit neointimal growth of vascular smooth muscle cells and prevent restenosis. The majority of multicenter RCTs investigated patients with femoropopliteal artery lesions suffering from intermittent claudication without tissue loss have proven the superiority of paclitaxel-coated balloons (PCBs) in late lumen loss, binary restenosis, and freedom from target lesion revascularization (TLR), providing a sufficient level of evidence to support equivalent or favorable mid-to-long-term outcomes for PCBs in comparison to POBA[28].

In a meta-analysis of randomized trials published in 2016 including 1609 patients (1403 subjects with claudication and 206 with CLI), high-quality evidence demonstrated a significant superiority of PCBs in reducing late lumen loss (LLL) [mean difference -0.89 mm; 95% confidence interval (CI): -1.14 to -0.64], less binary

restenosis (relative risk 0.47; 95%CI: 0.37 to 0.61), and re-interventions (relative risk 0.33; 95%CI: 0.22 to 0.49)[29].

In 2019, long-term 5-year outcomes from a multicenter RCT investigating a 3 μm/mm^2 PCB for femoropopliteal lesions demonstrated a sustained treatment effect with less re-interventions due to clinical relapse compared to POBA (target lesion revascularization 74.5% *vs* 65.3%, $P = 0.02$)[30]. However, in terms of clinical endpoints more specific for DF disease and ischemia such as wound healing, time to wound healing, and limb salvage, the superiority of PCBs over standard POBA has not been proven, as data remain limited and contradictive, especially for patients suffering from infrapopliteal disease.

More recently, data from three more RCTs investigating two different PCBs were made available with contradictive outcomes. The ACOART-BTK single-center RCT randomized 105 patients (nearly all diabetics) with CLI, and outcomes in the PCB group were superior to those in the POBA group for LLL, restenosis, and re-interventions at 6 mo follow-up. Most importantly, healing time, which is a highly significant clinical endpoint for the diabetic population under investigation, was also significantly improved in the PCB group (5.2 ± 2.7 mo *vs* 7.7 ± 3.9 mo, $P = 0.005$), while complete wound healing rate at 1 year was nearly significant in the PCB group (89.4% *vs* 74.5%, $P = 0.05$). Moreover, no major amputations were noted at the 1-year follow-up in both groups[31].

Lately, Del Giudice *et al*[31] conducted a prospective single-center cohort study that assessed the safety and efficacy of a new generation low-dose DCB with a reduced crystalline structure to treat below the knee (BTK) lesions in patients with CLI. To be more specific, immediate technical success was 97% (29/30), and primary safety outcome parameter was 94% (28/30). Angiographic follow-up was available in 20 patients. Results demonstrated primary angiographic patency 57% (12/21 lesions) and LLL 0.99 ± 0.68 mm at 6 mo. Moreover, freedom from TLR was 89% at 12 mo, and the rate of ulcer healing was 76% at 12 mo. Thus, ranger DCB balloons documented a positive trend with good safety outcome parameters for the treatment of CLI patients [31]. On the other hand, data from the larger multicenter Lutonix BTK RCT that randomized 442 patients (287 in group PCB and 155 group POBA) were not analogous, as the PCB under investigation failed to demonstrate superiority compared to POBA [32]. Similarly, outcomes of the multicenter, IN.PACT BTK randomized study to assess safety and efficacy of IN.PACT 014 *vs* PTA (50 CLI patients; 74% diabetics) reported no significant difference in LLL and re-intervention rate at 9-mo follow-up, although LLL was numerically lower in the PCB group[33].

However, a wide range of variability in study design, eligibility criteria, and outcome endpoints among RCTs was noted. Therefore, currently, there is no up-to-date available high-quality evidence to support the superiority of PCBs over POBA in reducing major amputations, and long-term randomized data are still in scarcity[34].

Moreover, significant safety issues have been raised following the publication of two meta-analyses of RCT that have reported an increased risk of death following the use of paclitaxel-eluting stents and PCBs in femoropopliteal lesions and decreased amputation-free survival following PCB use in the infrapopliteal arteries[35].

Nevertheless, the subject remains controversial, as following these findings, several large retrospective "real-life" studies have not confirmed these results. As available RCTs are contradictive and safety issues have been raised, the use of PCBs in infrapopliteal disease remains controversial, and further multicenter RCTs are required to support their use and safety in every-day clinical practice.

Pedal arch angioplasty and the angiosome approach for wound healing

A significant subgroup of diabetic patients with advanced PAD, especially those with concomitant end-stage renal disease, suffer from a diffuse steno-occlusive disease of the infrapopliteal and distal plantar vessels[36]. The main technical advantages of endovascular treatment over open bypass surgery include the possibility of revascularizing more than one infrapopliteal artery and, most importantly, treating outflow plantar artery disease reconstituting the pedal arch (arch-plasty), which is not amenable to surgical reconstruction[37]. Following revascularization of blood flow to the ischemic tissue, adequate blood reperfusion is established, relieving ischemic symptoms and promoting wound healing[38]. A 2009 landmark study by Manzi *et al* [39] compared infrapopliteal angioplasty with or without pedal arch angioplasty. This study referred to the pedal-plantar loop technique. The authors retrospectively analyzed outcomes following the recanalization of the pedal and plantar arteries and their anatomical anastomosis in 135 patients, aimed at the restoration of a direct arterial in-flow from both anterior and posterior tibial vessels (the pedal-plantar loop technique; first reported by Fusaro *et al*[40], in 2007). The acute success of the

technique was 85%, clinical improvement was maintained after a mean follow-up of 12 mo, while a significant improvement of $TCpO_2$ at 15 d was noted in the group with successful plantar arteries revascularization[39].

In 2017, the first large-scale multicenter retrospective analysis was published. The Retrospective Analysis for the Clinical Impact of Pedal Artery Revascularization Versus Non-Revascularization Strategy for Patients With Critical Limb Ischemia retrospective registry investigated a total of 257 CLI patients (with 187 or 72.8% diabetic patients) separated into two groups based on additional pedal angioplasty (n = 140) or not (n = 117). Wound healing (57.5% *vs* 37.3%, P = 0.003) and time to wound healing (211 d *vs* 365 d, P = 0.008) were notably better in the pedal angioplasty group compared to the no-pedal angioplasty group[41].

In 2019, a meta-analysis of below-the-ankle angioplasty (BTA) (10 studies, with 478 patients and 524 legs) was published by Huizing *et al*[42]. Pooled 1-year limb salvage and amputation-free survival rates were 92% and 78%, respectively, while no statistically significant difference was detected in these clinical endpoints following additional BTA angioplasty compared to standard infrapopliteal angioplasty only. However, the wound healing rate was superior when additional BTA angioplasty was performed, while for more severe pedal artery disease, wound healing results were also superior after BTA angioplasty. Notably, complete wound healing and time-to-wound healing are highly significant endpoints for the specific DFU population, as these correlate with the quality of life, hospitalization time, frequency of hospital visits, and, eventually, long-term limb salvage extending beyond 1 year, which is more frequently reported[42].

Further developments on tissue reperfusion techniques were initially published in 2011 by Alexandrescu *et al*[43], who reported the first results of the angiosome-guided infrapopliteal angioplasty. The fundamentals of angiosome theory are based on a wound-adjusted revascularization strategy, aiming to enhance wound healing and limb salvage. Despite the limitations of this initial study (a small number of participants, short-term follow-up, limitations in angiography interpretation, selection bias), additional pedal and plantar artery angioplasty of the branch directly supplying blood to the wound seemed to result in excellent limb salvage rate. Thus, angiosome-based revascularization improves wound perfusion and decreases time to wound healing, but there is a lack of solid evidence regarding limb salvage improvement[43].

Only recently, Ma *et al*[44] reported outcomes of a prospective single-center observational cohort study investigating the intraoperative quantification of parenchymal blood volume (BV) in different foot regions assessed by C-arm computed tomography before and after revascularization in 27 patients. Interestingly, direct revascularization, according to the angiosome approach, resulted in a 197% BV increase compared to a 39% increase following indirect revascularization (P = 0.028). The authors concluded that direct revascularization of the ischemic area results in superior tissue perfusion than indirect revascularization[44]. Despite the widespread use of the plantar arch and angiosome-guided angioplasty, triggered by clinical experience and available results (Figure 1), the current level of evidence regarding the clinical superiority of these techniques remains low, and their effect on overall amputation-free survival remains unclear[41]. Larger, carefully designed RCTs are required to determine the optimal endovascular treatment algorithm in diabetic patients with CLI.

Percutaneous deep venous arterialization

Percutaneous deep venous arterialization (pDVA) has been recently introduced as a novel technique to overcome ischemia in "no option" patients who lack a viable target vessel for either surgery or endovascular treatment. The technique is based on the concept that arterialization of the venous system could be considered as an alternative source of perfusion of the distal foot. In 2020, Schmidt *et al*[45] published the mid-term results of the largest available series, revealing a promising potential for this complex group of "no option patients". Specifically, investigators reported outcomes of a retrospective study of 32 consecutive patients (66% with type 2 DM) treated with pDVA using the LimFlow device in four vascular centers in Alkmaar (Netherlands), Leipzig (Germany), Paris (France), and Singapore (ALPS). The procedure aimed to create a fistula between a tibial artery and a tibial vein and provide pressurized arterial flow to the venous system of the foot. Considering the stage, extent, and prognosis of CLI in this group of patients, pDVA using the LimFlow device resulted in a high technical success rate (96.9%), very satisfactory limb salvage (79.8%), and complete wound healing (72.7%) at the 2-year follow-up. Therefore, according to currently available initial data, pDVA could provide an option in selected "no-option" CLI patients[45].

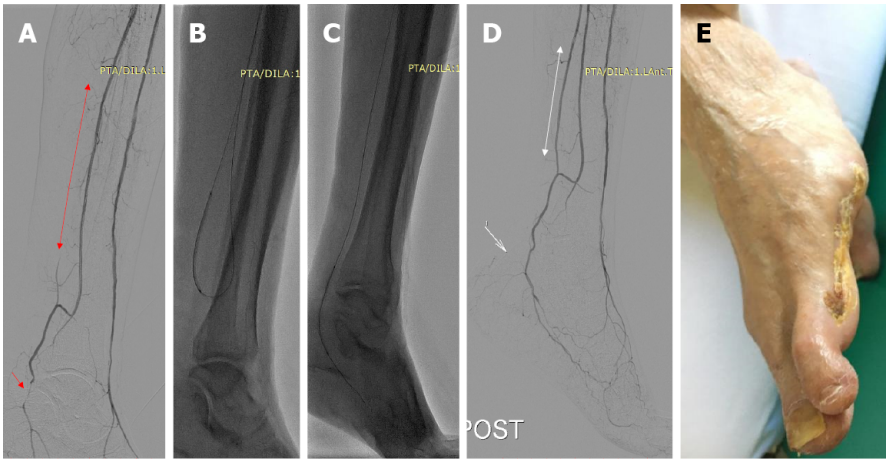

Figure 1 Wound-directed revascularization. An 81-year-old female patient with long-standing type II diabetes and non-healing wound following minor amputation of the 3rd, 4th, and 5th toe and respective metatarsals. A: Digital subtraction angiography (DSA) demonstrating patent anterior tibial and peroneal arteries, occlusion of the posterior tibial artery from its origin (red line with arrowheads), and significant stenosis of the distal below the ankle posterior tibial artery (red arrow), which supplies the area of the surgical wound. Note that wound healing was not satisfactory even though the anterior tibial artery was patent to the distal foot; B and C: Retrograde revascularization of the posterior tibial artery *via* the peroneal artery and balloon angioplasty followed by (C) antegrade balloon angioplasty of the below the ankle stenosis *via* the revascularized posterior tibial artery; D: Final DSA depicting excellent angiographic patency of the treated vessels; E: Complete wound healing noted at 3 mo follow-up.

NON-REVASCULARIZATION OPTIONS

Gene therapy

Hepatocyte growth factor: Hepatocyte growth factor (HGF), also known as scatter factor, is a paracrine cellular growth, motility, and morphogenic factor. It is a mesenchyme-derived pleiotropic angiogenetic growth factor that targets and acts primarily upon epithelial and endothelial cells and secondarily acts on hemopoietic progenitor cells and T cells. Recently, a novel therapeutic strategy for ischemic diseases using angiogenic growth factors to augment collateral artery development has been proposed[46].

HGF is implemented in the regulation of angiogenesis and has been shown to induce the formation of collateral vessels in rabbits. The first attempt to examine the pro-angiogenetic properties of HGF for limb ischemia in real-world patients was conducted by Nakagami and his colleagues[47] in 2005. Investigators performed intramuscular injection of naked plasmid DNA in the ischemic limbs of 6 patients, which demonstrated great potential. Although the study was designed to demonstrate the safety in a phase I/early phase IIa trial, these initial clinical outcomes using HGF gene transfer regarding its effectiveness as the sole therapy for CLI are optimistic[47].

Following the same concept, Gu *et al*[48] conducted a phase I clinical trial to evaluate the safety, tolerability, and preliminary efficacy of naked DNA therapy expressing two isoforms of hepatocyte growth factor (pCK-HGF-X7) in CLI patients. Improvement in wound healing was observed in 66.6% of patients with a baseline foot ulcer. These results supported the design of phase II RCT with pCK-HGF-X7[48,49].

Shigematsu and his colleagues[50], conducted a multicenter, randomized, double-blind, placebo-controlled trial in order to measure safety and efficacy of HFG plasmid DNA in patients with CLI. Efficacy was evaluated after 12 wk. Researchers report that overall improvement rate of the primary end point (improvement of rest pain or reduction of ulcer size) was 70.4% (19/27) in HGF group and 30.8% (4/13) in placebo group, showing a significant difference (P = 0.014). Furthermore HGF plasmid also improved quality of life. Thus, intramuscular injection of naked hepatocyte growth factor plasmid is safe and feasible for patients with CLI[50].

Two years later, the same group published a second multi-center study (the HGF-0205 trial). Powell *et al*[51] tried to explore further the safety and efficacy of HGF using a modified delivery technique. Patients classified as Rutherford-Becker categories 5 and 6 were enrolled. There was a significant improvement in the primary endpoint of the TBI and the secondary endpoint of rest pain assessment at 6 mo. Nonetheless, no significant difference was observed regarding wound healing and amputation rates. Recently, in a randomized, double-blinded, placebo-controlled phase II study of HGF published in 2019 by Yongquan *et al*[52], the NL003 DNA plasmid (pCK-HGF-X7) was investigated. This DNA plasmid encodes a genomic complementary DNA hybrid

human HGF sequence designed to express simultaneously two wild-type isoforms of HGF: HGF723and HGF728. According to the study design, 200 patients (Rutherford scale 4-5) were randomly assigned: Placebo (n = 50), low-dose (n = 50), middle-dose (n = 50), or high-dose NL003 (n = 50). The drug was administered to the affected limb on days 0, 14, and 28. No significant differences in the incidence of adverse events or serious adverse effects were found among the groups. Even though there were no statistically significant differences in $TcPO_2$, ABI, or TBI values between the four groups, this study reported the first effective evidence of significant improvement in complete ulcer healing, complete pain relief without analgesics, and safety for NL003 in patients with Rutherford stage 4–5[52].

Vascular endothelial growth factor: Vascular endothelial growth factor (VEGF) is a key factor in angiogenesis, stimulating the proliferation and migration of endothelial cells, which leads to the formation of new vessels. Today, several members of the VEGF family have been identified[53]. The VEGF-A is the main VEGF investigated in several clinical trials, as it has been recognized as a strong inducer of vascular permeability, with high angiogenic efficacy.

Another landmark study, published in 2002 by Mäkinen *et al*[54], first reported the possibility of VEGF transfer using an adenoviral vector. The authors conducted a phase II randomized, placebo-controlled, double-blind study evaluating local intra-arterial catheter-mediated AdVEGF165 gene therapy after percutaneous transluminal angioplasty. At 3 mo follow-up, DSA indicated increased vascularity in the VEGF-treated groups. There was also a numerical improvement in the Rutherford class and ABI values compared to the baseline, but this improvement was not significantly different from that observed in the placebo arm[54].

In 2003, Shyu *et al*[54] investigated the safety and efficacy of intramuscular gene therapy with vascular endothelial growth factor (VEGF 165) in patients with chronic CLI. Magnetic resonance angiography revealed qualitative evidence of improved distal flow in 19 limbs (79%). Ischemic ulcers healed or improved markedly in 12 limbs (75%). Rest pain was relieved or improved markedly in 20 limbs (83%). Complications were limited to transient leg edema in six limbs. As a result, this landmark study was among the first to show safety, efficacy, and feasibility of intramuscular gene therapy with VEGF (165) for patients with CLI[54].

In the Regional Angiogenesis with VEGF, also known as RAVE, trial published in 2003 by Rajagopalan *et al*[55], the intramuscular administration of VEGF was tested using different dose regimes *vs* placebo. In total, 105 patients with unilateral exercise-limiting intermittent claudication were enrolled to receive a low or high dose of AdVEGF121 or placebo by 20 intramuscular injections in a single session. However, patients receiving VEGF did not demonstrate significant improvement in the primary endpoint of peak walking time nor the secondary endpoints of ABI and quality of life measures compared to placebo. Furthermore, patients treated with VEGF developed peripheral edema, which may indicate its potential bioactivity[55].

Hypoxia-inducible factor 1a: Hypoxia-inducible factor (HIF) 1a is a transcriptional regulatory factor that orchestrates cellular responses to hypoxia. This agent has demonstrated an ability to increase collateral blood vessels, capillary density, and neovascularization in pre-clinical animal studies[56,57]. In a phase II prospective, randomized, double-blind, placebo-controlled, parallel-group, multicenter study conducted in 35 sites (27 in the United States, four in the United Kingdom, and four in Germany), a total of 289 patients with claudication were randomized in a double-blind manner to one of three doses of Ad2/HIF-1α/VP16 or placebo administered by 20 intramuscular injections to each leg. Unfortunately, HIF 1a failed to achieve significantly superior outcomes compared to placebo in the primary endpoint of peak walking time at 6 mo follow-up and in all secondary endpoints. Complementary studies to evaluate the potential usefulness of HIF-1a in CLI treatment are needed[58].

Stromal derived factor-1: Stromal-derived factor-1 (SDF-1), also known as CXC motif chemokine 12 (CXCL12), is a chemokine protein that in humans is encoded by the *CXCL12* gene on chromosome 10. SDF-1 has a pivotal role in the retention and homing of hematopoietic stem/progenitor cells into the bone marrow microenvironment[59,60]. Non-viral DNA plasmid encoding human stromal derived factor-1 (pSDF-1) enhance neovascularization at the micro-vascular level. In 2014 a promising phase IIa randomized double-blind placebo-controlled trial to Evaluate Plasmid Stromal Cell-Derived Factor-1 for Treatment of Critical Limb Ischemia (The STOP-CLI trial) was published that aimed to compare the effect of a biological agent *vs* placebo in the progression of CLI. Forty-eight CLI (Rutherford 4 or 5) patients who were poor candidates for surgical revascularization on stable medical therapy with ankle systolic

pressure ≤ 70 mmHg or toe systolic pressure ≤ 50 mmHg were enrolled into four cohorts (n = 12 each). The study aimed to evaluate the safety and tolerability of escalating doses of 1 mg/mL pSDF-1 (4 mg, 8 mg, 8 mg, or 16 mg) delivered *via* direct intramuscular injection (8 mg or 16 mg) to the ischemic limb. Interim results indicated the safety of SDF-1 and suggested its use for improving the clinical status of poor candidates for revascularization[61].

Recently, in 2019, Shishehbor *et al*[62] conducted a double-blind, placebo-controlled, phase IIB trial. Investigators randomized 109 patients (86 with DM) with CLI (Rutherford class V or VI) to 8 mg or 16 mg intramuscular injections of placebo *vs* a non-viral gene therapy that stimulates endogenous regenerative repair mechanisms known as JVS-100. Investigators set primary efficacy end point as a 3-mo wound healing score estimated by an independent wound core laboratory. The primary safety end point was major adverse limb events[62].

However, results from the three groups (placebo, 8 mg and 16 mg injections) revealed only 26% of wounds completely healed at 3 mo. Surprisingly, no variations among the three groups (26.5%, 26.5%, and 25%, respectively) were documented. Correspondingly, researchers notice no significant changes to TBI after 3 mo. Notable to mention that rates of major adverse limb events at 3 mo were 8.8%, 20%, and 8.3%, respectively[62].

Thus, while being safe, JVS-100 missed to improve wound healing or hemodynamic calibrations at 3 mo. A quarter, alone of CLI wounds was treated at 3 mo despite successful revascularization. These results point out the necessity for further research considering microcirculation augmentation therapies for PAD patients[62].

Basic fibroblast growth factor: Basic fibroblast growth factor (bFGF) (also known as FGF-β or FGF-2) is a growth factor and signaling protein that triggers a harmonized arteriogenic effect, activating several downstream signals such as VEGF and monocyte chemoattractant protein-1[63,64]. Until today, the level of evidence remains low as very few clinical trials have examined the role of bFGF in patients with PAD, while some clinical trials were prematurely terminated[65].

In 2009, Hashimoto *et al*[66] investigated the safety of selective and sustained delivery of bFGF using acidic gelatin hydrogel microspheres (AGHMs) for the treatment PAD in a single-arm prospective observational study in 8 patients with PAD. AGHM suspension containing 100 mg bFGF was infused into the artery of the affected limb. Evaluation of safety and changes in clinical symptoms, resting ABI measurement, and $TcPO_2$ as well as angiography was conducted at baseline and at various time points. No serious adverse events were observed. All cases demonstrated improvement of symptoms, although this was frequently temporary. The authors concluded that selective sustained delivery of bFGF by AGHMs was safe and well-tolerated in PAD[66].

Following the same concept, Kumagai *et al*[67] conducted an open-label, single-dose, phase I-IIa study that included 10 CLI patients to investigate the safety and efficacy of a sustained-release system of bFGF using biodegradable gelatin hydrogel. A single dose of 200 μg of bFGF-incorporated gelatin hydrogel microspheres was injected into the ischemic limb gastrocnemius. No serious procedure-related adverse events were recorded, while TcO_2 was significantly improved at 6 mo follow-up. Secondary endpoints (6 min walk, Rutherford class, rest pain, cyanotic scales) were also significantly improved. The authors concluded that a sustained release of bFGF from biodegradable gelatin hydrogel seems to induce angiogenesis and provide an effective alternative treatment option for CLI patients. However, more appropriately powered clinical investigations, especially in dose escalation, are needed[67].

Stem cells therapies

Over the last 2 decades, stem cell therapy (SCT) has emerged as a favorable alternative to traditional surgical and/or endovascular revascularization to treat ischemia in the DF. The primary benefit of SCT is to induce therapeutic neovascularization and generate collateral vessel formation to increase blood flow in the ischemic limb and soft tissue. Reported literature provides a solid rationale for ongoing in-depth studies aimed at advancing current SCT that may change the way PAD/CLI patients are treated.

The first landmark study was performed in 2002 by Tateishi-Yuyama *et al*[68]. The researchers recruited a mixed population of bone-marrow-derived $CD34^+$ and $CD34^-$ cells for no-option CLI patients. They conducted a pilot study and a subsequent larger, blinded RTC. Cells were only sorted and concentrated before limb implantation. Investigators reported a marginal increase in ABI values in treated limbs compared

with untreated limbs (approximately +0.1). Nonetheless, a noteworthy increase in $TcPO_2$ was reported. Notably, MR-angiography demonstrated an increased number of collateral vessels in the treatment group compared to the control group, and this was also correlated with clinical improvement[68].

The abovementioned promising results were partly reproduced in 2005 by Huang *et al*[69] This time, researchers focused completely on diabetic patients (both type 1 and type 2 DM). Authors selected peripheral blood mononuclear cells (MNCs) after mobilization *via* administration of granulocyte colony-stimulating factor. At the end of the 3-mo follow-up, lower limb pain and ulcers were significantly improved in patients included in the transplant group. Nevertheless, this was an open-label non-randomized trial, without a predetermined sample size[69]. Moreover, in both trials, mixed bone-marrow cell populations were used and whether mesenchymal stem cells or mixed MNCs contributed to the reported clinical benefit is not clear.

In 2011, Lu *et al*[70] conducted a single-center, three-arm, randomized, double-blind study to assess the effectiveness of stem cells therapy in CLI and to evaluate further the relative benefit of mixed bone marrow population and mesenchymal stem cells (MSCs), by comparing a mixed population of bone-marrow-derived MNCs and sorted bone-marrow MSCs with a placebo group of limbs, in which only normal saline was injected. Clinical benefit was reported over the control group for both treatments, with a more marked increase noted in limbs receiving MSCs. This benefit included a notable 100% ulcer healing and no amputation in the treated limbs. This study represents a milestone trial in cell therapy as it provided a high-level of evidence regarding the safety and effectiveness of MSCs therapy over bone marrow MNCs in increasing perfusion and promoting ulcer healing in diabetic patients with CLI[70]. Several other smaller studies have also confirmed these results[71-73].

The RESTORE-CLI, a multicenter, sponsor-initiated, double-blinded phase II RCT published in 2012, investigated a cellular product named Ixymielocel-T, crafted from each patient's bone marrow stem cells. Ixymielocel-T was a mixed population of MSCs and HSCs that underwent expansion by a proprietary procedure[74]. The study randomized 77 patients (Ixmyelocel-T: 48 patients and placebo 24 patients). Safety was demonstrated, and the efficacy endpoint of time to first occurrence of treatment failure was significantly longer for patients treated with Ixmyelocel-T ($P = 0.0032$). Moreover, a trend *vs* superior amputation-free survival was also noted in patients in the investigational group; however, this result did not reach statistical significance[74].

Summarizing the above-mentioned results, stem cell based therapies have proven to be safe and efficient to promote angiogenesis and blood flow in patients with CLI and especially those with no other options. Although initial results seem positive, variability between clinical trials is huge. As a result, there is an unresolved consensus regarding crucial factors such as cell doses, cell types or sources, administration routes, the parameters to define outcome efficacy, or the cohorts themselves. A lot of work needs to be done in order to translate the clinical benefits of SCT to a widely accepted model[75].

Hyperbaric oxygen therapy

Hyperbaric oxygen therapy (HBOT) for diabetic ulcers involves intermittent administration of 100% oxygen, usually in daily sessions of 90 min each, at pressures of 1.5 to 3.0 atmospheres absolute (ATA) in an airtight cabin. By increasing the blood oxygen content, HBOT creates a favorable gradient for the diffusion of oxygen into the tissues. In hypoxic tissues, the enhanced oxygen supply has multiple effects that may benefit wound healing. Additionally, by increasing the expression of VEGF and FGF, HBOT may enhance angiogenesis and fibroblast proliferation. Moreover, the resulting hyperoxia may cause vasoconstriction, thereby decreasing tissue edema. By reducing the expression of pro-inflammatory cytokines, HBOT reduces inflammation while simultaneously enhancing the bactericidal activity of leukocytes.

In 2014, Stoekenbroek *et al*[76] conducted a systematic review of RCTs to assess the additional value of HBOT in promoting the healing of DFU and preventing amputations. According to these results, some evidence of the effectiveness of HBOT in improving the healing of diabetic leg ulcers in patients with concomitant ischemia was reported.

Considering the low quality of current evidence, high costs of HBOT, and the burdensome nature of a full HBOT regimen, there is insufficient evidence to support the routine use of HBOT as an adjunct to standard wound care in diabetic patients with foot ulcers and more data are awaited[76].

Minimally invasive surgery

Minimally invasive preventive surgery at an early stage can be used to reduce focal points of pressure (off-loading) and to correct deformities (hammer and mallet toe) that may increase the risk for ulceration. Specifically, surgical off-loading is carried out by minimal percutaneous surgery, with specific well-established interventions such as distal metatarsal and phalanx osteotomies, tenotomies, and capsulotomies. The main objective of minimally invasive corrective foot surgery is to restore a stable foot during stance, which suggests that the head of the first and the fifth metatarsal as well as calcaneus are on the same plane. In addition, the aim is to minimize trauma without osteosynthesis, possibly decreasing the risk of infection and vascular and healing complications in diabetic patients. As a result, a subsequent more extensive surgery could be avoided. Similarly to endovascular techniques, minimally invasive surgery for DFU requires specific equipment (blades, high-speed burrs, high power machines) with fluoroscopy control and a far-reaching learning curve for devoted surgeons[77, 78].

CONCLUSION

DF is a challenging pathology with a broad spectrum of pathophysiological mechanisms and clinical manifestations. Prompt diagnosis of ischemia is crucial for timely treatment and rapid wound healing and should include detailed arterial assessment. Treatment of ischemia should be considered a medical emergency and decided in multidisciplinary team meetings. Open surgical, minimally invasive, or combined endovascular/surgical revascularization procedures should be readily available, and the choice of the optimal revascularization plan should be individualized. Both minimally invasive and surgical revascularization options have been reported to achieve satisfactory mid-to-long-term limb-salvage rates. Recently, highly specialized, large-volume vascular centers have endorsed the "endovascular-first" approach, which achieves similar limb salvage rates with open bypass, without precluding future surgical treatment options.

Various endovascular devices, mainly DES and DCB, have been used to reduce restenosis after endovascular treatment and minimize the need for reinterventions due to clinical relapse, while new revascularization techniques such as angiosome-guided angioplasty, pedal arch angioplasty, and pDVA have been endorsed by endovascular experts in everyday clinical practice in an attempt to optimize wound healing, time to wound healing, and limb salvage.

Multicenter randomized trials specifically focused on optimizing endovascular treatment options for DFU remain limited, and more high-quality data are expected. Gene and stem cell therapies have also been investigated mainly in "no option" CLI patients, not amenable to revascularization, and while initial data have been deemed positive, more evidence is required to justify their use. The authors speculate that soon these therapies combined with continuously improving endovascular revascularization techniques will optimize outcomes of DFU treatment.

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MINIREVIEWS

Omics era in type 2 diabetes: From childhood to adulthood

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Abstract

Parallel to the dramatic rise of pediatric obesity, estimates reported an increased prevalence of type 2 diabetes (T2D) already in childhood. The close relationship between obesity and T2D in children is mainly sustained by insulin resistance (IR). In addition, the cardiometabolic burden of T2D including nonalcoholic fatty liver disease, cardiovascular disease and metabolic syndrome is also strictly related to IR. Although T2D pathophysiology has been largely studied in an attempt to improve therapeutic options, molecular mechanisms are still not fully elucidated. In this perspective, omics approaches (including lipidomics, metabolomics, proteomics and metagenomics) are providing the most attractive therapeutic options for T2D. In particular, distinct both lipids and metabolites are emerging as potential therapeutic tools. Of note, among lipid classes, the pathogenic role of ceramides in T2D context has been supported by several data. Thus, selective changes of ceramides expression might represent innovative therapeutic strategies for T2D treatment. More, distinct metabolomics pathways have been also found to be associated with higher T2D risk, by providing novel potential T2D biomarkers. Taken together, omics data are responsible for the expanding knowledge of T2D pathophysiology, by providing novel insights to improve therapeutic strategies for this tangled disease. We aimed to summarize the most recent evidence in the intriguing field of the omics approaches in T2D both in adults and children.

Key Words: Omics; Diabetes; Children; Adults; Type 2 diabetes

Core Tip: Type 2 diabetes (T2D) represents an emerging health concern worldwide. Its

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cardiometabolic burden affects both adults and children. Given the alarming rise of pediatric obesity, a high prevalence of T2D has been reported already in childhood. Although lifestyle modifications and metformin represent the first-line therapy for T2D, several drugs are available and others are being studied. In this view, an attractive therapeutic tool derives from omics studies. Based on T2D pathophysiology, these analyses highlighted the role of different lipids and metabolites closely intertwined with insulin signaling pathways as potential biomarkers for T2D, by paving the way for novel treatment strategies.

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INTRODUCTION

Type 2 diabetes (T2D) is a global epidemic with an increasing prevalence both in adults and children[1,2]. Estimates have reported > 450 million T2D adult patients in 2019 with a potential rise to 700 million by the next three decades[2]. In children, the alarming T2D increase has been mainly linked to the concomitant obesity epidemic[3-5]. Recent data from the United States indicate an incidence of almost 5000 new pediatric T2D cases per year[1]. Similarly, an increased overall prevalence of T2D in adolescence has been observed over the past few years and is expected to be quadrupled in the next 40 years[6,7].

According to the American Diabetes Association, T2D criteria included fasting plasma glucose (FPG) ≥ 126 mg/dL or 2-h glucose concentration during an oral glucose tolerance test ≥ 200 mg/dL or hemoglobin A1C ≥ 6.5%[8].

Several factors (*e.g.*, genetic, epigenetic, metabolic and environmental) are involved in the complex pathophysiology of T2D, but insulin resistance (IR) and beta-cell dysfunction are recognized as key pathogenic players[1,4,5,9-12]. As result of various metabolic insults (*e.g.*, oxidative stress, vascular damage and lipotoxicity), different organs and systems (including heart, kidney, brain, liver, eyes and nervous system) are affected[10,11].

In particular, T2D consequences in adults are clustered in macrovascular and microvascular diseases. The former group (including stroke, myocardial infarction and peripheral artery disease) has been closely linked to hyperglycemia, hyperinsulinemia and dyslipidemia, while the latter (*i.e.*, retinopathy, kidney disease and neuropathy) has been related both to proinflammatory and prothrombotic effects of hyperglycemia and to cell lipid content changes[1,10].

The burden of comorbidities in pediatric T2D [including fatty liver, cardiovascular disease (CVD), kidney injury, and metabolic syndrome (MetS)] has been closely intertwined with obesity, representing the most important risk factor for T2D development at this age[1,13-15]. More specifically, IR represents the shared pathogenic feature of the entire spectrum of comorbidities, by underscoring its pivotal role in metabolic derangements[16].

Although several pharmacological options are currently available, lifestyle modifications and metformin remain the first-line therapy in adolescence[17]. In this perspective, new insights for T2D treatment have recently emerging from omics studies[18-21]. Indeed, this intriguing field (including metabolomics, proteomics, genomics and lipidomics) has currently provided evidence for a pathogenic role in several metabolic diseases[22-25].

Despite the large availability of data on T2D pathophysiology, less is known on molecular changes caused by hyperglycemia[26-31]. In an effort to enhance therapeutic strategies for this insidious disease, innovative recent studies focused on the pathophysiological significance of these modifications T2D-related through omics approaches.

We aimed to summarize the most recent evidence in this intriguing field both in children and in adults.

OMICS

As omics branches, different classes including genomics, proteomics, metabolomics and metagenomics provide a refined assessment by examining both quantitative and qualitative biomolecular characteristics.

Recently, a growing body of evidence has supported a pathogenic role of distinct lipid class including sphingolipids (in particular ceramides) for several metabolic disorders such as obesity, MetS, IR, CVD, nonalcoholic fatty liver disease (NAFLD) and T2D both in adults and children. Plasma ceramides levels have been closely linked to glucose homeostasis derangements and NAFLD through insulin signaling pathways impairment.

From a metabolic perspective, these new branches have allowed advances in the understanding of pathophysiology of beta-cell dysfunction leading to T2D development. Besides evidence from experimental models, there is a large amount of data in adult population and a still limited but compelling body of evidence in children.

Animal studies

A large body of evidence regarding omics approaches on T2D has been provided by experimental studies conducted on animal models (Table 1). Chen *et al*[32] investigated the role of lipids in diabetes development and evolution of diabetes using high-fat diet-streptozocin (HFD-STZ) induced diabetes in mice. In particular, the authors demonstrated this role in different organs such as heart, kidney and brain. Cardiac changes expressed as reduction in cardiolipin species with long chains were reported in the atrium and ventricle, while triglyceride (TG) levels were found to be decreased in the ventricle and increased in the atrium[32].

Likewise, renal changes were also demonstrated. Specifically, a reduction of TG species with shorter fatty chains and an increase of the long fatty chain TGs in the medulla were detected. Changes in the renal cortex were similar but involved longer fatty chains than in the medulla, by suggesting a targeted role of the lipidome in different regions of the same organ[32].

Lastly, cerebral change of HFD-STZ mice showed a higher reduction of cardiolipin levels, indicating a loss of mitochondrial function more severe compared to other organs[32].

As a consequence of the expanding knowledge on T2D pathophysiology through omics approaches, a pathogenic role of dyslipidemia (defined as elevated plasma triacylglycerol and cholesteryl esters levels) for microvascular disease development has been reported in animal models[33]. Indeed, Eid *et al*[33] found increased levels of several lipid species in the kidney and nerves and reduced overall lipid content in the retina of an experimental model of diabetic mouse.

Human studies

Omics techniques in human models have allowed identification of novel attractive therapeutic tools for several metabolic disorders including T2D. Indeed, these approaches have provided a better elucidation of the molecular pathogenic changes underpinning T2D and its comorbidities.

Evidence in adults

Most studies performing omics analyses have reported intriguing associations of different lipids and metabolites with T2D in adults (Table 2). Ge *et al*[34] studied the association between FPG levels and single nucleotide polymorphisms (SNPs)[34]; 76 out of 511 participants presented with increased FPG levels and 435 had decreased or fluctuant FPG concentrations. Nine SNPs in five genes (*RPL7AP27*, *SNX30*, *SLC39A12*, *BACE2* and *IGFL2*) were significantly associated with increased FPG levels[34]. Moreover, among the 24 identified glycan peaks (GPs), GPs 3, 8 and 11 showed a positive trend with increased FPG levels, while the opposite was found for GPs 4 and 14[34]. These findings provided evidence for novel potential biomarker for T2D through the combination of candidate SNPs and IgG glycomics[34].

Another study conducted on 1974 healthy subjects aged 50-70 years showed the role of sphingolipids as markers for incident T2D[35]. In fact, during the 6 years' follow-up, 529 participants developed T2D. In particular, 14 sphingolipids (of which 11 newly described) namely ceramides (d18:1/18:1, d18:1/20:0, d18:1/20:1 and d18:1/22:1), saturated sphingomyelins (C34:0, C36:0, C38:0 and C40:0), unsaturated sphingomyelins (C34:1, C36:1 and C42:3), hydroxyl-sphingomyelins (C34:1 and C38:3), and a hexosylceramide (d18:1/20:1), were positively associated with incident T2D[35].

Table 1 Main findings of the omics studies in animal models

Ref.	Experimental design	Main findings
Eid *et al* [33]	Examination of changes in glucose and lipid metabolism in the kidney, eye and nerve of leptin receptor KO BKS *db/db* mouse model	Glycolytic genes were uniformly upregulated in kidney and peripheral nerves; glycolytic metabolites were increased in kidney and retina but decreased in the peripheral nerve. Kidney and nerves showed an overall trend towards increased levels of different lipid species, while in the retina lipid content was decreased
Chen *et al*[32]	Evaluation of the characteristic of lipid species in serum and tissues in a diabetic mouse model fed a high fat diet and treated with streptozocin by using LC/HRMS and MS/MS	Brain and heart showed the largest reduction in cardiolipin levels, while the kidney had more alteration in triacylglycerol levels. Cardiolipin with highly polyunsaturated fatty acyls decreased only in the atrium but not in the ventricle; similarly, renal cortex showed longer fatty acyl chains both for increased and decreased triacylglycerol species than renal medulla
Guitton *et al*[25]	Systematic review about the role S1P in the development of T2D and obesity	SphK1 KO in rat pancreatic β-cells and in INS-1 cells resulted in both lowered glucose-stimulated insulin secretion and insulin content associated with decreased insulin gene expression. Conversely, SphK1 overexpression restored both insulin synthesis and secretion. HFD-fed SphK1 ko mice also showed a reduction of β-cells size, number and mass due to lipotoxic condition

T2D: Type 2 diabetes; HFD: High fat diet; LC/HRMS: Liquid chromatography high-resolution tandem; MS: Mass spectrometry.

The Weighted Gene Correlation Network Analysis generated five modules containing different species of sphingolipids, of which two clusters including saturated sphingomyelins showed the strongest association with increased T2D risk[35].

Omics approaches have also been used for examining the effect of certain drugs on tissue molecules. Peterson *et al*[36] investigated the effect of fenofibrate treatment on cardiac function in 65 T2D patients subdivided in treated (31 subjects) and placebo (34 subjects) groups. Fenofibrate is a fibric acid derivative, whose active metabolite is responsible for the primary pharmacodynamic drug effects, including reduction in total plasma cholesterol, low density lipoprotein cholesterol, TG, and very low-density lipoprotein concentrations and increase in high-density lipoprotein cholesterol and apolipoprotein AI and AII concentrations[37]. These effects are mediated by the activation of peroxisome proliferator-activated receptor-[36]. No significant changes in body mass index, diabetes control and hemodynamics were observed between the two groups. Fenofibrate treatment decreased plasma C24:0/C16:0 ceramide ratio (likely related to worsening in diastolic function) with slight changes in oxidative stress markers but no effect on inflammation[36]. It also seemed to be linked to diastolic function improvement through lowering TG plasma levels, but systolic or diastolic function did not significantly differ in both groups[36].

In addition, more data using a comprehensive omics approach (including lipidomics, metabolomics, and proteomics) support the close relationship of specific lipid class[38-40] and metabolites[41-44] with the metabolic milieu.

Evidence in children

Because of the pediatric obesity epidemic, an increasing prevalence of T2D in children has been reported over the last few decades[1]. In order to counteract the "diabesity" epidemic, novel therapeutic options are being studied. In this view, as observed in adults, meaningful data are provided by omics also in childhood[45-51] (Table 3).

Among lipid classes, the most interesting findings have been related to ceramides, representing important bioactive lipids belonging to the sphingolipid family produced from a fatty acid and sphingosine or by sphingomyelin hydrolysis affect cell signaling pathways involved in metabolic processes[22,24,28]. To date, these lipids are considered as the major players in IR development, as demonstrated by several works both in adults and children linking ceramides to various cardiometabolic diseases such as obesity, MetS, NAFLD, T2D, CVD and chronic kidney diseases[20,22,24,51].

Lopez *et al*[51] examined the role of ceramides and adiponectin in 28 female adolescents (14 healthy and 14 obese girls with T2D) aged 10–17 years. Higher C 18:0, C22:0 and C20:0 ceramides levels and decreased adiponectin concentrations were found in patients with T2D compared to healthy subjects.

A specific metabolomics signature has been demonstrated in children with metabolic derangements, by underscoring the pathogenic role of different metabolites affecting IR pathways.

Martos-Moreno *et al*[50] studied 100 prepubertal children with obesity (50 female/50 male, 50% IR and 50% non-IR for each group) by performing an oral glucose tolerance test for usual carbohydrate and lipid metabolism determinations. In IR obese children, impairments in the urea cycle, alanine metabolism and the glucose–alanine cycle were detected, suggesting a possible role of mitochondrial

Table 2 Main findings of the omics studies in adults

Ref.	Study design	Population (*n*)	Main findings
Ge *et al*[34]	Community-based case-control study	511 healthy adults, mean age 47.9 yr	76 patients had increased FPG and 435 had decreased or fluctuant FPG. Nine SNPs in five genes were significantly associated with increased FPG. Among the 24 glycan peaks identified, GPs 3, 8 and 11 had a positive trend with increased FPG levels, while opposite findings were found for GPs 4 and 14
Peterson *et al*[36]	Double-blind, randomized, placebo-controlled, parallel design study	65 adults aged 30-65 yr	Fenofibrate treatment lowered C24:0/C16:0 plasma ratio and minimally altered oxidative stress markers and correlated with worse diastolic function. Plasma TG lowering correlated with improvement in diastolic function
Yun *et al*[35]	Prospective study	1974 adults, aged 50-70 yr	During the 6 yr follow-up, 529 participants developed T2D. 14 sphingolipids (3 reported and 11 novel) were positively associated with incident T2D. WGCNA analysis generated 5 modules, containing different species of sphingolipids; of these, 2 modules containing saturated sphingomyelins showed the strongest association with increased T2D risk
Sun *et al*[38]	Systematic review	33 studies on the application of metabolomics to disease related-risk. 5 studies on the applications of metabolomics for disease prediction. 5 studies on the applications of metabolomics biomarkers for disease intervention. 8 studies about the integration of genomic and metabolomics data	The first 33 studies find out different metabolites associated with T2D, heart failure, IR and MetS. Studies about the disease prediction demonstrated that some metabolites (amino acids and lipids) were predictive for T2D. Studies about the applications of biomarkers investigated the effect of diet in reducing some risk factors. Studies on the integration of genomic and metabolomics data reported some allele positively associated with high levels of risk metabolites
Misra and Misra [2]	Systematic review	18 studies about heavy metals. 14 studies about persistent organic pollutants and pesticides. 7 studies about drugs and pharmaceuticals. 11 studies about atmospheric pollution	Heavy metals (*e.g.*, arsenic, lead, selenium and mercury) were positively associated with increased T2D risk. Some pollutants of the POPs and pesticides' family were directly associated with increased risk of developing T2D. Drugs such as antibiotics, antidepressant or antipsychotics were positively associated with increased T2D risk. Long exposure to atmospheric pollutants such as NO_2 and PM2.5 were directly associated with T2D
Zhang *et al*[39]	Cohort study	694 patients (491 HIV-infected and 203 HIV-uninfected) aged 35-55 yr	11 lipids species were identified and associated with T2D risk. No association of HIV status with higher T2D risk was found, while ART use was associated with 8 risk lipids (3 decreased-risk lipids and 5 higher-risk lipids)
Wang *et al*[40]	Systematic review complication	1 study about application of proteomics in T2D. 1 study about the application of metabolomics in T2D. 1 study about the application of metagenomics in T2D	Proteomics analyses on 62 Mexican T2D patients showed 113 proteins related to T2D risk; in particular, 3 of these have been associated with obesity and T2D while 1 was associated with anti-inflammatory pathways. Metabolomics analyses found 33 metabolites strongly related to T2D. Metagenomics analyses reported different gut microbiota profiles between fecal sample of T2D patients and control subjects
Gudmundsdottir *et al*[41]	Prospective cohort study	2916 European patients (789 diabetic patients and 2127 non diabetic patients at high T2D risk development)	55 modules of coexpressed genes in the whole blood of the nondiabetic cohort were found. These modules were associated with inflammation, fat tissues, glucose tolerance, insulin sensitivity, and C-reactive protein levels, and were also preserved between non-diabetic and newly diagnosed T2D cohort
Gu *et al*[42]	Observational study	72 patients (30 normal weight, 26 obese and 16 newly T2D diagnosed)	Obese patients showed upregulation of 78 metabolites and downregulation of 111 metabolites than lean subjects. T2D patients showed upregulation of 459 metabolites and downregulation of 166 metabolites compared to obese subjects. Several metabolites, including amino acids and amino acids metabolites, were identified as IR potential biomarkers
Diamanti *et al*[43]	Cohort study	42 subjects (12 healthy controls, 16 with prediabetes and 14 T2D subjects)	Plasma metabolomics profiling revealed a positive association of hepatic fat content with tyrosine and a negative relationship with lysophosphatidylcholine. Visceral and subcutaneous adipose tissue insulin sensitivity was positively associated with several lysophospholipids, while the opposite was found for branched-chain amino acids. Several metabolites were significantly higher in T2D subjects than normal/prediabetes subjects
Salihovic *et al*[44]		1424 adult subjects	Three out of 62 identified metabolites were associated with prevalent T2D (mainly lower urine levels of 3-hydroxyundecanoyl-carnitine). In participants without T2D at baseline, 6 metabolites improving T2D prediction were identified

T2D: Type 2 diabetes; FPG: Fasting plasma glucose; SNP: Single nucleotide polymorphism; TG: Triglycerides; IR: Insulin resistance; WGCNA: Weighted

gene coexpression network analysis.

Table 3 Main findings of the omics studies in children

Ref.	Study design	Population (*n*)	Main findings
Concepcion *et al*[45]	Cross-sectional study	90 children (30 healthy children, 30 obese children without T2D and 30 obese children with T2D) aged 13-19 yr	In urine samples of T2D patients, 22 metabolites (including succinylaminoimidazole carboxamide riboside (SAICA-riboside), betaine metabolites (betaine and dimethylglycine), branched chain amino acids (valine and leucine) and their direct catabolic derivatives (2-oxoisovaleric acid, 3-methyl-2-oxovaleric acid, 3-hydroxyisobutyrate) and aromatic amino acids (phenylalanine, tyrosine and tryptophan) were significantly associated in obese children. The metabolite pattern in OB and T2D groups differed between urine and plasma, suggesting that urinary BCAAs and their intermediates behaved as a more specific biomarker for T2D, while plasma BCAAs associated with obesity and IR independently of T2D
Perng *et al* [46]	Cross-sectional study	524 adolescents aged approximately 13 yr, grouped according to both obesity and glucose tolerance status	Five metabolite patterns differed with respect to phenotype: Factor 1 comprised long-chain fatty acids and was lower among non-OW/OB and high MetRisk *vs* non-OW/OB and low MetRisk. Factors 5 (branched chain amino acids; BCAAs), 8 (diacylglycerols) and 9 (steroid hormones) were highest among OW/OB and high MetRisk. Factor 7 (long-chain acylcarnitines) was higher among non-OWOB and high MetRisk and lower among OW/OB and low MetRisk
Gawlik *et al* [47]	Observational study	87 obese children divided in 2 groups (IR and Non-IR children) aged 8.5-17.9 yr old	31 steroid metabolites were quantified by GC-MS. IR was diagnosed in 20 (23%) of the examined patients. The steroidal IR signature was characterized by high adrenal androgens, glucocorticoids, and mineralocorticoid metabolites, higher 5a-reductase and 21-hydroxylase activity, and lower 11bHSD1 activity
Müllner *et al* [48]	Cross sectional study	81 adolescents aged > 10 yr, stratified into four groups based on BMI (lean *vs* obese), insulin responses (normal)	Two groups of metabolites were identified: (1) Metabolites associated with insulin response level: adolescents with HI (groups 3-4) had higher concentrations of BCAAs and tyrosine, and lower concentrations of serine, glycine, myo-inositol and dimethylsulfone, than adolescents with NI (groups 1-2); and (2) Metabolites associated with obesity status: obese adolescents (groups 2-4) had higher concentrations of acetylcarnitine, alanine, pyruvate and glutamate, and lower concentrations of acetate, than lean adolescents (group 1)
Mastrangelo *et al*[49]	Observational study	60 prepubertal obese children (30 girls/30 boys, 50% IR and 50% non-IR in each group, but with similar BMI)	47 metabolites out of 818 compounds were found to differ significantly between obese children with and without IR. Bile acids exhibit the greatest changes. The majority of metabolites differing between groups were lysophospholipids (15) and amino acids (17), indicating inflammation and central carbon metabolism as the most altered processes in impaired insulin signaling. Multivariate analysis (OPLS-DA models) showed subtle differences between groups that were magnified when females were analyzed alone
Martos-Moreno *et al* [50]	Observational study	100 prepubertal obese children (50 girls/50 boys, 50% IR and 50% non-IR in each group)	Twenty-three metabolite sets were enriched in the serum metabolome of IR obese children. The urea cycle, alanine metabolism and glucose-alanine cycle were the most significantly enriched pathways. The high correlation between metabolites related to fatty acid oxidation and amino acids (mainly branched chain and aromatic amino acids) pointed to the possible contribution of mitochondrial dysfunction in IR. The degree of BMI-standard deviation score excess did not correlate with any of the studied metabolomics components. Combination of leptin and alanine showed a high IR discrimination value in the whole cohort in both sexes. However, the specific metabolite/adipokine combinations with highest sensitivity were different between the sexes
Lopez *et al* [51]	Cross sectional study	28 children (14 obese female subjects with T2D and 14 lean healthy controls) aged 10-17 yr	Children with T2D had higher concentrations of C22:0 and C20:0 ceramides, with a 2-fold increase in C18:0 ceramide and C24:1 dihydroceramide. C22:0, C20:0 and C18:0 ceramide correlated with decreased adiponectin concentrations, increased HOMA-IR, BMI-SDS, triglyceride and fasting blood glucose concentrations. Plasma levels of C18:0, C20:0 and C22:0 ceramide, as well as C24:1 dihydroceramide were elevated in T2D obese female children and adolescents, probably due to tissue IR and low adiponectin levels

T2D: Type 2 diabetes; OB: Obesity; BMI: Body mass index; IR: Insulin resistance; GC-MS: Gas chromatography-mass spectrometry; HOMA-IR: Homeostatic model assessment for insulin resistance; MetRisk: Metabolic risk; 11bHSD1: 11β-Hydroxysteroid dehydrogenase type 1; OPLS-DA: Orthogonal partial least squares discriminant analysis.

dysfunction in IR[50]. Collectively, these findings supported the potential application of metabolomics analysis in clinical practice as a noninvasive tool to identify children at risk [50].

In this framework, a role of distinct lipid classes as potential mediators or biomarkers for several metabolic diseases has been widely recognized, but the putative pathophysiological mechanisms underpinning these associations are currently not fully elucidated. Although still limited, pediatric reports in this field are growing and promising.

CONCLUSION

The rising prevalence of the diabesity epidemic has highlighted the urgent need for more effective both prevention and treatment strategies. In this view, the growing knowledge regarding omics pathways affected by insulin signaling has favored the identification of novel potential biomarkers for this alarming epidemic.

Distinct metabolomics and lipidomics pathways have been recently linked to obesity, IR and T2D not only in adults but also in children, by allowing us to expand knowledge about the pathophysiology of several cardiometabolic diseases.

Given the unfavorable prognostic role of metabolic derangements in childhood, a better understanding, such as with omics profiles, of the pathophysiological mechanisms underlying beta-cell dysfunction is crucial. Findings from these studies are providing new insights into the intriguing field of molecular pathways related to IR as a predisposing factor for T2D. Therefore, novel attractive tools are emerging as potential therapeutic agents to counteract the risk of T2D and its related cardiometabolic burden already in childhood[31,33,34].

In particular, lipidomic profiling accompanied by experimental studies using pharmacological reagents to alter synthesis or metabolism of certain lipids, has given additional insights into mechanisms governing lipotoxicity and disease progression, by providing evidence about a role in several crucial cellular responses (*e.g.*, apoptosis, cell cycle and autophagy).

Recently, there has been significant progress in the understanding of the processes of insulin action and molecular defects determining IR, but many gaps according to the pathophysiology of metabolic disorders remain. Published data from studies conducted both on animals and humans have revealed a role for sphingolipids and metabolites in IR in different tissues such as skeletal muscle, liver and adipose tissue.

Among lipid classes, ceramides have gained remarkable attention as the major suspects in the development of IR. Therefore, changes in ceramide generation may become a desired therapeutic target, as shown in rodent models.

Further research is needed to identify the emerging role of both lipids and metabolites in the pathogenesis of cardiometabolic diseases in children in an attempt to provide novel clinical tools with potential therapeutic implications. Findings from the innovative omics era might pave the way for a noninvasive approach of personalized medicine for patients with a greater intrinsic cardiometabolic risk.

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MINIREVIEWS

Hypoglycemia in diabetes: An update on pathophysiology, treatment, and prevention

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Abstract

Hypoglycemia is a common complication in patients with diabetes, mainly in those treated with insulin, sulfonylurea, or glinide. Impairments in counterregulatory responses and hypoglycemia unawareness constitute the main risk factors for severe hypoglycemia. Episodes of hypoglycemia are associated with physical and psychological morbidity. The fear of hypoglycemia constitutes a barrier that impairs the patient's ability to reach good glycemic control. To prevent hypoglycemia, much effort must be invested in patient education regarding risk factors, warning signs, and treatment of hypoglycemia at an early stage, together with setting personalized goals for glycemic control. In this review, we present a comprehensive update on the treatment and prevention of hypoglycemia in type 1 and type 2 diabetic patients.

Key Words: Hypoglycemia; Diabetes mellitus; Insulin; Glucagon; Glucose; Continuous glucose monitoring

Core Tip: Hypoglycemia in diabetes is associated with increased morbidity and constitutes a barrier to glycemic control. Great effort must be invested in patient education on hypoglycemia prevention and management. Herein we present the recent data on the treatment and prevention of hypoglycemia in diabetes, with a focus on the benefits of treatment adjustment and the role of continuous glucose monitoring.

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INTRODUCTION

Hypoglycemia is defined as a condition where plasma glucose concentration is low, which may expose patients to possible harm. This is common amongst persons who have type 1 diabetes, with an annual incidence of severe hypoglycemia ranging from 3.3% to 13.5%[1]. While patients treated with insulin or insulin secretagogues (sulfonylureas and meglitinides) are generally at higher risk[2], severe hypoglycemia is less common in patients with type 2 diabetes.

Glucose-lowering medications that do not cause unregulated insulin secretion, such as dipeptidyl peptidase-4 inhibitors, metformin, glucagon-like peptide-1 receptor agonists, thiazolidinediones, and sodium-glucose cotransporter-2 inhibitors are associated with lower risk of hypoglycemia, unless used in combination with insulin or insulin secretagogues[3].

Historically, lowering the glycemic targets in diabetes in order to prevent microvascular and macrovascular complications has led to greater risk of hypoglycemia. In the United Kingdom Prospective Diabetes Study (UKPDS), when the HbA1c goal was 7% in the intensive treatment arm, the yearly incidence rate of severe hypoglycemia ranged from 0.7%-1.8% in type 2 diabetes patients receiving conventional treatment or treated with insulin, respectively[4]. In the ACCORD, ADVANCE, and VADT trials, significant increases in hypoglycemic episodes were observed in the intensive treatment as compared to the standard treatment groups (Figure 1)[5-8]. In the Diabetes Control and Complications Trial (DCCT), at least one episode of severe hypoglycemia during the follow-up period was experienced in ≤ 65% of type 1 diabetes patients in the intensive treatment arm[9]. Interestingly, observational studies point to a lack of significant reduction in the incidence of severe hypoglycemia over the last 20 years, albeit some recent studies have reported decreasing trends, especially among patients with type 2 diabetes[10-12].

In patients with diabetes, it is not easy to determine a specific plasma glucose concentration that is diagnostic of hypoglycemia, because the threshold for the appearance of hypoglycemia symptoms varies among patients. This threshold drops due to recurrent episodes of hypoglycemia and rises in individuals with uncontrolled diabetes.

The current classification of hypoglycemic episodes in diabetes includes three levels corresponding to the severity of hypoglycemia[13]:

Level 1 hypoglycemia: defined as plasma glucose concentration < 70 mg/dL but > 54 mg/dL. Plasma glucose of 70 mg/dL constitutes the threshold concentration below which neuroendocrine responses to hypoglycemia usually appear in individuals without diabetes. Many patients with diabetes suffer from impaired defense mechanisms against hypoglycemia and/or lack of hypoglycemia awareness; therefore, plasma glucose concentrations < 70 mg/dL are defined as clinically significant in diabetes and require intervention irrespective of symptom severity.

Level 2 hypoglycemia: defined as plasma glucose concentration below 54 mg/dL requiring immediate intervention to correct the hypoglycemia.

Level 3 hypoglycemia: defined as a serious event characterized by a change in the mental status or impairment in the patient's physical ability to function that requires intervention by another person to correct the glucose concentration.

Symptoms of hypoglycemia

Symptoms of hypoglycemia include autonomic symptoms and neuroglycopenic symptoms. These vary among patients according to age and diabetes duration. For example, children may demonstrate emotional and behavioral changes secondary to hypoglycemia in addition to classic autonomic and neuroglycopenic symptoms.

Autonomic symptoms include anxiety, tremor, palpitations, diaphoresis, paresthesia, and sensation of hunger.

Neuroglycopenic symptoms include lack of concentration, headache, blurred vision, dizziness, confusion, convulsions, speech disturbance, restlessness, and loss of consciousness.

Neuroglycopenic symptoms result from brain neuronal glucose deprivation. The glycemic threshold for neuroglycopenic symptoms is typically around 54 mg/dL[13]. Unlike autonomic symptoms, the onset of neuroglycopenic symptoms is usually not affected by counter-regulatory hormonal failure or previous episodes of hypoglycemia [14].

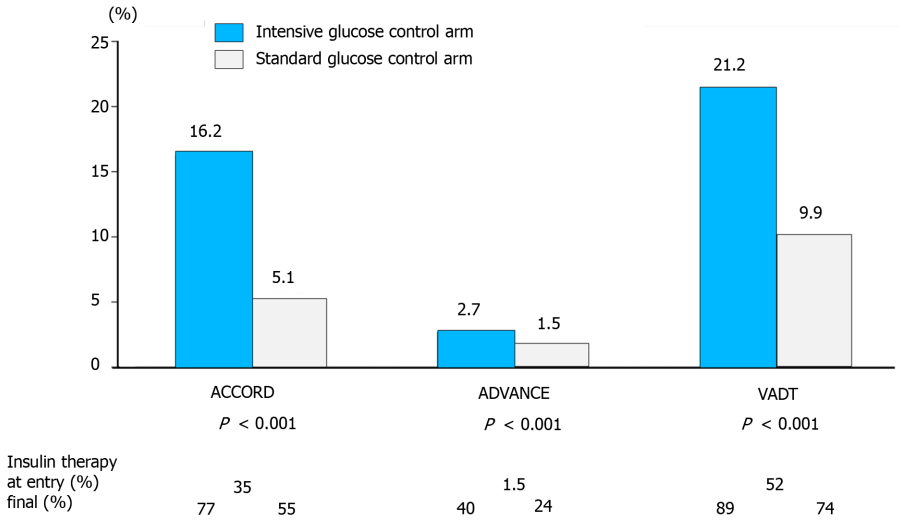

Figure 1 Percentage of severe hypoglycemic events in ACCORD, ADVANCE, and VADT. Adapted from Frier *et al*[8] with permission from the American Diabetes Association. Citation: Frier BM, Schernthaner G, Heller SR. Hypoglycemia and cardiovascular risks. *Diabetes Care* 2011; 34 Suppl 2: S132-S137. Copyright ©The American Diabetes Association.

RISK FACTORS FOR HYPOGLYCEMIA

Risk factors for hypoglycemia can be from therapeutic hyperinsulinemia or failure of "defense mechanisms" from a drop in plasma glucose concentration.

Conditions causing therapeutic hyperinsulinemia include: (1) Treatment with insulin, sulfonylureas, or glinides, if administered at high dose or with incorrect timing related to meal; (2) Lack of exogenous glucose, such as when eating a very low carbohydrate food portion, or during prolonged fasting; Lack of endogenic glucose production after drinking alcohol, (3) Increase in glucose consumption during or after physical exercise; (4) Increase in insulin sensitivity due to weight loss or physical exertion; and (5) Drop in insulin excretion under conditions such as renal failure, hepatic failure, and hypothyroidism[15] .

Impairment in counter-regulatory responses to hypoglycemia

A decrease in plasma glucose concentration may lead to two main responses in the body under normal conditions: (1) Increase in endogenous glucose production by glycogenolysis and gluconeogenesis; and (2) Behavioral changes leading to a sensation of hunger and food seeking[8].

In non-diabetic patients, the initial response to a drop in glucose concentration is reduced insulin secretion. This occurs while the glucose concentration is still within the low physiological range. An additional drop in glucose will cause increased secretion of glucagon and epinephrine (also cortisol and growth hormone, whose roles are less significant) so that lower glucose concentrations activate an intensive sympathoadrenal reaction leading to the appearance of relevant symptoms, with additional lowering of glucose concentration liable to cause cognitive deterioration and severe neurological effects (*e.g.*, convulsions, loss of consciousness)[16].

The above defense mechanisms are often impaired in patients with diabetes and significant beta-cell failure who lack an initial response to a drop in insulin. This leads to a delay in the secretion of glucose from the liver during hypoglycemia. The rate of hypoglycemic episodes increases with the duration of diabetes, perhaps due to the gradual lack of endogenous insulin, which occurs more rapidly in patients with type 1 diabetes and slower in those with type 2 diabetes[8].

In addition, although it is normal in the initial stages of diabetes, the glucagon reaction to hypoglycemia deteriorates over time in type 1 diabetes, and more slowly in persons with type 2 diabetes. In advanced stages, there is also a marked impairment in the sympathoadrenal reactions to hypoglycemia. The drop in the adrenal reaction is secondary to the reduction in the plasma glucose threshold required to activate this mechanism. In patients with type 1 diabetes, a combined reduction in glucagon and epinephrine reactions to hypoglycemia increases risk of hypoglycemia. These mechanisms also occur in the initial stages of type 2 diabetes but less as diabetes progresses[8].

Hypoglycemia unawareness

It is believed that the impaired sympathoadrenal response is secondary to repeated episodes of hypoglycemia that reduce the autonomic response to other hypoglycemic events. This exposes patients to a vicious cycle of frequent hypoglycemia events and shifts glycemic thresholds for symptoms to lower plasma glucose concentrations close to levels that cause cognitive failure. After 25 years of treatment, the prevalence of this phenomenon in patients with type 1 diabetes reached 50%, as compared to a prevalence of approximately 10% in type 2 diabetics. It is unclear whether this phenomenon develops in diabetic patients taking oral medications alone[8]. This condition was defined by Cryer as hypoglycemia-associated autonomic failure (HAAF)[17].

The defense mechanisms may be impaired by repeated hypoglycemia events, physical exercise, and sleep thus contributing to the development of hypoglycemia.

The presumed mechanisms of hypoglycemia unawareness are summarized in Figure 2[12].

Recurrent hypoglycemia can develop as a result of reduced autonomic response to hypoglycemia with attenuation of autonomic warning symptoms. The defective brain response is characterized by increased GLUT1 activity aimed at preserving brain function and altering glucose sensing in the ventromedial hypothalamus (VMH), mediated by elevated levels of Gamma-Aminobutyric Acid (GABA)[12]).

IMPLICATIONS OF HYPOGLYCEMIA IN DIABETES

Hypoglycemia causes physical and psychological morbidity in diabetic patients. Symptomatic hypoglycemia constitutes a concern and a distraction. It can impair judgment, performance of simple daily activities such as driving, and behavior. In more severe cases, hypoglycemia may result in convulsions and loss of consciousness. Sometimes transient neurological deficits may appear, and rarely, there may be permanent neurological damage.

In systematic follow-up of patients over 18 years from the DCCT/EDIC, no significant reduction in long-term cognitive function was demonstrated in patients with type 1 diabetes[18]. However, the data did not include elderly patients or children with diabetes. Other studies show evidence of a relationship between hypoglycemia and cognitive decline in patients with type 1 or 2 diabetes. In one study, a relationship was found between hypoglycemia and reduction in cognitive function in children, including linguistic abilities, working memory, and speed of non-verbal processing[19]. Other studies suggest that among elderly diabetic patients hypoglycemia had twice the risk of developing dementia[20].

Concern about hypoglycemia is a barrier to diabetes treatment and control, while patients experiencing recurrent episodes of hypoglycemia are also at risk of depression and anxiety.

In a meta-analysis of more than 900000 patients, a 2-fold increase in the risk of cardiovascular morbidity was observed amongst patients with type 2 diabetes and severe hypoglycemia. This phenomenon may be explained by the sympathoadrenal response and marked increase in the level of catecholamines in the blood, causing a direct effect on the myocardium and vascular system, platelet activation, and aggregation[21].

In more severe cases, hypoglycemia is liable to cause mortality, responsible for 4%-10% of mortality in patients with type 1 diabetes[22,23]. In patients with type 2 diabetes, the mortality rate from hypoglycemia is unknown.

Although severe, sustained hypoglycemia may cause brain death, with most cases of sudden death related to cardiac arrhythmias due to enhanced sympathoadrenal reaction causing QT prolongation[24]. Hypoglycemia may affect cardiovascular events by several mechanisms, as detailed in Figure 3[25].

Clinical and epidemiological studies based on tens of thousands of patients with type 1 and type 2 diabetes from a variety of health services in different world regions showed a 1.5- to 6-fold increase in the risk of cardiovascular events and mortality among patients who experienced severe hypoglycemia[26].

Hypoglycemia in pregnancy

It is very important to control diabetes in pregnant women to prevent maternal and fetal complications. The definition and diagnosis of hypoglycemia in pregnancy are challenging because glucose goals during pregnancy are 20% lower than prior to pregnancy[27]. A marked increase of up to 5-fold in the rate of severe hypoglycemia in

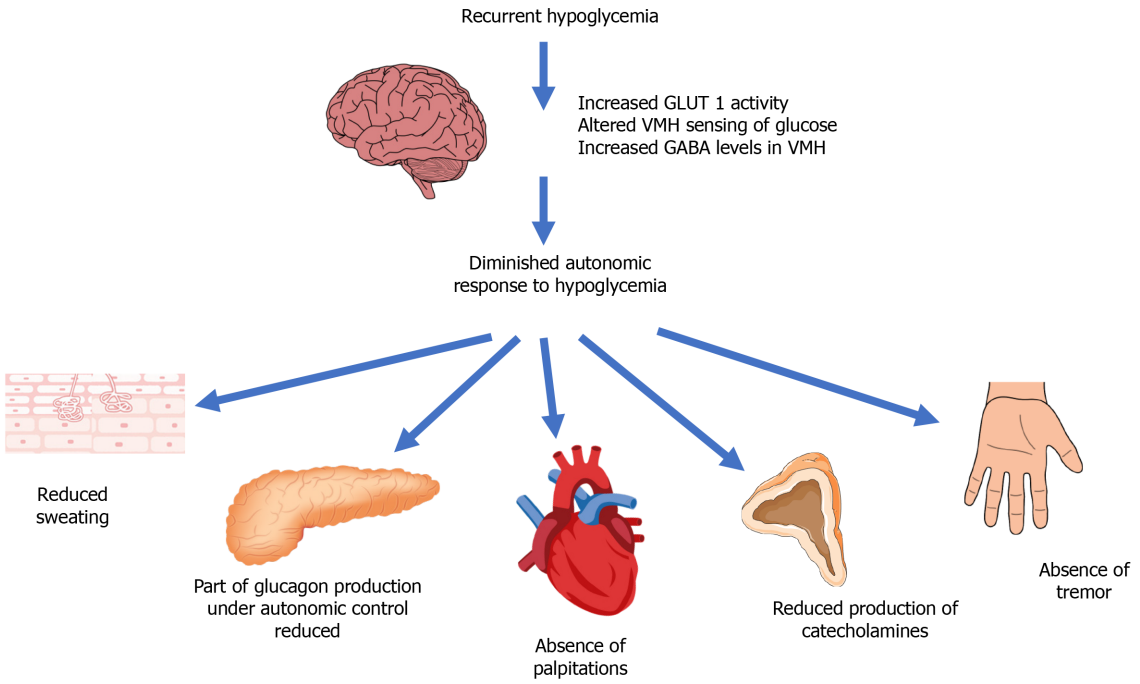

Figure 2 Putative mechanisms of hypoglycemia unawareness. Recurrent hypoglycemia results in a reduced autonomic response to hypoglycemia with attenuation of autonomic warning symptoms. The maladaptive response in the brain is characterized by increased glucose transporter 1 (GLUT1) activity in a bid to preserve brain function and alter glucose sensing in the ventromedial hypothalamus (VMH), mediated by elevated levels of gamma aminobutyric acid (GABA). Adapted from Iqbal *et al*[12] with permission from Elsevier. Citation: Iqbal A, Heller S. Managing hypoglycaemia. *Best Pract Res Clin Endocrinol Metab* 2016; 30: 413-430. Copyright © Elsevier.

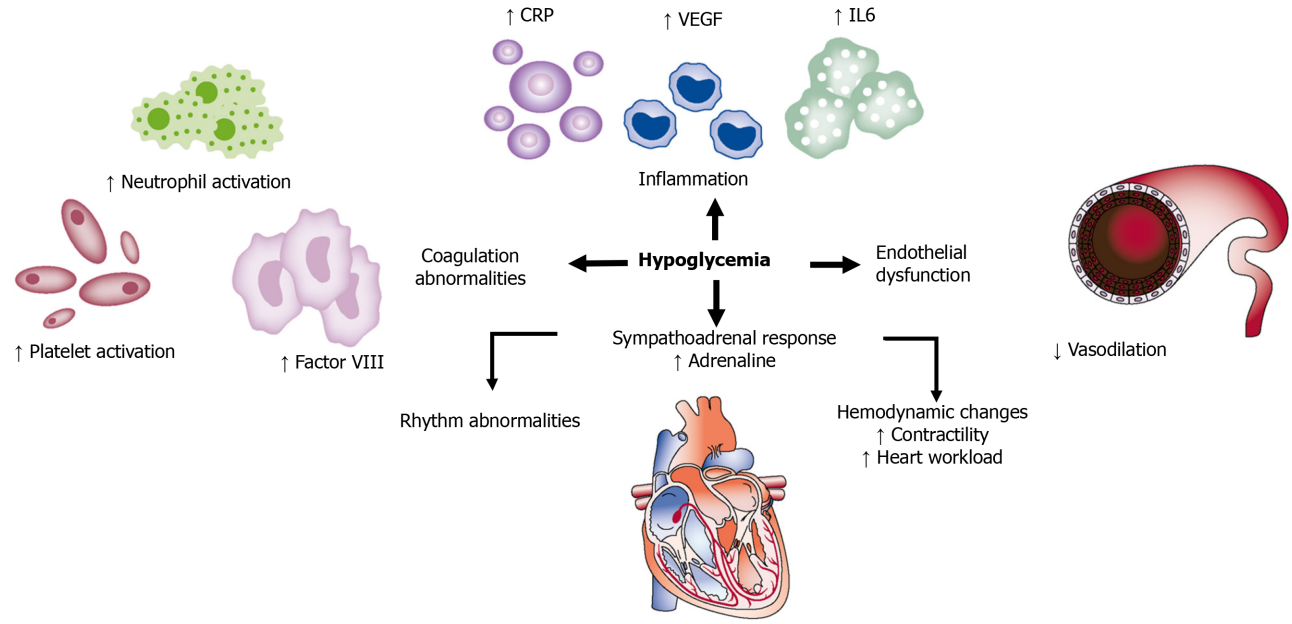

Figure 3 Mechanisms by which hypoglycemia may affect cardiovascular events. Hypoglycemic events may induce inflammation by stimulating the release of C-reactive protein (CRP), IL-6, and vascular endothelial growth factor (VEGF). Hypoglycemia also increases the activation of platelets and neutrophils. Sympathoadrenal response during hypoglycemia increases adrenaline release and may lead to arrhythmias and increased cardiac workload. Endothelial dysfunction may also contribute to cardiovascular risk. Adapted from Desouza *et al*[25] with permission from the American Diabetes Association. Citation: Desouza CV, Bolli GB, Fonseca V. Hypoglycemia, diabetes, and cardiovascular events. *Diabetes Care* 2010; 33: 1389-1394. Copyright ©The American Diabetes Association.

the first trimester among women with type 1 diabetes has been found[28].

In general, the fetus is not in danger from maternal hypoglycemia if the mother is not injured during the episode. The risk of hypoglycemia in diabetic women treated with insulin also increases with breastfeeding[29].

Hospitalized patients with hypoglycemia

At any given moment persons with diabetes constitute about 30 percent of hospitalized patients[30]. Attitudes towards glycemic control during hospitalization have altered considerably in the last decades, changing from a strict approach with tight control to a more lenient approach with less tight control. For the majority of critically and non-critically ill patients, a target glucose range of 140-180 mg/dL is recommended[31]. This change in recommendations is mostly based on observations that too much control often results in severe hypoglycemia and even endangers life, and thus, for diabetic patients, there is no improvement in morbidity or hospitalization parameters.

HYPOGLYCEMIA TREATMENT

Most self-monitoring diagnoses of episodes of symptomatic or asymptomatic hypoglycemia can be treated effectively by rapid-acting carbohydrate (approximately 20 g of glucose constitutes a reasonable dose in most cases) with an expectation of clinical improvement within 20 min.

The importance of giving long-acting carbohydrates after correction of glucose level should be emphasized, because in prolonged hyperinsulinemia effects of oral glucose last fewer than 2 h.

Table 1 presents the protocol for treating hypoglycemia, established mostly in accordance with the Joint British Diabetes Societies guidelines[32].

PREVENTION OF HYPOGLYCEMIA

The approach to hypoglycemia prevention includes patient education, appropriate dietary and exercise regimens, glucose monitoring, medication adjustment, and close clinical supervision[33].

Patient education

The patients and those around them should be educated to identify symptoms of hypoglycemia and given appropriate treatment as soon as possible. It is important to routinely discuss the dangers of developing hypoglycemia and how it should be treated in patients treated with insulin, sulfonylurea, or glinide. In every hypoglycemia documented, the circumstances of the episode should be investigated together with the patient to try to detect the reason, for example, a skipped meal/prolonged fasting, physical exertion, alcohol consumption, and injection of a high insulin dose.

Patients with diabetes who are at increased risk of hypoglycemia are requested to carry glucagon with them at all times. Family members and people in the environment of patients with diabetes should be instructed regarding the administration of glucagon to the patient; they should also know where the glucagon is kept. Glucagon products include a solution for subcutaneous or intramuscular injection and intranasal glucagon (FDA approved in 2019).

Dietary intervention

Dietary intervention includes instruction regarding the amount of carbohydrates at meals and its effect on blood glucose concentration and building a personalized regular meal plan. In patients treated with insulin, there should be an emphasis on the importance of giving insulin with appropriate dosage and timing in relation to meals. Patients at risk of hypoglycemia should be instructed to equip themselves with glucose or foods containing carbohydrates and to always keep them at hand. In some patients, especially those with type 1 diabetes or at high risk of nocturnal hypoglycemia, a bedtime snack can be recommended with the purpose of preventing overnight hypoglycemia.

Recommendations on physical exercise

Physical exercise increases glucose consumption and the risk of hypoglycemia. Risk factors include strenuous, prolonged physical exertion, and lack of energy source relative to the insulin in the body. By monitoring blood glucose before and after physical exercise, early steps can be taken to prevent hypoglycemia. Small meals should be eaten prior to physical exercise if there are drops in glucose concentration.

Table 1 Protocols for treating hypoglycemia

Steps	Procedure
	Adults who are conscious, orientated, and able to swallow
1	If the patient is receiving insulin (pump or IV infusion), stop it immediately
2	Give 15-20 g rapid-acting carbohydrate of the patient's choice where possible. Examples include: 15-20 g chewable glucose tablets, 150-200 mL orange juice, or 3-4 heaped teaspoons of sugar dissolved in water
3	Repeat capillary blood glucose measurement 10-15 min later. If it is still less than 70 mg/dL, repeat the previous step up to 3 times
4	If the capillary blood glucose remains less than 70 mg/dL after 30-45 min or three cycles of treatment, consider IV 200 mL of 10% glucose over 15 min or administration of 1 mg of glucagon IM
5	Once blood glucose is above 70 mg/dL and the patient has recovered, it is recommended to give a long-acting carbohydrate. Examples: one slice of bread, a 200-300 mL glass of milk, or two biscuits
	Adults who are conscious but confused, unable to cooperate but able to swallow
1	If the patient is receiving insulin (pump or IV infusion), stop it immediately
2	If the patient is uncooperative but is able to swallow, give a 15g tube of glucose (*e.g.*, Glucogel), squeezed into the mouth between the teeth and gums, or (if this is ineffective) glucagon 1mg IM
3	Repeat capillary blood glucose levels after 10-15 min. If it is still less than 70 mg/dL, repeat the previous step up to three times (glucagon injection should only be given once)
4	If the capillary blood glucose remains less than 70 mg/dL after 30-45 min (or three cycles of treatment), give IV 200 mL of 10% glucose over 15 min
5	Once blood glucose is above 70 mg/dL and the patient has recovered, giving a long-acting carbohydrate is recommended (as detailed above)
	Adults who are unconscious and/or having seizures
1	An urgent medical assessment is required. The following things should be checked and treated accordingly: Airway (administration of oxygen as appropriate), breathing, circulation (pulse), state of consciousness, blood glucose concentration, and body temperature
2	If the patient is receiving insulin (pump or IV infusion), stop it immediately
3	Request immediate assistance from medical staff
4	If IV access is available, give 100 mL of 20% glucose IV or 200 mL of 10% glucose over 15 min
5	If no immediate IV access is available, give 1mg glucagon IM. If no IV access is available initially, continue trying to obtain IV access as IM glucagon is less likely to be successful if required for a second time. If there is a need for prolonged treatment, IV administration of glucose is the treatment of choice
6	Capillary blood glucose test should be repeated after 10 min. If it is still less than 70 mg/dL repeat step 4 (or step 5 if IV access remains unavailable)
7	Once the blood glucose is greater than 70 mg/dL and the patient has recovered, give a long-acting carbohydrate (as previously detailed)

In an unconscious person with hypoglycemia, glucose may also be given as 20-50 mL of 50% glucose IV over 1-3 min in accordance with Diabetes Canada guidelines [33]. However, it is important to monitor the infusion, especially if given peripherally. The risk of extravasation during the administration of hypertonic glucose solution should be emphasized, as this may lead to significant tissue damage. It is important to note that glucagon may be less effective when administered repeatedly, in cases of sulfonylurea use, after alcohol consumption, and in patients with chronic liver disease. Individuals who received glucagon require a larger portion of complex carbohydrate (40 g) to replenish glycogen stores. Take into account that sometimes nausea appears after administration of glucagon. If hypoglycemia was secondary to sulfonylurea or long-acting insulin, the risk of hypoglycemia may persist 24-36 h following the last dose, especially in people with renal insufficiency.

Patients are recommended to equip themselves with rapid-acting carbohydrates during physical exercise. When physical exercise is planned, it is important to adjust the insulin dose.

Glucose monitoring

Self-monitoring of blood glucose (SMBG) and continuous glucose monitoring constitute essential tools to diagnose hypoglycemia in the early stages. SMBG constitutes an integral part of the efforts to prevent hypoglycemia. ADA recommendations for most patients on intensive insulin regimens (multiple daily injections (MDI) or pump) are to check glucose before meals and occasionally post-prandially, before

sleep and physical exercise, when there is a suspicion of low blood glucose, after treatment of hypoglycemia, and before certain activities requiring high concentration like driving[34].

There is insufficient information in the literature regarding the frequency of glucose self-monitoring required in patients who do not use intensive insulin regimens, including those with type 2 diabetes using basal insulin and/or oral agents. According to most authorities, monitoring should be less intensive with fasting measurements in the morning and sometimes before supper.

Continuous glucose monitoring (CGM), which measures the interstitial glucose in real-time, constitutes a potential tool to improve diabetes control and reduce hypoglycemic episodes. There are two types of CGM devices: real-time CGM that provides information about current glucose concentrations and trends to a receiver; and intermittently scanned CGM which requires passing a scanner over the transmitter to determine the glucose concentration.

CGM has been investigated in many studies, with the efficacy of CGM in diabetes control tested in some studies and the integration of CGM intending to reduce hypoglycemic episodes in other studies.

In patients with uncontrolled diabetes (type 1 or type 2), the use of CGM contributed to improved control and reduction of 0.3%-0.6% in HbA1c[35].

Most studies that investigated the use of CGM to prevent hypoglycemia in type 1 diabetes showed a significant reduction in time spent in hypoglycemia within the range of 54-70 mg/dL[35]. To this day, only limited evidence is available on the effectiveness of CGM in reducing level 3 hypoglycemia episodes.

In a study of 120 patients, children and adults, with type 1 diabetes and HbA1c < 7.5% were divided for 26 wk into a group under CGM monitoring and a control group. Patients using CGM spent less time in hypoglycemia per day and was accompanied by better control than the control group[36].

A study of 322 patients with type 1 diabetes treated with an intensive insulin regimen showed that adults > 25 years who used CGM had a decrease of 0.5% in HbA1c *vs* those who performed SMBG, without a significant difference in hypoglycemia rate. No significant difference was observed in HbA1c or hypoglycemia episodes in people < 25 years[37].

In a recent trial, CGM was effective in reducing hypoglycemia as compared with standard blood glucose monitoring in adults > 60 years with type 1 diabetes[38].

An additional study in patients with type 1 diabetes and initial HbA1c < 7% showed advantages to using CGM with regard to diabetes control and reducing hypoglycemia [39].

The HypoDE trial showed that the use of CGM significantly reduced hypoglycemia rate in adult patients with type 1 diabetes with a history of hypoglycemia unawareness or severe hypoglycemia who were treated with an MDI regimen[40]. Nevertheless, a significant reduction in episodes of severe hypoglycemia requiring medical intervention (administration of glucose or glucagon) was not observed when compared to the control group[40].

In the DIAMOND trial that was conducted in patients with type 1 diabetes, use of CGM for 24 wk led to improvement in diabetes control (decrease of 0.6% in HbA1c) with a significant decrease in glycemic variability and reduction in time spent in hypoglycemia, but without change in number of severe hypoglycemia episodes[41].

For patients with type 1 diabetes who experience recurrent episodes of hypoglycemia and/or hypoglycemia unawareness, CGM technology may be useful, though its long-term efficacy has not yet been determined.

An additional technology that has come into use in recent years is flash technology to monitor blood glucose, which works by scanning without the need for finger prick or calibration, which has been proven to significantly reduce hypoglycemia rate in adult patients with well-controlled type 1 diabetes[42].

In the recent ALERTT1 trial, adults with type 1 diabetes who switched from intermittently scanned CGM (devoid of alarms) to real-time CGM (with alarms) had improved glycemic control and lower rates of grade 3 hypoglycemia[43]. However, the ALERTT1 trial results might become less applicable as intermittently scanned CGM devices are being updated to include alarms[44]. Further research comparing real-time CGM with updated intermittently scanned CGM technology is needed.

There is insufficient information in the literature regarding CGM efficacy in preventing hypoglycemia in type 2 diabetes. In a recent meta-analysis, no significant advantage was observed for CGM over SMBG in preventing hypoglycemia among patients with type 2 diabetes treated with insulin. Nevertheless, it should be noted that no increase in the risk of hypoglycemia was observed in patients who used CGM, despite the more significant reduction in HbA1c[45]. Recently, Karter *et al*[46] followed

the outcomes of 3806 insulin-treated patients with diabetes (91% type 1, 9% type 2) who initiated real-time CGM. They demonstrated that patients with type 2 diabetes benefited from the use of CGM in terms of improved glycemic control and a significant decrease in the rate of hypoglycemia-related emergency department or hospital utilization. In a randomized clinical trial reported by Martens *et al*[47], CGM use in patients with type 2 diabetes treated with basal insulin resulted in better glycemic control with non-significant reduction of CGM-measured hypoglycemia.

However, further dedicated studies are needed to draw clear conclusions regarding CGM utility in hypoglycemia prevention among insulin-treated patients with type 2 diabetes.

Medication adjustment

Some hypoglycemia episodes in diabetes are associated with the treatment itself; therefore, it is important to use drugs with a low risk of hypoglycemia.

Metformin, DPP-4 inhibitors, GLP-1 analogs, SGLT-2 inhibitors, and pioglitazone are all associated with low risk of hypoglycemia in patients with type 2 diabetes. In contrast, sulfonylureas and glinides are associated with higher risk of hypoglycemia [48]; therefore, consideration of dose reduction or cessation of these drugs and switching to a different treatment is recommended in cases of recurrent hypoglycemia.

A decade ago, the transition to the use of long-acting basal insulin analogs (such as Detemir and Glargine U100) led to a significant reduction in episodes of nocturnal hypoglycemia compared to NPH insulin[49,50]. In patients with both type 1 and type 2 diabetes, the new ultra-long basal insulins Glargine U300 (300 units per mL) and Degludec have recently led to a significant additional reduction in the rate of nocturnal hypoglycemia[51-54].

The use of short-acting insulin analogs has also led to a significant reduction in rates of severe hypoglycemia as compared to human insulin[55].

In patients with type 2 diabetes, the combination of basal insulin and GLP-1 analog in one syringe at a fixed ratio showed a significant improvement in diabetes control without increased risk of hypoglycemia[56]. The approach to preventing hypoglycemia in patients with diabetes treated with insulin is detailed in Figure 4[57].

A 2010 study showed that the use of continuous subcutaneous insulin infusion (CSII) prevented hypoglycemic episodes and improved the threshold of hypoglycemia awareness in patients with type 1 diabetes who suffered from recurrent episodes of non-severe or severe hypoglycemia[58]. An earlier meta-analysis showed that, compared to the MDI regimen, the use of CSII reduced the rate of severe hypoglycemia, but this conclusion was based on three randomized controlled studies that used NPH or Lente insulin[59]. Data has so far been inconsistent for patients with either type 1 or type 2 diabetes; two meta-analyses concluded there was no advantage to using a CSII over MDI regimen in terms of reducing the risk of severe hypoglycemic events. CSII, especially sensor-augmented insulin pump, showed an advantage in terms of glycemic control in adults with type 1 diabetes mellitus[60,61].

In the ASPIRE trial, using a sensor-augmented insulin pump programmed to suspend insulin infusion in response to low glucose concentrations led to a significant reduction in episodes of nocturnal hypoglycemia in patients with type 1 diabetes, without an increase in HbA1c[62].

The HypoCOMPaSS trial, which included patients with type 1 diabetes, compared a group treated with MDI and SMBG with a group treated with CSII and real-time CGM, showing a similar reduction in episodes of severe hypoglycemia and improvement in hypoglycemia awareness in both groups; patient satisfaction was higher in the pump group[63].

In recent years, much effort has been invested in building an "artificial pancreas"- a closed-loop system combining a real-time CGM and CSII using glucose control and safety algorithms that manage insulin delivery in a glucose-responsive manner. The use of an artificial pancreas can reduce the burden on patients by automatically adjusting the delivery of insulin based on sensor glucose levels. Single-hormone (insulin-only) and dual-hormone (insulin and glucagon) systems have been developed. In the dual-hormone system glucagon is also delivered in a similar glucose-responsive manner.

In a recent systematic review and meta-analysis, it was shown that the use of the "artificial pancreas" technology constitutes effective and safe treatment for patients with type 1 diabetes and leads to improved diabetes control, and reduced time in hypoglycemia[64]. However, current evidence for artificial pancreas systems is limited by inconsistent reporting of outcomes and short follow-up times[64-67].

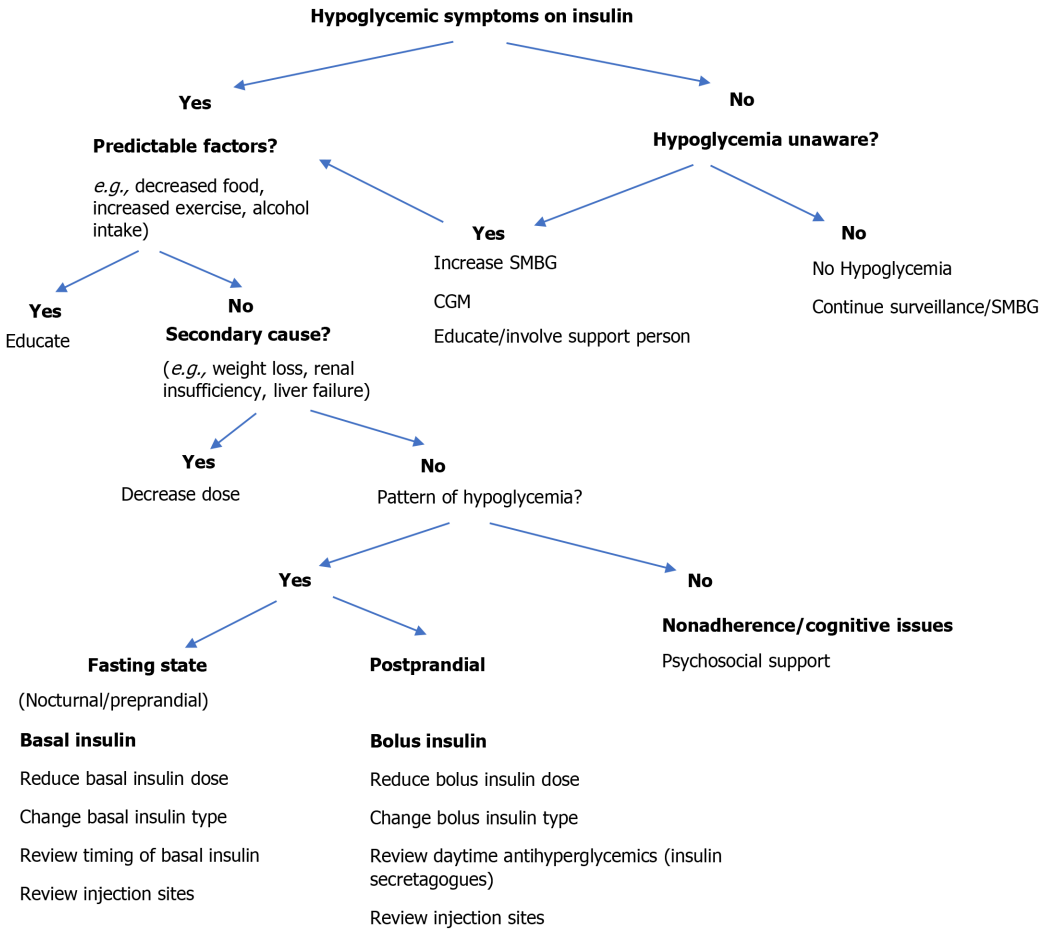

Figure 4 Algorithm of the approach to hypoglycemia. CGM: Continuous glucose monitoring; SMBG: Self-monitoring of blood glucose. Adapted from Blumer *et al*[57] with permission from Elsevier. Citation: Blumer I, Clement M. Type 2 Diabetes, Hypoglycemia, and Basal Insulins: Ongoing Challenges. *Clin Ther* 2017; 39: S1-S11. Copyright © Elsevier.

Pancreatic islet transplantation might be of great promise for patients with type 1 diabetes. Significant progress has been made to improve islet function and clinical outcomes after transplantation. Pancreatic islet transplantation has provided glycemic control, reduced episodes of hypoglycemia, and improved hypoglycemia awareness in patients with type 1 diabetes[68-70].

CONCLUSION

Hypoglycemia in diabetes is associated with increased morbidity and constitutes a barrier to glycemic control. Much effort must be invested in hypoglycemia prevention, including patient education, appropriate dietary and exercise regimens, adjustment of the treatment regimen, and implementation of glucose monitoring systems as appropriate.

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ORIGINAL ARTICLE

Basic Study

Inhibitory effect of maspinon neovascularization in diabetic retinopathy

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Abstract

BACKGROUND

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus. Retinal neovascularization is one of the main pathological features of proliferative DR, and inhibiting retinal neovascularization is a research focus.

AIM

The aim was to evaluate the effect of intravitreal injection of recombinant human maspin on neovascularization in DR.

METHODS

An oxygen-induced retinopathy (OIR) mouse model was used to simulate neovascularization in DR. New born C57BL/6J mice were randomly divided to a normal control group, a maspin injection OIR group, and an OIR group. The mice in the maspin injection OIR group were injected with recombinant human maspin in the bilateral vitreous cavity on postnatal day P12, and those in the OIR group were injected with sterile phosphate buffered saline. The protein expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) in the retina was measured by western blotting, and the mRNA expression of VEGF and HIF-1α was measured by real-time polymerase chain reaction. The vascular cell nuclei that broke through the inner limiting membrane (ILM) were counted in haematoxylin-eosin stained retinal sections.

RESULTS

It was found that the number of vascular cell nuclei breaking through the ILM was 31.8 ± 8.75 in the OIR group, which was significantly more than that in the normal control group (P < 0.001). The number of vascular cell nuclei breaking

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through the ILM was 6.19 ± 2.91 in the maspin injection OIR group, which was significantly less than that in OIR group ($P < 0.01$). The relative protein and mRNA expression of VEGF and HIF-1α was significantly lower in the retinas in the maspin injection OIR group than in those in the OIR group ($P < 0.01$).

CONCLUSION

Maspin inhibited neovascularization in DR by modulating the HIF-1α/VEGF pathway, which provides a potential and effective strategy for the treatment of DR.

Key Words: Maspin; Diabetic retinopathy; Neovascularization; Vascular endothelial growth factor; Hypoxia-inducible factor 1-alpha

Core Tip: The aim of our study was to evaluate the effectiveness of intravitreal injection of recombinant human maspin on neovascularization in diabetic retinopathy. A mouse model of oxygen-induced retinopathy was used to simulate neovascularization in diabetic retinopathy. Maspin inhibited neovascularization in this model by modulating the hypoxia-inducible factor 1-alpha/vascular endothelial growth factor pathway, which provides a potential and effective strategy for the treatment of diabetic retinopathy.

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INTRODUCTION

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus[1]. The prevalence of DR in patients with diabetes is 34.6% worldwide[2]. The global prevalence of diabetes is estimated to be 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045[3], and the prevalence of DR is expected to rise accordingly. DR progresses in stages from a non-proliferative to a more vision-threatening proliferative (PDR) form. Retinal neovascularization is a key pathological feature of PDR, and inhibiting retinal neovascularization is a research focus[4].

Maspin is a member of the serine protease inhibitor (serpin)family. Studies have shown that maspin, which is a class II tumour suppressor gene, can induce tumour cell apoptosis, reduce the movement of tumour cells, and increase adhesion to inhibit tumour invasion and metastasis. Maspin can also directly induce endothelial cell apoptosis and inhibit the endothelial cell signalling pathway to inhibit the development of tumour angiogenesis. Recombinant maspin inhibits corneal endothelial neovascularization by inhibiting the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor[5]. Recombinant maspin was found to inhibit tumour growth and angiogenesis in an animal model of prostate cancer[6]. Injection of adenovirus carrying maspin into the left ventricle was found to disrupt angiogenesis in developing and mature tumours[7]. Maspin is highly expressed during keratinocyte senescence and has antiangiogenic activity[8]. Maspin-mimetic nanostructures can inhibit angiogenesis in tubulogenesis assays with human umbilical vein endothelial cells and *in vivo* assays in the chick chorioallantoic membrane[9].

The mouse model of oxygen-induced retinopathy (OIR) has much in common with human ischemic retinopathy and effectively simulates retinal neovascularization *in vivo*. The model is widely used to study neovascularization in DR[10,11]. At present, the effect of maspin on retinal neovascularization in animal models is not clear. In this study, we investigated whether maspin could inhibit retinal neovascularization in a mouse OIR model. Through the results of this study, we hope to find agents that inhibit neovascularization in DR and provide a theoretical basis for clinical treatments.

MATERIALS AND METHODS

Ethical approval

All mouse procedures were performed following the guidelines of the Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals.

Animal groups

New born C57BL/6J mice were randomly divided into three groups of 25 each, a normal control group, a maspin injection OIR group, and an OIR group. The mice were housed in a specific pathogen free animal laboratory. On postnatal day P17, 10 mice (20 eyes) were randomly selected from each group for haematoxylin-eosin (HE) staining and 15 mice (30 eyes) were selected for RNA extraction from the left eye for real-time polymerase chain reaction (PCR) and protein extraction from the right eye for western blotting.

Mouse OIR model

The mouse OIR model was established as previous described[12]. On day P7 new-born C57BL/6J mice and their mothers were transferred to a constant hyper oxygen chamber with a volume fraction of 75% ± 2% and were then returned to normal air on day 12. Mice in the normal control group were housed in normal air.

Maspin administration

On day 12, 0.5 μL of 0.05 mg/mL recombinant human maspin was injected into the vitreous cavities of mice in the maspin injection OIR group mice with a microsyringe (Hamilton Company, Reno, NV, United States). In the OIR group, 0.5 μL of sterile phosphate buffered saline was injected.

Histological analysis of retinal sections

Ten mice (20 eyes) in each group were used to prepare tissue sections of eyeball specimens. The eyeballs were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sagittal sections parallel to the cornea and optic disc were prepared at a thickness of 6 μm. One of every six sections was selected at random; five sections were randomly selected from each eye. The sections were dewaxed and rehydrated in a graduated ethanol series for HE staining. The number of vascular cell nuclei that broke through the inner limiting membrane (ILM) was counted with an optical microscope, and the average number that broke through the ILM in each section was calculated. Vascular cell nuclei in the vitreous cavity that were not associated with the ILM were not counted).

Western blotting

Mouse retinal tissue was lysed in RIPA lysis buffer (Beyotime, Jiangsu, China) and the concentration of the extracted protein was determined with a bicinchoninic acid protein assay kit (Beyotime). Forty micrograms of total protein extract from each sample was separated by 5%–14% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). The membranes were incubated with primary antibodies (anti-VEGF, 1:500 dilution, BIOSS, Beijing, China; anti-HIF-1α, 1:500 dilution, Proteintech, Rosemont, IL, United States). Proteins were analysed with an enhanced chemiluminescence kit (Beyotime). β-actin served as an internal control.

Real-time PCR

mRNA was extracted from mouse retinal tissue with RNA pure total RNA extraction kits (Bioteke, Jiangsu, China). cDNA was generated with super M-MLV transcriptase (Bioteke). The primers were as follows: HIF-1α, forward: 5′-AGT GTACCC TAA CTA GCC GA-3′, reverse: 5′-CAC AAA TCAGCA CCA AGC -3′; VEGF, forward: 5′-ACA CACCCA CCC ACA TAC ATA-3′, reverse: 5′-ACT CAA GTCCAC AGC AGT CAA-3′. Relative expression of VEGF and HIF-1α mRNAs was calculated by the comparative cycle threshold method. β-actin served as an internal control.

Statistical analysis

The results were reported as means ± SD. All assays were repeated at least three times. SPSS software (Version 20.0, IBM, Armonk, NY, United States) was used for the statistical analysis. Between-group differences were compared by analysis of variance,

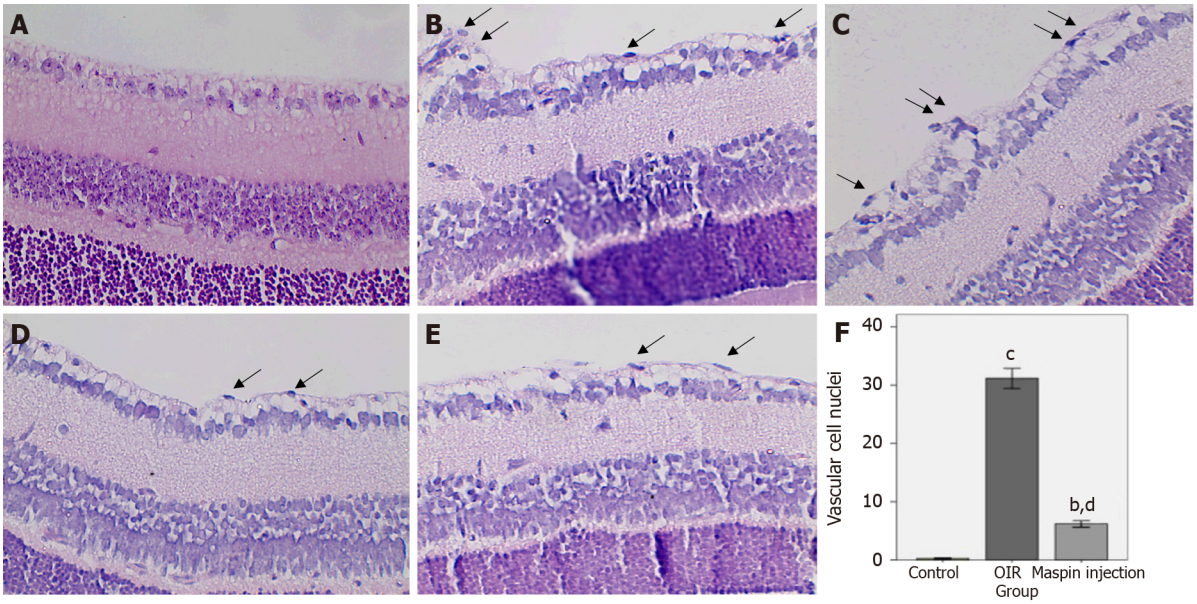

Figure 1 Retinal neovascularization was determined by counting the vascular cell nuclei (arrow) breaking through the inner limiting membrane on postnatal day 17. A: No nuclei were detected in the normal control group; B, C: There were many pathologic neovascular tufts beyond the inner limiting membrane (ILM) in the oxygen-induced retinopathy (OIR) group; D, E: Fewer vascular cell nuclei broke through the ILM in the maspin injection OIR group than in the OIR group but more than that in the normal control group. Magnification ×400; F: Data are means ± SD. $^{b}P < 0.01$ *vs* normal control group; $^{c}P < 0.001$ *vs* normal control group; $^{d}P < 0.01$ *vs* the OIR group. OIR: Oxygen-induced retinopathy.

and P value < 0.05 were considered to be statistically significant.

RESULTS

Number of preretinal neovascular nuclei

In the normal control group, the ILM was smooth, with vascular cell nuclei breaking through the ILM in only a few places (Figure 1A). Many pathologic neovascular tufts broke through the ILM in the OIR group (Figure 1B and C). The number of vascular cell nuclei in the maspin injection OIR group was significantly decreased compared with that in the OIR group (Figure 1D and E). In the normal control group, 0.52 ± 0.10 vascular cell nuclei broke through the ILM in each tissue section. The number that broke through the ILM in the OIR group was 31.8 ± 8.75, which was significantly higher than that in the normal control group ($P < 0.001$). In the maspin injection OIR group, 6.19 ± 2.91 vascular cell nuclei broke through the ILM in each tissue section, which was significantly less than that in OIR group ($P < 0.01$) and more than that in the normal control group ($P < 0.01$; Figure 1F).

VEGF and HIF-1α protein expression in the retina

The western blot results found that relative protein expression of VEGF and HIF-1α in retinas from the OIR group was significantly higher than that in the normal control group (both, $P < 0.001$). The expression of VEGF and HIF-1α protein in retinas from the maspin injection OIR group was significantly lower than that in the OIR group ($P < 0.01$), and higher than that in the normal control group ($P < 0.01$; Figure 2A, B, and C).

Expression of VEGF and HIF-1α mRNA in the retina

The relative mRNA expression of VEGF and HIF-1α mRNA in retinas from the OIR group was significantly higher than that in the normal control group (both $P < 0.001$). The relative mRNA expression of VEGF and HIF-1α mRNA in retinas from the maspin injection OIR group was significantly lower than that in the OIR group ($P < 0.01$), and higher than that in the normal control group ($P < 0.01$; Figure 2D and E).

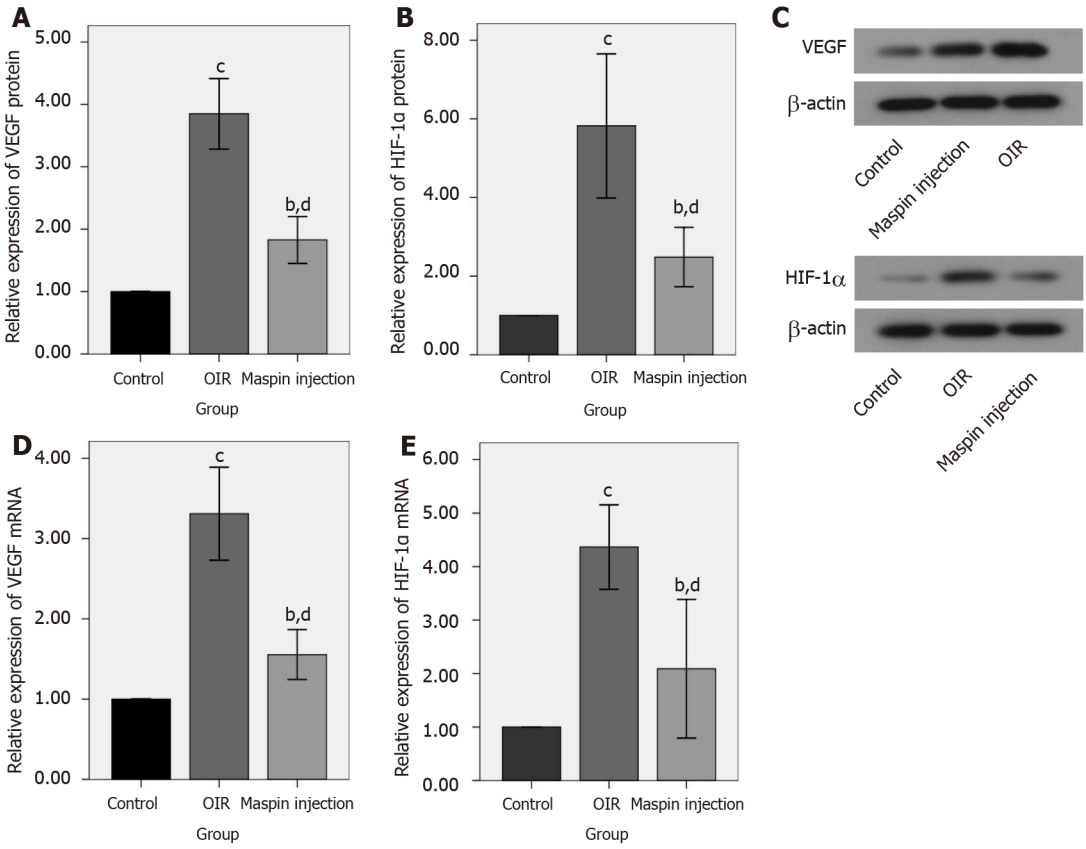

Figure 2 Maspin downregulated vascular endothelial growth factor and hypoxia-inducible factor 1-alpha expression in the maspin injection oxygen-induced retinopathy group. A, B, C: Relative protein expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) were determined by western blotting; D, E: Relative mRNA expression of VEGF and HIF-1α were assayed by real-time polymerase chain reaction. Data are means ± SD. $^{b}P < 0.01$ *vs* normal control group; $^{c}P < 0.001$ *vs* normal control group; $^{d}P < 0.01$ *vs* oxygen-induced retinopathy group. VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia-inducible factor 1-alpha; OIR: Oxygen-induced retinopathy.

DISCUSSION

Our previous studies showed that maspin inhibited high glucose-induced angiogenesis in human retinal microvascular endothelial cells[13]. The inhibitory effect of maspin on retinal neovascularization *in vivo* has not been reported. Intravitreal injection is the main method of studying and treating DR[4,14-16]. The mouse OIR model is widely used to simulate neovascularization and to study the prevention of neovascularization in ischemic retinal diseases such as DR. The retinal blood vessels of 7-d-old mice are not yet mature. Inhalation of high concentration oxygen stimulates retinal blood vessels to undergo reversible spasmodic changes[17]. Continuous hyperoxia causes small vessel occlusion and areas of retinal nonperfusion. After the mice returned to normal air on day P12, the central avascular retina was in a state of hypoxia, leading to both normal vessel regrowth and the formation of extraretinal pathological neovascularization[12,18]. On day P17, retinal neovascularization reached the most advanced stage[19-22]. The model shares many characteristics with DR, and the number of neovascular sites can be measured by counting the nuclei that break through the ILM in HE-stained retinal tissue sections[19,21,22]. We found that significantly fewer nuclei broke through the ILM in the maspin injection OIR group than in the OIR group, indicating that maspin inhibited the development of neovascularization in the DR model.

VEGF promotes the division and proliferation of vascular endothelial cells and increases vascular permeability[23] and is a key angiogenic factor that induces retinal neovascularization[24]. Clinical studies have found that inhibiting VEGF effectively inhibited retinal neovascularization; anti-VEGF therapy has become the main method of treating DR and other retinal neovascular conditions[25-27]. Under hypoxic conditions, HIF-1α is produced in the nucleus and binds to the HIF-1α binding site on the target gene to initiate transcription and promote angiogenesis[28]. HIF-1α can regulate the expression of VEGF, and is active in maintaining energy metabolism and angiogenesis of tumour cells. The activation of VEGF transcription and maintenance of

VEGF mRNA stability in hypoxic tissues is mainly regulated by HIF-1α. VEGF is one of the target genes of HIF-1α[29]. HIF-1α and VEGF are abnormally upregulated in PDR, and HIF-1α regulates the expression of VEGF and promotes retinal neovascularization[30-32]. Interfering RNA targeting VEGF and HIF-1a was effective in inhibiting retinal neovascularization[33,34]. Inhibiting VEGF and HIF-1α is an effective method to treat DR[35,36]. Our previous studies showed that maspin could inhibit HIF-1α and VEGF expression in HG-treated human retinal microvascular endothelial cells. In this study, the retinal expression of VEGF and HIF-1α in was significantly lower in the maspin injection OIR group than that in the OIR group, suggesting that maspin may inhibit neovascularization in DR by modulating the HIF-1α/VEGF pathway. In our study, we observed the inhibitory effect of one dose of recombinant human maspin on retinal neovascularization in OIR on day 17. The effect of different doses of maspin on retinal neovascularization in OIR at different times is planned in future research.

CONCLUSION

In conclusion, our study showed that maspin inhibited neovascularization in DR by modulating the HIF-1α/VEGF pathway, providing a potential and effective strategy for the treatment of DR.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus.

Research motivation

We used an experimental animal model to find a more effective strategy for the treatment of DR.

Research objectives

The study aim was to evaluate the effect of intravitreal injection of recombinant human maspin on neovascularization in DR.

Research methods

An oxygen-induced retinopathy (OIR) model in mice was used to simulate neovascularization in diabetic retinopathy. On postnatal day P12, 0.5 μL of 0.05 mg/mL recombinant human maspin was injected into the vitreous cavity of maspin injection OIR group mice. The protein and mRNA expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) in the retina were assayed. The numbers of vascular cell nuclei that broke through the inner limiting membrane were counted.

Research results

The results revealed that intravitreal injection of maspin inhibited neovascularization and reduced protein and mRNA expression of VEGF, HIF-1α in the retinal tissue of OIR model mice.

Research conclusions

Maspin inhibited neovascularization of DR by modulating the VEGF/HIF-1α pathway, providing a potential and effective strategy for the treatment of DR.

Research perspectives

Retinal neovascularization is one of the main pathological features of PDR. Inhibiting retinal neovascularization is a research focus.

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ORIGINAL ARTICLE

Basic Study

Molecular diagnosis of Kallmann syndrome with diabetes by whole exome sequencing and bioinformatic approaches

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Author contributions: Sun SS performed the research; Wang RX contributed to writing of the original draft, preparation of the paper for submission and revising of the manuscript for important intellectual content.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Shanxi Provincial People's Hospital.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: The data to support the findings in the study are available from the corresponding author upon reasonable request.

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed

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Abstract

BACKGROUND

Kallmann syndrome (KS) is a hypogonadotropic hypogonadism accompanied by anosmia or hyposmia. It is associated with the low secretion of gonadotropins which can lead to other abnormal endocrine metabolism disorders such as diabetes. Through genetic and molecular biological methods, more than 10 KS pathogenic genes have been found.

AIM

To identify the existing mutation sites of KS with diabetes and reveal the relationship between genotype and phenotype.

METHODS

We studied KS pathogenesis through high-throughput exome sequencing on four diabetes' patients with KS for screening the potential pathogenic sites and exploring the genotype-phenotype correlation. Clinical data and peripheral blood samples were collected from the patients. White blood cells were separated and genomic DNA was extracted. High-throughput sequencing of all exons in the candidate pathogenic genes of probands was performed, and the results obtained were analyzed.

RESULTS

Sequencing revealed mutations in the KLB p.T313M, ANOS1 p.C172F, and IGSF10 gene (p.Lys1819Arg and p.Arg1035Thr) at different sites, which may have been associated with disease onset.

CONCLUSION

The diagnosis of KS is challenging, especially in early puberty, and the clinical manifestations reflect physical delays in development and puberty. Timely diagnosis and treatment can induce puberty, thereby improving sexual, bone, metabolic and mental health.

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Core Tip: Kallmann syndrome is associated with low secretion of gonadotropins, which can also lead to other abnormal endocrine metabolism, such as diabetes. Sequencing revealed mutations in the KLB p.T313M, ANOS1 p.C172F, and IGSF10 gene (p.Lys1819Arg and p.Arg1035Thr) at different sites, which may have been associated with disease onset. Timely diagnosis and treatment can induce puberty, thereby improving sexual, bone, metabolic and mental health.

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INTRODUCTION

Idiopathic hypogonadotropic hypogonadism (IHH) is caused by congenital hypothalamic gonadotropin-releasing hormone (GnRH) neuron deficiency or dysfunction in GnRH synthesis or secretion, which leads to a reduction in gonadotropin secretion by the pituitary[1]. Low levels of gonadotropin secretion may consequently lead to insufficient gonadal function. This can manifest in the form of underdeveloped secondary sex characteristics, gamete synthesis disorders, and delayed bone closure, among other conditions. According to the clinical symptoms, there are two types: those with impaired sense of smell are called Kallmann syndrome (KS); those with normal sense of smell are called normosmic hypogonadotropic hypogonadism (nIHH) with normal sense of smell. Measurement of the levels of gonadotropins (luteinizing hormone, LH; follicle-stimulating hormone, FSH) and imaging examinations can help confirm the diagnosis of IHH and hormone replacement therapy can be used as a suitable therapeutic strategy.

KS is a hypogonadotropic hypogonadism accompanied by anosmia (cannot recognize any odor) or hyposmia(recognizable part of a strong pungent odor). It is a disease with clinical and genetic heterogeneity. KS can be familial or sporadic. There are three ways of inheritance: X-linked recessive inheritance, autosomal dominant inheritance, and autosomal recessive inheritance. The pathogenesis of KS is not fully understood. It is currently believed that GnRH neurons originating from the olfactory substrate cannot migrate normally and locate in the hypothalamus due to various reasons, resulting in complete or partial loss of the ability to synthesize and secrete GnRH, cause hypothalamic-pituitary-gonadal axis dysfunction and failure to activate puberty.

Long-term lack of sex hormones can also lead to other abnormal endocrine metabolism, such as calcium malabsorption, osteoporosis; abnormal glucose and lipid metabolism, insulin resistance, diabetes, hyperlipidemia, hypertension, *etc.*

The diagnosis of KS is challenging, especially in early puberty, and the clinical manifestations reflect physical delays in development and puberty. Timely diagnosis and treatment can induce puberty, thereby improving development in sexual, bone, metabolic and mental health.

In this study, we reported four cases of clinically confirmed KS with diabetes by investigating the mutation sites in these patients. The aim is to identify the existing mutation sites and reveal the relationship between genotype and phenotype.

MATERIALS AND METHODS

Patients

The diagnosis of KS is based on: (1) Males > 18-years-old (selecting 18-years-old can exclude some cases that did not enter puberty at the age of 14-18); (2) Clinical manifestations of hypogonadism; (3) LH, FSH, T Levels are low; (4) Thyroid axis function, adrenal axis function, growth hormone axis function and prolactin are normal; (5) Sellar MRI shows no organic abnormalities of the hypothalamus and pituitary; (6) Olfactory bulb/olfactory tract MRI: olfactory bulb, olfactory bundle dysplasia or underdevelopment; (7) Lagging bone age; and (8) Delayed response in GnRH excitability test, 9) normal chromosome karyotype.

Patient 1: A 35-year-old woman, diagnosed with diabetes, suffering from amenorrhea for 17 years was hospitalized in March 2018. The patient had menstrual cramps, underdeveloped breasts, sparse armpit and pubic hair, female secondary sexual characteristics, hyposmia, normal intelligence, pubic hair – Tanner II, and breast Tanner – V. Hospitalization was recommended for further diagnosis and treatment. Patient 2: A 24-year-old man diagnosed with diabetes and sexual underdevelopment 11 years prior was hospitalized in May 2018. The patient first observed at 13 years of age that his penis and testicles were underdeveloped. The patient suffered from azoospermia. Secondary sexual characteristics, such as pubic hair, axillary hair, laryngeal knot, and voice change, among others, were absent. No secondary sexual characteristics were observed. Bilateral breast enlargement was a feminine characteristic observed. Other characteristics included pubic hair – Tanner II, testicular development – Tanner II, and loss sense of smell. Patient 3: A 22-year-old man diagnosed with diabetes, who had hyposmia and found penis and testicles were underdeveloped, Secondary sexual characteristics, such as pubic hair, axillary hair, laryngeal knot, among others, were absent. Patient 4: An 18-year-old man diagnosed with diabetes, also found penis and testicles were underdeveloped, Secondary sexual characteristics, such as pubic hair, axillary hair, laryngeal knot, among others, were absent. and with loss of the sense of smell.

General clinical data collection

Data on the medical history of the patients and their family members were collected in detail and analyzed. Physical examination, routine electrocardiogram, and echocardiography were performed and the results were analyzed. Laboratory tests were performed on chemiluminescence instrument (MAGLUMI 4000 PLUS, China).

We also completed the GnRH stimulation test. Initial blood is drawn to check the basic values of LH and FSH. Next, the patient is injected with GnRH intravenously and the LH and FSH values are checked at 15 min, 30 min, 1 h, and 120 min later. The dynamic changes of these two hormones are observed.

Exome sequencing and bioinformatics analysis

DNA extraction and whole-exome sequencing: For DNA extraction, 2 mL of a venous blood sample was collected from each patient and their parents, which were treated with heparin to prevent coagulation. Genomic DNA was extracted according to the instruction provided by the Se Blood DNA kits (Omega Bio-Tek, Inc.), and the patients' DNA sample was dispatched to Aiji Taikang for whole-exome sequencing (WES).

Analysis of biological information of KS and nIHH related pathogenic genes: Search for KS and nIHH related pathogenic genes from published papers[2-5], analyze the intersection of the two related genes, and use kobas[6] to perform Gene Ontology and KEGG pathway enrichment analysis on the two related genes. The intersection and enrichment analysis graphs are drawn on the hiplot (https://hiplot.com.cn) platform. We then use STRING[7] to analyze the protein-related effects of KS and nIHH-related pathogenic genes.

Bioinformatics analysis of WES data

The raw sequence data obtained was subjected to quality control using FastQC[8] and the clean reads were aligned with the human reference genome (hg19) using bwa[9]. The duplicate reads were labeled using SAMBLASTER[10]. We used the GATK HaplotypeCaller[11] for variant calling, dbNSFP[12] databases were used to annotate the variant sites.

Filter of candidate genes' variants

The following were filtered from the data: population with a mutation frequency greater than 1% (gnomAD data from dbNSFP), mutation in the dbSNP database, and nonsense mutation sites (intron regions, synonymous mutations, and other mutations that do not affect protein function). To predict the effect of variation on protein function(MutationTaster, SIFT_pred, Polyphen2_HDIV methods from dbNSFP).

Verification of candidate sites

In order to verify the variants of candidate genes, we extracted DNA from the blood of their parents and verified the genotype of these variants with Sanger sequencing, the primer pairs used for PCR see Table 1.

RESULTS

Laboratory data

In patient 1, the level of 25-hydroxyvitamin D was found to be 11.78 ng/mL. Liver function, renal function, erythrocyte sedimentation rate, and the levels of electrolytes, calcium phosphate, parathyroid hormone, and C-reactive protein were normal. Blood osmotic pressure was 297 mOsm/kg·H20 and urine osmotic pressure was 620 mOsm/kg·H20. The growth hormone level was found to be 0.895 ng/mL. No abnormalities were observed in the thyroid function test. The cortisol circadian rhythm pattern was as follows: 08:00 120 ng/mL; 16:00 106.6 ng/mL; 00:00 13.93 ng/mL. The 24-h urine-free cortisol level was 188.40 μg/24 h. Color Doppler ultrasound examination revealed that the uterus size was small. The levels of the following sex hormones were measured: blood prolactin 94.67 μIU/mL; estradiol 13.7 pg/mL; progesterone 0.303 ng/mL; testosterone 0.13 ng/mL; LH 0.66 mIU/mL; FSH 0.88 mIU/mL.

The GnRH stimulation test revealed that the peak values of LH and FSH exceeded 1 mIU/mL, which indicated stimulation, see Table 2. MRI scan of the pituitary and CT scan of the adrenal glands revealed no abnormalities. No abnormal lesions were observed in the uterus and breasts.

The following observations were made in patient 2: ACTH 08:00 91.32 pg/mL; alanine aminotransferase 12.58 IU/L; aspartate aminotransferase 12.81 IU/L; blood creatinine 43.75 μmol/L; urine osmotic pressure 898 mOsm/kg·H20; blood osmotic pressure 291 mOsm/kg·H20.

The GnRH stimulation test revealed that the basal values of LH and FSH secretion were low. Both peaked at 120 min, see Table 2. The levels of the following sex hormones were tested: blood prolactin, 201.1 μIU/mL; estradiol 14.12 pg/mL; progesterone 0.479 ng/mL; testosterone 0.361 ng/mL; LH 0.23 mIU/mL; FSH 0.69 mIU/mL. The cortisol rhythm was found to be normal, and MRI scan of the pituitary and CT scan of the bilateral adrenal glands revealed no obvious abnormalities.

In patient 3, Urine protein 2+; urine glucose 3+, ketone body 3+, blood ketone 2.8mmol/L, the growth hormone level was found to be 0.301 ng/mL. No abnormalities were observed in the thyroid function test. The cortisol circadian rhythm pattern was as follows: 08:00 103.1 ng/mL; 16:00 29.67 ng/mL; 00:00 37.39 ng/mL. The levels of the following sex hormones were measured: blood prolactin 350.6 μIU/mL; estradiol 21.17 pg/mL; progesterone 1.07 ng/mL; testosterone 0.46 ng/mL; LH 0.63 mIU/mL; FSH 0.85 mIU/mL.

The GnRH stimulation test revealed that the peak values of LH and FSH exceeded 1 mIU/mL, which indicated stimulation, see Table 2.

In patient 4, The growth hormone level was found to be 0.175 ng/mL. No abnormalities were observed in the thyroid function test. The cortisol circadian rhythm pattern was normal. The levels of the following sex hormones were measured: blood prolactin157.2 μIU/mL; estradiol 5 pg/mL; progesterone 0.13 ng/mL; testosterone 0.46 ng/mL; LH 0.68 mIU/mL; FSH 0.78 mIU/mL.

The GnRH stimulation test revealed that the peak values of LH and FSH exceeded 1 mIU/mL, which indicated stimulation, see Table 2.

KS and nIHH related genes

KS and nIHH related genes can be seen in Table 3. There were 6 genes both in the KS and nIHH. There were 31 genes only in nIHH, however, there were 17 genes in KS (Figure 1). KS related genes were enrichment in Hypogonadotropic hypogonadism, hypothalamus and pituitary gland diseases, endocrine and metabolic diseases, which

Table 1 List of primer pairs used for polymerase chain reaction

Patient	Gene	Template ID	Forward Primer	Reverse Primer	Amp Size (bp)
Patient 1	IGSF10	chr4:39435930-39435950	ACATTTCCGCCCACATCAGAAG	TCAGCTGTGCCTCTCATCTCAT	246
Patient 2	IGSF10	chr3:151161270-151161285	TAACAGGTGGTGCTGCAATGAC	AAGCACTGTGGAACTGAAGTGC	251
Patient 3	KLB	chr3:151164660-151164670	AGCAATGTCAGCTTTGGGGAAG	GCTTTGGGAGGCAGAGGAAAAT	260
Patient 4	ANOS1	chrX:8565100-8565108	TGTGACACTGCATGTGTCTTCAC	TGACCAGCTGTGAGTTCCTCAA	236

Table 2 Gonadotropin-releasing hormone stimulation test result

	Dose (mIU/ml)	0	15 min	30 min	60 min	120 min
Patient 1	FSH	0.88	1.33	1.72	2.47	3.07
	LH	0.66	0.98	1.22	2.31	2.67
Patient 2	FSH	0.69	1.19	1.26	1.61	3.07
	LH	0.23	0.32	0.38	0.64	2.61
Patient 3	FSH	0.85	1.29	1.64	1.88	2.42
	LH	0.63	0.84	1.02	1.36	1.53
Patient 4	FSH	0.78	1.30	1.44	1.84	2.02
	LH	0.68	0.94	1.29	1.33	1.63

FSH: Follicle-stimulating hormone; LH: Luteinizing hormone.

Table 3 List of gene related to Kallmann syndrome and normosmic hypogonadotropic hypogonadism

Disease	Gene (Phenotype MIM number)	Count
Kallmann syndrome and normosmic hypogonadotropic hypogonadism	FGFR1(147950), FGF8 (612702), PROK2 (610628), CHD7 (612370), WDR11 (614858)	5
Normosmic hypogonadotropic hypogonadism	LEP (614962), LEPR (614963), NR0B1 (300200), SRA1, GNRHR (146110), GNRH1 (614841),KISS1R (614837), KISS1 (614842), TACR3 (614840), TAC3 (614839), NR5A1, HESX-1, LHX3, SOX2, FSHB (229070), LHB (228300), PC1, PNPLA6 (215470), RNF216, OTUD4, STUB1, POLR3A (607694), POLR3B (614381), RAB3GAP1, RAB3GAP2, RAB18, TBCID20, DMXL2, KISS1R(614837), NDNF (618841)	30
Kallmann syndrome	ANOS1 (308700), FGF17 (615270), IL17RD (615267), DUSP6 (615269), SPRY4 (615266), FLRT3 (615271),, KLB, PROKR2 (244200), SEMA3A (614897), SEMA3E, SOX10, HS6ST1 (614880), CCDC141, FEZF1 (616030), IGSF10, SMCHD1, NELF (614838), SOX3	18

may be related to fibroblast growth factor receptor signaling pathway[13] (Figure 2). However, nIHH related genes were enrichment with Neuroactive ligand-receptor interaction, GnRH signaling pathway, RNA polymerase, ovarian steroidogenesis, Cytosolic DNA-sensing pathway (Figure 3).

FGF8, CHD7, GNRH1 genes were located in the center of the protein interaction network of KS and nIHH related genes (Figure 4).

Genetic testing

Quality control of raw WES data: Quality control analysis of the raw WES data (using FastQC) of the four samples is illustrated in Table 2. The average quality of bases was greater than 30 (accuracy greater than 99.9%), and the sequence quality was satisfactory. See Figure 5.

Sequence alignment and sequencing depth: Exome sequencing of the four samples yielded 39M paired-end reads, of which 99% (mapped reads) sequences could be matched to the human reference genome, and the proportion of duplicate reads was approximately 15%. The average sequencing depth (mean depth) exceeded 130X. See Table 4.

Table 4 Sequence alignment and sequencing depth

Sample	Patient 1	Patient 2	Patient 3	Patient 4
Raw reads (PEM)	36.83373	39.8752	39.856	39.975
reads mapping rate (%)	99.59	99.58	99.64	99.65
Target duplication rate (%)	14.15	14.32	14.59	14.75
Target mean depth	130.55	139.46	151.43	145.43
T 10X coverage rate (%)	99.57	99.6	99.45	99.27
T 20X coverage rate (%)	99.04	99.13	99.26	99.04
T 30X coverage rate (%)	97.98	98.23	98.57	98.35

Table 5 Distribution of single-nucleotide polymorphisms

Sample	Patient 1	Patient 2	Patient 3	Patient 4
Total	82986	84748	84579	84731
dbsnp, *n* (%)	82205 (99.06)	83887 (98.98)	83752 (99.02)	83835 (98.94)
1000g_EAS, *n* (%)	77404 (93.27)	78717 (92.88)	78737 (93.09)	78824 (93.03)
ExAC_EAS, *n* (%)	45558 (54.90)	45630 (53.84)	45773 (54.12)	45936 (54.21)
GnomAD_exome_EAS, *n* (%)	45617 (54.97)	45698 (53.92)	45866 (54.23)	46025 (54.32)
GnomAD_genome_EAS, *n* (%)	81924 (98.72)	83549 (98.59)	83441 (98.65)	83536 (98.59)
Exonic, *n* (%)	22847 (27.53)	23016 (27.16)	23100 (27.31)	22921 (27.05)
Splicing, *n* (%)	239 (0.29)	246 (0.29)	251 (0.30)	259 (0.31)
UTR3, *n* (%)	3103 (3.74)	3161 (3.73)	3152 (3.73)	3084 (3.64)
UTR5, *n* (%)	2234 (2.69)	2310 (2.73)	2305 (2.73)	2333 (2.75)
Intronic, *n* (%)	48754 (58.75)	50084 (59.10)	49683 (58.74)	50121 (59.15)
Intergenic, *n* (%)	1902 (2.29)	2108 (2.49)	2184 (2.58)	2145 (2.53)
Upstream, *n* (%)	816 (0.98)	881 (1.04)	882 (1.04)	827 (0.98)
Downstream, *n* (%)	371 (0.45)	379 (0.45)	379 (0.45)	366 (0.43)
Ncrna_exonic, *n* (%)	764 (0.92)	726 (0.86)	730 (0.86)	790 (0.93)
Ncrna_splicing, *n* (%)	6 (0.01)	8 (0.01)	4 (0.00)	3 (0.00)
Ncrna_intronic, *n* (%)	1888 (2.28)	1764 (2.08)	1848 (2.18)	1823 (2.15)
Synonymous SNV, *n* (%)	11529 (13.89)	11613 (13.70)	11559 (13.67)	11518 (13.59)
Nonsynonymous SNV, *n* (%)	10755 (12.96)	10727 (12.66)	10815 (12.79)	10749 (12.69)
Stopgain, *n* (%)	85 (0.10)	92 (0.11)	94 (0.11)	84 (0.10)
Stoploss, *n* (%)	10 (0.01)	10 (0.01)	11 (0.01)	8 (0.01)
Unknown, *n* (%)	483 (0.58)	589 (0.70)	635 (0.75)	576 (0.68)

Extent of variation: The bioinformatics analysis revealed that the sample from patient 1 had 82,986 SNPs and 13,495 INDELs. Through dbSNP annotation, 99.06% of the SNPs and 91.15% of the INDELs could be annotated (Tables 5-6).

Patient 2 had 84,748 SNPs, and 13,931 INDELs. Through dbSNP annotation, 98.98% of SNPs and 90.98% of the INDELs could be annotated (Tables 5-6).

Patient 3 had 84, 579 SNPs, and 13, 760 INDELs. Through dbSNP annotation, 99.02% of SNPs and 90.99% of the INDELs could be annotated (Tables 5-6).

Patient 4 had 84,731 SNPs, and 13,794 INDELs. Through dbSNP annotation, 98.94% of SNPs and 90.99% of the INDELs could be annotated (Tables 5-6).

Table 6 Indel distribution

Sample	Patient 1	Patient 2	Patient 3	Patient 4
Total	13495	13931	13760	13794
dbsnp, *n* (%)	12301 (91.15)	12675 (90.98)	12538 (91.12)	12551 (90.99)
1000g_EAS, *n* (%)	8269 (61.27)	8454 (60.68)	8378 (60.89)	8410 (60.97)
ExAC_EAS, *n* (%)	5560 (41.20)	5547 (39.82)	5562 (40.42)	5637 (40.87)
GnomAD_exome_EAS, *n* (%)	5306 (39.32)	5284 (37.93)	5286 (38.42)	5355 (38.82)
GnomAD_genome_EAS, *n* (%)	12567 (93.12)	12991 (93.25)	12760 (92.73)	12812 (92.88)
Exonic, *n* (%)	708 (5.25)	733 (5.26)	707 (5.14)	694 (5.03)
Splicing, *n* (%)	199 (1.47)	182 (1.31)	201 (1.46)	192 (1.39)
UTR3, *n* (%)	669 (4.96)	676 (4.85)	667 (4.85)	661 (4.79)
UTR5, *n* (%)	375 (2.78)	384 (2.76)	364 (2.65)	373 (2.70)
Intronic, *n* (%)	10469 (77.58)	10845 (77.85)	10712 (77.85)	10767 (78.06)
Intergenic, *n* (%)	297 (2.20)	312 (2.24)	309 (2.25)	307 (2.23)
Upstream, *n* (%)	160 (1.19)	187 (1.34)	170 (1.24)	184 (1.33)
Downstream, *n* (%)	51 (0.38)	65 (0.47)	66 (0.48)	68 (0.49)
Ncrna_exonic, *n* (%)	103 (0.76)	93 (0.67)	102 (0.74)	99 (0.72)
Ncrna_splicing, *n* (%)	0 (0.00)	2 (0.01)	4 (0.03)	1 (0.01)
Ncrna_intronic, *n* (%)	407 (3.02)	398 (2.86)	400 (2.91)	391 (2.83)
Frameshift insertion, *n* (%)	94 (0.70)	103 (0.74)	96 (0.70)	100 (0.72)
Frameshift deletion, *n* (%)	135 (1.00)	124 (0.89)	132 (0.96)	137 (0.99)
Nonframeshift insertion, *n* (%)	198 (1.47)	216 (1.55)	200 (1.45)	181 (1.31)
Nonframeshift deletion, *n* (%)	217 (1.61)	215 (1.54)	202 (1.47)	203 (1.47)
Stopgain, *n* (%)	7 (0.05)	8 (0.06)	9 (0.07)	10 (0.07)
Stoploss, *n* (%)	1 (0.01)	1 (0.01)	0 (0.00)	1 (0.01)
Unknown, *n* (%)	99 (0.73)	106 (0.76)	111 (0.81)	103 (0.75)

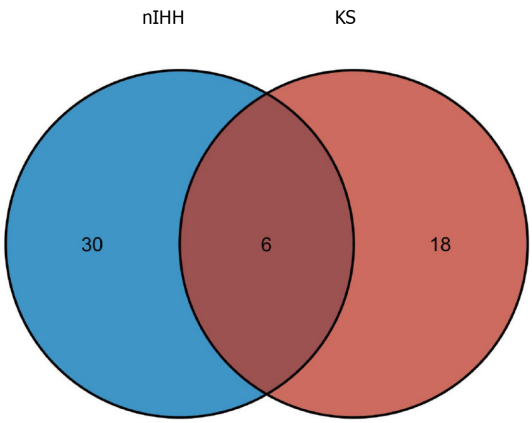

Figure 1 Intersection analysis of Kallmann syndrome and normosmic hypogonadotropic hypogonadism pathogenic genes. nIHH: Normosmic hypogonadotropic hypogonadism; KS: Kallmann syndrome.

Analysis of candidate gene mutations: The gene mutations were filtered according to the following criteria: (1) The mutation should be located in the exon; (2) The mutation should not be synonymous; (3) Population frequency should be greater than 0.001; and (4) Gene in the KS and nIHH related genes list.

Table 7 Specific information about IGSF10, KLB, ANOS1 mutation

Type	Patient 1	Patient 2	Patient 3	Patient 4
Chr.Start.End	chr3.151161279.151161279	chr3.151164665.151164665	chr4.39435942.39435942	chrX.8565101.8565101
Vcf_mut	T/C	C/G	C/T	C/A
GT	0/1	0/1	0/1	1/1
AD	51/53	30/31	34/47	0/86
AAChange.HGVS	IGSF10:NM_178822.4:5/6:c.5456A>G:p.(Lys1819Arg)	IGSF10:NM_178822.4:4/6:c.3104G>C:p.(Arg1035Thr)	KLB:NM_175737:exon2:c.C938T:p.T313M	ANOS1:NM_000216:exon4:c.G515T:p.C172F
cytoBand	3q25.1	3q25.1	4p14	Xp22.31
InterVar_automated	Uncertain significance	Uncertain significance	Uncertain significance	Uncertain significance
ACMG(missense only)	PM1,PM2,BP4	.	PM1,BP4	PM1,PM2,PP3
gnomAD_exome_ALL	.	0.0004	0.0001	0
SIFT_pred	T	D	T	D
Polyphen2_HDIV_score	0.011	0.981	0.036	1
Polyphen2_HDIV_pred	B	D	B	D
Polyphen2_HVAR_score	0.056	0.69	0.016	1
Polyphen2_HVAR_pred	B	P	B	D
LRT_score	0.039	0	0.59	0
LRT_pred	N	D	N	D
MutationTaster_score	0.808	1	1	1
MutationTaster_pred	D	N	N	D
MutationAssessor_score	0.15	2.47	2.535	4.455
MutationAssessor_pred	N	M	M	H
FATHMM_score	-0.27	-0.65	1.43	-5.61
FATHMM_pred	T	T	T	D
CADD_raw	0.585	1.94	-0.287	6.358
CADD_phred	8.054	15.84	0.711	29.4
fathmm-MKL_coding_score	0.039	0.257	0.068	0.967
fathmm-MKL_coding_pred	N	N	N	D

GERP++_RS	0.193	5.46	-7.48	4.51

After filtering, only two variants were detected (one each in IGSF10 and CHD7 genes) for patient 1, whereas four variants were detected (one each in DMXL2, IGSF10, and ANOS1 genes) for patient 2, one variant was detected in the KLB for patient 3, one variant were detected in the ANOS1 for patient 4. Variants in the IGSF10 gene were common to both patients, see Table 7 and Figure 6. The mutations of ANOS1 are located in the region encoding the WAP domain(Figure 6A), The mutations of KLB are located in the region encoding the Glyco_hydro_1 domain (Figure 6B). The two mutations of IGSF10 are located in the region encoding the immunoglobulin I-set domain and in the non-domain region (Figure 6C). Furthermore, the literature search revealed that the genes CHD7, ANOS1, IGSF10, and DMXL2 were also related to IHH.

Verification of candidate sites: To verify the pathogenic sites in the four patients, we compared the parental genotypes and found that IGSF10 (p.Lys1819Arg), KLB p.T313M and ANOS1 p.C172F may harbor the pathogenic site, see Figure 7. Population data did not reveal the presence of a mutation at this site, and the mutation frequency of p. Arg1035Thr in the gnomAD database (from dbNSFP) was found to be 0.0004. and the mutation frequency of p.T313M in the gnomAD database was found to be 0.0001. and the mutation frequency of p.C172F in the gnomAD database was found to be 0.

MutationTaster (from dbNSFP) predicted Lys1819Arg to be a harmful mutation, whereas SIFT_pred (from dbNSFP) and Polyphen2_HDIV (from dbNSFP) predicted Arg1035Thr to be a harmful mutation. Whereas SIFT_pred predicted p.C172F to be a harmful mutation.

DISCUSSION

In this study, the mutant genes and loci of 4 KS patients were analyzed by WES, and four potential pathogenic loci of IGSF10 gene (p.lys1819Arg and p.arg1035Thr) , KLB p.T313M and ANOS1 p.C172F were identified. These loci are new loci that have not been reported.

With the further research on KS genetics, some genes related to KS pathogenesis have been found, such as KAL1, FGFRI, FGF8, PROKR2, PROK2. The function of these genes may be related to the normal migration of GnRH neurons and the development of the olfactory bulb. However, only 30% of the incidence of Kallmann syndrome is related to the above genes, suggesting that there are other disease-related genes of KS that have not been found.

In this study, WES was performed to analyze the mutant genes and loci in four patients with KS and two potential pathogenic loci of the IGSF10 gene (p. Lys1819Arg and p. Arg1035Thr) were identified. According to the analysis of the IGSF10 gene

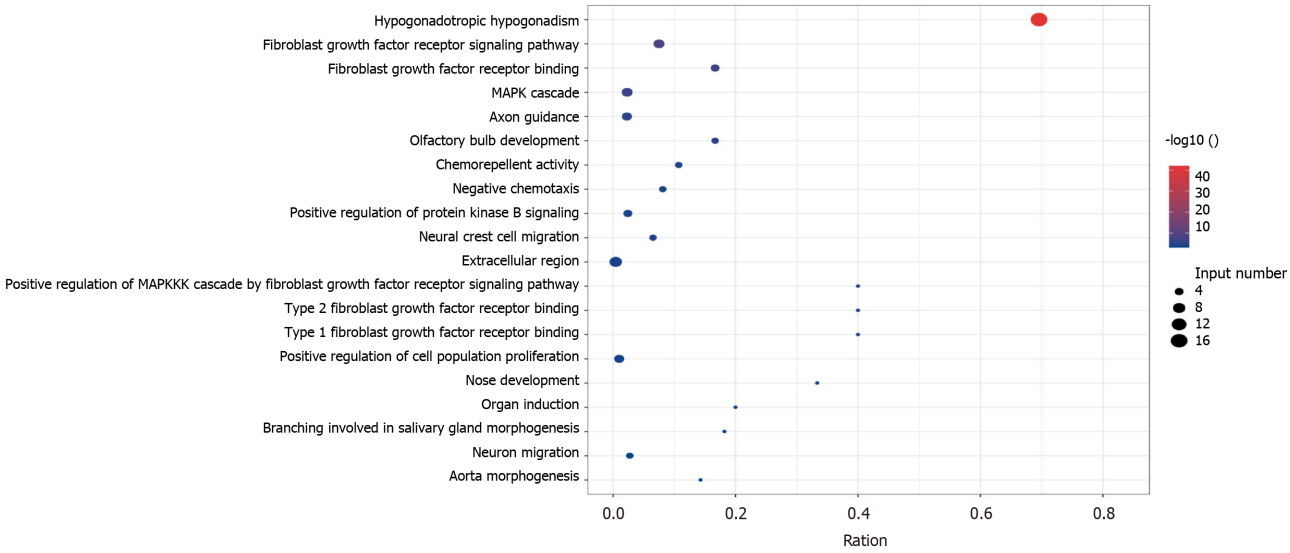

Figure 2 Kallmann syndrome pathogenic gene enrichment pathway and gene ontology, the x-axis represents gene ratio, and the y-axis represents gene ontology term; the size of the dot represents the number of genes, and the color of the dot represents the level of *P* value.

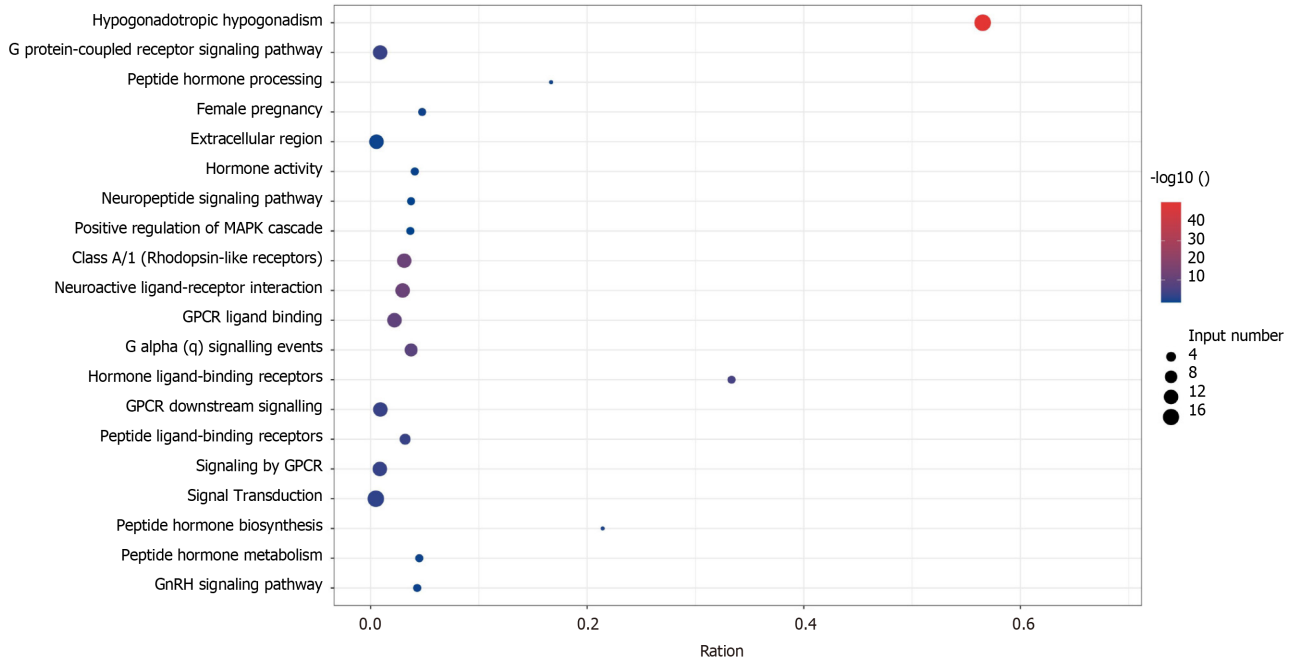

Figure 3 Normosmic hypogonadotropic hypogonadism pathogenic gene enrichment pathway and gene ontology, the x-axis represents gene ratio, and the y-axis represents gene ontology term; the size of the dot represents the number of genes, and the color of the dot represents the level of *P* value.

mutations in the two patients, the variations included alteration of the amino acid at the 1819th position from lysine to arginine and at the 1035th position from arginine to threonine. The Lys1819Arg site is located in the I-set domain of the protein which is primarily associated with immune function and angiogenesis. The discovery of this site can improve our understanding of KS pathogenesis and serve as a novel target in further studies on KS.

IGSF10 is a member of the immunoglobulin superfamily[14]. While its exact function is yet to be clarified, studies have shown that IGSF10 expression is associated with combined pituitary hormone deficiency. It is also considered a novel prognostic biomarker for breast and lung cancers and has been associated with various diseases, such as primary ovarian insufficiency and endometrial cancer. Mutations in the IGSF10 gene are reportedly associated with abnormal regulation of the migration of

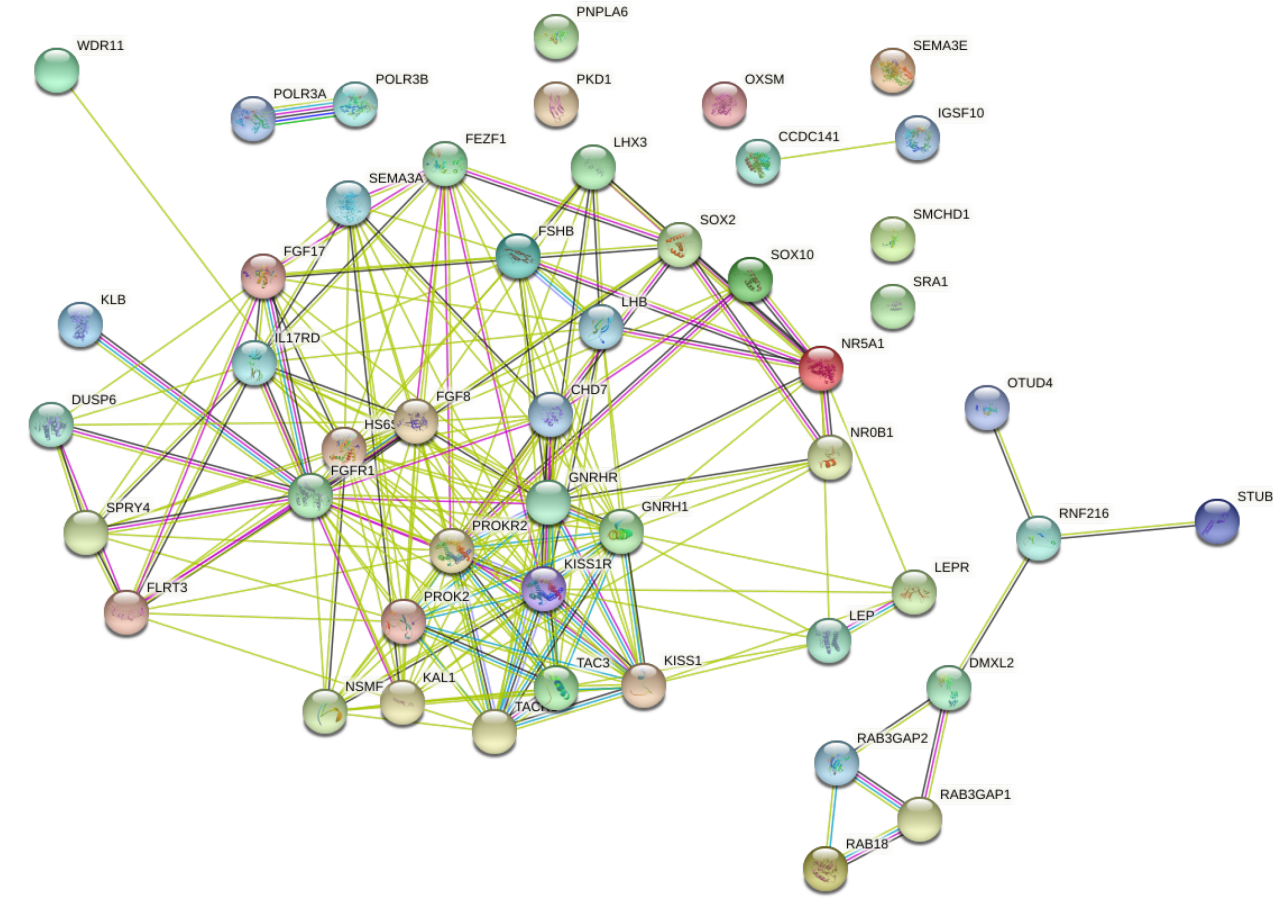

Figure 4 Normosmic hypogonadotropic hypogonadism interacts with Kallmann syndrome pathogenic gene proteins.

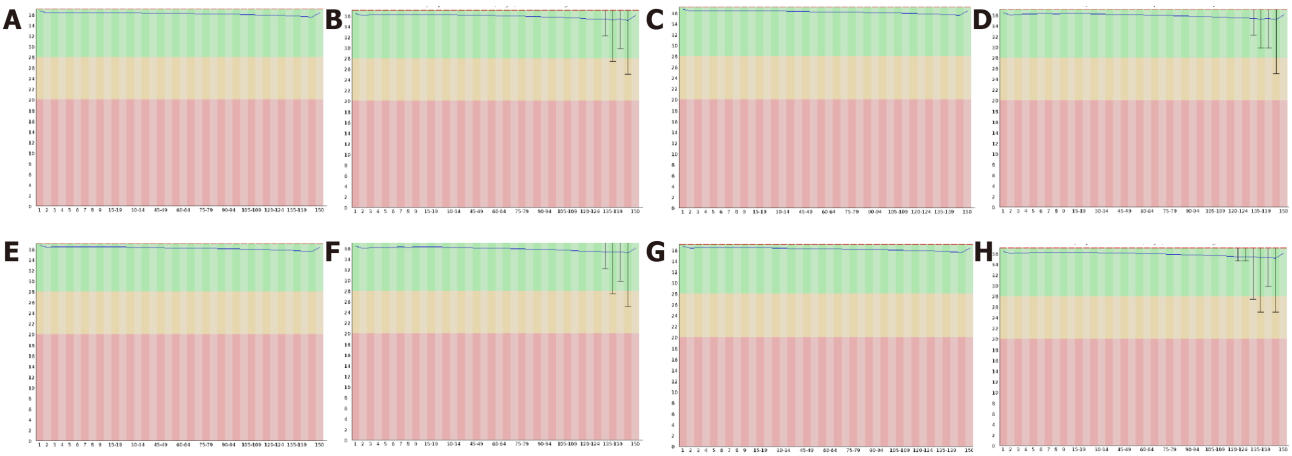

Figure 5 Original results of exome sequencing. A and B: The sequence quality distribution map of reads 1 and 2 of patient 1; C and D: The sequence quality distribution map of reads 1 and 2 of patient 2; E and F is the sequence quality distribution map of reads 1 and 2 of patient 3; G and H is the sequence quality distribution map of reads 1 and 2 of patient 4.

GnRH neurons, which may delay puberty and other developmental processes.

Among KS patients, about 90% of the patients, lack pubic and armpit hair. Bone age lagged behind chronological age in some patients. Some patients have anosmia or hyposmia. Some males have breast hyperplasia, a small penis, cryptorchidism and a lack of vas deferens. Some patients can also be accompanied by other body or organ abnormalities, such as facial cranial midline deformity, nervous system abnormalities, musculoskeletal system abnormalities and other systemic abnormalities.

KLB and KL genes have homology. It is highly expressed in metabolic tissues and especially in fat tissues. FGF21 is an endocrine FGF that is mainly secreted by the liver, which regulates the main metabolic processes such as glucose and lipid metabolism.

Figure 6 The location of four mutation sites.

Endogenous FGF21 regulates the physiological response of starvation and various other metabolic stresses. FGF21 signals through the KLB/FGFR1c receptor complex in a tissue-specific manner. KLB enhances the binding of FGF21-FGFR1c, thereby promoting FGF21 signal transduction by binding FGF21 and FGFR1c to itself through two different sites at the same time. In addition, the competitive binding of FGF8 and b-Klotho to the same site of FGFR1 will facilitate the binding to endocrine FGF21 and inhibit the binding and signal transduction of paracrine FGF8. Most patients with KLB mutations exhibit KS and metabolic defects, such as overweight, diabetes, and dyslipidemia and are consistent with the metabolic effects of this pathway[15-20]. Patient 3 was diagnosed with Kallmann syndrome with diabetes, which belongs to the KLB gene mutation, which also confirmed the above view. It suggests that Kallmann syndrome with KLB gene mutation should pay attention to the change of blood sugar and the occurrence of diabetes

ANOS1 gene is the pathogenic gene found to cause x-linked KS. It is located on the X chromosome (Xp22.3), contains 14 exons, adjacent to the pseudo-autosomal 1 region

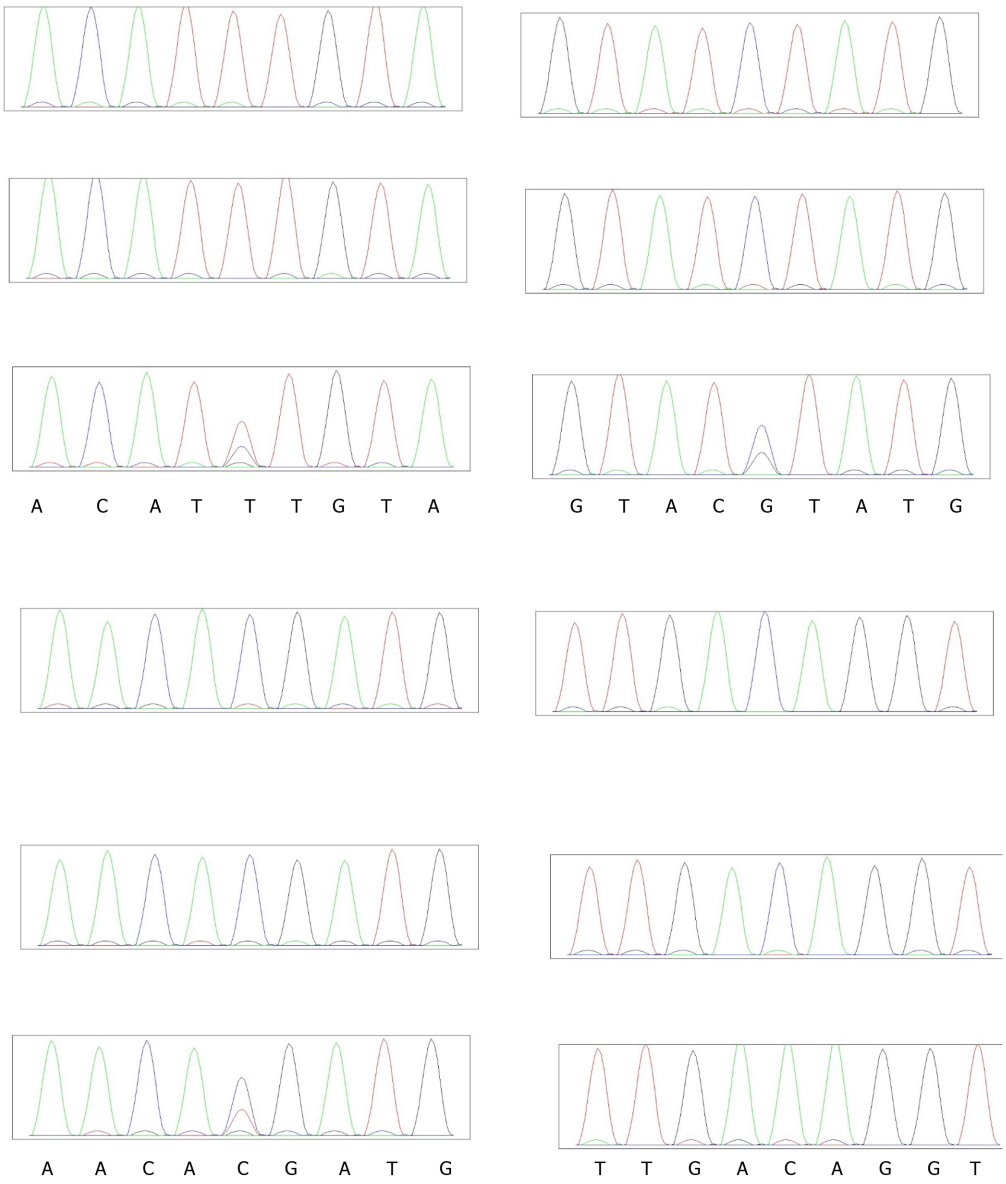

Figure 7 Sanger sequencing of 4 Kallmann syndrome family results.

(PAR1), which is a highly variable and unstable region on the chromosome. ANOS1 encodes anosmin-1, an extracellular matrix protein. Anosmin-1 consists of a cysteine-rich region (CR domain), a whey acidic protein (WAP)-like domain similar, four consecutive fibronectin type III domains and a C-terminal region rich in basic histidines and prolines. Anosmin-1 promotes neuronal cell adhesion, neurite outgrowth, axon guidance and CNS projection neuron branching. In addition, it is also involved in the migration of many types of neural precursors, including GnRH-producing neurons and oligodendrocyte precursors. The ANOS1 mutation is found in patients with familial and sporadic KS. The ANOS1 gene mutation has a low incidence in patients with sporadic KS, but a high incidence in patients with familial KS. In KS patients with ANOS1 mutations, the loss or dysplasia of cryptorchidism and olfactory bulb is high.

In our study, another two potential pathogenic loci of the KLB p.T313M and ANOS1 p.C172F were identified, It suggests that KS disease with KLB mutation should be alert to the risk of diabetes, and KS disease with ANOS1 mutation is related to X-linked recessive inheritance. Although our analysis is limited to 4 patients with KS, it supports the previous view and found new mutation sites to facilitate follow-up research.

Because KS hyposmia can be manifested in different degrees, sometimes it is not easy to distinguish KS and nIHH, especially in patients with hypogonadism, often without careful evaluation of olfactory function. There is genetic evidence that the genes encoding GnRH and Kisspeptin receptors are related to nIHH, but not related to

the migration of GnRH neuroendocrine cells (KS patients may have abnormal migration of GnRH neuroendocrine cells), suggesting that KS and nIHH may have different inheritance background and pathogenesis.

Exon sequencing can be used for studying various diseases. It is useful as a diagnostic tool owing to its low cost and high throughput. The method can be used to detect all mutations in human exons simultaneously. With technological advancements, exon capture has emerged as a useful method. Currently, the chip used has been up to 60M, which can include multiple introns and untranslated regions, and provides valuable information for the study of disease-causing sites.

CONCLUSION

In four Kallmann syndrome patients with diabetes, sequencing revealed mutations in the KLB p.T313M, ANOS1 p.C172F,and IGSF10 gene (p.Lys1819Arg and p.Arg1035Thr) at different sites, which may have been associated with disease onset. The diagnosis of KS is challenging, especially in early puberty, and the clinical manifestations reflect physical delays in development and puberty. Timely diagnosis and treatment can induce puberty, thereby improving sexual, bone, metabolic and mental health.

ARTICLE HIGHLIGHTS

Research background

Kallmann syndrome is a hypogonadotropic hypogonadism accompanied by anosmia or hyposmia. Through genetic and molecular biological methods, more than 10 KS pathogenic genes have been found.

Research motivation

The diagnosis of KS is challenging, especially in early puberty, and the clinical manifestations reflect physical delays in development and puberty.

Research objectives

To identify the existing mutation sites of Kallmann syndrome with Diabetes and reveal the relationship between genotype and phenotype.

Research methods

We studied KS pathogenesis through high-throughput exome sequencing on four diabetes' patients with KS for screening the potential pathogenic sites and exploring the genotype-phenotype correlation. The results obtained were analyzed.

Research results

Sequencing revealed mutations in the KLB p.T313M, ANOS1 p.C172F,and IGSF10 gene (p.Lys1819Arg and p.Arg1035Thr) at different sites, which may have been associated with disease onset.

Research conclusions

The diagnosis of KS is challenging. Timely diagnosis and treatment can induce puberty, thereby improving sexual, bone, metabolic and mental health.

Research perspectives

Exon sequencing can be used for studying various diseases. It is useful as a diagnostic tool owing to its low cost and high throughput and it is very helpful for the diagnosis and treatment of KS.

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ORIGINAL ARTICLE

Case Control Study

Genome-wide association study reveals novel loci for adult type 1 diabetes in a 5-year nested case-control study

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Author contributions: Xu M and Zhang ZL designed the present study; Gao Y, Gu WY, Fang C, Huang YT, Lu Y and Su J performed the sample and data collection; Gao Y, Chen S and Xu M finished the analysis of data; Chen S and Gao Y wrote the article; Wu M, Zhang J, Gao Y, Xu M and Wu M mainly supported the costing of GWAS analysis.

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Abstract

BACKGROUND

Type 1 diabetes (T1D) is a severe and prevalent metabolic disease. Due to its high heredity, an increasing number of genome-wide association studies have been performed, most of which were from hospital-based case-control studies with a relatively small sample size. The association of single nucleotide polymorphisms (SNPs) and T1D has been less studied and is less understood in natural cohorts.

AIM

To investigate the significant variants of T1D, which could be potential biomarkers for T1D prediction or even therapy.

METHODS

A genome-wide association study (GWAS) of adult T1D was performed in a nested case-control study (785 cases *vs* 804 controls) from a larger 5-year cohort study in Suzhou, China. Potential harmful or protective SNPs were evaluated for T1D. Subsequent expression and splicing quantitative trait loci (eQTL and sQTL) analyses were carried out to identify target genes modulated by these SNPs.

RESULTS

A harmful SNP for T1D, rs3117017 [odds ratio (OR) = 3.202, 95% confidence interval (CI): 2.296-4.466, $P = 9.33 \times 10^{-4}$] and three protective SNPs rs55846421 (0.113, 0.081-0.156, 1.76×10^{-9}), rs75836320 (0.283, 0.205-0.392, 1.07×10^{-4}), rs362071 (0.568, 0.495-0.651, 1.66×10^{-4}) were identified. Twenty-two genes were further identified as potential candidates for T1D onset.

CONCLUSION

We identified a potential genetic basis of T1D, both protective and harmful, using a GWAS in a larger nested case-control study of a Chinese population.

Key Words: Type 1 diabetes; Genome-wide association study; Nested case-control study; Polymorphism

Core Tip: Type 1 diabetes (T1D) is a severe and prevalent metabolic disease. Due to its high heredity, an increasing number of genome-wide association studies have been performed, most of which were from hospital-based case-control studies with a relatively small sample size. The aim of this study was to investigate the significant variants of T1D, which could be potential biomarkers for T1D prediction or even therapy. The effects of different polymorphisms in Chinese T1D patients were determined in a healthy population cohort study. The results showed 4 novel variants highly associated with the onset of T1D, namely rs3117017, rs55846421, rs75836320, and rs362071.

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INTRODUCTION

Diabetes mellitus, a prevalent endocrine system disease worldwide, is characterized by high blood glucose level and can be life-threatening. Diabetes can be pathologically classified as type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes and other unclassified diabetes[1]. According to the International Diabetes Federation (IDF), T1D accounts for approximately 5%-10% of all diabetes[2]. Globally, the

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incidence of T1D is increasing, with an overall annual increase of 2%-5%[3,4]. There is also substantial regional disparity, ranging from 0.5% in the Caribbean to 7.0% in Africa[5]. In the IDF 9th edition, the assumption of T1D prevalence to incidence ratio was 6.3 for countries with no available age-specific incidence rates[2]. In developed countries, severe burden in patients and the entire healthcare system are expected as the incidence of T1D is estimated to double every 20 years[6,7]. According to current research, T1D is associated with increased mortality in the population: Its standardized mortality ratio ranges from 0 to 854 in different countries[8]. This implies that T1D patients have 3- to 4-times higher mortality than the general population[4,9].

With regard to the autoimmune feature of T1D, insulin-producing β cells in the pancreas are destroyed by the body due to insufficient islet secretion instead of insulin resistance in T2D[10]. T1D pathobiology is multifactorial, which includes genetic factors, environmental factors, and their potential interactions[11]. It has been demonstrated that first degree relatives of T1D patients had an approximately 6% risk of T1D, which is 15-times higher than the risk in the general population[12]. This finding suggests that genetic factors can be strong determinants of T1D. In China, existing observational studies indicated that approximately two-thirds of new T1D cases were reported in adults over 20 years old. T1D onset in adults is not rare in the Chinese population[13]. However, the exact mechanism of T1D onset in adults remains unknown[14].

Single nucleotide polymorphisms (SNPs) are potential genetic factors of T1D. SNPs have been investigated in population studies of both Caucasian and Chinese populations[15-18]. SNPs of the human leucocyte antigen (HLA) class II gene are identified as major risk factors for T1D[15]. However, these studies are mostly hospital-based case-control studies, which are inevitably prone to sampling bias[19,20]. The profile of T1D-related SNPs in a larger population is rarely addressed or investigated. Consequently, sampling bias in hospital-based studies can have a substantial impact on the subsequent genome-wide association study (GWAS), and may lead to unreliable results.

In order to overcome the challenge due to sampling bias, we performed a GWAS of adult T1D in a nested case-control study from a large 5-year cohort study in Suzhou, China. We aim to comprehensively investigate and quantify the association between T1D and SNPs in the general population. In addition, we will identify novel potential genes that are regulated by the identified SNPs, and evaluate their roles in the pathogenesis of adult T1D.

MATERIALS AND METHODS

Participants, materials and methods

Ethical compliance: This study was reviewed and approved by the Research Ethics Committee of Jiangsu Provincial Center for Disease Control and Prevention (No. 2012025). The study was in full compliance with the Declaration of Helsinki.

Each participant, either in the case or control group, was informed of the nature of this study. Blood samples from the participants were collected after they signed the written informed consents.

Participants and background information on the study: A nested case-control study within a larger cohort study was performed. The original larger cohort included 3466780 residents in Suzhou, China. The complete demographic, clinical, and epidemiological data of the cohort were collected from the Suzhou medical and social insurance system between January 2012 and December 2018. To exclude latent autoimmune diabetes in adults, T2D and other types of diabetes, all corresponding medical records were cross validated by physicians at the First Affiliated Hospital of Soochow University and the Second Affiliated Hospital of Soochow University. Follow-up visits were performed at the Suzhou Center for Disease Prevention and Control, following the procedures recommended by Park *et al*[21].

According to the medical records, a total of 1088 patients were diagnosed with T1D during this period, of whom 966 were adult patients more than 20 years old at the time of diagnosis. Fifty-eight patients were further excluded in the pre-collection stage for the following reasons: (1) 2 patients passed away before blood sample collection; (2) 21 patients moved and were unable to be contacted; (3) 34 patients declined to participate in the follow-up investigation; and (4) 1 patient was diagnosed with systemic lupus erythematosus with severe hematopoietic dysfunction, and was not eligible for blood sample collection. The T1D patients were divided into the testing and validation

groups (414 and 494 patients, respectively), according to their residence.

For GWAS analysis, routine quality control steps included removing SNPs with imputation quality (INFO) scores < 0.4, with minor allele frequency (MAF) < 0.05, and without a valid Hardy-Weinberg test result ($P < 10^{-6}$). SNPs on mitochondrial DNA and sex chromosomes were also excluded. Five patients were removed from the group. In addition, we further excluded 118 participants with close familial relationship after checking their genetic relationships. Finally, 785 adult T1D patients were included in the study and formed the nested case group. The complete screening process and quality control of T1D patients enrolled in the study are shown in Figure 1.

In the control group, a similar number of age-, gender- and residence-matched participants without metabolic system diseases were randomly selected. A total of 804 participants were further divided into the testing (377) and validation (427) groups.

Genotype imputation: Ungenotyped data were imputed using IMPUTE2 software (V2.3.2)[22]. 920636 SNPs were eventually filtered for the GWAS analysis, after excluding invalid imputed SNPs with INFO < 0.4. SHAPEIT V2 software was used to improve the imputation performance following the execution of IMPUTE2. The 1000 Genomes Project Phase III database (http://mathgen.stats.ox.ac.uk/impute/impute_v2.html) was used as the reference dataset to determine population bias.

Gene regulatory network construction: Gene ontology functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of host genes of the polymorphisms were carried out with the R package clusterProfiler (R version 3.6.0). The Bayesian algorithm was chosen to optimize the regulatory network model and to identify the most probable gene regulation pathway in diabetes.

STRING software (version 11.0) was used to analyze functional interaction networks of potential downstream regulated genes of potential SNPs.

In Silico bioinformatics analysis: Expression quantitative trait loci (eQTLs) in GTEx (http://gtexportal.org/home/) was used to assess the impacts of SNPs on T1D. QTL overlapping was applied when the SNPs had a high linkage disequilibrium (LD) ($r^2 > 0.8$) with the top QTL genes for SNPs.

Statistical analyses: The χ^2 test was performed to evaluate gender differences in the nested case-control study. Age and body mass index (BMI) were compared using the student *t*-test. After adjusting for age, gender, and BMI, the R package GWAS tools was for statistical analyses. Following existing GWAS publications, $P < 5 \times 10^{-8}$ was set as the detection limit to account for Bonferroni adjustment in this study[23].

RESULTS

Characteristics of participants in the nested case-control study

Detailed demographic, clinical, and epidemiological characteristics of the 785 adult T1D cases and 804 control participants are provided in Table 1. There were no statistically significant differences in age, gender and BMI between the two groups in both the testing and validation stage of GWAS.

Novel T1D-related SNPs from GWAS

Among all SNPs examined, four novel loci significantly associated with T1D were identified (Table 2): rs55846421 at 14q21 ($P_{Bonferroni\text{-}adjust} = 4.28 \times 10^{-11}$), rs3117017 at 6q13 ($P_{Bonferroni\text{-}adjust} = 6.36 \times 10^{-5}$), rs75836320 at 12q14 ($P_{Bonferroni\text{-}adjust} = 0.011$), and rs362071 at 22q11.2 ($P_{Bonferroni\text{-}adjust} = 4.5 \times 10^{-4}$). *P*-values of these four SNPs were significantly lower than the threshold of genome-wide significance (*P*-value after Bonferroni adjust should be less than 0.05, which usually means original *P*-value < 10^{-8}). LD analysis further confirmed the independence of these SNPs. In the validation stage, the diabetic susceptibilities of these four loci between 404 adult T1D cases and 427 controls showed similar trends as in the testing stage. After combining participants in both stages, these four SNPs were still significantly associated with the onset of adult T1D: rs55846421 ($P_{Bonferroni\text{-}adjust} = 1.76 \times 10^{-9}$), rs3117017 ($P_{Bonferroni\text{-}adjust} = 9.33 \times 10^{-4}$), rs75836320 ($P_{Bonferroni\text{-}adjust} = 1.07 \times 10^{-4}$), and rs362071 ($P_{Bonferroni\text{-}adjust} = 1.66 \times 10^{-4}$).

eQTL and sQTL analyses of candidate SNPs

As there were no specific data for islet tissue or diabetes in the GTEx database, we comprehensively analyzed these SNPs in all types of tissues (Table 3). Of the four

Table 1 Characters of participants in the present type 1 diabetes genome-wide association study investigation

	Discovery stage			Validation stage			Combination		
	Cases (*n* = 381)	Controls (*n* = 377)	*P*	Cases (*n* = 404)	Controls (*n* = 427)	*P*	Cases (*n* = 785)	Controls (*n* = 804)	*P*
Sex									
Male	197	200	0.711[a]	209	223	0.887[a]	406	423	0.722[a]
Female	184	177		195	204		379	381	
Age (yr)									
mean ± SD	28.45 ± 7.70	28.99 ± 8.01	0.338[b]	28.53 ± 7.95	28.91 ± 7.67	0.496[b]	28.49 ± 7.51	28.94 ± 7.98	0.248[b]
< 25	109	101	0.637[a]	118	114	0.704[a]	227	215	0.494[a]
[25, 35)	180	174		188	203		368	377	
≥ 35	92	102		98	110		190	212	
BMI (kg/m^2)									
mean ± SD	21.77 ± 4.10	22.10 ± 4.00	0.266[b]	21.93 ± 4.26	21.52 ± 3.70	0.146[b]	21.85 ± 4.18	21.79 ± 3.85	0.773[b]
< 18.5	60	46	0.371[a]	51	66	0.500[a]	111	112	0.985[a]
[18.5, 25)	270	279		303	311		573	590	
≥ 25	51	52		50	50		101	102	
Glucose level (mmol/L)									
mean ± SD	12.83 ± 2.35	5.43 ± 1.33	< 0.0001[b]	13.42 ± 2.48	5.53 ± 1.30	< 0.0001[b]	13.13 ± 2.44	5.49 ± 81.32	< 0.0001[b]

[a]Two-sided chi-square test.
[b]Students' *t* test. BMI: Body mass index.

Table 2 Identification of type 1 diabetes risk loci in a Chinese population

SNPs	Location	Stage	Genotype distribution		OR_{add} (95%CI)	*P*[a] for Bonferroni
			Cases (*n* = 785)	Controls (*n* = 804)		
rs55846421	Chr14. 50310401	Discovery	10/149/222	0/6/371	0.024 (0.010-0.056)	4.28×10^{-11}
		Validation	8/124/272	2/37/385	0.232 (0.159-0.337)	8.73×10^{-8}
		Combination	18/273/494	2/43/756	0.113 (0.081-0.156)	1.76×10^{-9}
rs3117017	Chr6. 33095275	Discovery	2/40/339	36/117/224	4.958 (3.107-7.912)	6.36×10^{-5}
		Validation	6/32/365	32/98/297	3.202 (2.296-4.466)	4.51×10^{-4}
		Combination	8/72/704	68/215/521	3.794 (2.984-4.823)	9.33×10^{-4}
rs75836320	Chr12. 65156128	Discovery	8/77/296	1/12/356	0.153 (0.082-0.285)	0.011
		Validation	8/63/333	4/27/396	0.429 (0.289-0.638)	3.3×10^{-4}
		Combination	16/140/629	5/39/752	0.283 (0.205-0.392)	1.07×10^{-4}
rs362071	Chr22. 20811645	Discovery	82/193/104	46/101/230	0.445 (0.348-0.570)	4.5×10^{-4}
		Validation	100/173/131	79/149/199	0.705 (0.588-0.846)	0.0002
		Combination	182/366/235	125/250/429	0.568 (0.495-0.651)	1.66×10^{-4}

[a]Two-sided chi-square test. CI: Confidence interval; OR: Odds ratio.

novel SNPs, rs55846421 is located on the intron of NEMF gene. However, based on our eQTL results, rs55846421 showed a potential causal effect on LRR1, RP11-596C23.6, RHOQP1, KLHDC1 and VCPKMT genes, instead of NEMF.

Table 3 The expression and splicing quantitative trait loci analysis of target single nucleotide polymorphisms

SNP name	Influence type	Gene symbol	Expression tissues	*P* value
rs55846421	eQTLs	ARF6	Cells-cultured fibroblasts	0.00015
		KLHDC1	Brain-cerebellum	0.0000022
			Lung	0.000034
			Thyroid	0.00021
		LRR1	Adipose-subcutaneous	8.00×10^{-10}
			Adipose-visceral	5.20×10^{-7}
			Breast-mammary tissue	8.70×10^{-7}
			Esophagus-muscularis	0.000054
			Lung	0.000077
			Nerve-tibial	0.00013
			Thyroid	0.00016
		RHOQP1	Adipose-subcutaneous	3.30×10^{-8}
		RP11-596C23.6	Artery-tibial	1.1×10^{-9}
			Esophagus-mucosa	2.3×10^{-9}
			Artery-aorta	6.0×10^{-8}
			Testis	2.9×10^{-7}
			Esophagus-muscularis	0.0000019
			Esophagus-gastroesophageal junction	0.0000025
			Muscle-skeletal	0.0000027
			Small intestine-terminal ileum	0.0000042
			Lung	0.000019
			Colon-sigmoid	0.00002
			Spleen	0.000024
			Artery-coronary	0.000031
			Thyroid	0.000061
			Heart-atrial appendage	0.000062
	sQTLs	RPL36AL	Liver	0.0000063
			Brain-nucleus accumbens	0.0000071
rs3117017	eQTLs	COL11A2	Thyroid	2.0×10^{-8}
		HCG24	Brain-nucleus accumbens	5.0×10^{-25}
			Brain-putamen	6.4×10^{-17}
			Brain-cortex	3.0×10^{-14}
			Brain-caudate	3.4×10^{-14}
			Brain-frontal cortex	4.3×10^{-11}
			Brain-anterior cingulate cortex	0.000022
			Brain-hippocampus	0.000025
		HLA-DOA	Whole blood	0.00005
		HLA-DPA1	Cells-cultured fibroblasts	0.000071
		HLA-DPB1	Testis	0.000028
		HLA-DPB2	Brain-nucleus accumbens	1.3×10^{-18}
			Brain-caudate	2.4×10^{-13}

			Brain-putamen	3.4×10^{-11}
			Brain-cortex	3.5×10^{-8}
			Brain-hypothalamus	3.6×10^{-7}
			Brain-cerebellum	0.000057
		HSD17B8	Thyroid	1.0×10^{-13}
			Muscle-skeletal	3.5×10^{-11}
			Esophagus-muscularis	1.3×10^{-8}
			Artery-tibial	5.6×10^{-7}
			Esophagus-gastroesophageal junction	0.0000011
			Colon-sigmoid	0.0000018
			Heart-left ventricle	0.0000028
			Testis	0.0000032
			Adipose-visceral	0.000016
			Artery-aorta	0.000018
			Esophagus-mucosa	0.000029
			Skin-not sun exposed	0.00003
			Cells-cultured fibroblasts	0.000065
		RING1	Pancreas	0.0000059
		RPS18	Artery-tibial	0.000032
			Whole blood	0.000084
		WDR46	Lung	0.000018
	sQTLs	COL11A2	Thyroid	3.5×10^{-8}
			Brain-spinal cord	9.0×10^{-7}
			Pituitary	0.0000084
		HLA-DPB1	Skin-sun exposed (lower leg)	6.6×10^{-7}
			Muscle-skeletal	8.5×10^{-7}
			Skin-not sun exposed (suprapubic)	8.6×10^{-7}
		HLA-DPB2	Brain-cerebellum	1.9×10^{-12}
			Skin-sun exposed (lower leg)	6.6×10^{-7}
			Muscle-skeletal	8.5×10^{-7}
			Skin-not sun exposed (suprapubic)	8.6×10^{-7}
			Brain-spinal cord	0.000004
			Colon-transverse	0.000014
			Artery-tibial	0.000063
			Testis	0.00012
rs75836320	-	-	-	-
rs362071	eQTLs	MED15	Artery-tibial	3.1×10^{-13}
			Nerve-tibial	1.2×10^{-11}
			Esophagus-muscularis	5.4×10^{-11}
			Lung	1.4×10^{-9}
			Adipose-subcutaneous	6.5×10^{-9}
			Thyroid	8.2×10^{-9}
			Spleen	1.4×10^{-8}

		Whole blood	3.6×10^{-8}
		Artery-aorta	9.0×10^{-8}
		Colon-transverse	1.2×10^{-7}
		Adrenal gland	2.0×10^{-7}
		Adipose-visceral	5.0×10^{-7}
		Prostate	5.8×10^{-7}
		Liver	9.7×10^{-7}
		Esophagus-gastroesophageal junction	0.0000046
		Pancreas	0.0000063
		Skin-sun exposed (lower leg)	0.000015
		Cells-cultured fibroblasts	0.000052
sQTLs	KLHL22	Skin-sun exposed (lower leg)	0.000003
		Skin-not sun exposed (suprapubic)	0.0000049
	MED15	Muscle-skeletal	3.0×10^{-7}

eQTLs: Expression quantitative trait loci; sQTLs: Splicing quantitative trait loci.

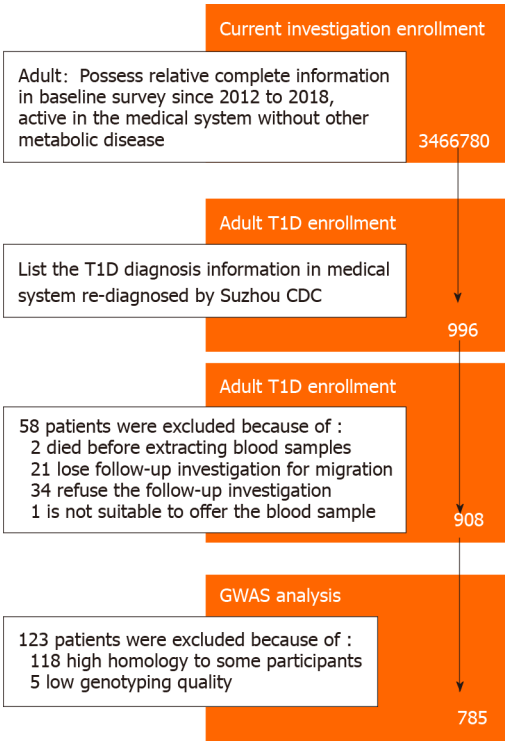

Figure 1 Study workflow. T1D: Type 1 diabetes; GWAS: Genome-wide association study; CDC: Center for Disease Prevention and Control.

rs3117017 is located on a haplotype block, including part of the HLA-DPB2 gene. HLA-DPB2 is considered the key gene in both T1D and T2D metabolism[24]. In addition, expression of COL11A2, HSD17B8, HCG24, HLA-DOA, HLA-DPB2, HLA-DPA1, RPS18, and RING1 genes are also associated with rs3117017 in different tissues.

rs75836320 is located on the promoter region of TBC1D30 gene, a key gene for insulin processing and secretion. However, no GTEx data were available for this SNP due to its low frequency (MAF < 1%) in the samples.

rs362071 is located on the intron of KLHL22 gene, which has not been previously reported to be associated with diabetes. The expression of MED15 and KLHL22 genes may be highly correlated with this SNP, according to the outcome of eQTL prediction.

Bioinformatics network analysis of possible influenced genes of SNPs

Gene ontology (GO) and KEGG pathway analyses were performed on the target genes listed in Table 3. As shown in Figure 2, these genes are mainly involved in enriched GO terms of "protein monoubiquitination", "antigen processing and presentation of exogenous peptide antigen *via* major histocompatibility complex (MHC) class II", "antigen processing and presentation of peptide antigen *via* MHC class II", "antigen processing and presentation of peptide or polysaccharide antigen *via* MHC class II" in biological process (Figure 2A), and "MHC class II receptor activity", "immune receptor activity", "estradiol 17-beta-dehydrogenase activity", and "Ribosomal large subunit binding" in molecular function (Figure 2B).

The results of KEGG analysis are shown in Figure 3. The top five pathways were "Asthma", "Allograft rejection", "Graft-versus-host diseases", "Type 1 diabetes mellitus" and "intestinal immune network for IgA production" shown in Figure 3A. The enrichment map of KEGG in potential target genes is shown in Figure 3B.

For the association among potential downstream regulated genes of these SNPs, the protein-protein interaction network is shown in Figure 4. KLHDC1, NEMF, RPS18, HSD17B8, RING1, COL11A2, HLA-DPA1 and HLA-DOA genes form a complicated regulation pathway. The corresponding SNPs on this pathway may have an interactive effect in T1D. In addition, LRR1, KLHL22 and MED15 genes also form a straightforward regulation chain, echoing the eQTL finding of rs362071 in both diabetes-related genes KLHL22 and MED15.

DISCUSSION

Through a comprehensive nested case-control study in a Chinese cohort, we identified 4 novel SNPs (one harmful and three protective) with a significant association with T1D *via* GWAS. This study has established the genetic basis of T1D in the Chinese Han population in Suzhou, one of the largest and fastest growing cities in southeast China.

According to the eQTL and sQTL results, rs55846421, the most significant SNP in this study, is highly associated with the expression of six genes (ARF6, KLHDC1, LRR1, RHOQP1, RP11-596C23.6 and VCPKMT) and splicing quantity of one gene (RPL36AL). However, only ARF6 is currently known to be associated with diabetes or diabetes-related diseases[25-27]. As a type of small G-protein, ARF6 is involved in transporting and docking insulin granules on plasma membrane for exocytotic insulin secretion[27]. rs3117017 is located on the HLA-DPB2 gene region and is highly associated with HLA-DPB2 gene expression, which belongs to the HLA genes. Several studies have demonstrated the importance of HLA class II genes, including HLA-DQA1, HLA-DR, and HLA-DQB1[28,29]. A recent hospital-based GWAS analysis of T1D identified a novel T1D-related SNP rs1770 on the 6p21.3 region of HLA-DQB1 [15]. rs1770 is especially important for T1D in the Chinese population. Our findings on rs3117017 also suggest that another HLA class II gene - HLA-DPB2 - is associated with T1D susceptibility and pathogenesis in the Chinese population. As of now, there is no record of rs75836320 regulating downstream gene expression in either the GTEx database or GEO database. The last SNP identified in this study, rs362071, belongs to the KLHL22 gene as an intron variant, and we first report its association with T1D. KLHL22 is usually not associated with diseases, but the product of KLHL22 could be polymerized with CUL3. CUL3/KLHL2 is a notable E3 ubiquitin ligase gene[30-32] and its expression will trigger amino acid-dependent mTORC1 signaling, which may be involved in aging, cancer and diabetes[33,34].

In addition to genetic basis, we suggest that economic growth in the study region (Suzhou, China) may have influenced people's diet preference, which increased the incidence of T1D. Studies have shown that T1D incidence in adolescents may be associated with gross domestic product (GDP) in Poland[17]. While GDP showed a steady increasing trend in Suzhou from 2012 to 2018, the incidence of adult T1D remained relatively stable during the same period (Supplementary Figure 1, provided by the Suzhou Statistical Yearbook since 2012 to 2018). Therefore, adult T1D is not significantly associated with economic growth in Suzhou, or may have a lag effect which warrants further investigation. In addition, diet may not play an important role in adult T1D in the Chinese population.

Furthermore, during the preprocessing stage of this study, we excluded 118 patients due to their close familial heredity to other T1D patients. Genetic correlation of T1D was more than 13% in our study sample. However, according to studies based on the Caucasian population[16,35], the degree of familial cluster was much lower than in the Chinese population. Another study confirmed a significant difference in GWAS results

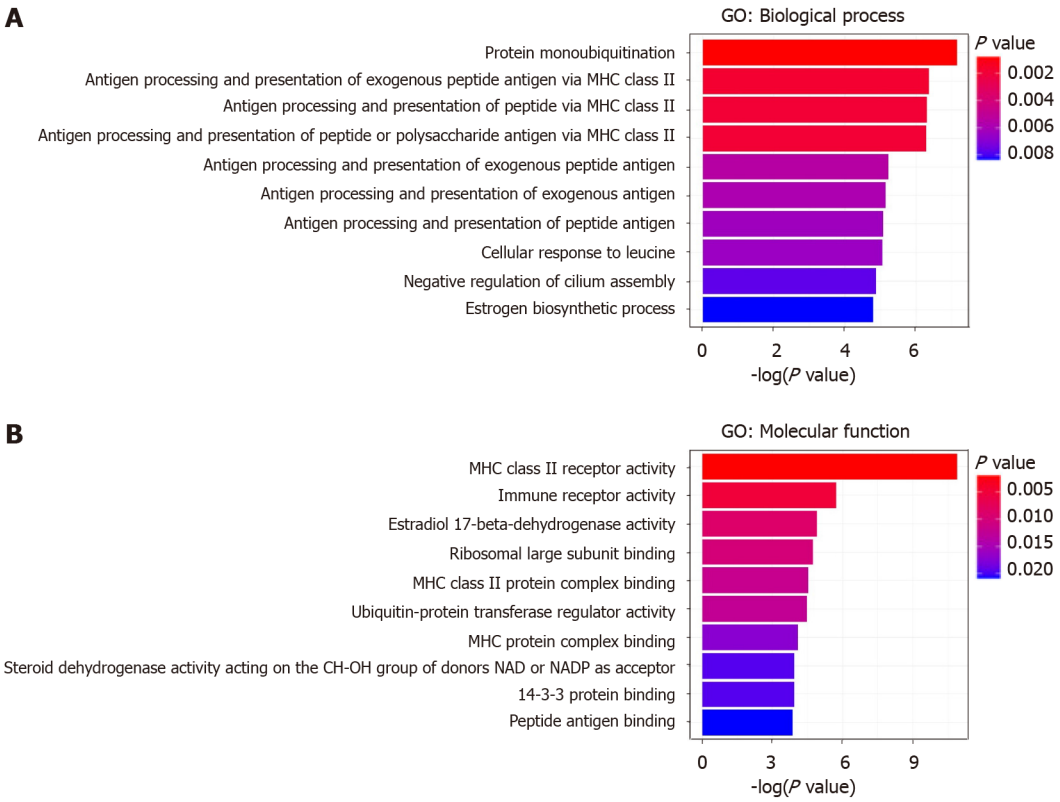

Figure 2 Gene ontology analysis of potential expression quantitative trait loci genes of genome-wide association study outcomes. A: Biological process analysis for expression quantitative trait loci (eQTL) genes; B: Gene ontology molecular function analysis for eQTL genes. GO: Gene ontology; MHC: Major histocompatibility complex.

between Chinese and Caucasian populations[15]. Based on previous studies in both populations and our current study, we suggest that the genetic basis and pathogenesis of T1D may vary between Chinese and Caucasian populations. These distinctions can have profound implications on T1D risk estimation, diagnosis, and treatment across different ethnic groups.

There are some limitations in our study. First, unlike a hospital-based case-control study, the sample size of T1D patients (cases) in our nested case-control study was relatively small due to the low incidence of T1D in the natural cohort. Therefore, the results of this study need to be further validated in larger studies. Second, we only investigated the contribution of genetic factors (SNPs) in this study. Other factors, for example, dietary preferences and habits, could also be important in T1D pathogenesis. We will investigate the influence of these factors on T1D in future studies. Third, T1D is a disease with high heritability. In our study, we excluded individuals with high homology, following the protocol of GWAS. However, this exclusion may conceal the actual effects of some relevant SNPs in T1D.

CONCLUSION

To the best of our knowledge, this is the first study to conduct a GWAS for adult T1D in a nested case-control study. We identified 4 novel SNPs as genetic biomarkers for adult T1D onset. Subsequent basic pedigree can further complement and strengthen our research.

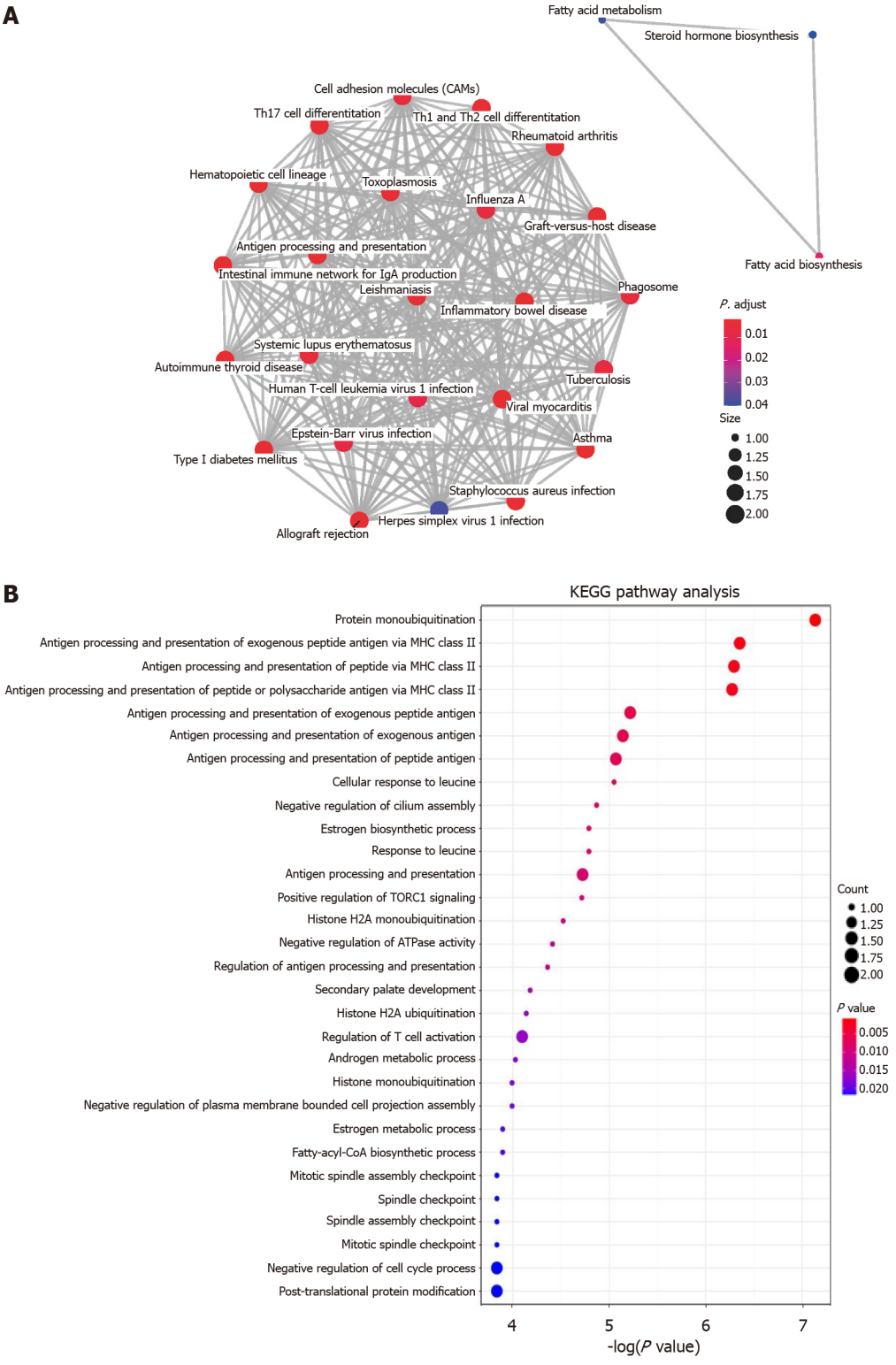

Figure 3 Kyoto Encyclopedia of Genes and Genomes pathway for expression quantitative trait loci genes of genome-wide association study outcomes. A: Network of pathways involved in A; B: The potential pathways for expression quantitative trait loci genes. KEEG: Kyoto Encyclopedia of Genes and Genomes.

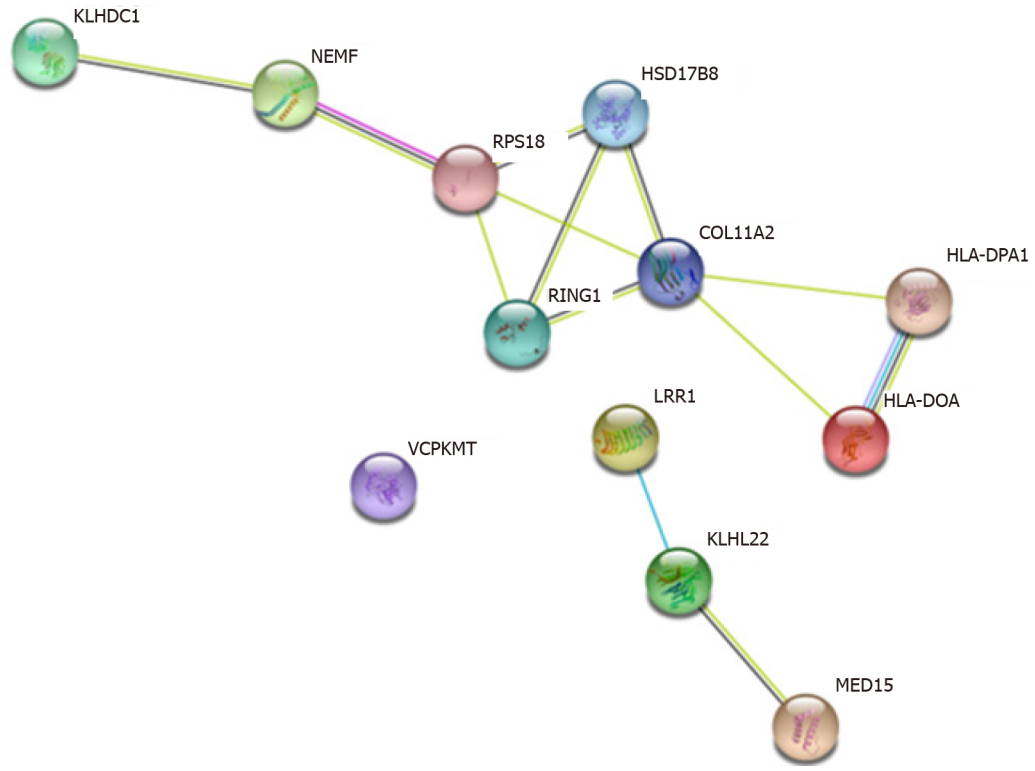

Figure 4 The predictive interaction of expression quantitative trait loci genes in diabetes onset.

ARTICLE HIGHLIGHTS

Research background

The genome-wide association study (GWAS) of type 1 diabetes (T1D) is valuable.

Research motivation

We hoped to add data from a natural cohort to fill gaps in the knowledge of T1D susceptibility.

Research objectives

We conducted a cohort study to evaluate the variants of genes in order to adjust the bias in hospital-based research.

Research methods

The GWAS analysis used in our research reflected the associations between single nucleotide polymorphisms (SNPs) and T1D with high-throughput sequencing.

Research results

In T1D patients, rs3117017 displayed its damaging role in the onset of T1D, while rs55846421, rs75836320, and rs362071 displayed their protective roles in T1D.

A larger nested case-control study of a Chinese population will be helpful in validating our findings.

Research conclusions

The GWAS analysis from a hospital-based population was a little different to that from normal population.

Research perspectives

The specific function of these SNPs should be investigated in the future.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy of omarigliptin, once-weekly dipeptidyl peptidase-4 inhibitor, in patients with type 2 diabetes

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Institutional review board statement: This study's protocol has been reviewed and approved by the ethics committee of Shin-Koga hospital.

Informed consent statement: To collected the retrospective data patients did not provide their verbal or written informed consent

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Abstract

BACKGROUND

Omarigliptin is one of several once-weekly dipeptidyl peptidase-4 inhibitors (DPP-4is). Despite the high frequency of switching from various daily DPP-4is to omarigliptin in actual clinical practice, data regarding its efficacy in patients with type 2 diabetes (T2D) after switching are limited.

AIM

To analyze the efficacy of omarigliptin in Japanese patients with T2D who had previously received treatment with other glucose-lowering agents.

METHODS

Forty-nine T2D patients treated for the first time with omarigliptin were recruited retrospectively and divided into four groups defined as either add-on or switched from daily DPP-4is: switched from linagliptin, switched from sitagliptin, and switched from vildagliptin. During a 3-mo follow-up, the clinical parameters among these groups were assessed and compared, with the impact of the switch on glycemic variability as measured by continuous glucose monitoring also being evaluated in the switched groups.

RESULTS

Hemoglobin A1c levels saw a significant decrease of -0.32% ± 0.41% in the add-on group (P = 0.002). However, the other groups' variables depended on the pre-switch daily DPP-4i: switched from linagliptin, -0.05% ± 0.22%; switched from sitagliptin, -0.17% ± 0.33%; and switched from vildagliptin, 0.45% ± 0.42%, which saw significant worsening (P = 0.0007). Multivariate logistic regression analysis revealed that switching from vildagliptin to omarigliptin was independently associated with worsening glycemic control (P = 0.0013). The mean and standard

deviation of sensor glucose value, the mean amplitude of glycemic excursions, and the mean of daily difference significantly improved when switching the patient from either linagliptin or sitagliptin to omarigliptin. However, in patients switched from vildagliptin, not only did the glucose variability indices see no improvements, the mean of daily difference even underwent significant worsening.

CONCLUSION

Administering omarigliptin as add-on therapy or switching to it from sitagliptin and linagliptin, but not vildagliptin, improves glycemic control and thus should help in decision making when selecting DPP-4is for T2D patients.

Key Words: Omarigliptin; Once-weekly dipeptidyl peptidase-4 inhibitor; Real-world practice; Retrospective study; Type 2 diabetes

Core Tip: This paper reported on the efficacy of omarigliptin in Japanese patients with type 2 diabetes who had previously received treatment with other glucose-lowering agents. The present study demonstrated that administering omarigliptin as add-on therapy or switching to it from sitagliptin and linagliptin, but not vildagliptin, provides more effective glycemic control. Ultimately, these findings should help decision-making in the actual clinical setting when selecting and using dipeptidyl peptidase-4 inhibitors in patients with type 2 diabetes.

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INTRODUCTION

Type 2 diabetes (T2D) is a chronic metabolic disease characterized by hyperglycemia, predominantly associated with varying degrees of β-cell dysfunction, and insulin resistance. Although medical nutrition therapy and exercise are central to managing T2D, many patients require pharmacological treatment to achieve their glycemic targets. Over the past decade, we have witnessed a rapid development in antidiabetic agents, including, but not limited to, once-weekly medications. Among a range of currently available oral hypoglycemic agents, the most frequently prescribed in Japan belong to the dipeptidyl peptidase-4 inhibitors (DPP-4is) class of medication, with more than 70% of patients with oral hypoglycemic agents receiving DPP-4is[1].

Recently, several once-weekly DPP-4is, which may improve patient adherence to a medication regimen due to a lower medication burden, received approval in Japan. Omarigliptin is one such once-weekly DPP-4i, whose non-inferiority in efficacy and safety as add-on therapy to glucose-lowering agents has been demonstrated in comparison with daily DPP-4is[2]. However, despite the high frequency of switching from daily DPP-4is to omarigliptin in actual clinical practice, data regarding its efficacy in patients with T2D after switching are limited. Therefore, we carried out the present retrospective study in real-world practice to explore the efficacy of omarigliptin as add-on therapy to other oral hypoglycemic agents or in switching to omarigliptin from daily DPP-4is in patients with T2D. Additionally, we examined the impact of the switch from daily DPP-4is to omarigliptin on glycemic variability using a continuous glucose monitoring (CGM) devise.

to join the study but were instead allowed to refuse participation according to the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects and the local Institutional Review Board Approval due to its retrospective nature. As for the continuous glucose monitoring study, all patients provided informed written consent prior to enrollment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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MATERIALS AND METHODS

Patients

Forty-nine T2D patients who received treatment with omarigliptin for the first time, either with or without other hypoglycemic agents for a course lasting at least 3 mo, were retrospectively recruited. The study consisted of 35 males and 14 females, with the mean age at examination and mean duration of diabetes being 68.2 ± 11.9 and 12.4 ± 7.8 years, respectively. All patients received omarigliptin 25 mg on an outpatient basis and had no changes made to their diabetes treatment (*e.g.*, medical nutrition therapy, exercise, medication) for at least 3 mo after being administered omarigliptin. Patients were divided into four groups according to add-on to other glucose-lowering agents or switched from daily DPP-4is: add-on (AO, n = 18), switched from linagliptin 5 mg (L→Om, n = 6), switched from sitagliptin 50 mg (S→Om, n = 10), and switched from vildagliptin 100 mg (V→Om, n = 15). The clinical characteristics of these groups are described in Table 1.

To analyze the effect switching from daily DPP-4is to omarigliptin has on glucose variability, an additional ten outpatients with T2D treated with daily DPP-4is (sitagliptin, linagliptin, and vildagliptin) for a course greater than 3 mo were enrolled in the CGM study. These additional subjects consisted of 6 males and 4 females, with the mean age at examination, mean duration of diabetes, and mean hemoglobin A1c (HbA1c) being 66.1 ± 10.6 years, 13.2 ± 7.1 years, and 7.0% ± 0.8 %, respectively. Of these additional patients, 3 had been treated with sitagliptin 50 mg, 2 with linagliptin 5 mg, and 5 with vildagliptin 100 mg. All patients received a written or verbal explanation of the study before providing informed consent. This study's protocol has been approved by the ethics committee of Shin-Koga Hospital.

Assessment of treatment efficacy

In evaluating the efficacy of omarigliptin, clinical and laboratory data were collected through a review of electronic medical records at baseline and after 1, 2, and 3 mo of omarigliptin treatment. The study's primary objective was to evaluate the efficacy of omarigliptin as add-on to or switched from daily DPP-4is (sitagliptin, linagliptin, or vildagliptin) over 3 mo by assessing the change in HbA1c from baseline. In one analysis, we divided the patients into three groups: improved (more than 0.3% decrease in HbA1c), worsened (more than 0.3% increase in HbA1c), and stable (-0.3% < change in HbA1c < 0.3%) and compared the clinical characteristics. Additionally, we examined the parameters affecting the therapeutic response to omarigliptin in the patients who were switched from daily DPP-4is.

Assessment of glucose variability using CGM

The impact of switching from daily DPP-4is to omarigliptin on glycemic variability as measured by CGM, FreeStyle Libre Pro™ (Abbott Diabetes Care, Alameda, CA, United States) was also assessed in the additional 10 T2D patients treated with daily DPP-4is (sitagliptin, linagliptin, or vildagliptin). On day 1, using a self-adhesive pad, the FreeStyle Libre Pro™ was placed on the back of the patient's upper arm and worn for 14 d. From days 1 to 7, all ongoing diabetes treatments using daily DPP-4is were maintained. On day 8, while still being assessed by CGM, daily DPP-4is were replaced with 25 mg of omarigliptin and administered once weekly.

Since sensor glucose values as determined by FreeStyle Libre Pro™ from days 2 to 14 have been reported to be comparable in accuracy to self-monitoring blood glucose devises in obtaining capillary blood glucose levels[3], the FreeStyle Libre Pro™ was used to collect and analyze the variability of daily DPP-4is data from days 2 to 7. Data were also collected to analyze the glucose variability of omarigliptin; however, to eliminate any residual effects of daily DPP-4is, the data was only taken from days 9 to 14. We then compared the mean sensor glucose levels, standard deviation (SD) of glycemic variability, mean amplitude of glycemic excursion (MAGE), and the mean of daily difference (MODD) for each period using the Glycemic Variability Analyzer Program in MATLAB (MathWorks, Natick, MA, United States).

Statistical analysis

Data are presented as a mean ± SD and as n (%) for frequencies unless otherwise specified. Where appropriate the prevalence was compared using the ^{2} test or Fisher's exact test, and differences in nonparametric data were tested using the Mann-Whitney U test or Kruskal-Wallis test. A comparison of different time points within the same group was made using Friedman's analysis of variance test for repeated measures, and multiple logistic regression analysis was used to determine the parameters affecting

Table 1 Clinical characteristics of patients with type 2 diabetes who received omarigliptin divided according to add-on to or switched from daily dipeptidyl peptidase-4 inhibitor

	AO (*n* = 18)	L→Om (*n* = 6)	S→Om (*n* = 10)	V→Om (*n* = 15)
Male : Female	13 : 5	4 : 2	5 : 5	13 : 2
Age (yr)	66.8 ± 12.1	75.2 ± 9.0	63.6 ± 15.7	70.1 ± 8.7
Duration (yr)	12.0 ± 10.1	15.5 ± 10.7	11.2 ± 5.6	12.9 ± 4.8
BMI (kg/m^2)	24.1 ± 3.3	24.9 ± 1.6	23.5 ± 2.4	23.7 ± 3.4
eGFR (mL/min/1.73 m^2)	63.6 ± 19.5	45.0 ± 22.2[a]	76.1 ± 17.5	63.3 ± 15.7
HbA1c (%)	7.48 ± 1.28	6.33 ± 0.79	6.88 ± 0.37	7.14 ± 0.66
Metformin use	8 (44%)	1 (17%)	4 (40%)	6 (40%)
Insulin secretagogues[1] use	5 (28%)	0 (0%)	4 (40%)	8 (53%)
Insulin use	2 (11%)	0 (0%)	1 (10%)	3 (20%)

[1]Insulin secretagogues include sulfonylurea and glinide, but not dipeptidyl peptidase-4 inhibitor.

Data are shown as mean ± SD or *n* (%) unless otherwise indicated. AO: Add-on; BMI: Body mass index; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c; L→Om: Switch from linagliptin to omarigliptin; S→Om: Switch from sitagliptin to omarigliptin; V→Om: Switch from vildagliptin to omarigliptin. [a]$P < 0.05$ *vs* S→Om group (multiple comparison).

the therapeutic response to omarigliptin. Additionally, changes in glycemic variability parameters before and after administering omarigliptin were analyzed using the Wilcoxon signed-rank test within the groups, with *P* values of less than 0.05 being considered statistically significant. Statistical analysis for this study was performed using StatView statistical software (version 5.0, SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

No significant difference was observed among the four groups (AO, L→Om, S→Om, and V→Om) relating to sex, age at examination, duration of diabetes, body mass index, or HbA1c (Table 1). Furthermore, there were no significant differences regarding the frequency of metformin, insulin secretagogues, or insulin use among the four groups. However, the mean estimated glomerular filtration rate at baseline in the L→Om group was significantly lower than the S→Om group due to the tendency for linagliptin to be used in patients with renal impairment since the elimination of this DPP-4i is primarily through non-renal routes.

Efficacy of omarigliptin

As shown in Figure 1, HbA1c levels improved significantly in the AO group (P = 0.002), with the maximum change from baseline after administering omarigliptin being -0.32% ± 0.41%. At the same time, however, there was some variability in the other three groups depending on the pre-switch daily DPP-4is; -0.05% ± 0.22% in the L→Om group, -0.17% ± 0.33% in the S→Om group, and 0.45% ± 0.42% in the V→Om group, which saw significant worsening (P = 0.0007). The change in HbA1c levels among the four groups revealed a statistically significant difference (Figure 2).

Three months after being administered omarigliptin, 13 patients (26.5%) showed an increase in HbA1c levels of more than 0.3% (worsened group). On the other hand, 36 patients (73.5%) revealed either no change or decreased HbA1c levels compared with the baseline value (improved/stable group). Of note, 10 of the 13 patients (76.9%) in the worsened group belonged to the V→Om group (Figure 3), indicating that vildagliptin may be more effective than omarigliptin concerning glycemic control.

The varying clinical characteristics of the improved/stable and worsened glycemic-control patients after switching from vildagliptin to omarigliptin are shown in Table 2. There was no significant difference in sex, age at examination, duration of diabetes, body mass index, HbA1c, estimated glomerular filtration rate, or the frequency of metformin, insulin secretagogues, and insulin use between the two groups (Table 2). Furthermore, multivariate logistic regression analysis revealed that switching from

Table 2 Clinical characteristics of patients with type 2 diabetes between the improved/stable and worsened glycemic control after switching from vildagliptin to omarigliptin

	Improved/stable (*n* = 5)	Worsened (*n* = 10)	*P* value
Male : Female	4 : 1	9 : 1	NS
Age (yr)	67.8 ± 10.2	71.2 ± 8.1	NS
Duration (yr)	9.8 ± 4.1	14.5 ± 4.5	NS
BMI (kg/m^2)	25.1 ± 4.3	23.0 ± 2.8	NS
eGFR (mL/min/1.73 m^2)	71.2 ± 19.2	59.4 ± 12.9	NS
HbA1c (%)	7.48 ± 0.97	6.97 ± 0.41	NS
Metformin use	2 (40%)	4 (40%)	NS
Insulin secretagogues[1] use	3 (60%)	5 (50%)	NS
Insulin use	2 (40%)	1 (10%)	NS

[1]Insulin secretagogues include sulfonylurea and glinide but not dipeptidyl peptidase-4 inhibitor.
Data are shown as mean ± SD or *n* (%) unless otherwise indicated. BMI: Body mass index; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c; NS: Not significant.

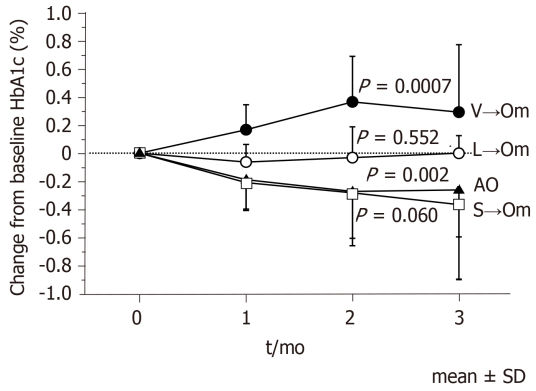

Figure 1 Changes in hemoglobin A1c levels in the subgroups at 3 mo follow-up. A comparison of different time points within the same group was made using Friedman's analysis of variance test for repeated measures. AO: Add-on; HbA1c: Hemoglobin A1c; L→Om: Switch from linagliptin to omarigliptin; S→Om: Switch from sitagliptin to omarigliptin; V→Om; Switch from vildagliptin to omarigliptin; SD: Standard deviation.

vildagliptin to omarigliptin was independently associated with worsening glycemic control (P = 0.0013, Table 3).

Change of glucose variability after switching from daily DPP-4is to omarigliptin

In determining the efficacy in glucose variability after switching from daily DPP-4is to omarigliptin, CGM analyses were performed in the 10 additional subjects who had been treated with either sitagliptin 50mg (n = 3), linagliptin 5mg (n = 2), or vildagliptin 100mg (n = 5). As shown in Figure 4, both the mean and SD of the sensor glucose value, MAGE, and MODD significantly improved when patients were switched from either linagliptin or sitagliptin to omarigliptin. However, except in the case of MODD, which worsened significantly after switching, no other significant changes were observed in any of the glucose variability indices when patients were switched from vildagliptin to omarigliptin.

DISCUSSION

In the present study, we demonstrate that: (1) Treatment using omarigliptin as add-on to other glucose-lowering agents decreased HbA1c levels; (2) There was a distinct drug effect regarding the efficacy of omarigliptin when switching from daily DPP-4is; and (3) Vildagliptin was more effective than omarigliptin for glycemic control.

Table 3 Multivariate logistic regression analysis on worsening of glycemic control after omarigliptin administration

	Odds ratio	95%CI	*P* value
Sex (male)	0.837	0.03-24.30	NS
Age	1.004	0.82-1.23	NS
Insulin secretagogues[1] use	0.575	0.04-7.72	NS
Insulin use	0.038	0.001-1.460	NS
BMI at omarigliptin administration	0.757	0.45-1.26	NS
eGFR at omarigliptin administration	0.941	0.84-1.06	NS
HbA1c at omarigliptin administration	5.862	0.86-39.80	NS
Switching from vildagliptin	146.62	7.00-3072.60	0.0013

[1]Insulin secretagogues include sulfonylurea and glinide, but not dipeptidyl peptidase-4 inhibitor.
BMI: Body mass index; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c; NS: Not significant; CI: Confidence interval.

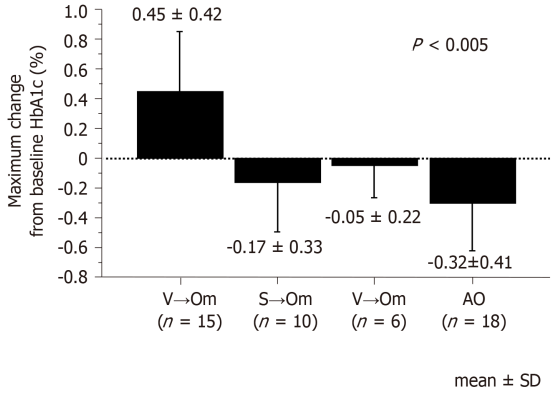

Figure 2 Maximum changes in hemoglobin A1c from baseline in the subgroups after omarigliptin administration. The Kruskal-Wallis test was used to determine the differences and degree of significance ($P < 0.05$). AO: Add-on; HbA1c: Hemoglobin A1c; L→Om: Switch from linagliptin to omarigliptin; S→Om: Switch from sitagliptin to omarigliptin; V→Om; Switch from vildagliptin to omarigliptin; SD: Standard deviation.

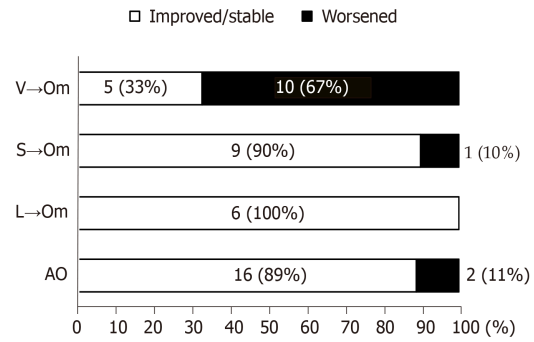

Figure 3 The prevalence of the improved/stable and worsened glycemic control after omarigliptin administration. AO: Add-on; L→Om: Switch from linagliptin to omarigliptin; S→Om: Switch from sitagliptin to omarigliptin; V→Om; Switch from vildagliptin to omarigliptin.

DPP-4is that ameliorate β-cell dysfunction with low hypoglycemic risk are now widely used in the glycemic control of patients with T2D and are rapidly becoming a first-line antidiabetic drug in Japan. Moreover, recent technological advances have enhanced existing drugs and enabled prolonged actions such as in once-weekly DPP-4is. Due to the lower medication burden of once-weekly DPP-4is, there may also be improved patient adherence and satisfaction to these medication regimens[4]. In recent publications, omarigliptin, one such once-weekly DPP-4i medication, has been determined to be safe and effective as monotherapy or add-on therapy to other glucose-lowering agents[2,5-9]. Furthermore, reports have shown that T2D patients

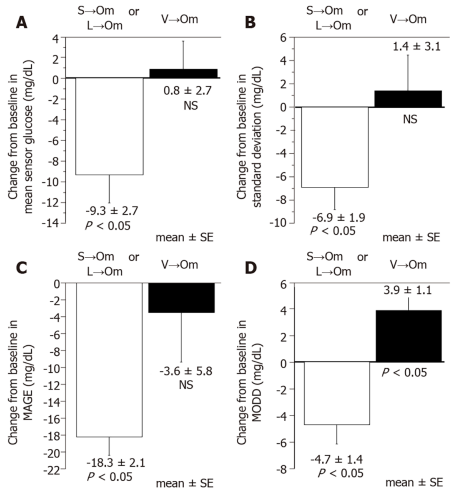

Figure 4 Change of glucose variability indices from baseline. A: Mean sensor glucose; B: Standard deviation of sensor glucose; C: Mean amplitude of glycemic excursion (MAGE); D: Mean of daily difference (MODD). The Wilcoxon signed rank test was used to determine the differences and degree of significance. L→Om: Switch from linagliptin to omarigliptin; S→Om: Switch from sitagliptin to omarigliptin; V→Om; Switch from vildagliptin to omarigliptin; NS: Not significant; SE: Standard error.

with non-alcoholic related fatty livers or non-alcoholic steatohepatitis are positively affected by omarigliptin through improved insulin resistance and reduced inflammation[10].

As current drug adherence rates among diabetic patients following a daily medication regimen are reported to be less than 70%[11], it is conceivable that switching from daily DPP-4is to once-weekly DPP-4is may lead to better glycemic control. However, data regarding the efficacy of omarigliptin after switching from various daily DPP4is in patients with T2D are still limited. While the present study is not definitive and did not reach statistical significance (P = 0.06), it demonstrates a decrease from baseline in mean HbA1c after switching patients from sitagliptin to omarigliptin exists and sets a precedent for future studies. Additionally, the mean and SD of the sensor glucose value, and the value of MAGE and MODD significantly improved when either linagliptin or sitagliptin was switched to omarigliptin (Figure 4). In contrast, switching from vildagliptin to omarigliptin resulted in significantly aggravated glycemic controls (P = 0.0007), no improvement in the glucose variability indices, and even significantly worsened MODD (Figure 4). These results indicate that vildagliptin may be more effective than omarigliptin concerning glycemic control.

DPP-4is possess distinct chemical structures categorized into three binding patterns (classes 1, 2, 3) based on the inhibitor binding subsites known as the S1, S2, S1′, S2′, and S2 extensive subsites[12-14]. The binding patterns of the DPP-4is used in this study are as follows: (1) Vildagliptin binds to S1 and S2 subsites (class 1); (2) Linagliptin binds to S1 and S2 as well as S1′ and/or S2′ subsites (class 2); while (3) Sitagliptin and omarigliptin bind to the S1, S2, and S2 extensive subsites (class 3)[15]. According to the previous study, the increased inhibitory activity of DPP-4is on DPP-4 tends to correlate with an increased number of binding subsites[13]. Furthermore, a recent meta-analysis revealed that the factor that explains most of the variance in HbA1 was baseline HbA1c levels: higher baseline HbA1c levels were associated with the greater fall in HbA1c seen after administering various DPP-4is[16]. However, contrary to this, the present study showed that the HbA1c levels increased after switching from class 1 DPP-4i (vildagliptin) with fewer binding subsites to class 3 DPP-4i (omarigliptin) with multiple binding subsites and that baseline HbA1c was similar among the V→Om, L→Om, and S→Om groups. Similar results published by other research groups have shown that switching from class 1 to class 3 DPP-4i worsened HbA1c levels by 0.33% in patients with T2D[17]. These results suggest the estimated reduction in the HbA1c levels does not correlate with the inhibitory activity of each DPP-4i and alludes to the number of binding subsites utilized by the various DPP-4is being a factor in determining selectivity between DPP-4 and other related enzymes.

This study has several limitations to report. To begin with, the number of patients in each study group was relatively small, and it was carried out retrospectively in the actual clinical setting and did not include a control group. Furthermore, we were

unable to include some daily DPP-4is such as anagliptin, alogliptin, teneligliptin, and saxagliptin due to a lack of availability in our facility. Finally, no evaluation of medical nutrition therapy was carried out. Therefore, a further prospective study using a larger cohort that includes a control group and the effects of medical nutrition therapy is warranted to investigate the efficacy and safety of all types of daily DPP-4is.

CONCLUSION

In conclusion, the current study revealed that the change in HbA1c variables is dependent on the daily DPP-4i medication regimen followed before switching to a once-weekly DPP-4i. This study's findings should help physicians in decision making regarding the selection and use of DPP-4is in patients with T2D by bringing awareness to the possibility of worsening glycemic control when switching from vildagliptin to omarigliptin.

ARTICLE HIGHLIGHTS

Research background

Dipeptidyl peptidase-4 inhibitors (DPP-4is) have become standard medications for glycemic control in patients with type 2 diabetes (T2D). Despite the high frequency of switching from various daily DPP-4is to once-weekly DPP-4is in actual clinical practice, data regarding its efficacy in patients with T2D after switching are limited.

Research motivation

Compound-specific effects can be present and influence the efficacy of daily DPP-4is in patients with T2D.

Research objectives

The authors analyzed the efficacy of omarigliptin, one of several once-weekly DPP-4is, in Japanese patients with T2D who had previously received treatment with other glucose-lowering agents.

Research methods

The 49 patients in this study were divided into four groups defined as either add-on or switched from daily DPP-4is (linagliptin, sitagliptin, and vildagliptin), and the clinical parameters among these groups were assessed and compared during a 3-mo follow-up. Additionally, glycemic variability measured by continuous glucose monitoring was also assessed in the switched groups.

Research results

The glycemic control saw significant improvement in the add-on group, while the switched from vildagliptin to omarigliptin group experienced significant worsening. Multivariate logistic regression analysis revealed that switching from vildagliptin to omarigliptin was independently associated with worsening glycemic control (P = 0.0013). However, the mean of daily difference significantly improved when the patient was switched from either linagliptin or sitagliptin to omarigliptin but significantly worsened when patients were switched from vildagliptin.

Research conclusions

Administering omarigliptin as add-on therapy or switching from sitagliptin and linagliptin, but not vildagliptin, provides more effective glycemic control. These results should help in decision-making regarding the selection and use of DPP-4is in patients with T2D.

Research perspectives

To investigate the efficacy and safety of all types of daily DPP-4is, a prospective study using a larger cohort and inclusive of a control group should be conducted in the future.

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ORIGINAL ARTICLE

Retrospective Study

Sodium ozagrel and atorvastatin for type 2 diabetes patients with lacunar cerebral infarction

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Author contributions: Yu Y and Ma HY designed the research study; Wang L and Yu Y performed the research; Zhu X, Liu YF and Ma HY analyzed the data and wrote the manuscript; and all authors have read and approve the final manuscript.

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Informed consent statement: All study participants provided informed written consent prior to study enrollment.

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Provenance and peer review:

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Abstract

BACKGROUND

The main pathological factor of cerebral infarction is atherosclerosis, which is the pathological process of chronic inflammatory diseases such as vascular smooth muscle hyperplasia, inflammatory cell infiltration, extracellular matrix increase, and thrombosis. At present, the focus of clinical treatment is anti-platelet aggregation and improving blood status, and current research is limited to improving symptoms only.

AIM

To observe the effect of sodium ozagrel and atorvastatin on type 2 diabetes patients with lacunar cerebral infarction.

METHODS

Eighty-two patients with type 2 diabetes and lacunar cerebral infarction admitted to our hospital from January 2018 to February 2020 were equally categorized into two groups according to their treatment method. The control group was administered atorvastatin, and the observation group was administered sodium ozagrel combined with atorvastatin. The National Institutes of Health stroke scale (NIHSS) score, activities of daily living (ADL) score, blood glucose, lipid levels, inflammatory factors, high-mobility group box 1 (HMGB1) levels, paraoxonase-1 (PON-1) levels, erythrocyte sedimentation rate (ESR), and macrophage migration inhibitory factor (MIF) levels were recorded before and after treatment. The total effective rate and adverse reaction rate of the two groups were analyzed.

RESULTS

The total effective rate of the observation group (94.00%) was significantly higher than that of the control group (80.00%) ($\chi^2 = 3.998$; $P = 0.046$). The blood glucose indexes, total cholesterol levels, triglyceride levels, low-density lipoprotein cholesterol levels, high-sensitivity C-reactive protein levels, interleukin-1β levels, tumor necrosis factor-α levels, HMGB1 Levels, ESR, MIF levels, platelet

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aggregation rates, and plasma viscosity of the two groups decreased after treatment; however, high-density lipoprotein cholesterol and PON-1 Levels increased after treatment. After treatment, the blood glucose indexes; blood lipid indexes; inflammatory factors; HMGB1, PON-1, and MIF levels; ESR; platelet aggregation rate; and plasma viscosity of the observation group were better than those of the control group ($P < 0.05$). After treatment, all patients in the observation group had higher ADL scores and lower NIHSS scores than those in the control group ($P < 0.05$).

CONCLUSION
Sodium ozagrel with atorvastatin can reduce inflammatory reactions; regulate ESR and HMGB1, PON-1, and MIF levels; control blood glucose and lipid indexes; and alleviate nerve injury without increasing adverse effects of atorvastatin alone.

Key Words: Sodium ozagrel; Atorvastatin; Type 2 diabetes; Lacunar infarction; Inflammatory response; Nerve damage

Core Tip: This study was performed to observe the effect of sodium ozagrel combined with atorvastatin on high-mobility group protein B1 and high-sensitivity C-reactive protein in patients with type 2 diabetes mellitus and lacunar infarction. The purpose was to find a treatment plan that can effectively inhibit the pathological mechanism, alleviate clinical symptoms, improve the prognosis, and guide clinical treatment.

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INTRODUCTION

Type 2 diabetes is a common metabolic disease that is often complicated by abnormal lipid metabolism[1]. Abnormal lipid metabolism is one of the pathological causes of cerebrovascular disease. Consequently, type 2 diabetes mellitus complicated with cerebral infarction is commonly encountered in clinical settings[2]. Lacunar infarction refers to lesions and occlusion of the small perforating artery in the deep part of the cerebral hemisphere or brainstem. It results in minor harm because the perforating artery supplies only a small area. Active symptomatic treatment can help reduce the degree of disability. Statins can effectively regulate blood lipids and reduce the degree of vascular lesions[3]. However, the effect of statins alone is not ideal.

As an antiplatelet drug and thromboxane A inhibitor, sodium ozagrel is widely used to treat ischemic cerebrovascular diseases. It has been reported that sodium ozagrel combined with atorvastatin calcium is effective for treating type 2 diabetes mellitus with lacunar infarction without increasing adverse drug reactions[4]. However, research has been limited to the improvement of symptoms only, and its mechanism of action has not been investigated in depth. This study aimed to observe the effects of sodium ozagrel combined with atorvastatin on high-mobility group protein B1 (HMGB1) and high-sensitivity-C reactive protein (hs-CRP) in patients with type 2 diabetes mellitus and lacunar infarction.

MATERIALS AND METHODS

General information

Eighty-two patients with type 2 diabetes mellitus and lacunar infarction treated at our hospital from January 2018 to February 2020 were categorized into two groups according to the method of treatment (41 patients in each group). There were 24 males

and 17 females in the control group. Their ages ranged from 34 to 68 years (58.69 ± 9.22 years). The mean body mass index (BMI) was 24.15 ± 2.03 kg/m^2. Type 2 diabetes had been diagnosed between 1 and 10 years previously (5.69 ± 1.74 years). The mean National Institutes of Health Stroke Scale (NIHSS) score was 15.23 ± 2.33. In the observation group, there were 22 males and 19 females. Their ages ranged from 38 to 69 years (59.17 ± 10.45 years). The mean BMI was 24.09 ± 2.14 kg/m^2. Type 2 diabetes had been diagnosed 1 to 10 years previously (5.61 ± 1.85 years). The mean NIHSS score was 14.96 ± 2.17. There were no significant differences in the general data of the two groups ($P > 0.05$).

Inclusion and exclusion criteria

Inclusion criteria were as follows: Type 2 diabetes meeting the criteria of the Chinese guidelines for the prevention and treatment of type 2 diabetes mellitus[5]; lacunar infarction conforming to the criteria of the European treatment guidelines for acute cerebral infarction and confirmed by cranial computed tomography and/or magnetic resonance imaging[6]; age 18 years or older and younger than 70 years; and complete clinical data.

Exclusion criteria were as follows: Mental illness or serious communication disorders; recent history of surgery or diseases with bleeding tendencies; serious diseases of the heart, liver, kidney, and other organs; malignant tumors and systemic infection; pregnancy and lactation; and allergies.

Medications and instruments

The following medications and instruments were used: atorvastatin calcium tablets (10 mg; H19990258; Beijing Jialin Co., Ltd.); sodium ozagrel (20 mg; H20093400; Guangdong Pidi Pharmaceutical Co., Ltd.); ELX800 multifunctional enzymometer (Berten Company); BS634 platelet aggregation instrument (Beijing Biochemical Instrument Factory); and HT-100B blood rheometer (Hengtuo, Zibo).

Treatment and study design

Patients in both groups were administered symptomatic treatment to control blood glucose and blood pressure, nourish brain cells, and maintain water and electrolyte balance. Patients in the control group were treated with oral atorvastatin calcium tablets 10-20 mg once per day. The observation group was administered intravenous sodium ozagrel combined with atorvastatin. Atorvastatin was administered in the same dosage as that of the control group. Sodium ozagrel 80 mg was added to intravenous 0.9% sodium chloride and 500 mL intravenous drip twice per day. All patients were treated continuously for 2 wk to evaluate the curative effect.

Observation index and assessment methods

Changes in the NIHSS score; activities of daily living (ADL) score; blood glucose index; blood lipid index; inflammatory factors; HMGB1, paraoxonolipase-1 (PON-1), and macrophage migration inhibitor (MIF) levels; erythrocyte sedimentation rate (ESR); platelet aggregation rate; and plasma viscosity that occurred in the two groups were recorded before and after treatment. The total effective rate and adverse reaction rate of the two groups were calculated.

Fasting venous blood was obtained before treatment and 2 wk after treatment and divided into five samples. One sample was used to test the blood glucose and blood lipids using a Hitachi 7600 automatic biochemical analyzer. One sample was centrifuged at a rotational speed of 3500 rpm for 10 min, and hs-CRP, interleukin (IL)-1 β, tumor necrosis factor (TNF)-α, HMGB1, PON-1, and MIF in the serum were assayed using an enzyme-linked immunosorbent assay (American Boteng Company ELX800 multi-function enzyme label instrument; Nanjing Jiancheng Bioengineering Research Institute). One sample was tested using the Wechsler method to determine the ESR. One sample was tested using the BS634 platelet aggregation instrument (Beijing Biochemical Instrument Factory) to assess the platelet aggregation rate. One sample was tested using the Zibo Hengtuo HT-100B hemorheology instrument to assess plasma viscosity.

Curative effects

The NIHSS score[7,8] was used to determine the curative effect. If the NIHSS score decreased by ≥ 90% after treatment, then the condition was considered cured. If the NIHSS score decreased between 45% and < 90% after treatment, then the condition was considered to have made significant progress. If the NIHSS score decreased between 18% and < 45% after treatment, then the condition was considered to have

made progress. If the NIHSS score decreased < 18% or if it increased, then no change or deterioration in the condition was considered. The total effective rate was calculated by adding the basic cure rate, significant progress rate, and progress rate.

NIHSS and ADL scores

The higher the NIHSS score, the more serious the degree of neurological impairment. NIHSS scores < 7 indicate mild defects, NIHSS scores 7-15 indicate moderate defects, and NIHSS scores > 15 indicate severe defects[9,10].

The ADL score[11,12] is determined based on the ability to independently defecate, urinate, perform basic grooming, eat, transfer from sitting to standing position, dress, climb stairs, and bathe. The total possible score is 100.

Statistical analysis

SPSS version 19.0 was used to process and analyze the data collected in this study. The NIHSS score; ADL score; blood glucose index; blood lipid index; inflammatory factors; HMGB1, PON-1, and MIF levels; ESR; and other measurements were evaluated. First, a normal distribution test was performed. Then, the measurements that were normally distributed or approximately normally distributed were evaluated using the χ^2 test. An independent sample t test was used for comparisons between groups, and a paired t test was used for comparisons within groups. Sex and the incidence of adverse reactions were expressed as percentages. The χ^2 test was used for comparisons between groups, and statistical significance was considered when P value was < 0.05.

RESULTS

After treatment, of the 41 patients in the control group, 8 cases (19.51%), 14 cases (34.15%), 8 cases (19.51%), and 11 cases (26.83%) were considered cured, to have made significant progress, to have made progress, and to have experienced no change or deterioration, respectively. Of the 41 patients in the observation group, 13 cases (31.71%), 17 cases (41.46%), 7 cases (17.07%), and 4 cases (9.76%) were considered cured, to have made significant progress, to have made progress, and to have experienced no change or deterioration, respectively, after treatment. The total effective rate of the observation group (94.00%) was higher than that of the control group (80.00%), and this difference was statistically significant ($\chi^2 = 3.998$; $P = 0.046$) (Figure 1).

Comparison of the blood glucose indexes between groups

Before treatment, the mean fasting plasma glucose (FPG) and plasma blood glucose (PBG) of the observation group were 8.81 ± 1.27 mmol/L and 11.24 ± 1.27 mmol/L, respectively. The mean FPG and PBG of the control group were 8.78 ± 1.23 mmol/L and 11.32 ± 1.05 mmol/L, respectively. There was no significant difference between the groups ($P > 0.05$). After treatment, the mean FPG and PBG of the observation group were 6.91 ± 0.79 mmol/L and 9.53 ± 0.88 mmol/L, respectively; the mean FPG and PBG of the control group were 7.32 ± 0.96 mmol/L and 10.23 ± 1.07 mmol/L respectively. The FPG and PBG of the observation group were lower than those of the control group, and the difference was statistically significant ($P < 0.05$). The FPG and PBG of the two groups decreased significantly after treatment ($P < 0.05$) (Figure 2A and B).

Comparisons of blood lipid indexes, platelet maximum aggregation rates, and plasma viscosity between groups

The high-density cholesterol levels of the two groups increased after treatment; furthermore, that of the observation group was higher than that of the control group after treatment. Total cholesterol levels, triglyceride levels, low-density cholesterol levels, maximum platelet aggregation rates, and plasma viscosity were lower after treatment, and all these values were lower in the observation group than in the control group ($P < 0.05$) (Table 1).

Comparison of NIHSS scores and ADL scores between groups

Before treatment, the mean NIHSS score and ADL score of the observation group were 14.96 ± 2.17 and 51.89 ± 7.54, respectively, and those of the control group were 15.23 ± 2.33 and 54.25 ± 6.36, respectively. There was no significant difference between the two groups ($P > 0.05$). After treatment, the NIHSS score and ADL score of the observation

Table 1 Comparison of blood lipid indexes, platelet maximum aggregation rate, and plasma viscosity between the two groups (mean ± SD)

Group		Control group (*n* = 41)	Observation group (*n* = 41)
TC (mmol/L)	Before treatment	6.52 ± 0.47	6.40 ± 0.56
	After treatment	5.17 ± 0.41[a]	4.79 ± 0.32[a,c]
TG (mmol/L)	Before treatment	2.92 ± 0.41	2.89 ± 0.45
	After treatment	2.05 ± 0.36 [a]	1.68 ± 0.31[a,c]
HDL-C (mmol/L)	Before treatment	0.96 ± 0.31	0.95 ± 0.36
	After treatment	1.67 ± 0.37[a]	1.95 ± 0.42[a,c]
LDL-C (mmol/L)	Before treatment	4.25 ± 0.56	4.21 ± 0.53
	After treatment	3.37 ± 0.45[a]	2.89 ± 0.39[a,c]
Maximum platelet aggregation rate(%)	Before treatment	75.92 ± 9.64	78.40 ± 10.22
	After treatment	46.48 ± 7.26[a]	58.30 ± 7.74[a,c]
Blood plasma viscosity (mPa·s)	Before treatment	1.86 ± 0.48	1.94 ± 0.43
	After treatment	1.40 ± 0.33[a]	1.61 ± 0.35[a,c]

[a]$P < 0.05$ *vs* before treatment.
[c]$P < 0.05$ *vs* the control group.
TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

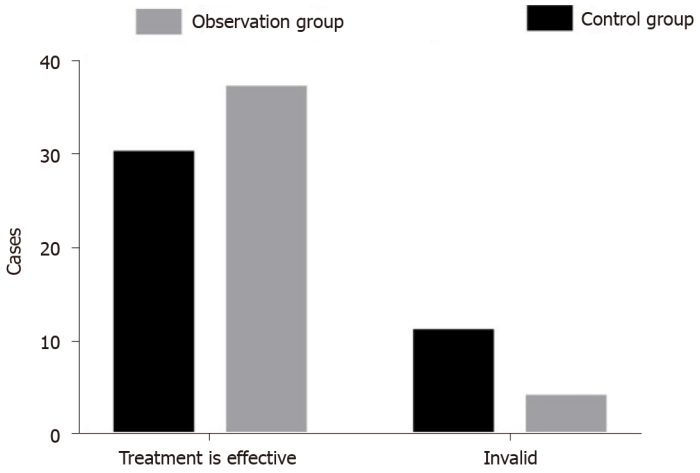

Figure 1 Graph of effective and ineffective treatment of cases in both groups.

group were 8.79 ± 1.65 and 78.26 ± 9.22, respectively, and those of the control group were 10.23 ± 2.05 and 67.89 ± 7.98, respectively. This difference was statistically significant ($P < 0.05$). The ADL scores increased and NIHSS scores decreased after treatment in both groups, and the difference was statistically significant ($P < 0.05$).

Comparisons of inflammatory factors and immune levels between groups

The PON-1 Levels increased after treatment and were higher in the observation group than in the control group. Furthermore, the hs-CRP, IL-1β, TNF-α, HMGB1, and MIF levels and ESR decreased after treatment and were lower in the observation group than in the control group ($P < 0.05$) (Table 2).

Comparison of adverse reactions between groups

The rate of adverse reactions was 21.95% for the observation group and 17.07% for the control group ($\chi^2 = 0.311$; $P = 0.577$).

Table 2 Comparison of inflammatory factors and immune levels between the two groups (mean ± SD)

Group		Control group (*n* = 41)	Observation group (*n* = 41)
hs-CRP (mg/L)	Before treatment	10.63 ± 2.58	10.49 ± 2.44
	After treatment	7.23 ± 1.96[a]	5.48 ± 1.67[a,c]
TNF-α (pg/mL)	Before treatment	58.69 ± 6.32	59.02 ± 7.44
	After treatment	46.58 ± 5.24[a]	37.02 ± 4.63[a,c]
HMGBl (μg/L)	Before treatment	5.84 ± 0.56	5.81 ± 0.59
	After treatment	3.23 ± 0.52[a]	2.94 ± 0.43[a,c]
PON-1 (U/L)	Before treatment	135.23 ± 25.21	130.58 ± 22.17
	After treatment	159.63 ± 25.74[a]	174.25 ± 31.02[a,c]
ESR (mm/h)	Before treatment	32.15 ± 5.27	30.96 ± 6.42
	After treatment	19.36 ± 3.78[a]	13.02 ± 4.11[a,c]
MIF (ng/mL)	Before treatment	84.96 ± 5.87	86.02 ± 8.11
	After treatment	76.36 ± 6.14[a]	68.21 ± 5.47[a,c]

[a]$P < 0.05$ *vs* before treatment.
[c]$P < 0.05$ *vs* the control group.
hs-CRP: High-sensitivity-C reactive protein; TNF-α: Tumor necrosis factor-alpha; HMGBl: High-mobility group protein B1; PON-1: Paraoxonase-1; ESR: Erythrocyte sedimentation rate; MIF: Macrophage migration inhibitory factor.

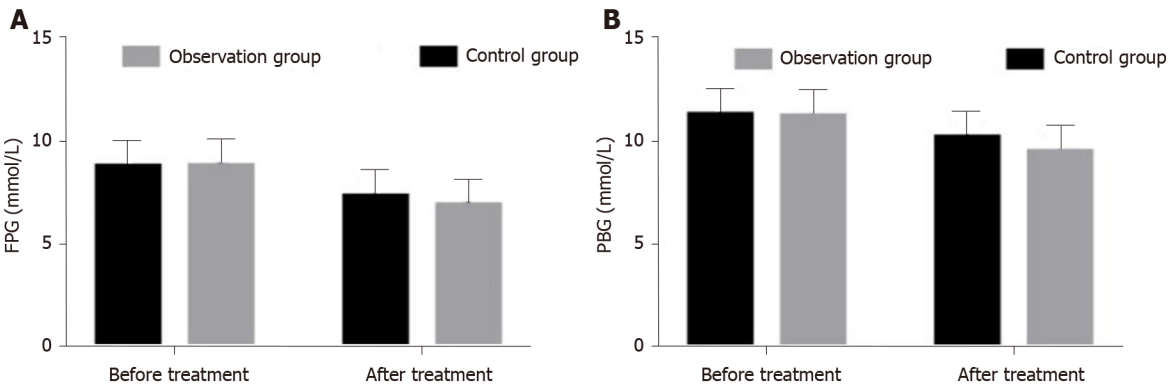

Figure 2 Fasting plasma glucose and plasma blood glucose levels of both groups. A: Fasting plasma glucose levels; B: Plasma blood glucose levels. FPG: Fasting plasma glucose; PBG: Plasma blood glucose.

DISCUSSION

Relationship between diabetes and lacunar cerebral infarction

Diabetes can cause metabolic disorders that seriously affect the quality of life[13-15]. Abnormal lipid metabolism caused by diabetes leads to atherosclerotic plaque in blood vessels and may cause cerebral infarction if there is atherosclerotic plaque in cerebral vessels[16-18]. Gap infarction is the most common type of cerebral infarction in diabetic patients and is one of the main causes of death[19]. Lacunar cerebral infarction occurs in the deep part of the cerebral hemisphere or the small perforating artery of the brain stem[20]. With long-term hypertension, vascular wall lesions occur, resulting in lumen occlusion and the formation of cystic lesions 0.2-15 mm in diameter; this diameter is slightly larger than the vascular diameter, thereby causing embolism, which is common in the elderly and especially in diabetic patients. Lacunar cerebral infarction mainly occurs in the putamen, caudate nucleus, internal capsule, thalamus, and pons. Because of the limited range of arterial blood supply, occlusion of a single branch cause only a small area of ischemic necrosis of brain tissue and lacunar infarction[21]. Clinically, lacunar infarctions are more common in patients with type 2 diabetes, and the treatment effect is poor. Re-infarction or other major vascular complications are prone to occur. Therefore, early detection and prevention are partic-

ularly important[22].

Relationships between inflammation, diabetes, and lacunar cerebral infarction

Diabetes is an inflammatory disease[23]. The important pathological causes of lacunar cerebral infarction are small cerebral artery atherosclerosis and atherosclerosis, which is a chronic vascular inflammatory disease[24]. hs-CRP, IL-1β, TNF-α, and MIF are classical markers related to inflammation[25-32] and reflect the degree of inflammation in the body. When inflammation occurs in the body, serum levels of these markers increase. HMGB1 is a type of delayed inflammatory factor that can increase insulin resistance, lead to impaired glucose tolerance, promote tumor metastasis, and affect the blood-brain barrier permeability[33,34]. PON-1 is a calcium-dependent aromatic esterase that can hydrolyze lipid peroxides, protect low-density lipoprotein cholesterol from oxidative modification, and protect against cardiovascular and cerebrovascular diseases[35]. Lacunar infarctions often occur in the putamen, caudate nucleus, internal capsule, thalamus, pons, and other areas. Small perforator vessel wall lesions, stenosis, and occlusion form a small focus of infarction that only causes a small area of brain tissue damage and forms a "cavity" attributable to long-term hypertension and hyperlipidemia. Type 2 diabetes is an independent risk factor for cerebral infarction, and these two conditions often occur together. Common clinical manifestations include vertigo, limb numbness, and memory loss, resulting in a low degree of disability; however, these manifestations can occur repeatedly because the degree of neurological impairment associated with lacunar infarction is mild. During clinical treatment, attention should be focused on the regulation of lipids and improvement in microcirculation[36-39].

Use of atorvastatin

Actively administering symptomatic and supportive treatment is helpful for reducing the degree of disability. Statins can effectively regulate blood lipids and reduce the degree of vascular lesions[40,41]. Atorvastatin[42] selectively inhibits the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase and reduces total cholesterol and low-density lipoprotein cholesterol levels, thereby affecting the deformation and oxygen-carrying capacity of red blood cells, improving microcirculation, and promoting the recovery of neurological function. This drug has good lipid-regulating effects and pharmacological effects, such as protecting the vascular endothelium and antioxidative and anti-inflammatory properties[43-46].

Use of sodium ozagrel

Sodium ozagrel is an antiplatelet drug and thromboxane A inhibitor that can resist platelet aggregation, reduce blood viscosity, promote vasodilation to alleviate the blood hypercoagulable state, reduce thrombosis, and improve brain metabolism and microcirculation[46-49]. Ozagrel has an important role in the treatment of ischemic cerebrovascular disease and concomitant limb dyskinesia[47]. During this study, the total effective rate for patients treated with sodium ozagrel combined with atorvastatin was higher than that for patients treated with atorvastatin alone; also, the improvements in blood glucose and blood lipid indexes of patients treated with sodium ozagrel combined with atorvastatin were better than those of patients treated with atorvastatin alone[48]. Additionally, the ADL scores of patients treated with sodium ozagrel combined with atorvastatin were higher than those of patients treated with atorvastatin alone[49]. Moreover, the NIHSS scores of patients treated with sodium ozagrel combined with atorvastatin were lower than those of patients treated with atorvastatin alone. These results suggest that sodium ozagrel combined with statins may be better for controlling blood glucose and blood lipids, reducing the degree of nerve injury, and improving the self-care ability of patients with type 2 diabetes mellitus with lacunar cerebral infarction than treatment with statins alone. Ozagrel can selectively inhibit thromboxane synthase and prevent prostaglandin H2 from synthesizing thromboxane A2 to inhibit platelet aggregation and dilate blood vessels. As a result, ozagrel can increase the local perfusion in brain tissue and improve the abnormal energy metabolism caused by ischemia and hypoxia, thus reducing defects in neurological function. Blood hypercoagulability and high viscosity are risk factors for lacunar infarction. During this study, we assessed the platelet maximum aggregation rate and plasma viscosity and found that sodium ozagrel combined with atorvastatin for the treatment of type 2 diabetes mellitus with lacunar infarction can correct blood hypercoagulability and high viscosity and reduce the risk of lacunar infarction.

Inflammation is always present during the pathological process of type 2 diabetes and lacunar cerebral infarction. hs-CRP is a sensitive indicator of inflammation, and the hs-CRP serum level can reflect the degree of inflammation. Additionally, hs-CRP is associated with the severity of cardiovascular and cerebrovascular diseases. IL-1β and TNF-α are both classical proinflammatory factors that can not only directly cause tissue inflammatory damage but also expand the inflammatory response by promoting the release of other proinflammatory factors. HMGB1 is a highly conserved nuclear protein that has an important proinflammatory role in inflammation. ESR is a routine index associated with the active stage of inflammation that reflects the sedimentation rate of red blood cells[25]. MIF is a marker associated with inflammation that releases proteolytic enzymes under the actions of cytokines and growth factors, promotes atherosclerosis, and affects plaque stability. PON-1 is a calcium-dependent aromatic esterase that can hydrolyze lipid peroxides, protect low-density lipoprotein cholesterol from oxidative modification, reduce the level of oxidized low-density lipoprotein, reduce the uptake of oxidized low-density lipoprotein by macrophages, reduce the formation of foam cells, and exert protective effects on cerebrovascular vessels. The levels of hs-CRP, IL-1β, TNF-α, HMGB1, and MIF and ESR of patients treated with ozagrel combined with atorvastatin were lower than those of patients treated with atorvastatin alone. Moreover, their PON-1 Levels were lower than those of patients treated with atorvastatin alone. These results suggest that sodium ozagrel combined with statins is better for reducing inflammation and inhibiting atherosclerosis to treat type 2 diabetes mellitus with lacunar cerebral infarction than treatment with statins alone. This is because ozagrel can promote the conversion of prostaglandin H2 by endothelial cells to prostaglandin I2, regulate the balance of thromboxane A2 and prostaglandin I2, and reduce inflammation.

There was no significant difference in the rates of adverse reactions between groups. These results suggest that sodium ozagrel combined with statins does not increase the risk of adverse reactions. Type 2 diabetes mellitus with lacunar cerebral infarction is common in clinical settings. Although statins alone can alleviate the disease to a certain extent, they alone cannot achieve the ideal effect. The antiplatelet drug ozagrel was administered based on the routine treatment and lipid regulation of statins. Ozagrel is beneficial for regulating blood glucose and blood lipids, and reducing nerve injury. During this study, through the assessment of serum inflammatory indicators, it was clear that reducing inflammation and inhibiting atherosclerosis are important mechanisms for treating type 2 diabetes mellitus with lacunar cerebral infarction.

Although ozagrel has been used in combination with statins in previous clinical studies[50], most studies assessed only blood glucose or a single blood index. We assessed blood glucose, blood lipids, blood coagulation, ESR, NIHSS score, ADL score, inflammatory factors, and specific indicators, namely, HMGB1, PON-1, and MIF in a comprehensive analysis; we found that the combination of these two drugs has a good effect on blood glucose and blood lipids, reduces nerve injury, and reduces inflammation. Inhibition of atherosclerosis is an important mechanism for the treatment of type 2 diabetes mellitus complicated with lacunar infarction. Some limitations of our study should be recognized. These include the small sample size, the short follow-up time, and the lack of long-term curative effect observation. The results need to be verified with further larger scale studies and include other statins in combination with ozagrel.

CONCLUSION

Sodium ozagrel combined with atorvastatin for the treatment of type 2 diabetes mellitus with lacunar cerebral infarction can reduce inflammatory reactions and regulate the expression levels of HMGB1, PON-1, and MIF and ESR. Additionally, ozagrel can effectively control blood glucose and blood lipid indexes and reduce nerve injury, without increasing adverse reactions when compared to treatment with atorvastatin alone.

ARTICLE HIGHLIGHTS

Research background

Type 2 diabetes is a common metabolic disease that is often complicated by abnormal lipid metabolism.

Research motivation
As an antiplatelet drug and thromboxane A inhibitor, sodium ozagrel is widely used to treat ischemic cerebrovascular diseases.

Research objectives
We want to observe the effects of sodium ozagrel combined with atorvastatin on high-mobility group protein B1 (HMGB1) and high-sensitivity-C reactive protein (hs-CRP) in patients with type 2 diabetes mellitus and lacunar infarction.

Research methods
Eighty-two patients with type 2 diabetes mellitus and lacunar infarction treated were categorized into two groups according to the method of treatment (41 patients in each group).

Research results
After treatment, the blood glucose indexes; blood lipid indexes; inflammatory factors; HMGB1, paraoxonase-1, and macrophage migration inhibitory factor levels; erythrocyte sedimentation rate; platelet aggregation rate; and plasma viscosity of the observation group were better than those of the control group.

Research conclusions
Sodium ozagrel with atorvastatin can reduce inflammatory reactions.

Research perspectives
The results need to be verified with further larger scale studies and include other statins in combination with ozagrel.

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ORIGINAL ARTICLE

Observational Study

Rates and associates of influenza and pneumococcus vaccination in diabetes mellitus: A nationwide cross-sectional study (TEMD vaccination study)

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Institutional review board statement: The study was reviewed and approved by the TC

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Abstract

BACKGROUND

Vaccination against influenza and pneumococcus is effective in reducing morbidity and mortality in patients with diabetes.

AIM

To investigate the prevalence of influenza and pneumococcal vaccinations and to search for the independent associates of vaccination in Turkish patients with diabetes.

METHODS

In this cross-sectional, nationwide, multicenter study, adult patients with type 1 diabetes (T1DM) (n = 454) and type 2 diabetes (T2DM) (n = 4721), who were under follow-up for at least a year in the outpatient clinics, were consecutively enrolled. Sociodemographic, clinical, and laboratory parameters of patients were recorded. Vaccination histories were documented according to the self-statements of the patients.

RESULTS

Patients with T1DM and T2DM had similar vaccination rates for influenza (23.6% *vs* 21.2%; P = 0.240) and pneumococcus (8% *vs* 7%; P = 0.451) vaccinations. Longer diabetes duration and older age were the common independent associates of having vaccination for both types of diabetes patients. Higher education level, using statin treatment, and having optimal hemoglobin A1c levels were the common independent associates of influenza and pneumococcal vaccination in patients with T2DM.

CONCLUSION

TEMD Vaccination Study shows that patients with T1DM and T2DM had very low influenza and pneumococcal vaccination rates in Turkey. The lower rates of vaccination in certain populations urges the necessity of nationwide vaccination strategies targeting these populations.

Key Words: Diabetes; Influenza; Pneumococcus; Vaccination; Type 1 diabetes; Type 2 diabetes

Core Tip: The TEMD Vaccination Study is a cross-sectional, multicenter survey, which was carried out between April 1 and June 30, 2017, in 68 tertiary endocrine units from 37 cities throughout Turkey. The study revealed that the vaccination rates for pneumococcus and influenza were very low in patients with diabetes. Only 6.6% patients with type 1 diabetes (T1DM) and 5.8% patients with type 2 diabetes (T2DM) received both vaccines. Older age and longer diabetes duration were the common independent associates of vaccination in patients with T1DM and T2DM. The common independent associates of vaccination rates for T2DM were using statins, higher education and the lower hemoglobin A1c levels.

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INTRODUCTION

Patients with diabetes are prone to influenza and pneumococcal infections with a more

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severe clinical course[1-3]. Deaths due to influenza and pneumococcal infections are two to three times higher in patients with diabetes compared to non-diabetic patients [4]. Moreover, the need for hospitalization due to influenza and pneumococcal infections is also higher in patients with diabetes[2]. Various observational studies reported that vaccination against influenza and pneumococcus is effective in reducing morbidity and mortality in patients with diabetes[5,6]. Therefore, many national and international guidelines recommend annual influenza vaccination, and one or two doses of pneumococcal vaccination depending on age under or over 65 years, for both type 1 (T1DM) or type 2 diabetes mellitus (T2DM)[7-9].

Despite all these recommendations, the vaccination rates in patients with diabetes are unsatisfactory around the world[10-12]. To establish better immunization policies, there is a need to find out the regional or national barriers to vaccination. So far, various factors such as low availability of the vaccine, healthcare costs, physician and patient attitudes, racial disparities, social influences, and misbeliefs were mentioned in various reports as the causes of low vaccination rates[13,14]. However, there is hardly enough data about the rates and predictors of influenza and pneumococcal vaccination in Turkish patients with diabetes[15,16].

The recently published national survey "Turkish Nationwide SurvEy of Glycemic and Other Metabolic Parameters of Patients with Diabetes Mellitus (TEMD Study)" reported the clinical and demographical factors predicting the glycemic and metabolic targets in Turkish patients with diabetes[17]. TEMD Vaccination Study was carried out by using the TEMD database to investigate the prevalence of influenza and pneumococcal vaccinations and to search for the independent associates of vaccination in Turkish patients with T1DM and T2DM.

MATERIALS AND METHODS

Study design

The TEMD Study is a cross-sectional, nationwide, multicenter survey, which was carried out between April 1 and June 30, 2017, in 68 tertiary endocrine units from 37 cities throughout Turkey. The study centers were selected according to the 12 Nomenclature of Territorial Units for Statistics (NUTS) regions of the country. Both local and central ethics committees approved the study. The ClinicalTrials.gov registration number is NCT03455101. All patients signed informed consent forms before data collection.

Patients with either T1DM or T2DM who were under follow-up in the same center for at least one year were consecutively enrolled in the original study[17]. Patients were excluded if they were pregnant, younger than 18 years, had decompensated liver disease, psychiatric disorders interfering with cognition or compliance, had bariatric surgery, or were undergoing renal replacement therapy.

Patients were asked to fill specifically designed questionnaires about sociodemographic characteristics (age, marital status, education, occupation, and income), concomitant diseases, medications, macro-and microvascular complications, family history, lifestyle, personal diabetes management (diet, exercise, smoking, self-monitoring of blood glucose, and frequency of hypoglycemia), data of outpatient care standards (dietitian visits, diabetes nurse interviews, foot, and dental examinations, and vaccinations), treatment regimens, and current and previous laboratory data. The history of pneumococcal vaccination after diagnosis of diabetes and influenza vaccination in the last year was obtained from the patient's own statement.

Anthropometrics

The height, weight, and waist circumference recordings were performed according to the standard protocol with the patients in their underwear. The ratio of weight to the square of height (kg/m^2) was given as body mass index (BMI). Arterial blood pressure (ABP) was recorded using automatic blood pressure (BP) monitors (Omron M2, HEM-7121-E) in a sitting position after at least 5 min of rest. Three consecutive measurements were conducted on the same arm, and the mean was recorded.

Definitions

Hypertension was defined as the presence of high office BP recordings or currently undergoing antihypertensive treatment. For patients who were not under antihypertensive medication, an average office BP > 140/90 mmHg in two different visits was defined as hypertension. Dyslipidemia was triglycerides (TG) > 150 or low-density lipoprotein cholesterol (LDL-C) > 100, or low high-density lipoprotein cholesterol

(HDL-C; men < 40, women < 50 mg/ dL), or receiving medications for dyslipidemia. The BMI values ≥ 30 kg/m^2 were defined as obesity[18]. Treatment targets were defined as glycosylated hemoglobin A1c (HbA1c) < 7% (< 53 mmol/mol), office ABP < 140/90 mmHg, and LDL-C < 100 mg/dL. Regular exercise was defined as performing physical activity on more than two days a week, with each episode lasting for more than 30 min. Low income was self-reported monthly earnings below the minimum wage level declared in 2017. A low education level was defined as receiving less than 8 years of formal education. Macrovascular complications were either self-reported, having a history of coronary artery disease, angina, heart attack, cerebrovascular event, or peripheral artery disease, or recorded by the physicians according to findings such as non-palpable extremity pulses, and low ankle-brachial index (ABI ≤ 0.9), positive findings on coronary or peripheral arteriography, and carotid or peripheral arterial duplex ultrasound examination. Retinopathy was self-reported by the patients when asked whether they have been told in eye examinations that they have any problem related to diabetes mellitus. Nephropathy was defined as the presence of albuminuria or decreased estimated glomerular filtration rate[19]. Neuropathy was defined as the presence of symptoms related to bilateral distal symmetrical neuropathy or other autonomous neuropathies attributed to diabetes mellitus.

Statistical analysis

Statistical analysis was performed in SPSS 18.0 (SPSS Inc., Chicago, IL, United States). Normality of distribution was tested using the Shapiro-Wilk test. Data are presented as the mean ± SD for continuous variables or as number (percentage) for categorical variables. An independent sample t-test was used for comparisons among continuous variables, and a Chi-square test was employed for categorical variables. Uni- and multivariate logistic regressions were studied to identify independent variables associated with receiving influenza and pneumococcal vaccination. Having statistical significance (P < 0.05) in the univariate analysis, as well as the clinical rationale for a potential association with vaccination, were the criteria for inclusion in the model for these variables, which were gender, age (years), diabetes duration (years), BMI (kg/m^2), HbA1c (%), BP on target (< 140/90 mmHg *vs* higher), having microvascular and macrovascular complications, education level, smoking, exercise (≤ 2 d/wk *vs* higher), statin treatment, insulin use, hypertension, dyslipidemia, follow-up center type (private center *vs* government hospital), and monthly income (in two categories). Odds ratios (ORs) with 95% confidence intervals (CIs) are given in Figures 1 and 2. The P value was two-tailed with a significance level of ≤ 0.05.

RESULTS

Patients with T1DM (n = 454) and T2DM (n = 4721) were included. The clinical and sociodemographic characteristics of patients who have been vaccinated and not vaccinated for influenza and pneumococcus are given in Tables 1 and 2, respectively. The ratio of receiving both vaccines was 6.6% (n = 30) in patients with T1DM and 5.8% (n = 274) in patients with T2DM.

In patients with T1DM, the rate of influenza vaccination was 23.6% (n = 107) and pneumococcus vaccination was 8.0% (n = 36). Compared to patients who were not vaccinated for influenza, patients who were vaccinated were older, had longer diabetes duration, higher BMI levels, higher rates of dyslipidemia and statin use, higher private care center follow-up rates, lower rates of smoking, lower HbA1c levels and LDL-C target achievement rates (P < 0.05 for all). Patients who received pneumococcal vaccination were also older, with longer diabetes duration, higher rates of dyslipidemia, and lower LDL-C target achievement rates (P < 0.05 for all) (Table 1).

In patients with T2DM, the rate of influenza vaccination was 21.2% (n = 1003) and pneumococcus vaccination was 7.0% (n = 330). Patients vaccinated against influenza and/or pneumococcus were predominantly male, older, with longer diabetes duration, lower BMI, diastolic BP, HbA1c, LDL-C, and TG levels (for the influenza group), higher rates of macro and microvascular complications, higher education levels, higher income, a higher rate of private center follow-up, exercising regularly, smoking less, more frequently use statins and higher rate of achieve metabolic targets for HbA1c, BP(for pneumococcus) and LDL-C P < 0.05 for all) (Table 2).

Table 1 The comparison of the clinical and sociodemographic characteristics of patients with type 1 diabetes mellitus with and without seasonal influenza and pneumococcal vaccination status

	Patients with T1DM, *n* = 454					
	Influenza vac (+), *n* = 107 (23.6%)	Influenza vac (-), *n* = 346 (76.4%)	*P* value	Pneumo. vac (+), *n* = 36 (8.0%)	Pneumo. vac (-), *n* = 415 (92.0%)	*P* value
Sex (female)	72 (67.3)	207 (59.8)	0.165	23 (63.9)	254 (61.2)	0.751
Age (yr)	38.48 ± 13.61	32.18 ± 11.58	**< 0.001**	42.34 ± 14.51	32.94 ± 11.89	**< 0.001**
Diabetes duration (yr)	18.20 ± 11.20	12.19 ± 9.33	**< 0.001**	18.17 ± 11.52	13.23 ± 9.92	**0.005**
BMI (kg/m^2)	24.67 ± 4.26	23.61 ± 4.08	**0.021**	24.44 ± 3.70	23.83 ± 4.18	0.399
SBP office (mm Hg)	118.44 ± 13.83	117.65 ± 15.35	0.635	119.64 ± 15.30	117.68 ± 14.99	0.452
DBP office (mm Hg)	74.44 ± 9.60	74.17 ± 9.44	0.796	75.22 ± 9.43	74.15 ± 9.51	0.518
HbA1c (%) (mmol/mol)	8.24 ± 1.77 (66.61 ± 19.33)	8.79 ± 2.04 (72.56 ± 22.33)	**0.014**	8.11 ± 2.05 (65.19 ± 22.42)	8.71 ± 1.99 (71.70 ± 21.72)	0.095
LDL-C (mg/dL)	112.75 ± 39.00	105.18 ± 35.67	0.072	117.12 ± 35.19	106.04 ± 36.65	0.086
TG (mg/dL)	110.83 ± 74.83	116.32 ± 170.15	0.754	132.29 ± 82.02	113.41 ± 158.08	0.486
HDL-C (mg/dL)	60.23 ± 18.70	56.32 ± 17.36	0.056	57.78 ± 19.49	57.01 ± 17.49	0.805
Macrovascular complications, *n* (%)	12 (11.2)	25 (7.2)	0.188	4 (11.1)	33 (8.0)	0.508
Microvascular complications, *n* (%)	42 (39.3)	125 (36.1)	0.558	17 (47.2)	149 (35.9)	0.177
Higher education, *n* (%)	82 (76.6)	255 (75.0)	0.732	31 (86.1)	304 (74.3)	0.116
Private care center, *n* (%)	18 (16.8)	23 (6.6)	**0.001**	6 (16.7)	35 (8.4)	0.099
Lower-income, *n* (%)	18 (21.4)	69 (25.7)	0.423	4 (14.3)	83 (25.7)	0.180
Current smoking, *n* (%)	19 (17.8)	98 (28.5)	**0.027**	9 (25.0)	109 (26.3)	0.862
Regular exercise, *n* (%)	30 (28.3)	67 (19.5)	0.055	9 (25.0)	88 (21.4)	0.616
Obesity, *n* (%)	13 (12.3)	26 (7.5)	0.130	4 (11.1)	35 (8.5)	0.590
Hypertension, *n* (%)	33 (30.8)	82 (23.8)	0.147	14 (38.9)	102 (24.7)	0.062
Dyslipidemia, *n* (%)	84 (83.2)	232 (70.9)	**0.015**	31 (88.6)	284 (72.6)	**0.040**
Statin treatment, *n* (%)	23 (21.5)	31 (9.0)	**< 0.001**	7 (19.4)	47 (11.3)	0.150
Insulin pump, *n* (%)	25 (23.8)	64 (18.6)	0.236	6 (16.7)	82 (19.9)	0.827
Achieving metabolic targets, *n* (%)						
HbA1c on target (< 7%), (< 53 mmol/mol)	16 (15.2)	52 (15.4)	0.971	7 (20.6)	61 (15.0)	0.385
BP on target (< 130/80 mm Hg)	98 (91.6)	308 (89.5)	0.536	32 (89.9)	371 (89.8)	0.858
LDL-C on target (< 100 mg/dL)	35 (35.7)	162 (49.1)	**0.020**	10 (28.6)	186 (47.6)	**0.031**

T1DM: Type 1 diabetes mellitus; vac: Vaccination; Pneumo.: Pneumococcal, BMI: Body mass index; SBP and DPB: Systolic and diastolic blood pressures; HbA1c: Glycosylated hemoglobin A1c; LDL-C and HDL-C: Low and high density lipoprotein cholesterol; TG: Triglycerides.

Multivariable associations of receiving influenza and pneumococcal vaccinations in patients with T1DM

In the multivariable model, longer diabetes duration (OR: 1.04, 95%CI: 1.01-1.07), being followed up in private care center (OR: 2.44, 95%CI: 1.13-5.28) and not smoking (OR: 0.52, 95%CI: 0.27-0.98) were significantly associated with being vaccinated for influenza. Age was the only significant associate of pneumococcus vaccination (OR: 1.05, 95%CI: 1.02-1.09) (Figure 1).

Table 2 The comparison of the clinical and sociodemographic characteristics of patients with type 2 diabetes mellitus with and without seasonal influenza and pneumococcal vaccination status

	Patients with T2DM, *n* = 4721					
	Influenza vac (+), *n* = 1003 (21.2%)	Influenza vac (-), *n* = 3718 (78.8%)	*P* value	Pneumo. vac (+), *n* = 330 (7.0%)	Pneumo. vac (-), *n* = 4366 (93.0%)	*P* value
Sex (female)	525 (52.3)	2251 (60.5)	**< 0.001**	180 (54.5)	2581 (59.1)	**< 0.001**
Age (yr)	62.22 ± 9.93	57.44 ± 10.36	**< 0.001**	63.97 ± 9.83	58.02 ± 10.38	**< 0.001**
Diabetes duration (yr)	13.62 ± 8.36	10.08 ± 7.09	**< 0.001**	14.10 ± 8.84	10.57 ± 7.36	**< 0.001**
BMI (kg/m^2)	31.60 ± 6.28	32.39 ± 6.65	**0.004**	31.08 ± 5.94	32.21 ± 6.61	**< 0.001**
SBP office (mm Hg)	133.32 ± 18.15	132.34 ± 18.40	0.138	133.83 ± 17.63	132.46 ± 18.40	0.191
DBP office (mm Hg)	79.56 ± 10.84	80.81 ± 10.79	**0.001**	78.90 ± 10.28	80.67 ± 10.86	**0.004**
HbA1c (%) (mmol/mol)	7.54 ± 1.59 (58.95 ± 17.41)	7.79 ± 1.78 (61.62 ± 19.49)	**< 0.001**	7.44 ± 1.59 (57.80 ± 17.33)	7.76 ± 1.76 (61.29 ± 19.20)	**0.001**
LDL-C (mg/dL)	109.27 ± 35.76	115.13 ± 36.15	**< 0.001**	107.80 ± 35.82	114.32 ± 36.14	**0.002**
TG (mg/dL)	169.75 ± 100.06	185.77 ± 136.46	**0.001**	172.21 ± 111.17	183.20 ± 131.21	0.144
HDL-C (mg/dL)	46.81 ± 12.55	46.46 ± 13.04	0.464	47.63 ± 11.91	46.43 ± 12.97	0.109
Macrovascular complications, *n* (%)	317 (31.6)	827 (22.2)	**< 0.001**	119 (36.1)	1013 (23.2)	**< 0.001**
Microvascular complications, *n* (%)	509 (50.7)	1734 (46.6)	**0.021**	175 (53.0)	2058 (47.1)	**0.039**
Higher education, *n* (%)	461 (46.7)	1331 (36.3)	**< 0.001**	155 (47.7)	1630 (37.9)	**< 0.001**
Private care center, *n* (%)	154 (15.4)	319 (8.6)	**< 0.001**	154 (15.4)	319 (8.6)	**< 0.001**
Lower income, *n* (%)	237 (29.8)	981 (33.5)	0.052	54 (23.9)	1157 (33.2)	**0.004**
Current smoking, *n* (%)	108 (10.8)	491 (13.3)	**0.039**	26 (7.9)	570 (13.1)	**0.006**
Regular exercise, *n* (%)	226 (22.9)	688 (18.8)	**0.003**	87 (26.7)	824 (19.2)	**0.001**
Obesity, *n* (%)	548 (55.2)	2180 (59.4)	**0.016**	173 (52.9)	2538 (58.9)	**0.034**
Hypertension, *n* (%)	768 (77.0)	2433 (65.9)	**< 0.001**	258 (78.7)	2924 (67.4)	**< 0.001**
Dyslipidemia, *n* (%)	939 (95.0)	3457 (95.1)	0.989	310 (94.8)	4061 (95.1)	0.835
Statin treatment, *n* (%)	531 (52.9)	1333 (35.9)	**< 0.001**	196 (59.4)	1659 (38.0)	**< 0.001**
Achieving targets, *n* (%)						
HbA1c on target (< 7%) (53 mmol/mol)	434 (44.2)	1417 (39.1)	**0.004**	165 (50.8)	1677 (39.4)	**< 0.001**
BP on target (< 130/80 mg/dL)	700 (70.4)	2563 (69.6)	0.636	249 (75.9)	2999 (69.4)	**0.013**
LDL-C on target (< 100 mg/dL)	422 (43.6)	1245 (35.5)	**< 0.001**	141 (43.9)	1517 (36.7)	**0.010**

T2DM: Type 2 diabetes mellitus; vac: Vaccination; Pneumo.: Pneumococcal, BMI: Body mass index; SBP and DPB: Systolic and diastolic blood pressures; HbA1c: Glycosylated hemoglobin A1c; LDL-C and HDL-C: Low and high density lipoprotein cholesterol; TG: Triglycerides.

Multivariable associations of receiving influenza and pneumococcal vaccinations in patients with T2DM

In the multivariable model, female gender (OR: 0.73, 95%CI: 0.60-0.89), age (OR: 1.03, 95%CI: 1.02-1.05), diabetes duration (OR: 1.05, 95%CI: 1.03-1.06), higher education (OR: 1.50, 95%CI: 1.22-1.83), having optimal HbA1c levels (OR: 1.36, 95%CI: 1.13-1.64) and using statins (OR: 1.57, 95%CI: 1.31-1.89) were significantly associated with receiving influenza vaccination.

The significant associates of receiving pneumococcal vaccination were age (OR: 1.05, 95%CI: 1.03-1.07), longer diabetes duration (OR: 1.04, 95%CI: 1.02-1.06), higher education (OR: 1.50, 95%CI: 1.06-2.12), followed up in a private care center (OR: 2.27, 95%CI: 1.47-3.49), having optimal HbA1c levels (OR: 1.69, 95%CI: 1.24-2.30) and statin

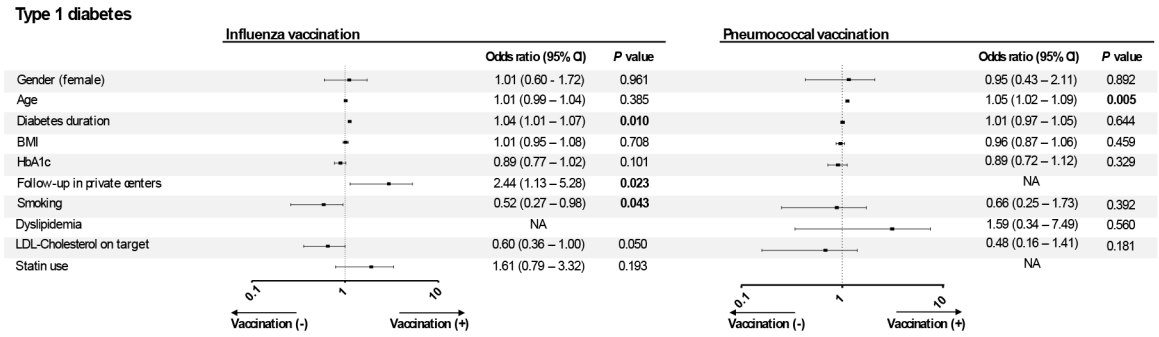

Figure 1 Multivariable logistic regression analysis of vaccination among patients with type 1 diabetes mellitus (dependent variable: vaccination of influenza and pneumococcus). BMI: Body mass index; HbA1c: Glycosylated hemoglobin A1c; LDL-C: Low density lipoprotein cholesterol; CI: Confidence interval; OR: Odds ratio.

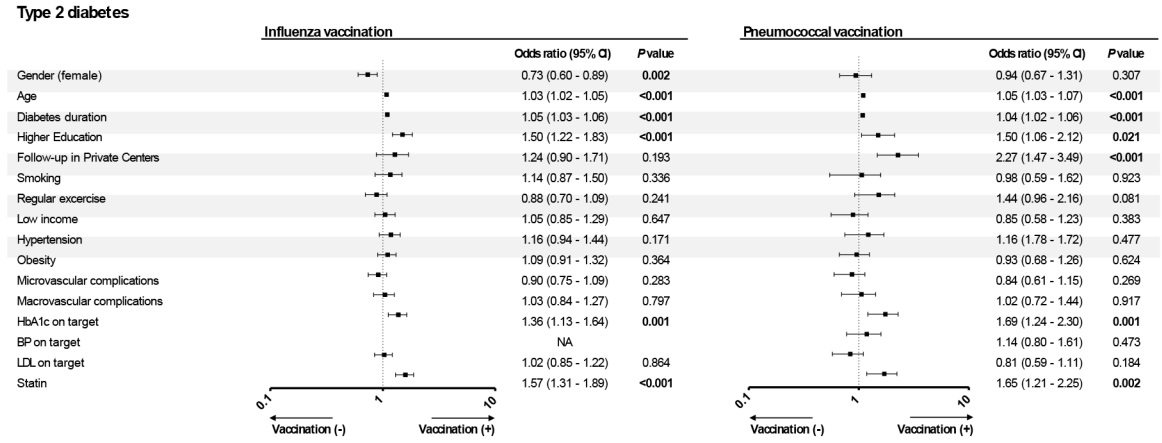

Figure 2 Multivariable logistic regression analysis of vaccination among patients with type 2 diabetes mellitus (dependent variable: vaccination of influenza and pneumococcus). HbA1c: Glycosylated hemoglobin A1c; BP: Blood pressure; LDL: Low density lipoprotein cholesterol; CI: Confidence interval; OR: Odds ratio.

treatment (OR: 1.65, 95%CI: 1.21-2.25) (Figure 2).

The comparison of the vaccination rates in age specific subgroups

The rates of vaccination were higher in patients with T2DM who were over 65 years old for both influenza (36.4% *vs* 23.2%, $P < 0.001$) and pneumococcus (27.3% *vs* 7.4%, $P < 0.001$). (Figure 3) We did not compare the vaccination rates in patients with T1DM since there were few patients over 65 years in this group.

DISCUSSION

The results of the present study showed that Turkish patients with diabetes had very low vaccination rates for influenza and pneumococcus infections. There were no significant differences between the vaccination rates in patients with T1DM and T2DM and the influenza vaccination was much more common in both types. Longer diabetes duration and older age were the common independent associates of having vaccination for both types of patients. On the other hand, higher education levels, having optimal glycemic control, and using statins were the common independent associates of vaccination in patients with T2DM. Regarding the growing awareness of the importance of vaccination, the implications of these findings are discussed below.

Prevention of influenza and pneumococcal infections with routine vaccination decreased mortality and morbidity and the hospitalization rates in patients with diabetes[5,6]. The importance of having vaccinations against influenza and pneumococcus has become more evident during the outbreak of coronavirus disease 2019. Several studies show that influenza and pneumococcal vaccinations may protect against symptomatic cases of infection and death by cross-reactivity with severe acute

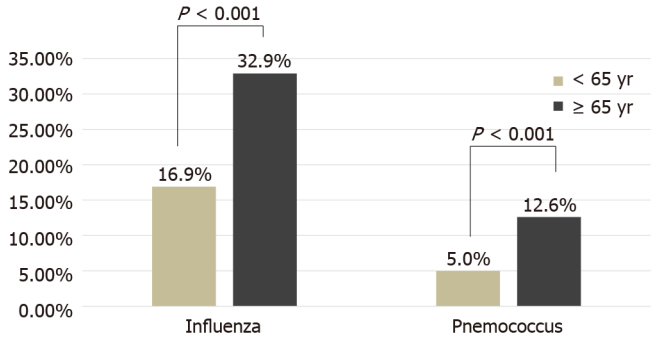

Figure 3 The comparison of the vaccination rates in patients with type 2 diabetes mellitus (according to age).

respiratory syndrome coronavirus 2 antigens[20,21]. On the other hand, the prevention of influenza and pneumococcal infections will help to reduce diagnostic dilemmas, and inappropriate management in terms of antiviral therapy and infection control[22]. National and international guidelines strongly recommend routine vaccinations of influenza and pneumococcal pneumonia in patients with diabetes[7,9]. But the overall vaccination rates are not at the desired levels in many countries. Studies in different populations reported influenza vaccination rates between 40% and 60%[12,23-25]. In terms of pneumococcal vaccine, the situation is slightly worse, and the rate of vaccination in various countries varies between 18% and 53% in patients with diabetes [26-28]. Moreover, there are various reasons for the lower vaccination rates in studies. The most common reason for low vaccination rates in these studies seems to be the low vaccination awareness of both patients and healthcare providers. To produce effective nationwide vaccination policies, it is necessary to know the regional factors that resulted in low vaccination rates.

TEMD Vaccination study showed that the vaccination rates in Turkey are much lower than those of the other countries in patients with T1DM and T2DM. These findings are in line with the results of a previous report about the vaccination rates in Turkish patients with diabetes, which mentioned the rate of receiving influenza and pneumococcus vaccinations in patients with T2DM as 27% and 9.8%, respectively[15]. Another previous single-center study reported the vaccination rates for influenza and pneumococcus as 14.6% and 3.8% in patients with diabetes[16]. Considering that the patients enrolled in the current study were followed up in tertiary endocrine or diabetes units, it is seen that the current vaccination rates are quite low and there is no significant increase in these rates compared to previous studies. In this regard, the low vaccination rates even in this patient group, which includes patients with significantly complicated and advanced diabetic ages, suggest that healthcare givers do not pay sufficient attention to vaccination even in the tertiary care centers for diabetes.

TEMD Vaccination Study also gives us data about the sociodemographical characteristics of patients vaccinated for influenza and pneumococcal infections. Especially the older age and longer diabetes duration were common determinants in patients with both T1DM and T2DM. Patients with a longer duration of diabetes are likely to be more complicated, and therefore, they are likely to visit health centers more frequently and may have a higher chance to get advice for vaccination from healthcare professionals. In this regard, a Spanish study showed that physician visits increase the probability of receiving the vaccination[29]. The multivariable analysis also showed that age had a significant impact on vaccination rates of patients with diabetes. The rates of vaccination for influenza and pneumococcus more than doubled in patients with T2DM over 65 years. The effect of age on vaccination was also reported in many other studies of the diabetes population[10-12]. The reason why age was an important factor for receiving vaccination in many studies may be that patients of older ages are more complicated, have a higher number of comorbidities, and therefore, apply to health centers more frequently. Another reason may be that in many countries, including Turkey, influenza and pneumococcal vaccines are recommended routinely for older ages, regardless of diabetes[30-32].

Being followed up in private centers appear to be another important factor for the higher vaccination rates of patients with diabetes. The reason for this association may be that healthcare providers working in private centers can devote more time to patients and thus may be much more concerned about preventive measures such as recommending vaccination. In this regard, inadequate knowledge provided by the healthcare professionals to patients and/or the clinical inertia of physicians to prevent

diseases has been shown as the main obstacles to increasing vaccination rates[33]. In patients with T2DM, optimal glycemic control was also associated with receiving influenza and pneumococcal vaccination. Similarly, higher education level was also a determinant of receiving influenza and pneumococcal vaccination in these patients. In our previous study, high education level was found to be an important predictor for optimal glycemic control[17]. The higher rates of vaccination in educated patients with better glycemic regulation can be explained by the better self-care and higher demand for preventive health measures in these patients. Overall, when the parameters determining vaccination in both types of diabetes are evaluated together, it is seen that especially young patients and patients with shorter diabetes duration are less likely to be vaccinated.

There may be several limitations of the TEMD Vaccination Study. First of all, the cross-sectional design of the study may preclude the casual relationship between patients' characteristics and receiving influenza and pneumococcal vaccination. Additionally, there may be a possibility of selection bias in the present study. Patients enrolled in this study are under follow-up in the tertiary endocrine or diabetes units, therefore, they are more likely to have multiple comorbidities and complications. For this reason, the study population may not reflect entirely the general population with diabetes. Also, the design of the current study does not include the beliefs and attitudes of physicians about vaccination of patients. However, the large study population, multicenter design, and the presentation of the results for patients with T1DM and T2DM in separate are the strengths of the present study.

CONCLUSION

In conclusion, the findings of the TEMD Vaccination study indicate that Turkish patients with diabetes have very low influenza and pneumococcal vaccination rates. Considering that this study was conducted in tertiary endocrine or diabetes units, the physicians focused only on the treatment of the disease, and consequently, stay away from preventive medicine. The lower rates of vaccination in some special populations, such as younger patients and patients with short duration of diabetes, suggest that specific vaccination strategies should be developed for these populations. Finally, the TEMD study showed that not only metabolic control but also preventive measures are not sufficient enough for people with diabetes living in Turkey.

In conclusion, this study identified demographic and clinical factors related to low influenza and pneumococcus vaccination rates among the adult population with diabetes. Regarding that this nationwide survey was held in tertiary endocrine or diabetes centers, it is highly likely that the rates of vaccination could be much lower in the overall country. These results should mandate urgent measures to increase vaccination rates including the efforts to improve health awareness in patients and prevent inertia in physicians caring for patients with diabetes.

ARTICLE HIGHLIGHTS

Research background

The prevalence of diabetes is increasing worldwide, and increased diabetes frequency means an increase in the incidence of diabetes-related mortality and morbidity. Turkey stands as the country with the highest diabetes mellitus prevalence in Europe. Since commonly seen infections are associated with significantly increased morbidity and mortality, vaccination programs are now among the standard of care for diabetes mellitus. Vaccination for influenza and pneumococcal infections has gained broad acceptance worldwide.

Research motivation

Although current guidelines emphasize the importance of influenza and pneumococcal vaccination in diabetic patients and that physician acceptance has been reported to increase, the reported vaccination rates still remain low in many countries with different economic development. The rates of vaccination in patients with diabetes mellitus in Turkey have not been systematically evaluated so far.

Research objectives

The main objective of the current study was to perform a nationwide survey to explore the vaccination status for two major diseases, pneumococcus and influenza, among patients with diabetes mellitus. The secondary objective was to determine which patients tend to get vaccinated or not vaccinated.

Research methods

In a multicenter, cross-sectional survey design, the TEMD Vaccination Study enrolled 454 patients with type 1 diabetes mellitus (T1DM) and 4721 patients with type 2 diabetes mellitus (T2DM), who were under followed up in 68 tertiary endocrinology clinics. Vaccination status was assessed by self-reports and medical records.

Research results

The study found 23.6% and 8% vaccination rates for influenza and pneumococcus, respectively, in patients with T1DM. The rates were 21.2% and 8% in patients with T2DM. Vaccination for both conditions was recorded in 6.6% of patients with T1DM and 5.8% of patients with T2DM. Older age and longer diabetes duration were the most common associates of vaccination for both types of diabetes. Among patients with T2DM, higher education level, statin use, and lower HbA1c level were also independently associated with higher vaccination status.

Research conclusions

This study showed for the first time that patients with T1DM and T2DM had very low influenza and pneumococcal vaccination rates in Turkey. The findings warrant new and improved strategies to increase the awareness of vaccination among the partners involved in different levels of diabetes care, from patients to policymakers and healthcare professionals.

Research perspectives

As vaccination programs are cost-saving by reducing diabetes-related mortality and morbidity, there is an unmet need to identify the barriers and obstacles against the acceptance of vaccination programs by the patients and healthcare programs in this population. Additionally, potential difficulties in implementing the vaccination programs at the system level need to be identified. Finally, increasing the number of patients with diabetes mellitus who are vaccinated should be prioritized as these patients are considered much defenseless against opportunistic infections.

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ORIGINAL ARTICLE

Observational Study

Skeletal muscle loss is associated with diabetes in middle-aged and older Chinese men without non-alcoholic fatty liver disease

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Author contributions: Chen LY did the study design and literature research, wrote the manuscript, and made critical editing; Chen LY and Lin HD made definition of intellectual content and did data analysis; Chen LY, Xia MF, Wu L, Li Q, Hu Y, Ma H, Gao X, and Lin HD participated in the data acquisition and clinical study; Gao X and Lin HD contributed to study concept and did the critical review and editing of the manuscript; Gao X was the guarantor of integrity of the entire study; Lin HD contributed to statistical analysis; all authors approved the final draft of the manuscript and agreed to submit it for publication.

Institutional review board statement: The present study was approved by the Ethics Committee of Zhongshan Hospital of Fudan University (No. 2008-119). The research was carried out in accordance with the World Medical Association Declaration of Helsinki.

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Abstract

BACKGROUND

Skeletal muscle, a key insulin target organ, has been reported to be associated with diabetes mellitus (DM). Compared to non-diabetic patients, diabetic patients have decreased muscle mass and a higher prevalence of sarcopenia, and patients with sarcopenia may be at increased risk of developing diabetes. In individuals with nonalcoholic fatty liver disease (NAFLD), sarcopenia is associated with the severity of fibrosis and steatosis. Previous studies have demonstrated that NAFLD is strongly associated with DM and sarcopenia.

AIM

To determine the relationship between skeletal muscle mass and DM in Chinese middle-aged and elderly men, and whether the association is affected by NAFLD.

METHODS

Skeletal muscle mass was calculated as appendicular skeletal muscle mass (ASM) in kg/body weight × 100%. Liver fat content (LFC) was measured using a quantitative ultrasound method.

RESULTS

As the ASM decreased, fasting blood glucose (FBG), 2-h postprandial blood glucose (2hBG), and LFC increased in both genders, as did the prevalence of DM and NAFLD. Spearman analysis showed that the ASM was negatively correlated with the FBG, 2hBG, and LFC. Stepwise logistic regression analysis showed that after adjustments, the ASM quartile was negatively associated with the presence of DM in males, but not in females. Subgroup analysis showed that the ASM

Informed consent statement: All participants provided written informed consent before their inclusion in the study.

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quartiles remained negatively correlated with the presence of DM in the non-NAFLD population (including males and females), but no correlation was found between ASM quartiles and the presence of DM in the NAFLD population. When stratified by LFC quartiles, ASM was negatively correlated with the presence of DM in the first and second LFC quartiles in males.

CONCLUSION

Skeletal muscle mass loss was shown to be associated with the presence of DM in males, but not in females; NAFLD weakens this association. The results suggested that the stratified management of diabetes mellitus should be considered according to skeletal muscle mass and NAFLD.

Key Words: Diabetes mellitus; Liver fat content; Non-alcoholic fatty liver disease; Skeletal muscle mass

Core Tip: Aging is becoming more severe in China. The present study showed that decreased skeletal muscle mass is associated with the presence of diabetes mellitus in males but not in females; non-alcoholic fatty liver disease weakens this association. The results suggested that stratified management of diabetes mellitus should be considered according to skeletal muscle mass and non-alcoholic fatty liver disease.

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INTRODUCTION

The progressive decrease in skeletal muscle mass, strength, and function is known as sarcopenia. Sarcopenia significantly increases with age[1]. With the growth of the aging society, sarcopenia has become a major focus of worldwide public health research and public policy[2]. Skeletal muscle loss reduces mobility in the elderly and increases the risk of fractures and falls[3,4]. In addition, skeletal muscle loss is closely related to metabolic disorders, tumors, and other chronic diseases[5-8]. As the largest non-fat component of the human body, skeletal muscle is responsible for 80% of postprandial glucose disposition[9]. As an important insulin target organ for glucose uptake and utilization, skeletal muscle loss leads to a systemic metabolic disorder, which is closely related to diabetes mellitus (DM)[9,10]. Compared to non-diabetics, patients with DM have lower muscle mass and a high prevalence of sarcopenia[11-13]. Conversely, reduced skeletal muscle mass may also increase the risk of DM[14].

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease caused by abnormal accumulation of fat in the liver. Previous studies have shown that NAFLD often coexists with the occurrence and progression of type 2 DM or is associated with an increased risk of type 2 DM[15]. A meta-analysis showed that 28%-70% of type 2 DM patients have NALFD[16]. Another summarization of data concluded that 22.5% of NAFLD patients have type 2 DM[17]. Taken together, the above findings suggest that interactions exist between NAFLD and type 2 DM. Because skeletal muscle mass loss may also increase the risk of DM[14], skeletal muscle may indirectly affect the development of NAFLD. Indeed, previous studies have shown that age-related skeletal muscle mass reduction is associated with NAFLD[18,19]. Other studies have reported that sarcopenia is associated with the severity of fibrosis and steatosis independent of inflammation, insulin resistance, and obesity in patients with NAFLD and metabolic disorders[20,21]. Although large population studies are needed to assess the impact of interactions between sarcopenia, DM, and NAFLD progression, no such research has been conducted to determine the relationship between sarcopenia, DM, and NAFLD progression in a Chinese community population. In the

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present study, we recruited participants ≥ 45 years of age from Changfeng Community in Shanghai to conduct a large-scale community population study to determine the association between skeletal muscle mass (SMM), DM, and NAFLD, and to provide new evidence for the prevention and treatment of NAFLD and DM.

MATERIALS AND METHODS

Participants

A total of 5626 residents ≥ 45 years of age were enrolled from Changfeng community in Shanghai between May 2010 and December 2012 according to the Shanghai Changfeng Study which has been reported elsewhere[22]. The inclusion criteria were as follows: (1) The subjects were 45 years of age and older; (2) people with autonomous capacity were able to cooperate with the research; and (3) without acute diseases such as myocardial infarction, acute stroke, and acute infection. Participants meeting the following criteria were excluded: (1) Lacking biochemical and liver fat content (LFC) data; (2) lacking dual energy X-ray absorptiometry (DXA) data; and (3) viral hepatitis and excessive alcohol consumption. Following application of the inclusion and exclusion criteria, 3969 subjects were included in the study (1370 males and 2599 females).

The details of the research were explained to all participants and written informed consent was obtained from all of them. The study was approved by the Ethics Committee of Zhongshan Hospital of Fudan University (No. 2008-119).

Data collection

All participants were interviewed and the medical histories were recorded by trained researchers using a standard questionnaire. Then, standing height and body weight were measured without shoes and outer clothing. The body mass index (BMI) was calculated as the weight in kg divided by the height in m squared (kg/m^2). Resting blood pressure (BP), including systolic BP (SBP) and diastolic BP (DBP), were measured three times with an electronic BP monitor (Omron Model HEM-752 FUZZY; Omron Co., Dalian, China) and the average data were calculated.

Blood samples were collected after overnight fasting for at least 10 h. Biochemical indices, including fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), and high-density lipoprotein-cholesterol (HDL-C), were measured with an automated bio-analyzer (Hitachi 7600; Tokyo, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation. The 2-h postprandial blood glucose (2hBG) level was determined following a 75-g oral glucose load for non-diabetics or a 100-g steamed bread meal for patients diagnosed with DM. An electrochemiluminescence immunoassay was used to measure the serum insulin concentration. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated by multiplying the FBG (mmol/L) times fasting insulin (mU/L) and dividing by 22.5.

Hepatic ultrasonography scanning was performed by an experienced technician using a GE Logiq P5 scanner (GE Healthcare, Milwaukee, WI, United States) with a 4-MHz probe. The liver ultrasound images were analyzed with ImageJ 1.41o (National Institutes of Health, Bethesda, MD, United States) and standardized using a tissue-mimicking phantom (Model 057; Computerized Imaging Reference Systems, Norfolk, VA, United States). The participants' details were blinded to the technician. LFC was measured according to the method described in detail elsewhere[23]. The LFC was calculated using the following equation: LFC (%) = 62.592 × standardized US hepatic/renal ratio + 168.076 × standardized US hepatic attenuation rate - 27.863.

Body composition, including lean mass and fat mass (FM), was measured using DXA (Lunar iDXA; GE Healthcare). All measurements were carried out by a single, trained technician at a single clinical center. Manual DXA analysis software was used to analyze all DXA scans. The FM percentage (FM%) was calculated by dividing FM by total body mass. The SMM was calculated as weight adjusted by the appendicular skeletal muscle mass (ASM) [ASM% = ASM (kg)/body weight (kg) × 100%][20].

Definitions

Obesity was defined as a BMI ≥ 28 kg/m^2 according to Chinese criteria[24]. DM was defined as a FBG ≥ 7.0 mmol/L or a 2hBG ≥ 11.1 mmol/L based on oral glucose tolerance test by the 1999 WHO criteria[25], or self-reported current hypoglycemic treatment. NAFLD was diagnosed when the LFC exceeded a cut-off value of 9.15% by ultrasonography, excluding excessive alcoholic intake and virus hepatitis[23].

Statistical analysis

All statistical analyses were performed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL, United States). Continuous data are presented as the mean ± SD except for skewed variables, which are presented as the median with the inter-quartile range in parentheses (25%-75%). All subjects were divided into four groups according to gender-specific quartiles of ASM% as follows: Males (Q1, ≥ 32.0%; Q2, ≥ 30.5%-< 32.0%; Q3, ≥29.0%-< 30.5%; and Q4: < 29.0%); females (Q1, ≥ 26.8%; Q2, ≥25.5%-< 26.8%; Q3, ≥ 24.3%-< 25.5%; and Q4, < 24.3%). Analysis of variance or the Kruskal-Wallis test was used for inter-group comparisons of continuous data, whereas the chi-squared test was used for comparisons of categorical variables. The Spearman analysis was performed to assess the relationships between the ASM% and blood glucose concentration, as well as other clinical parameters. Multivariate logistic regression analyses were performed to determine the association of ASM% quartiles with DM after adjusting for age, smoking, DM family history, FM, interaction between FM and ASM% quartiles, obesity, BP, serum TG, HDL-C, and HOMA-IR (in order). The interaction between ASM% and FM was included in the multiple regression models because there were significant correlations between ASM%, FM, and blood glucose concentration. To further determine whether NAFLD affects the relationship between SMM and DM, subgroup analysis was performed based on the presence of NAFLD and LFC quartiles. *P*-values < 0.05 were considered statistically significant.

RESULTS

Characteristics of subjects

A total of 3969 subjects were included; the mean age was 63.3 years and the mean BMI was 24.1 kg/m^2. The characteristics of the subjects are shown in Table 1. All subjects with a lower ASM% were older and had a higher body weight and BP (specifically, a higher BMI, FM, FM%, SBP, and DBP). The lipid disorders were aggravated in subjects with a lower ASM%, who had higher TC, TG, and LDL-C concentrations, and a lower HDL-C concentration. The most noteworthy findings were that the FBG, 2hBG, HOMA-IR, and LFC levels increased gradually, as well as the prevalence of DM and NAFLD, with ASM% decreasing in both male and female participants (P < 0.001).

Effects of SMM on glucose metabolism and other metabolic parameters

The Spearman analysis showed that in addition to age, body composition, and metabolic parameters, including BMI, FM, FM%, TG, LDL-C, SBP, and DBP, the ASM% was negatively correlated with the FBG, 2hBG, HOMA-IR, and LFC levels (P < 0.001; Table 2).

To further determine whether a low ASM% was associated with the presence of DM, we performed logistic stepwise regression analysis with ASM% quartiles as independent variates and the presence of DM as a dependent variate. As shown in Table 3, a crude analysis showed that the odds ratios (ORs) for DM were 0.665 [95% confidence interval (CI): 0.592-0.746) in males and 0.775 (95%CI: 0.710-0.840) in females. The relationship remained significant in males after adjusting for age, smoking, family history of DM, FM, FM × ASM%, obesity, SBP, TG, HDL-C, and HOMA-IR in order (OR = 0.537; 95%CI: 0.312-0.923), but the association was not apparent in females (OR = 0.985; 95%CI: 0.614-1.580; Table 3).

Effect of NAFLD on relationship between SMM and DM

NAFLD increases the prevalence and risk of type 2 DM[15]. Indeed, we showed that the ASM% was negatively associated with LFC. Thus, we performed logistic analysis to determine the effect of NAFLD on the relationship between SMM and the presence of DM, as shown in Table 4. Among the 2658 non-NAFLD participants, the ASM% quartile was negatively correlated with the presence of DM in males and females before adjustment. After multiple adjustments, the negative association remained significant in males (OR = 0.330; 95%CI: 0.157-0.694) but not in females (OR = 0.800; 95%CI: 0.416-1.537). Among the 1311 NAFLD patients, the correlation between the ASM% and DM was absent after adjustments in both genders.

Because NAFLD was diagnosed by LFC in the present study, LFC was displayed as a continuous variable. We further stratified the population by LFC quartiles from low to high. In the first three LFC quartiles in males and the first two LFC quartiles in females before adjustments, the ASM% quartiles were negatively correlated with the presence of DM. The relationship remained significant in the first and second quartiles

Table 1 Characteristics of male participants according to appendicular skeletal muscle mass (%) quartiles, *n* (%)

Male	Quartiles of ASM%				*P* value	Female	Quartiles of ASM%				*P* value
	Q1	Q2	Q3	Q4			Q1	Q2	Q3	Q4	
	n = 343	*n* = 343	*n* = 343	*n* = 342			*n* = 649	*n* = 651	*n* = 649	*n* = 650	
Age (yr)	61.6 ± 8.8	63.2 ± 8.9[a]	63.2 ± 8.9[a]	69.4 ± 10.1[a]	< 0.001	Age, yr	60.8 ± 8.7	61.7 ± 8.8[a]	62.9 ± 9.4[a]	64.8 ± 9.7[a]	< 0.001
BMI (kg/m^2)	22.7 ± 2.9	24.3 ± 3.4[a]	24.3 ± 3.4[a]	25.6 ± 2.9[a]	< 0.001	Weight, kg	55.1 ± 8.1	58.0 ± 8.3[a]	59.8 ± 8.6[a]	62.6 ± 9.7[a]	< 0.001
WC, cm	80.6 ± 9.1	85.8 ± 7.7[a]	85.8 ± 7.7[a]	91.7 ± 8.5[a]	< 0.001	BMI, kg/m^2	22.0 ± 2.7	23.5 ± 2.9[a]	24.4 ± 3.1[a]	26.0 ± 3.7[a]	< 0.001
FM, kg	17.9 ± 4.6	20.3 ± 4.3[a]	20.3 ± 4.3[a]	24.5 ± 4.9[a]	< 0.001	WC, cm	76.4 ± 7.9	80.5 ± 8.6[a]	82.8 ± 8.5[a]	87.5 ± 9.4[a]	< 0.001
FM%	22.4 ± 4.2	27.2 ± 3.2[a]	27.2 ± 3.2[a]	33.1 ± 3.3[a]	< 0.001	FM, kg	20.0 ± 4.1	22.5 ± 4.2[a]	24.8 ± 4.8[a]	27.6 ± 5.8[a]	< 0.001
ASM%	32.6 ± 1.9	30.6 ± 1.0[a]	30.6 ± 1.0[a]	27.2 ± 1.6[a]	< 0.001	FM%	32.2 ± 3.9	36.3 ± 2.9[a]	38.8 ± 3.0[a]	42.4 ± 3.4[a]	< 0.001
SBP, mmHg	129 ± 18	134 ± 17[a]	134 ± 17[a]	141 ± 19[a]	< 0.001	ASM%	27.7 ± 2.0	25.7 ± 0.8[a]	24.5 ± 0.6[a]	22.8 ± 1.1[a]	< 0.001
DBP, mmHg	76 ± 11	78 ± 11[a]	78 ± 11[a]	77 ± 10	0.014	SBP, mmHg	129 ± 20	132 ± 19[a]	135 ± 20[a]	138 ± 19[a]	< 0.001
FBG, mmol/L	5.4 ± 1.4	5.7 ± 1.7[a]	5.7 ± 1.7[a]	6.0 ± 1.7[a]	< 0.001	DBP, mmHg	72 ± 10	74 ± 10[a]	75 ± 9	76 ± 10[a]	< 0.001
2hBG, mmol/L	6.7 ± 2.5	7.6 ± 2.9[a]	7.6 ± 2.9[a]	8.9 ± 3.7[a]	< 0.001	FBG, mmol/L	5.3 ± 1.0	5.4 ± 1.2[a]	5.5 ± 1.3[a]	5.7 ± 1.7[a]	< 0.001
HOMA-IR	1.2 (0.8-1.9)	1.8 (1.2-2.5)[a]	1.8 (1.2-2.5)[a]	2.5 (1.7-3.9)[a]	< 0.001	2hBG, mmol/L	6.8 ± 2.6	7.2 ± 2.5[a]	7.7 ± 4.0[a]	8.3 ± 3.4[a]	< 0.001
TG, mmol/L	1.2 (0.9-1.8)	1.4 (1.0-2.0)[a]	1.4 (1.0-2.0)[a]	1.6 (1.2-2.2)[a]	< 0.001	HOMA-IR	1.6 (1.0-2.3)	1.8 (1.2-2.6)[a]	2.1 (1.4-3.2)[a]	2.3 (1.6-3.6)[a]	< 0.001
TC, mmol/L	4.6 ± 0.8	4.7 ± 0.8[a]	4.7 ± 0.8[a]	4.7 ± 0.8[a]	0.049	TG, mmol/L	1.2 (0.9-1.8)	1.4 (1.0-2.0)[a]	1.5 (1.1-2.1)	1.6 (1.2-2.2)[a]	< 0.001
HDL-C, mmol/L	1.40 ± 0.37	1.26 ± 0.29[a]	1.26 ± 0.29[a]	1.20 ± 0.25[a]	< 0.001	TC, mmol/L	5.2 ± 0.8	5.3 ± 0.9	5.3 ± 0.9	5.4 ± 1.0[a]	< 0.001
LDL-C, mmol/L	2.59 ± 0.72	2.75 ± 0.74	2.75 ± 0.74	2.72 ± 0.72[a]	0.004	HDL-C, mmol/L	1.64 ± 0.42	1.54 ± 0.37[a]	1.48 ± 0.34	1.46 ± 0.33[a]	< 0.001
LFC%	3.9 (1.9-7.8)	5.0 (2.3-10.8)[a]	5.0 (2.3-10.8)[a]	6.3 (2.6-13.3)[a]	< 0.001	LDL-C, mmol/L	2.86 ± 0.75	3.02 ± 0.79[a]	3.04 ± 0.83[a]	3.14 ± 0.86[a]	< 0.001
NAFLD *n* (%)	68 (19.9)	107 (31.2)[a]	107 (31.2)[a]	141 (41.2)[a]	< 0.001	LFC, %	4.8 (2.5-9.7)	5.8 (2.7-11.6)[a]	5.9 (2.7-11.8)[a]	7.1 (3.2-13.6)[a]	< 0.001
DM *n* (%)	42 (12.3)	81 (23.6)[a]	81 (23.6)[a]	123 (35.8)[a]	< 0.001	NAFLD *n* (%)	171 (26.3)	214 (32.9)[a]	219 (33.7)[a]	275 (42.3)[a]	< 0.001
						DM *n* (%)	88 (13.6)	106 (16.3)	152 (23.4)[a]	158 (24.3)[a]	< 0.001

[a]*P* < 0.05, compared with Q1. The quartiles of ASM% were divided as follows: Male, (Q1, ≥ 32.0%; Q2, ≥ 30.5%-32.0%; Q3, ≥ 29.0%-< 30.5%; Q4: < 29.0%) and female, (Q1, ≥ 26.8%; Q2, ≥ 25.5% -< 26.8%; Q3, ≥ 24.3%-< 25.5%; Q4, < 24.3%).

BMI: Body mass index; WC: Waist circumference; FM: Fat mass; ASM: Appendicular skeletal muscle mass; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; 2hBG: 2-h postprandial blood glucose; HOMA-IR: Homeostasis model assessment for insulin resistance; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol; LFC: Liver fat content; NAFLD: Non-alcoholic fatty liver disease; DM: Diabetes mellitus.

in males after adjustment; however, the correlation no longer existed after adjustments in the third and fourth quartiles in males and in all quartiles in females (Table 5).

DISCUSSION

Several studies have determined the relationship between SMM and DM. Specifically, SMM loss increases the risk of DM in the middle-aged and elderly[10-14]. A cohort study conducted by Korean researchers also showed that a lower ASM% increases the risk of DM, even in the young and middle-aged[26]. The results of our study not only confirmed these findings, but also showed a gender difference in the relationship between muscle loss and DM. In the current study, the FBG, 2hBG, and HOMA-IR increased with the prevalence of DM while ASM% decreased in male and female participants. The SMM, as measured by ASM%, was negatively associated with the

Table 2 Spearman analysis of appendicular skeletal muscle mass (%) and other clinical parameters

	ASM%	
	Male (*r*, *P* value)	Female (*r*, *P* value)
Age (years)	-0.317, < 0.001	-0.164, < 0.001
BMI (kg/m$^{2)}$	-0.347, < 0.001	-0.441, < 0.001
WC (cm)	-0.452, < 0.001	-0.448, < 0.001
FM (kg)	-0.605, < 0.001	-0.590, < 0.001
FM (%)	-0.792, < 0.001	-0.799, < 0.001
FBG (mmol/L)	-0.177, < 0.001	-0.106, < 0.001
2hBG (mmol/L)	-0.254, < 0.001	-0.201,< 0.001
HOMA-IR	-0.385, < 0.001	-0.264, < 0.001
TG (mmol/L)	-0.198, < 0.001	-0.193, < 0.001
TC (mmol/L)	-0.049, 0.071	-0.087, < 0.001
HDL-C (mmol/L)	0.213, < 0.001	0.162, < 0.001
LDL-C (mmol/L)	-0.075, 0.006	-0.119, < 0.001
SBP (mm Hg)	-0.244, < 0.001	-0.192, < 0.001
DBP (mm Hg)	-0.075, 0.006	-0.139, < 0.001
LFC (%)	-0.149, < 0.001	-0.113, < 0.001

ASM: Appendicular skeletal muscle mass; BMI: Body mass index; WC: Waist circumference; FM: Fat mass; FBG: Fasting blood glucose; 2hBG: 2-h postprandial blood glucose; HOMA-IR: Homeostasis model assessment for insulin resistance; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LFC: Liver fat content.

Table 3 Multivariate-adjusted associations of appendicular skeletal muscle mass (%) quartiles with diabetes mellitus

	Male	Female
	OR (95%CI, *P* value)	OR (95%CI, *P* value)
Unadjusted	0.665	0.775
	(0.592-0.746, < 0.001)	(0.710-0.847, < 0.001)
Model 1	0.527	0.505
	(0.336-0.826, 0.005)	(0.342-0.745, 0.001)
Model 2	0.640	0.728
	(0.401-1.020, 0.051)	(0.481-1.101, 0.133)
Model 3	0.537	0.985
	(0.312-0.923, 0.024)	(0.614-1.580, 0.950)

Model 1: Adjusted for age, cigarette smoking, diabetes family history, FM, and FM × ASM% quartiles, and obesity. Model 2: Adjusted for covariates in Model 1 plus SBP, TG, and HDL-C. Model 3: Adjusted for covariates in Model 3 plus HOMA-I. OR: Odds ratio; FM: fat mass; ASM: Appendicular skeletal muscle mass; SBP: Systolic blood pressure; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance.

blood glucose concentration, but logistic stepwise regression analysis showed that SMM loss may be associated with the presence of DM in males. The dissociation of SMM loss and DM in women is noteworthy, especially after adjustment by FM and lipid parameters. The reason underlying this interesting phenomenon is not apparent; however, one reason may be that the subjects in the present study were elderly. A previous study showed that as age increases, the body fat percentage gradually increases, which is more pronounced in older women[26]. Another possible

Table 4 Multivariate-adjusted associations of appendicular skeletal muscle mass (%) quartiles with diabetes mellitus in participants with or without non-alcoholic fatty liver disease

	Male	Female
	OR (95%CI, *P* value)	OR (95%CI, *P* value)
	Non-NAFLD (*n* = 2658)	
Unadjusted	0.635	0.770
	(0.548-0.736, < 0.001)	(0.682-0.870, < 0.001)
Model 1	0.403	0.581
	(0.221-0.735, 0.003)	(0.344-0.981, 0.042)
Model 2	0.455	0.807
	(0.246-0.842, 0.012)	(0.463-1.409, 0.452)
Model 3	0.330	0.800
	(0.157-0.694, 0.003)	(0.416-1.537, 0.503)
	NAFLD (*n* = 1311)	
Unadjusted	0.789	0.845
	(0.650-0.956, 0.016)	(0.739-0.966, 0.014)
Model 1	1.259	0.508
	(0.542-2.924, 0.592)	(0.265-0.975, 0.042)
Model 2	1.954	0.710
	(0.788-4.851, 0.148)	(0.355-1.418, 0.332)
Model 3	1.328	1.106
	(0.435-4.055, 0.619)	(0.485-2.523, 0.810)

Model 1: Adjusted for age, cigarette smoking, diabetes family history, FM, FM × ASM% quartiles, and obesity. Model 2: Adjusted for covariates in Model 1 plus SBP, TG, and HDL-C. Model 3: Adjusted for covariates in Model 3 plus HOMA-IR. OR: Odds ratio; FM: Fat mass; ASM: Appendicular skeletal muscle mass; SBP: Systolic blood pressure; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance.

explanation is the difference in body fat distribution between genders. As reported, age and gender are important factors influencing plasma lipid levels, such as TC, LDL-C, and HDL-C, and females are more likely to have insulin resistance and lipid disorders than males as age increases[27]. Although the body fat percentage of females is higher than age-matched males, and the accumulation of intra- and inter-muscular fat is more significant in females than in males as age increases[28], females have more type I muscle fibers than males, which contributes to stronger oxidative function in skeletal muscle[29]. In addition, hormones, especially estrogen, can influence TG and free fatty acid metabolism[30]. The estrogen decreases with aging, especially in postmenopausal women, which may result in a TG reduction, and is associated with a reduced risk of DM[31]. In addition, in the process of aging, the decline in SMM is more remarkable in males than in females, which also contributes to the more significant effect of SMM loss on the risk of DM in males[32]. This physiologic differences of females from males might neutralize the effect of SMM reduction on DM, and thus reduce its association with the presence of DM. The results of our study suggested that gender-stratified management of DM according to the SMM should be considered. Indeed, increased SMM might have a more beneficial effect on improving glucose metabolism in males.

NAFLD is an important risk factor for DM[15-17], and several previous studies have demonstrated that low SMM is also closely associated with NAFLD[18-21]. In the present study, the SMM was negatively associated with LFC, which was in agreement with previous results[18,19]. Whether LFC influences the relationship between sarcopenia and DM is unknown. As an important risk factor for DM, excessive liver fat accumulation could lead to insulin resistance, mitochondrial dysfunction, and hyperlipidemia[33,34]. Reducing the LFC may be more important with respect to improving DM in patients with NAFLD[15-17]. Our results showed that a relationship

Table 5 Multivariate-adjusted associations of appendicular skeletal muscle mass (%) quartiles with diabetes mellitus in participants with different liver fat content, *n* (%)

LFC	DM	OR (95%CI, *P* value)	OR (95%CI, *P* value)
Male		Unadjusted	After adjusted
Q1 (*n* = 389)	82 (21.1)	0.635 (0.504-0.799, < 0.001)	0.308 (0.102-0.932, 0.032)
Q2 (*n* = 350)	66 (18.8)	0.540 (0.416-0.700, < 0.001)	0.184 (0.049-0.685, 0.012)
Q3 (*n* = 306)	74 (24.2)	0.760 (0.599-0.963, 0.023)	0.678 (0.158-2.908, 0.601)
Q4(*n* = 325)	116 (35.7)	0.830 (0.665-1.035, 0.099)	1.561 (0.440-5.539, 0.491)
Female			
Q1 (*n* = 605)	84 (13.9)	0.828 (0.672-1.021, 0.077)	0.964 (0.311-2.990, 0.949)
Q2 (*n* = 642)	92 (14.3)	0.745 (0.609-0.913, 0.004)	0.517 (0.170-1.578, 0.247)
Q3 (*n* = 685)	118 (17.2)	0.739 (0.615-1.887, 0.091)	0.750 (0.274-2.048, 0.574)
Q4 (*n* = 667)	210 (31.5)	0.871 (0.750-1.011, 0.070)	1.122 (0.445-2.830, 0.808)

The quartiles of LFC were divided as follows: Q1: < 2.53%; Q2: ≥ 2.53%-< 5.52%; Q3: ≥ 5.52%- < 11.61%; Q4: ≥ 11.61%. Adjusted factors: Age, cigarette smoking, diabetes family history, FM, FM × ASM% quartiles, obesity, SBP, TG, HDL-C, and HOMA-IR. LFC: Liver fat content; DM: Diabetes mellitus; OR: Odds ratio; FM: Fat mass; ASM: Appendicular skeletal muscle mass; SBP: Systolic blood pressure; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance.

between SMM and DM existed in the non-NAFLD male population and was not present in the NAFLD population. Further analysis in our study revealed an association between SMM and DM that persisted in males with an LFC < 5.52%, which was similar to a histopathologic diagnosis of fatty liver. The findings also indicate that in males with a LFC < 5.52%, increasing SMM may prevent DM. In the male non-NAFLD population, SMM enhancement might facilitate DM treatment.

The mechanism underlying the relationship between low SMM and DM is not fully understood. It is known that insulin resistance and systemic inflammation play important roles in the development of both SMM reduction and DM[9,10,35,36]. As an important target organ of insulin action, skeletal muscle plays an important role in maintaining glucose metabolism stability[9,10]. Decreased SMM, which is often accompanied by intermuscular fat accumulation, increases macrophage infiltration, mitochondrial dysfunction, and inflammatory factors release, thus contributing to insulin resistance and reducing glucose uptake and utilization[37,38]. The current study also showed that in the male population, age-related SMM loss is independently associated with the presence of DM after adjustment for obesity, HOMA-IR, and all components of metabolic syndrome, which suggest that there may be other mechanisms to account for this association. Although it is unclear whether SMM loss is the cause or consequence of DM, direct crosstalk between skeletal muscle and glucose metabolism has been demonstrated. Previous studies have shown that skeletal muscles secrete a variety of cytokines, such as IL-6 and irisin, that regulate insulin sensitivity, promote glucose uptake by skeletal muscle cells, reduce liver gluconeogenesis, and improve glucose metabolism by acting on adipose tissue, the liver, and other tissues [39,40]. Impairment of muscle secretary function due to muscle loss may contribute to the development of DM.

The current study is the first to assess the influence of NAFLD on the association of DM with gender- and age-related SMM in a large-scale community population. Our findings may develop a new perspective for prevention of DM, especially in the male non-NAFLD population. There were also several limitations in the current study. First, the study had a cross-sectional design, which cannot demonstrate a causal relationship between SMM and DM. Thus, it is necessary to further verify our findings in a prospective cohort study. Second, the association between SMM loss and DM only existed in the first and second LFC quartiles, and the cut-off point for LFC should be further conformed. Third, several serum myokines were not detected in the current study, which might help explore the mechanisms underlying the relationship between low SMM and DM. Finally, this study was not able to collect/analyze current DM prevalence data for these patients with non-diabetic sarcopenia in 2010-2012.

CONCLUSION

In conclusion, SMM loss was shown to be associated with the presence of DM in Chinese middle-aged and elderly males without NAFLD. Our results suggest a new practical strategy to facilitate personalized intervention of DM by increasing SMM in males without NAFLD.

ARTICLE HIGHLIGHTS

Research background

Aging is getting worse in China. Sarcopenia has become a major focus of public health research on aging.

Research motivation

There seems to be a close relationship between non-alcoholic fatty liver disease (NAFLD), diabetes mellitus (DM), and skeletal muscle mass (SMM).

Research objectives

We tried to determine the association between SMM, DM, and NAFLD in a Chinese population.

Research methods

Three thousand nine hundred and sixty-nine participants > 45 years of age from Changfeng Community in Shanghai were recruited to conduct a large-scale community population study. All participants were interviewed and the medical histories were recorded by trained researchers using a standard questionnaire. Blood samples were collected after overnight fasting for at least 10 h from each participant. The data related to SMM, DM, and NAFLD were analyzed.

Research results

In the current study, the fasting blood glucose, 2-h postprandial blood glucose, and homeostasis model assessment for insulin resistance increased with the prevalence of DM while appendicular skeletal muscle mass (ASM)% decreased in male and female participants. The SMM, as measured by ASM%, was negatively associated with the blood glucose concentration, but logistic stepwise regression analysis showed that SMM loss may be associated with the presence of DM in males. The dissociation of SMM loss and DM in women is noteworthy, especially after adjustment for fat mass and lipid parameters.

Research conclusions

SMM loss was shown to be associated with the presence of DM in Chinese middle-aged and elderly males without NAFLD.

Research perspectives

Our results suggest a new practical strategy to facilitate personalized intervention of DM by increasing SMM in males without NAFLD.

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