# ADVANCES OF ENDOCRINE AND METABOLIC CARDIOVASCULAR OUTCOMES: FROM BASIC TO CLINICAL SCIENCE

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-656-1
DOI 10.3389/978-2-83250-656-1

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# ADVANCES OF ENDOCRINE AND METABOLIC CARDIOVASCULAR OUTCOMES: FROM BASIC TO CLINICAL SCIENCE

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**Citation:** Jin, S., Ding, Y., Xu, C., Lu, Q., eds. (2022). Advances of Endocrine and Metabolic Cardiovascular Outcomes: From Basic to Clinical Science. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-656-1

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frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 28 July 2021
doi: 10.3389/fendo.2021.721213

# The Protective Effect of Metformin on Abdominal Aortic Aneurysm: A Systematic Review and Meta-Analysis

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**Background:** Type 2 diabetes mellitus (T2DM) patients have a lower risk of abdominal aortic aneurysm (AAA) and its comorbidities, which might be associated with the usage of metformin. The objective of the study was to evaluate the role of metformin in the process of AAA development.

**Method:** PubMed, Embase and Cochrane Library were searched up to May 15th, 2021. We implemented several methods including the risk of bias graph, GRADE system and funnel plot to assess the quality and possible bias of this study. Subgroup analysis and sensitivity analysis were applied to address quality differences and validate the robustness of the final results.

**Result:** Ten articles were enrolled after screening 151 articles searched from databases. The pooled results showed that, compared with T2DM patients without metformin, metformin prescription was associated with a slower annual growth rate of the aneurysm (mean difference (MD) -0.67 cm [95% confidence interval (CI) -1.20 ~ -0.15 cm]). Besides, metformin exposure was associated with a lower frequency of AAA events (odds ratio (OR) 0.61 [95% CI 0.41-0.92]).

**Conclusion:** Metformin alleviated both annual expansion rate and aneurysm rupture frequency in AAA patients with T2DM.

**Systematic Review Registration:** PROSPERO, identifier https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=217859 (CRD42020217859).

**Keywords: abdominal aortic aneurysm, metformin, expansion rate, aneurysm rupture, inflammation**

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***Edited by:***
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***Specialty section:***
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

***Received:*** *06 June 2021*
***Accepted:*** *12 July 2021*
***Published:*** *28 July 2021*

***Citation:***
*Yuan Z, Heng Z, Lu Y, Wei J and Cai Z (2021) The Protective Effect of Metformin on Abdominal Aortic Aneurysm: A Systematic Review and Meta-Analysis. Front. Endocrinol. 12:721213. doi: 10.3389/fendo.2021.721213*

## INTRODUCTION

Abdominal aortic aneurysm (AAA) is characterized by the permanent dilation of the infrarenal segment of the aorta (1). The prevalence of AAA varied from 3.9 to 7.7% among developed countries in the 1980s and 1990s, and was decreased to 1-2% in recent years (2). In developing countries, however, AAA incidence has been increasing for the past few decades (2). Due to the

insidious nature of AAA, AAA is usually discovered accidentally by ultrasonography (3) or presents with catastrophic results like rupture, which accounts for 50-80% of mortality (4). According to the current guidelines, aneurysm repair (including open surgery and endovascular aneurysm repair) is indicated in patients with AAA larger than 5.5 cm in diameter or the onset of symptoms such as abdominal/back/flank pain. Annual screening is suggested for asymptomatic patients with minor AAA (5–7). However, there is no medical therapy available for asymptomatic patients with AAA so far (8).

Type 2 diabetes mellitus (T2DM) is a well-established risk factor of various cardiovascular diseases (CVDs) due to its detrimental effect on microcirculation and median-sized vessels such as coronary arteries (9). Interestingly, researchers found DM was conversely related to the prevalence, incidence, and annual growth rate of AAA (10–12). A multicenter cohort study involving 1.9 million subjects also confirmed T2DM is associated with a lower incidence of AAA (13). There are several different hypotheses on this anomalous phenomenon. Raffort et al. suggested that this resulted from the direct effect of DM on aortic walls, such as mural neo-angiogenesis, intraluminal thrombus formation, inflammation, glycation, extracellular matrix (ECM) remodeling, and vascular smooth muscle homeostasis (10). However, other studies showed that glucose-lowering therapies also had an inhibitory impact on AAA formation (14), which may provide a new medical treatment strategy for asymptomatic patients with AAA.

Metformin, a biguanide-class antidiabetic drug, is the first-line pharmacologic treatment for T2DM, which has been proved to decrease the incidence of cardiovascular events and all-cause mortality in DM patients (14). Besides its role in reducing blood glucose, metformin was also influential in several other areas such as cancer, longevity, and gastrointestinal disorders (15, 16). Recently, metformin was reported to attenuate AAA development and decrease the risk of aneurysm rupture in murine studies and human randomized controlled trials (17–19). Metformin, therefore, may be a potential medical choice for asymptomatic AAA patients. The purpose of this systemic review is to evaluate the effectiveness of metformin in suppressing AAA among patients with T2DM.

# METHOD

## Literature Search Strategy

Studies were enrolled by exploring electronic databases and scanning reference lists of articles for additional analyses. A comprehensive literature search of PubMed, EMBASE and Cochrane Central Register of Controlled Trials (CENTRAL) was performed to screen qualified articles up to May 2021. The search terms were based on the combination of Medical Subject Heading terms and free words (synonym) and limited English language. The following Medical Subject Heading (MeSH) terms and various text words were used: "Aneurysm, Abdominal Aortic", "Abdominal Aortic Aneurysm", "Aortic Aneurysm,

**TABLE 1 |** Characteristics of the included studies.

| Author, year | Region | Study design | Non-metformin | Metformin | Age-y | Outcomes | Study duration | Mean follow-up-y |
|---|---|---|---|---|---|---|---|---|
| Sutton, 2020 (20) | USA | Retrospective cohort | 43,073 | 24,361 | Non-metformin: 72.27 ± 7.85<br>Metformin: 69.65 ± 7.15 | Surgery and/or death | 2000-2019 | NA |
| Golledge, 2019 (21) | Australia | Prospective cohort | 105 | 129 | Non-metformin: 74.2 ± 7.2<br>Metformin: 72.4 ± 6.5 | Surgery and/or death as a result of AAA rupture | 2.5 ± 3.1y | 2.5 |
| Kristensen, 2020 (22) | Denmark | Case control study | 10,375 | 415 | 75 (69–80) | AAA rupture | 1996-2016 | NA |
| Kristensen,2017 (23) | Denmark | Nested case-control | 2,857 | 1,125 | 74 (68–79) | AAA rupture | 1995-2017 | NA |
| Hsu, 2016 (24) | Taiwan | Nested case-control | 5,337 | 3,599 | AAA: 67.5 ± 47.3<br>non-AAA: 67.5 ± 47.3 | Diagnosis of AAA | 2000-2013 | NA |
| Golledge, 2017 (25) | New Zealand and Australia | Retrospective cohort | 132 | 173 | NA | Infrarenal aortic diameters growth (ultrasound in cohort 1; CT in cohorts 2 and 3) | Cohort 1:2002-2015<br>Cohort 2: 2002-2015<br>Cohort 3: 2009-2015 | Cohort 1:3.6<br>Cohort 2: 2.9<br>Cohort 3: 1 |
| Unosson, 2020 (26) | Sweden | Retrospective cohort | 33 | 65 | Non-metformin: 70.1 ± 6.9<br>Metformin: 68.5 ± 5.4 | Infrarenal aortic diameters growth determined by ultrasonography | 2005-2017 | 3.2 |
| Itoga, 2019 (27) | USA | Retrospective cohort | 8,392 | 5,492 | 69.8 ± 7.8 | Infrarenal aortic diameters growth determined by radiographic reports | 2003-2013 | 4.2 |
| Fujimura, 2016 (19) | USA | Retrospective cohort | 43 | 15 | 72 (56–90) | Infrarenal aortic diameters growth determined by CT | 2006-2009 | 2.6 |
| Wang, 2018 (28) | USA | Retrospective cohort | 34 | 50 | Non-metformin: 69.6<br>Metformin: 69.5 | $CD4^+$ lymphocyte phenotyping, plasma cytokine, antigen and antibody quantification | 2015-2017 | NA |

*NA, Not applicable.*

Abdominal", "Metformin", "Dimethylbiguanidine", "Glucophage" and "Dimethylguanylguanidine".

## Eligible Criteria of Reference

Cohort studies, either prospective or retrospective, and randomized controlled trials were included if they met the following criteria: (a) studies included T2DM patients who were prescribed with metformin; (b) reported data of annual aneurysm growth rate, the incidence of rupture of AAA; and (c) published in English. Studies containing duplicate data or overlapping participates were excluded.

## Data Extraction and Quality Assessment

Concerning studies eligible for inclusion, we extracted data to a prespecified table (**Table 1**), which included the first author of the study, year and country of publication, sample size, baseline patient characteristics, primary outcomes, follow-up duration, and endpoint data. Two reviewers independently verified the extracted data.

After the initial assessment, two authors independently assessed the eligibility of studies identified for potential inclusion. Two authors also completed data extraction and quality assessment independently. The discrepancy was eliminated after discussion. This meta-analysis was conducted in concordance with PRISMA standards of quality for reporting meta-analysis (29).

## Outcome Measures

The primary outcomes of the study were the annual growth rate of AAA and incidence of aneurysm rupture or death.

## Statistical Analysis

The role of metformin in alleviating the annual growth rate of AAA and preventing aneurysm events was investigated. We extracted data information from the eligible trials and use a weighted mean difference with its 95% confidence intervals (CI) to demonstrate the effect of metformin on aneurysm expansion. To compare the incidence of aneurysm events from different studies, we adopted the adjusted odds ratio (OR) with its 95% CI to compute a pooled OR.

The $I^2$ index was provided to indicate whether the total variation (in percentage) across studies was attributable to heterogeneity rather than chance. $I^2$ >50% indicated substantial heterogeneity. To figure out the origin of heterogeneity, we evaluated covariates that may contribute to heterogeneity with subgroup analysis, based on study scale regarding the number of participants, region, study design, method of imaging studies, and follow-up duration. We also performed a sensitivity analysis to address quality differences and validate the robustness of the final result. We implemented a funnel plot to investigate the publication bias for this study.

Data were analyzed with the Stata MP, version 16.0 software (STATA, College Station, TX) and Review Manager (RevMan) 5.4 (The Nordic Cochrane Centre, The Cochrane Collaboration).

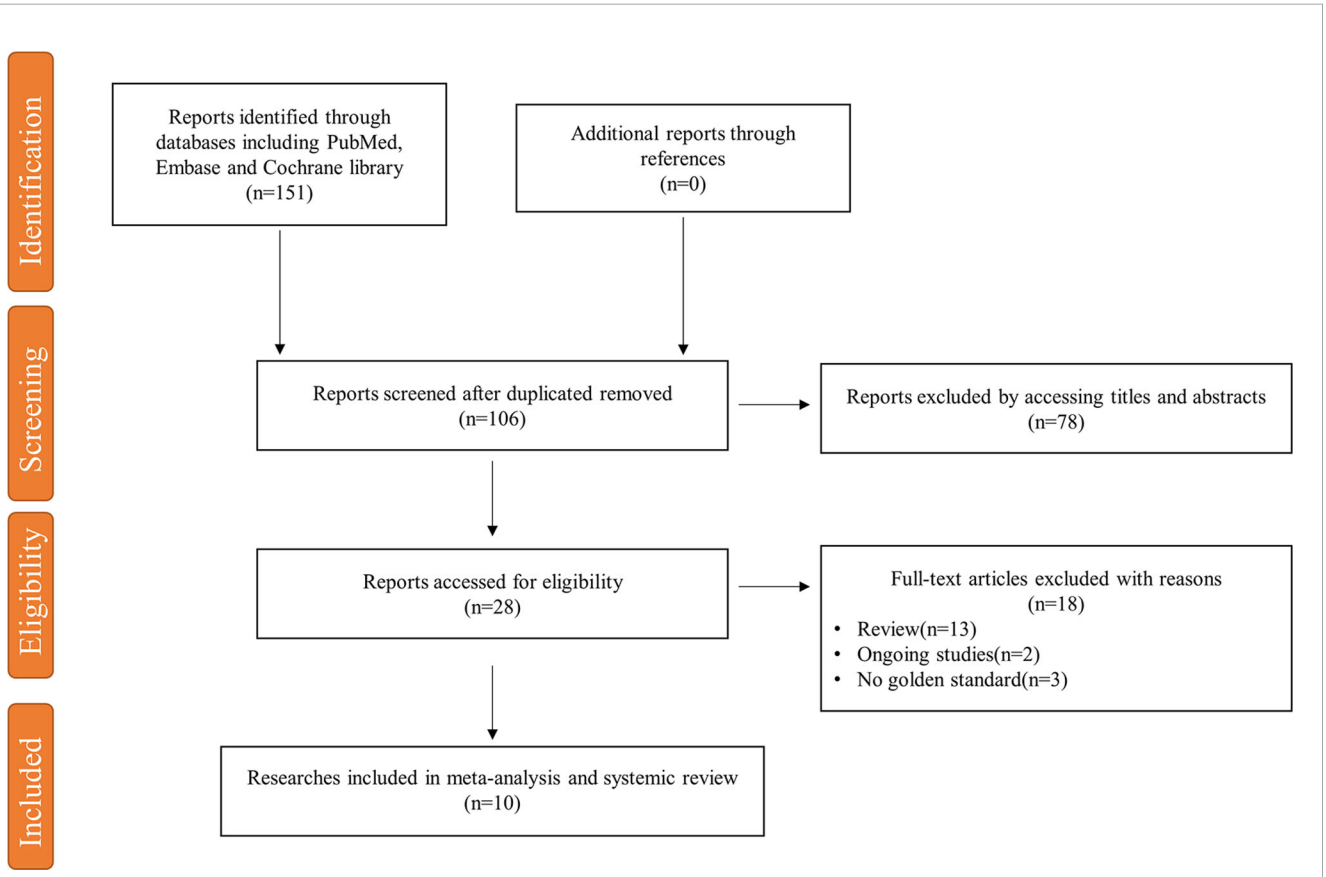


**FIGURE 1** | Flowchart of selected studies for inclusion in the meta-analysis.

# RESULTS

## Identification of Studies

The initial literature search identified 151 articles and retained 106 studies after excluding duplicates. After a review of the titles and abstracts, 78 articles were excluded. An additional 18 studies were deleted due to various reasons, including improper article type, unpublished studies, or without golden diagnostic standards. The whole process was shown in the study flow chart (**Figure 1**). There were 10 studies finally included in this study, of which six trials out of 4 studies explored the effect of metformin on attenuating annual aneurysm growth rate, three studies identified its role in decreasing incidence of AAA events, and the others were about other relationships between metformin and AAA, such as anti-inflammation and morbidity. We added the grading of recommendations Assessment, Development and Evaluation (GRADE system) to estimate the evidence quality of analysis on annual AAA growth rate and incidence of events (**Figure 2**).

**A**

| Certainty assessment | | | | | | | № of patients | | Effect | | Certainty | Importance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| № of studies | Study design | Risk of bias | Inconsistency | Indirectness | Imprecision | Other considerations | metformin | no metformin | Relative (95% CI) | Absolute (95% CI) | | |
| **Annual expansion rate** | | | | | | | | | | | | |
| 5 | randomised trials | not serious | not serious | not serious | not serious | none | 281 | 180 | - | MD **0.84 lower** (1.3 lower to 0.37 lower) | ⨁⨁⨁⨁ HIGH | IMPORTANT |

**B**

| Certainty assessment | | | | | | | № of patients | | Effect | | Certainty | Importance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| № of studies | Study design | Risk of bias | Inconsistency | Indirectness | Imprecision | Other considerations | Metformin | no metformin | Relative (95% CI) | Absolute (95% CI) | | |
| **AAA event morbidity** | | | | | | | | | | | | |
| 3 | randomised trials | not serious | not serious | not serious | not serious | none | 306/1669 (18.3%) | 5527/13337 (41.4%) | **OR 0.72** (0.55 to 0.95) | **77 fewer per 1,000** (from 134 fewer to 12 fewer) | ⨁⨁⨁⨁ HIGH | IMPORTANT |

**FIGURE 2** | Grading of recommendations Assessment, Development and Evaluation (GRADE) plot. **(A)** Evaluation for annual AAA growth rate analysis. **(B)** Evaluation for incidence of AAA events. CI, confidence interval; MD, mean difference; OR, odds ratio.

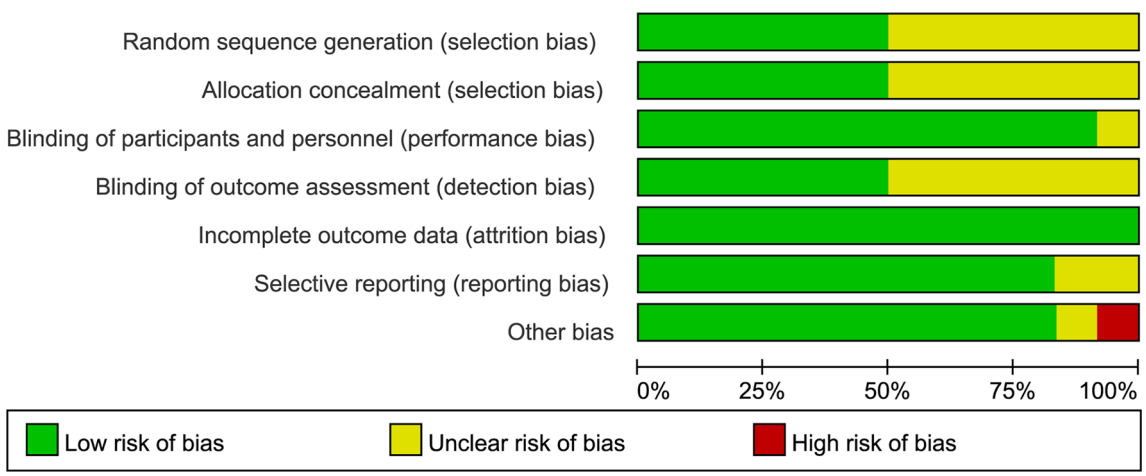


**FIGURE 3** | Risk of bias and applicability concerns summary.

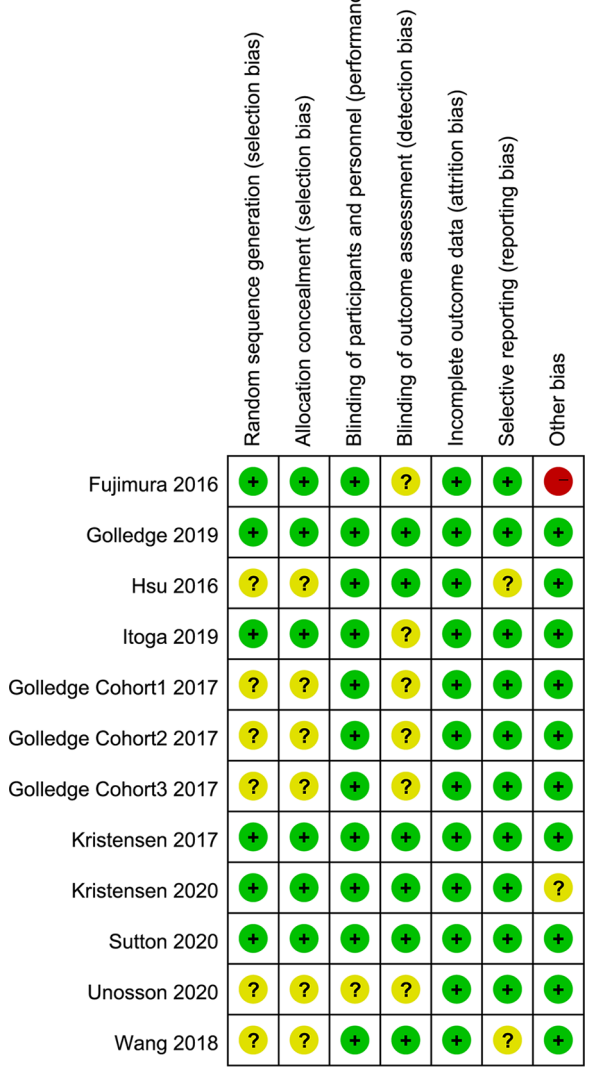


**FIGURE 4** | Risk of bias and applicability concerns graph.

## Risk of Bias

We evaluate the quality of enrolled studies with the risk of bias graph and summary (**Figures 3**, **4**), which proved relatively low concern of applicability of these articles. As these diagrams showed, the included studies had a limited risk of bias. One trial was considered to contain a high risk of other bias in the reporting because they indicated their subjects divided into the metformin group were taking or had ever taken metformin (28). Unclear risk of bias was established if there was limited information to exclude associated risk.

## Metformin and AAA Annual Growth Rate

We extracted four studies consisting of 6 trials to illustrate the association between metformin exposure and the annual growth rate decline of AAA (19, 25–27) (**Figure 5**). The integrated analysis showed that metformin usage significantly decreased the aneurysm expansion speed, in which the mean difference reached -0.67 cm with a 95% CI from -1.20 to -0.15 cm (p=0.01). However, the $I^2$ was 87%. Since the result of Fujimura et al. might be confounded by the inclusion of subjects who had ever taken metformin and the excessively large population of Itoga's study compared with others, we further pooled data without these two reports. The mean difference turned into -0.35 cm and a 95% CI from -0.42 to -0.29 cm (p<0.00001), with an $I^2$ = 0 (**Figure 6**).

## Metformin and Incidence of AAA Events

Two trials reported the risk of AAA events including death, aneurysm rupture, and need for surgery in T2DM patients (21, 23). The frequency of AAA events is remarkably lower in the metformin group. The pooled adjusted odds ratio of these three studies was 0.61 (95% CI, 0.41-0.92), with $I^2$ = 50% (**Figure 7**). Another case-control study demonstrated that metformin did not influence the frequency of AAA events among the general population (22).

## Other Impacts of Metformin on AAA

We also analyzed several other aspects that metformin might influence AAA development from some trials. Hsu et al. conducted a nested case-control analysis using the database extracted from Taiwan's National Health Insurance Research Database, in which a total of 4468 cases and matched controls

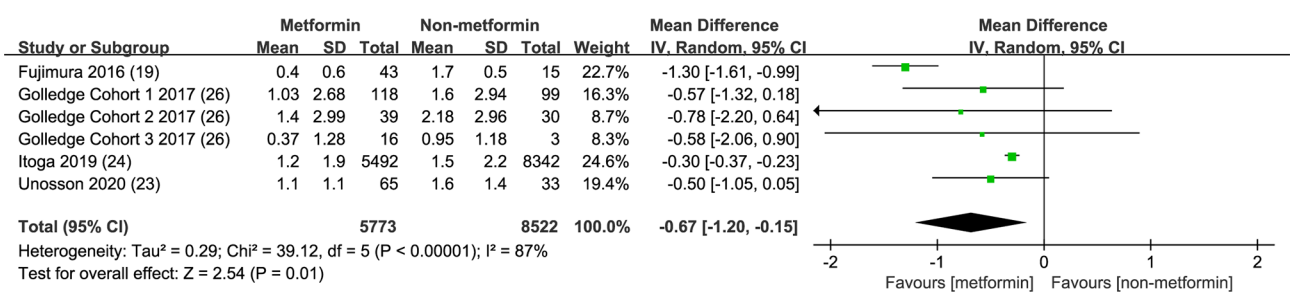


**FIGURE 5** | Assessment of the effect of metformin on annual AAA growth rate. Forrest plot of studies assessing the effect of metformin on annual AAA growth rate.

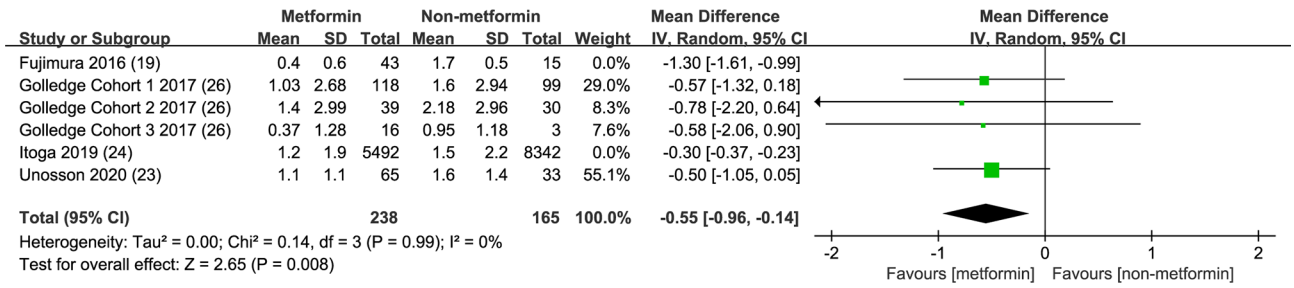


**FIGURE 6** | Assessment of the effect of metformin on incidence of AAA. Forest plot of studies assessing the effect of metformin on incidence of AAA events.

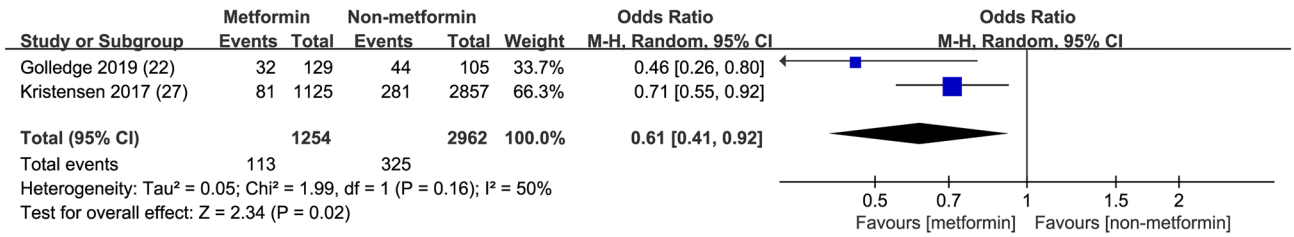


**FIGURE 7** | Assessment of the effect of metformin on incidence of AAA after excluding Fujimura 2016. AAA. Forest plot of studies assessing the effect of metformin on incidence of AAA events after excluding Fujimura 2016. *P < 0.05.

were involved (24). This study concluded that metformin prescription was associated with decreased hazard of aneurysm formation. A retrospective cohort study directed by Sutton et al. showed that patients with T2DM had a lower risk of aneurysm repair than subjects without T2DM. Still, they had higher imminence of perioperative mortality (20). However, the mortality was lower in T2DM patients with metformin than patients without T2DM (20). Besides, these patients also had a lower possibility of death in the first ten years after AAA diagnosis (20). Golledge et al. found AAA events might be reduced in T2DM patients with metformin rather than other anti-diabetic therapies compared with those without T2DM (21).

Two articles explored the relationship between metformin and inflammation in AAA patients. Wang et al. collected peripheral blood from patients diagnosed with AAA. They found there was no significant difference of inflammatory cells and cytokines, such as interferon-γ and interleukins, between patients taking metformin or not (28). However, Unosson et al. analyzed samples from 240 patients with AAA and discovered chemokine expression was significantly decreased in those using metformin (26), but the correlation between chemokine level and aneurysm growth rate was not clear. More studies need to be done to prove the association between metformin prescription and AAA expansion and inflammation.

## Publication Bias

Publication bias of AAA growth rate was assessed with a funnel plot by observing the symmetry of study distribution (**Figure 8A**). As mentioned above, Itoga 2019 and Fujimura 2016 were excluded with reasons, and the funnel plot for the rest of studies was illustrated in **Figure 8B**. No publication bias was noted in the corrective study.

## Subgroup Analysis and Sensitivity Analysis

To identify if the effect of metformin on AAA annual growth rate varies among different study characteristics, we conducted a subgroup analysis by dividing the included articles into several subgroups depending on subject numbers, study regions, method of imaging studies, and follow-up years (**Table 2**). The result clarified that metformin can downregulate the annual growth rate in diabetic patients independent of study properties. We also implemented sensitivity analysis for these trials, which indicated no significant quality difference among the included studies (**Table 3**).

# DISCUSSION

In this paper, we analyzed the role of metformin in AAA development among diabetic patients from distinctive aspects. According to the pooled results, we found that metformin was prone to alleviate the annual growth rate of the aneurysm and reduce the incidence of fatal AAA events, including aneurysm rupture or death. The inverse association between annual growth rate and metformin prescription was observed independent of study populations, regions, imaging methods, or follow-up years.

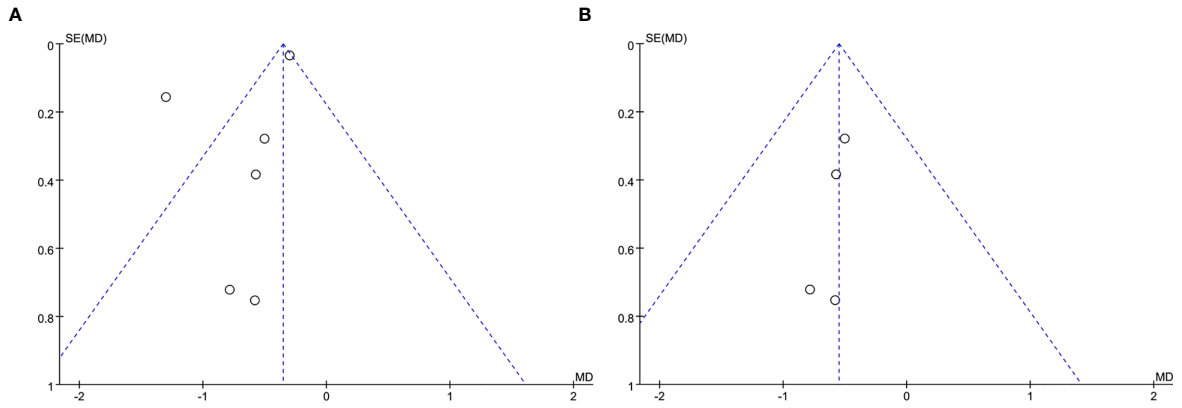


**FIGURE 8** | **(A)** Funnel plot of publication bias among studies analyzing AAA growth rate. **(B)** Funnel plot of publication bias after deleting questionable studies.

**TABLE 2** | Subgroup analysis results.

| Subgroup | Population | Study number | Metformin | Non-metformin | Mean difference | Confidendence interval | P value |
|---|---|---|---|---|---|---|---|
| All combined | Overall | 6 | 5773 | 8522 | -0.67 | [-1.20, -0.15] | 0.01 |
| Subject number | <100 | 4 | 163 | 81 | -0.89 | [-1.44, -0.34] | 0.001 |
| | ≥100 | 2 | 5610 | 8441 | -0.3 | [-0.37, -0.23] | <0.00001 |
| Region | New Zealand and Australia | 3 | 173 | 132 | -0.61 | [-1.22, -0.00] | 0.05 |
| | Others | 2 | 5600 | 8390 | -0.7 | [-1.41, 0.01] | 0.05 |
| Imaging | Ultrasound | 2 | 183 | 132 | -0.52 | [-0.97, -0.08] | 0.02 |
| | CT | 3 | 98 | 48 | -1.25 | [-1.54, -0.95] | <0.00001 |
| | Mixed | 1 | 5492 | 8342 | -0.3 | [-0.37, -0.23] | <0.00001 |
| Follow-up | <3 years | 3 | 98 | 48 | -0.52 | [-0.97, -0.08] | 0.02 |
| | ≥3 years | 3 | 5675 | 8474 | -0.31 | [-0.37, -0.24] | <0.00001 |

**TABLE 3** | Sensitivity analysis results.

| Exclued study | Metformin | Non-metformin | Mean difference | Confidendence interval | P value |
|---|---|---|---|---|---|
| Fujimura, 2016 (19) | 5730 | 8507 | -0.31 | [-0.37, -0.24] | <0.00001 |
| Itoga, 2019 (27) | 281 | 180 | -0.84 | [-1.30, -0.37] | 0.0004 |
| Golledge Cohort 1, 2017 (25) | 5655 | 8423 | -0.7 | [-1.29, -0.10] | 0.02 |
| Golledge Cohort 2, 2017 (25) | 5734 | 8492 | -0.66 | [-1.22, -0.11] | 0.02 |
| Golledge Cohort 3, 2017 (25) | 5757 | 8519 | -0.68 | [-1.24, -0.13] | 0.02 |
| Unosson, 2020 (26) | 5708 | 8489 | -0.72 | [-1.35, -0.08] | 0.03 |

Besides, metformin was related to decreased morbidity of AAA formation in patients with T2DM (24). Prescription of metformin was also accompanied by a lower risk of AAA rupture. Lastly, researchers demonstrated that metformin might down-regulate the inflammatory response such as chemokine production in samples from AAA patients, although it remained to be proved by more studies. These findings indicated that metformin might be a potential medical treatment for asymptomatic patients with AAA.

Metformin is commonly used as an antidiabetic drug that acts in several ways, including suppressing hepatic gluconeogenesis, activating AMP-activated protein kinase, and increasing insulin sensibility in the gut lumen (30, 31). The mechanism that metformin can benefit AAA patients is controversial. Wang et al. implicated that metformin repressed the pathogenesis of aneurysm formation by inhibiting the activation of PI3K/AKT/mTOR/autophagy pathway, which is an important signal pathway regulating cell growth, proliferation, apoptosis, and autophagy of vascular smooth muscle cells (VSMCs) in aortic tissues (18). VSMCs are the main cellular component of aortic walls. Impaired functions of VSMCs might lead to decreased aortic contractility, increased vulnerability to inflammatory cells, and a higher risk of rupture (32, 33). Besides, metformin was shown to be eligible to reduce the formation of atherosclerotic plaques with downregulated serum high-sensitivity C-reactive protein and the activation of NF-κB pathway in the vascular wall, and in the meantime protect vascular endothelial cells (34). Owing to the phenomenon that AAA development is commonly accompanied by aortic atherosclerotic changes within the vascular lumen, metformin may attenuate local inflammatory cell accumulation and hemodynamic changes. Vasamsetti et al. found metformin limited plaque formation and aortic aneurysm

in *Apoe*$^{-/-}$ mice by reducing monocyte infiltration (35). Raffort et al. concluded in their review that metformin prescription is related to changes in the expression of ECM proteins such as alpha1 type IV collagen, alpha2 type XVIII collagen, gamma1, and beta2 laminin (17). These findings might explain why metformin has a protective effect on AAA expansion and rupture.

Studies exploring the mechanism that metformin is beneficial for AAA patients are limited. Although an article claimed that metformin could downregulate serum chemokines in those taking metformin, it demonstrated little correlation between the level of chemokines and aneurysm growth rate (26). The effect of metformin on AAA in patients without T2DM remained unknown. Due to the relatively low prevalence of AAA among diabetic patients, it still lacks solid evidence to show the direct impact of metformin on aortic walls. Some researchers try to identify the effect of metformin on wild-type murine studies. Fujimura et al. found metformin significantly relieved AAA progression with medial elastin and smooth muscle preservation, and suppressed aortic mural macrophage, $CD8^+$ T cell infiltration without influencing blood-glucose levels (19). A non-published clinical trial (NCT03507413) showed that metformin could reduce the annual growth rate of AAA in patients without T2DM. Metformin for Abdominal Aortic Aneurysm Growth Inhibition (MAAAGI) Trial is an ongoing multicenter, randomized prospective trial with blinded outcome assessment to evaluate whether metformin reduces AAA growth in non-diabetic patients over five years, of which primary efficacy will be estimated by the difference of AAA diameter compared to baseline (17). There are also several ongoing randomized controlled trials (NCT04224051, NCT03507413, etc.) which may help to identify whether metformin is efficient to prevent AAA development in non-diabetic patients. If the positive associations between metformin using and the prognosis of AAA patients without T2DM can be established, it may become a new medical treatment strategy for these patients.

Several limitations exist in this meta-analysis. AAA development depends on lifestyles, races, and male gender, while this paper did not standardize the included subjects, which may result in confounding bias in part of this meta-analysis. Besides, it should be known that all of the included studies in this article are conducted among subjects with T2DM.

In conclusion, this meta-analysis suggests that metformin can significantly decrease the annual growth rate of AAA compared with patients not taking metformin. Furthermore, metformin is prone to reduce the prevalence of AAA formation and incidence of aneurysm rupture or death from comorbidities. More studies should be encouraged to explore mechanisms of the protective role in AAA patients, which may produce more preventive therapeutic interventions in the future.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

ZY and HZ collected data and performed analysis. ZY and ZC wrote the manuscript. ZY, YL, and ZC made revision of the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by funding from the National Natural Science Foundation of China (No. 81970396 to ZC, and No. 81900416 to YL), the Zhejiang Provincial Natural Science Foundation for Distinguished Young Scholars (No. LR20H020002 to ZC), and the Zhejiang Provincial Natural Science Foundation (No. LY19H040012 to JW).

## ACKNOWLEDGMENTS

The authors appreciated Dr. Jifan Chen for technical assistance.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 30 July 2021
doi: 10.3389/fendo.2021.713849

# Treatment Outcomes of Clopidogrel in Patients With ACS and Diabetes Undergoing PCI-Analysis of Beijing Municipal Medical Insurance Database

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**Edited by:**
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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *25 May 2021*
**Accepted:** *05 July 2021*
**Published:** *30 July 2021*

**Citation:**
*Wang W, Wang X, Zhang L, Zhang J, Man F, Pan Q and Guo L (2021) Treatment Outcomes of Clopidogrel in Patients With ACS and Diabetes Undergoing PCI-Analysis of Beijing Municipal Medical Insurance Database. Front. Endocrinol. 12:713849. doi: 10.3389/fendo.2021.713849*

**Background and Aims:** Several clinical trials have proved the efficacy of clopidogrel treatment for patients with percutaneous coronary intervention. There are few large-scale studies to identify the mortality associated with different durations of treatment of clopidogrel in patients with diabetes and ACS undergoing PCI in the Chinese population. The objective of this analysis was to determine the efficacy of long-term clopidogrel therapy (≥12 months) *versus* short-term use (<12 months) in Chinese patients with diabetes after PCI.

**Methods and Results:** We used the Beijing Municipal Medical Insurance Database provided by the Beijing Municipal Medical Insurance Bureau. The Beijing Municipal Medical Insurance Database contained medical data of about 16 million people, including about 990,000 patients with diabetes and a history of taking antidiabetic medicines. Patients were divided into two groups, one group of 9,116 patients receiving consecutive clopidogrel for one year or more, and another group of 3290 patients receiving consecutive clopidogrel for less than one year. The primary outcomes of this analysis were the risk of all-cause death, myocardial infarction, and revascularization. In patients with diabetes after PCI, long-term clopidogrel treatment was associated with a reduced risk of all-cause death (HR, 0.57[95%CI, 0.49-0.67], P<0.0001), myocardial infarction (HR, 0.79[95%CI, 0.68-0.93], P=0.0035) and an increased risk of angina (HR, 1.18[95%CI, 1.10-1.27], P<0.0001]) and revascularization (HR, 1.07[95%CI, 1.01-1.13], P=0.02]). There was no significant difference in the prevalence of all-cause re-hospitalization, diabetes-related re-hospitalization, and cerebrovascular re-hospitalization.

**Conclusion:** The present study concluded that long-term dual antiplatelet therapy including clopidogrel and aspirin could decrease the risks of all-cause death, myocardial infarction. But it could increase the risks of angina and revascularization. Further studies should interpret the cause of this question.

**Keywords: clopidogrel, mortality, diabetes, PCI, medical insurance database**

# INTRODUCTION

Several clinical trials have proved the efficacy of clopidogrel treatment for patients with percutaneous coronary intervention (PCI) (1, 2). For patients with PCI, current guidelines suggest clopidogrel treatment for at least 12 months (3). Non-adherence with clopidogrel after coronary stent implantation could be related to some adverse effects like increased mortality (4). Diabetes is one of the four significant non-communicable diseases and is a major cause of premature death and disability. Among patients with diabetes, the adherence therapy of clopidogrel after myocardial infarction leads to a lower induction in the risk of death (all-cause death and cardiovascular death) (5) compared with it in patients without diabetes. It is well known that patients accompanied by diabetes and acute coronary syndrome (ACS) undergoing PCI are at higher risk for some adverse effects like death (6). The prevalence of diabetes in China has increased 10-fold in the past decade and reached 114 million, making it the country with the highest diabetic population in the world (7, 8). Among Chinese patients with ACS, 37.6% accompanied diabetes or possible diabetes. Even in patients with diabetes younger than 45 years old, 26.9% were accompanied by diabetes or possible diabetes (9). There are few large-scale studies to identify the mortality associated with different durations of treatment of clopidogrel in patients with diabetes and ACS undergoing PCI in the Chinese population.

All citizens with medical insurance are registered with a personal number in China. Since the establishment of China's medical insurance system, there has been little relevant large-scale clinical data analysis. The Beijing Municipal Medical Insurance Database covers the medical data of thousands of hospitals and community clinics.

In China, the time range of clopidogrel therapy is consistent with guidelines. We conducted large-scale research of 12406 PCI-treated patients with diabetes to evaluate the effect of different durations of treatment of clopidogrel on mortality and other indicators.

# METHODS

## Data Source

In China, all medicare citizens are registered with a personal number in the Medical Insurance System. The Beijing Municipal Medical Insurance Bureau holds all information on all outpatients and hospitalizations in Beijing. The Medical Insurance Databse records all the prescriptions and diagnosis information dispensed from hospitals and clinics in Beijing. For calculating the expenses of medicare, all the treatment and use of drugs are registered in the Medical Insurance Databse in China. The study was approved by the ethics committee of Beijing Hospital.

## Population

The population included in this study were all enrolled in the Beijing Municipal Medical Insurance Bureau with available treatment records from 2012-2016. First of all, patients with diabetes diagnoses were selected in Medical Insurance Databse. Then among the patients with diabetes, PCI treatment was identified to locate the patients diagnosed with acute coronary syndrome and diabetes. Patients with survival days of less than 30 days were excluded. Diabetic patients who had at least one time PCI treatment were eligible for further selection. Then patients who have continuous treatment (≥1 year) of aspirin were selected for further investigation in this research. The data extracted from the Medical Insurance Databse contains all medical prescriptions and surgery history.

## Medication Therapy

The Beijing Municipal Medical Insurance Bureau provided medications and diagnoses used from 2012-2016 in patients with diabetes after PCI. Since the drug name appearing in the Medical Insurance Databse may be the chemical name or trade name, we classified the drugs according to the clinical guidelines, such as metformin, sulfonylurea, DPP-4 inhibitors, thiazolidinediones (TZDs), α-glucosidase inhibitors, and glinides (hypoglycemic drugs), diuretics, CCBs, ARB/ACEI, β-receptor inhibitors (antihypertensive drugs). We tracked the dates of prescription of aspirin and clopidogrel up to 4 years after PCI. Patients were considered as not taking aspirin or clopidogrel if the prescription lapsed over 30 days from the last day of the supply. Clopidogrel use was defined as either long-term (≥12 months therapy after PCI) or short-term (<12 months therapy after PCI) therapy.

## Outcomes

Clinical outcomes in patients with diabetes after PCI were identified through the Medical Insurance Databse until December 2016. These outcomes included all-cause death, myocardial infarction, all-cause re-hospitalization, diabetes-related re-hospitalization, cerebrovascular re-hospitalization, angina, and revascularization.

## Statistical Analysis

Quantitative variables were expressed as mean ± SD and categorical variables as frequencies and percentages. Continuous variables with normal distribution were compared between the two groups by the t–test and continuous variables without normal distribution by the Wilcoxon rank–sum test. Categorical variables were compared between groups using the chi-square test or Fisher's exact probability method (when the expected frequency of cells greater than 25% is less than 5). Events were summarized with Kaplan–Meier curves and estimates at three years. Hazard ratios (HRs) comparing treatment groups were derived from univariate Cox regression models. Subgroups were analyzed with a Cox model, including subgroup, treatment, and the subgroup-by-treatment interaction. Multivariate Cox regression models were derived for the event by the use of a backward selection algorithm. The significance level for staying in the model was set to

0.05 two-sides. All data were prospectively analyzed using SAS, version 9.4.

# RESULTS

## Population Selection

The selection process was shown in **Figure 1**. The Beijing Municipal Medical Insurance Database contains medical data of about 16 million people, including about 990,000 patients with diabetes and a history of taking antidiabetic medicines. Among them, 18,799 patients had a record of PCI surgery in 2014-2016. Among them, 13,693 patients have continuous aspirin withdrawal records in the database. After excluding patients with survival time of less than one year or no continuous clopidogrel medication withdrawal records, the patients were divided into two groups, one group of 9,116 patients receiving consecutive clopidogrel for one year or more, and another group of 3290 patients receiving consecutive clopidogrel less than one year. All the personal numbers of included participants were masked by desensitization in our research.

## Demographics of Drugs Treatment

We summarized the medication situation of the two groups of patients as shown in **Table 1**. Classifications of drugs contain antidiabetic medicine (Thiazolidinediones, α-glucosidase inhibitors, metformin, sulfonylureas, DPP-4 inhibitors, glinides, insulin), antihypertensive medicine (ARB/ACEI, CCB, β-receptor inhibitors, diuretic), related cardiovascular medicine (statin, nitrate, proton pump inhibitors).

## Mortality and Prevalence of Recurrent Myocardial Infarction and Hospitalization

The mortality was lower in patients treated with clopidogrel for more than one year compared with the group treated with clopidogrel less than one year (4.6% *vs* 7.7%, HR, 0.57 [95%CI, 0.49-0.67], P<0.0001) (**Figure 2**). The prevalence of myocardial infarction was lower in patients treated with clopidogrel for more than one year compared with patients treated with clopidogrel less than one year (8.2% *vs* 10.1%, HR, 0.79[95%CI, 0.68-0.93], P=0.0035) (**Figure 3**). However, there were no significant differences in the prevalence of all-cause re-hospitalization (P=0.7529), diabetes-related re-hospitalization (P=0.9727), and cerebrovascular re-hospitalization (P=0.2958) (**Figures 4–6**).

## Prevalence of Angina and Revascularization

The rate of angina and revascularization was 35.8% and 54.5% in long-term dual antiplatelet therapy group compared with 31.1% and 51.8% in placebo group (HR, 1.18[95%CI, 1.10-1.27], P<0.0001]) (HR, 1.07[95%CI, 1.01-1.13], P=0.02]) (**Figures 7** and **8**). A long-term combination of aspirin and clopidogrel could cause higher risks of angina and revascularization.

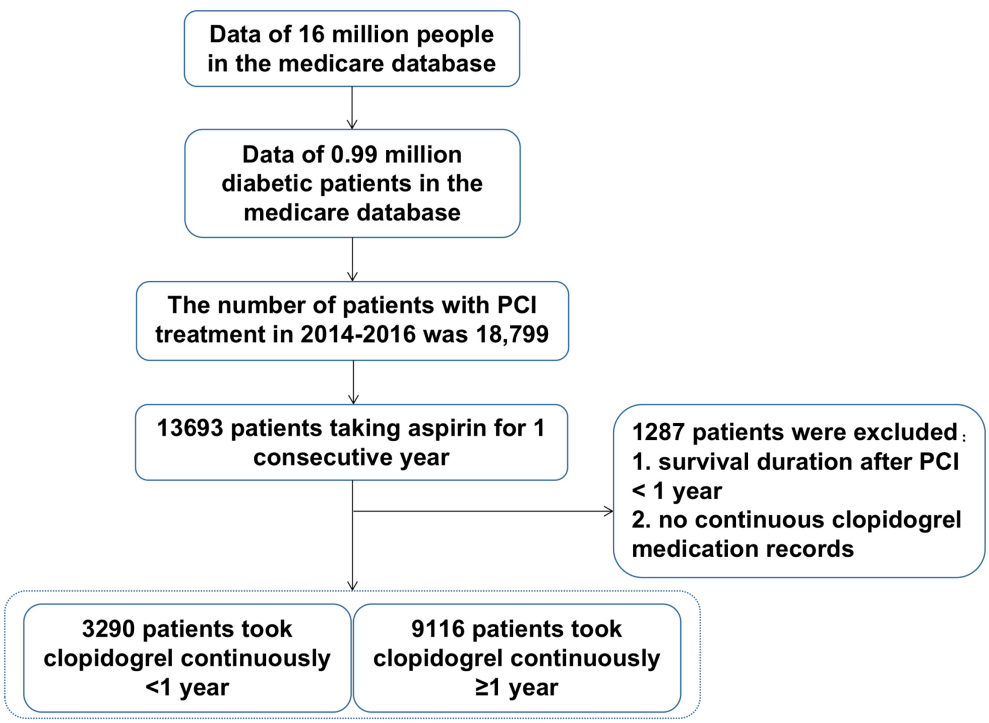


**FIGURE 1** | The selection process of included patients.

**TABLE 1** | Demographics of drug treatment.

| Drug names | Duration of drug treatment | Nubmer of patients with clopidogrel <1 year (%) | Number of patients with clopidogrel ≥1 year (%) | *P* value |
|---|---|---|---|---|
| Metformin | none | 1089 (33.5) | 3032 (33.3) | 0.0346 |
| | < 1year | 755 (23.2) | 1940 (21.3) | |
| | ≥ 1 year | 1407 (43.3) | 4144 (45.5) | |
| α-glucosidase inhibitor | none | 791 (24.3) | 2126 (23.3) | 0.1094 |
| | < 1year | 723 (22.2) | 1925 (21.1) | |
| | ≥ 1 year | 1737 (53.4) | 5065 (55.6) | |
| thiazolidinedione (TZD) | none | 2878 (88.5) | 8076 (88.6) | 0.3075 |
| | < 1year | 227 (7.0) | 679 (7.4) | |
| | ≥ 1 year | 146 (4.5) | 361 (4.0) | |
| Sulfonylureas | none | 1873 (57.6) | 5252 (57.6) | 0.4824 |
| | < 1year | 559 (17.2) | 1496 (16.4) | |
| | ≥ 1 year | 819 (25.2) | 2368 (26.0) | |
| Glinides | none | 2776 (85.4) | 7759 (85.1) | 0.8629 |
| | < 1year | 265 (8.2) | 743 (8.2) | |
| | ≥ 1 year | 210 (6.5) | 614 (6.7) | |
| DPP-4 inhibitor | none | 3056 (94.0) | 8589 (94.2) | 0.3037 |
| | < 1year | 178 (5.5) | 497 (5.5) | |
| | ≥ 1 year | 17 (0.5) | 30 (0.3) | |
| insulin | none | 1835 (56.4) | 5193 (57.0) | 0.5542 |
| | < 1year | 351 (10.8) | 923 (10.1) | |
| | ≥ 1 year | 1065 (32.8) | 3000 (32.9) | |
| ticagrelor | none | 3122 (96.0) | 9029 (99.0) | < 0.0001 |
| | < 1year | 114 (3.5) | 85 (0.9) | |
| | ≥ 1 year | 15 (0.5) | 2 (0.1) | |
| ARB/ACEI | none | 499 (15.3) | 1463 (16.0) | 0.0077 |
| | < 1year | 690 (21.2) | 1706 (18.7) | |
| | ≥ 1 year | 2062 (63.4) | 5947 (65.2) | |
| CCB | none | 922 (28.4) | 2461 (27.0) | < 0.0011 |
| | < 1year | 757 (23.3) | 1915 (21.0) | |
| | ≥ 1 year | 1572 (48.4) | 4740 (52.0) | |
| β receptor blocker | none | 317 (9.8) | 883 (9.7) | < 0.0001 |
| | < 1year | 566 (17.4) | 1190 (13.1) | |
| | ≥ 1 year | 2368 (72.8) | 7043 (77.3) | |
| diuretic | none | 1802 (55.4) | 5110 (56.1) | 0.6018 |
| | < 1year | 827 (25.4) | 2238 (24.6) | |
| | ≥ 1 year | 622 (19.1) | 1768 (19.4) | |
| statin | none | 11 (0.3) | 29 (0.3) | < 0.0001 |
| | < 1year | 202 (6.2) | 249 (2.7) | |
| | ≥ 1 year | 3038 (93.4) | 8838 (97.0) | |
| nitrate | none | 377 (11.6) | 808 (8.9) | < 0.0001 |
| | < 1year | 1248 (38.4) | 2841 (31.2) | |
| | ≥ 1 year | 1626 (50.0) | 5467 (60.0) | |
| PPI | none | 1288 (39.6) | 3700 (40.6) | 0.0597 |
| | < 1year | 1427 (43.9) | 3797 (41.7) | |
| | ≥ 1 year | 536 (16.5) | 1619 (17.8) | |

## DISCUSSION

From 2012 to 2016, we screened a cohort of 12,406 patients who underwent PCI surgery among 990,000 diabetic patients in Beijing. The results showed that clopidogrel could reduce all-cause mortality and the probability of recurrent myocardial infarction after more than one year's regular treatment, but had no significant effect on all-cause readmission, cerebrovascular readmission, and diabetes-related readmission. But long-term dual antiplatelet treatment including aspirin and clopidogrel could increase the risks of angina and revascularization. In the past published articles in the Veterans Health Administration database, in patients with diabetes mellitus who received drug-eluting stents, prolonged clopidogrel (more than 12 months) was associated with a reduced risk of death (10). This result is consistent with the long-term use of clopidogrel in patients without diabetes (4, 11). However, similar studies on the Medical Insurance Database in Chinese population are still lacking.

A high risk of poor clinical outcomes was observed in patients with diabetes after PCI (12, 13). The DAPT study concluded that the treatment of thienopyridine beyond one year could decrease the risks of stent thrombosis, and major cerebrovascular and cardiovascular events (14). As expected, long-term dual antiplatelet therapy could increase the risk of bleeding. That was why our results showed that over 50% of patients had used PPI. However, other research showed that different stents (15) and P2Y12 inhibitors (16) had been related to different rates of

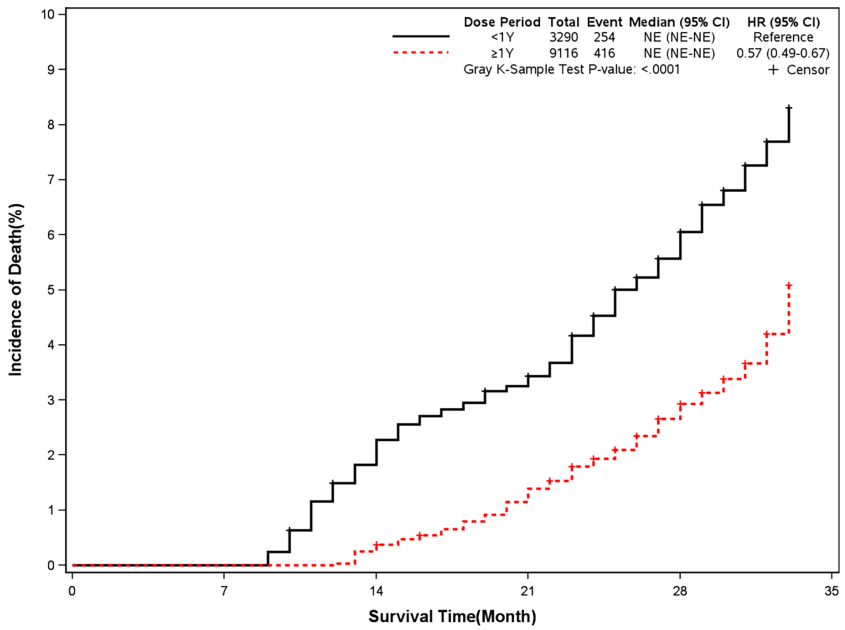


**FIGURE 2** | Incidence of all-cause death in two groups. Blackline: patients took clopidogrel continuously < 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

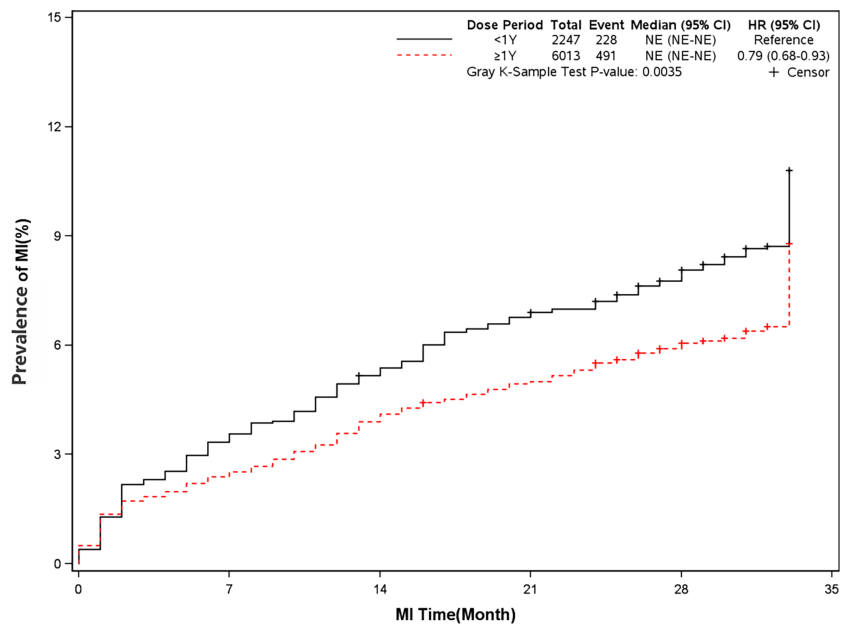


**FIGURE 3** | Prevalence of recurrent myocardial infarction in two groups. Blackline: patients took clopidogrel continuously < 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

stent thrombosis and myocardial infarction. Our research has not focused on this difference as it is inconvenient to distinguish different stents in the China medicare database. The rate of all-cause death and myocardial infarction in the DAPT study was 2.0% and 2.1% in the long-term thienopyridine treatment and 1.5%, 4.1% in the placebo group. But in our study, the rate of all-cause death and myocardial infarction in the long-term dual antiplatelet therapy group was 4.6% and 8.1% compared with

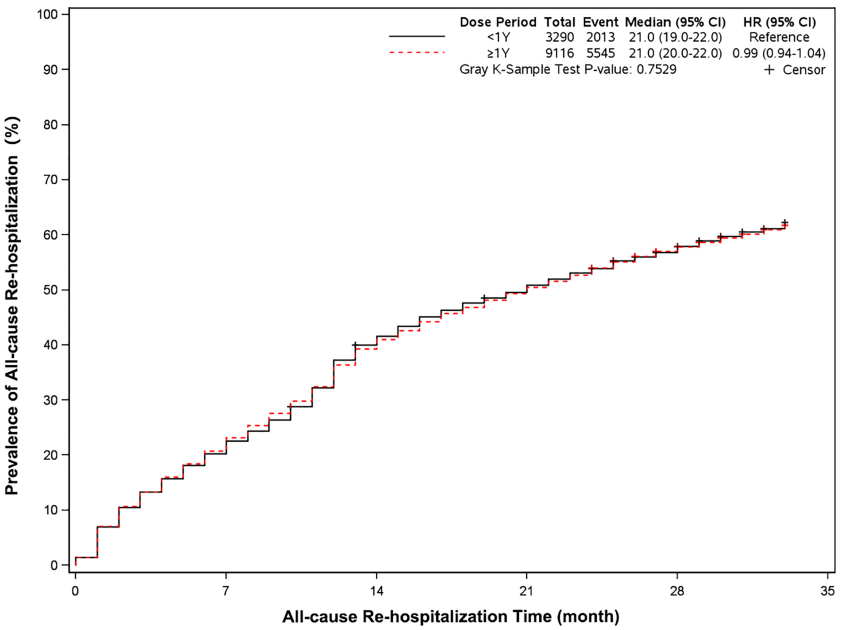


**FIGURE 4** | Prevalence of all-cause rehospitalization in two groups. Blackline: patients took clopidogrel continuously 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

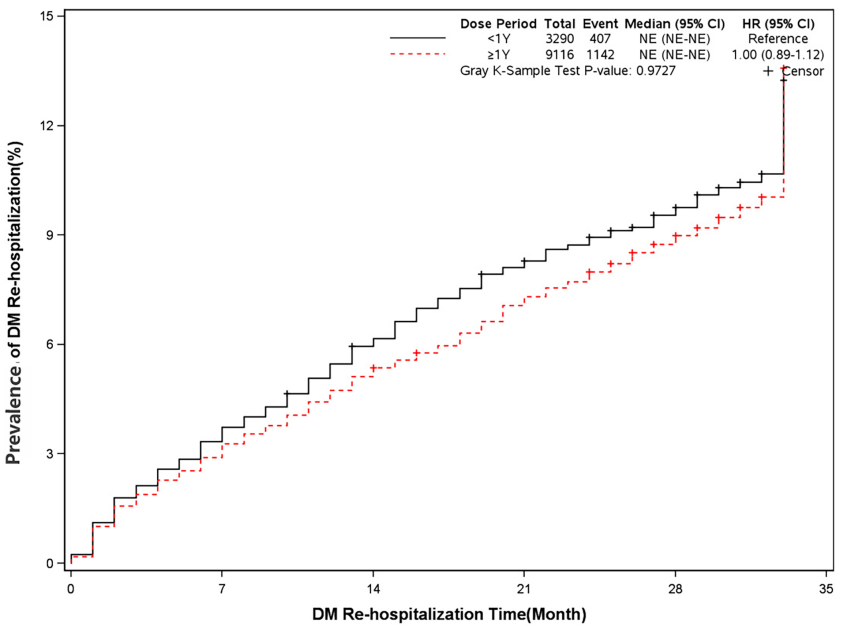


**FIGURE 5** | Prevalence of diabetes-related rehospitalization in two groups. Blackline: patients took clopidogrel continuously 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

7.7% and 10.1% in the placebo group. This was a big difference between the DAPT study and our results. This result also proved that patients with diabetes after PCI had a higher risk of poor clinical outcomes compared with patients without diabetes. The same aspect between the two studies was that long-term therapy did not affect cerebrovascular outcomes.

Another finding is that long-term dual antiplatelet therapy could increase the risks of angina and revascularization in

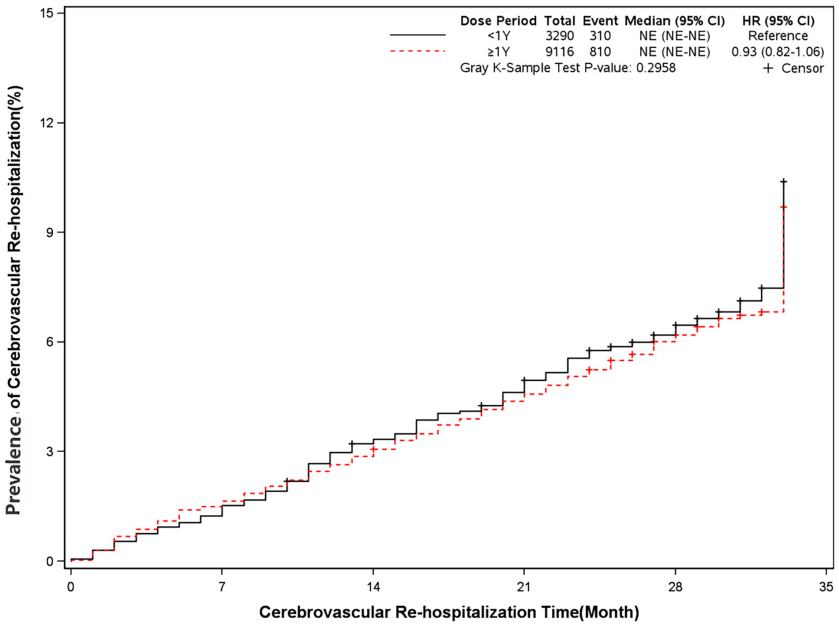


**FIGURE 6** | Prevalence of cerebrovascular rehospitalization in two groups. Blackline: patients took clopidogrel continuously < 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

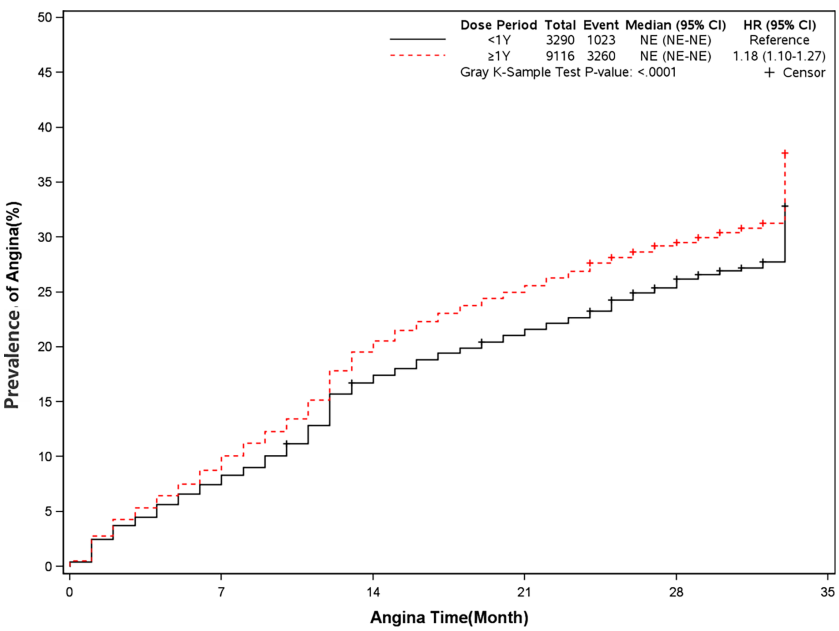


**FIGURE 7** | Prevalence of angina in two groups. Blackline: patients took clopidogrel continuously < 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

patients with diabetes undergoing PCI. Controversies still exist with the combination use of clopidogrel and PPI following coronary stenting. The previous meta-analysis (17) showed that the continuous use of clopidogrel and PPI was associated with higher MACES (OR:1.27, 95%CI[1.13-1.42]). Another meta-analysis (18) found that long-term dual therapy leads to increased MACEs, myocardial infarction, stent thrombosis, and target vessel revascularization. Our results showed that

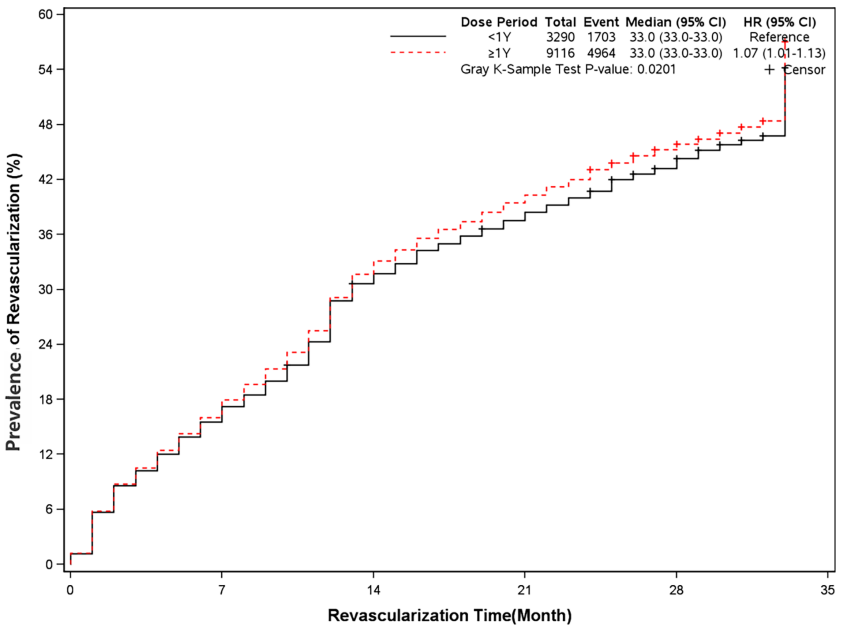


**FIGURE 8** | Prevalence of revascularization in two groups. Blackline: patients took clopidogrel continuously < 1 year; Red line: patients took clopidogrel continuously ≥ 1 year.

17.8% of patients with long-term dual therapy and 16.5% of patients with short-term dual therapy had used PPI for more than one year. This may be the cause of increased rates of angina and revascularization. The reason for increased adverse effects may be that PPI was involved in the same metabolic pathway (CYP2C19 isoenzyme and so on) as that of clopidogrel (19). However, several studies found different results. From the results of Guthrie Health Off-label Stent (GHOST) research, the combination of PPI and clopidogrel was not related to any increased risk in MACEs outcomes after PCI (20). The specific conclusion still needs further investigation.

Ticagrelor is the first reversibly binding direct P2Y12 inhibitor. It does not need enzymatic activation compared with clopidogrel and prasugrel. It also inhibits platelets faster, better, and more stable than clopidogrel (21, 22). Meanwhile, regardless of revascularization or not, ticagrelor could reduce the risk of all-cause death with no significant increase in the risk of overall bleeding compared with clopidogrel (23). Our results showed that over 95% of diabetic patients after PCI surgery never used ticagrelor. The most possible reason is that ticagrelor has not been included in the medical insurance catalog until 2017 in China.

At the same time, we also summarized the medication situation of patients with diabetes after PCI surgery. We found that the most commonly used antidiabetic drug was alpha-glucosidase inhibitors, followed by metformin. According to the guidelines, most of the lipid-lowering drugs selected for patients with diabetes after PCI were statins (3, 24), which was consistent with our results. More than 90% of patients have used statins for more than one year. But recent research found that treatment with fenofibrate and metformin produces the cardioprotective effect in a rat model with acute myocardial infarction and diabetes (25). And the possible mechanism may act through the PPARα activation.

## LIMITATIONS

There was no specific classification of drugs and diseases in the Beijing Municipal Medical Insurance Database. Researchers completed the classification of all drugs and diseases. There were also no basic demographic characteristics and laboratory indicators in the database. It was hard to distinguish the different types of stents to go through further investigation. This study also did not include more potent antiplatelet agents. Although this study was a large-scale investigation, it was only generated from a single city's database.

## CONCLUSION

The present study concluded that long-term dual antiplatelet therapy including clopidogrel and aspirin could decrease the risks of all-cause death, myocardial infarction and did not affect the prevalence of all-cause re-hospitalization, diabetes-related re-hospitalization, and cerebrovascular re-hospitalization in patients with diabetes after PCI. But it could increase the risks of angina and revascularization.

## DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of the policy request. Requests to access the datasets should be directed to the corresponding author LG.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

WW, XW, and LZ made substantial contributions to study design, data collection, data analysis, and manuscript writing. QP and LG made substantial contributions to study design and intellectual direction. JZ and FM made contributions to data collection and analysis. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was funded by the National Natural Science Foundation of China (Grant No. 81670763 and 81471050).

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 September 2021
doi: 10.3389/fendo.2021.728493

# Association of Urinary Sodium Excretion and Left Ventricular Hypertrophy in People With Type 2 Diabetes Mellitus: A Cross-Sectional Study

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***Specialty section:***
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

***Received:*** *21 June 2021*
***Accepted:*** *10 September 2021*
***Published:*** *28 September 2021*

***Citation:***
*Liu J, Yang X, Zhang P, Guo D, Xu B, Huang C, Xue Y and Zhang H (2021) Association of Urinary Sodium Excretion and Left Ventricular Hypertrophy in People With Type 2 Diabetes Mellitus: A Cross-Sectional Study. Front. Endocrinol. 12:728493. doi: 10.3389/fendo.2021.728493*

**Background:** It has been well documented that left ventricular hypertrophy (LVH) is highly associated with the incidence of cardiovascular disease (CVD). Evidence indicated that high sodium intake was closely related with LVH in general population. However, information is not available regarding the association between urinary sodium excretion and LVH in patients with type 2 diabetes mellitus (T2DM). This study aimed to explore the association between urinary sodium excretion and LVH in patients with T2DM.

**Methods:** This cross-sectional analysis included baseline data from 1,556 individuals with T2DM enrolled in the NanFang Prospective Diabetes Study (NFPDS). Urinary sodium excretion levels were measured from 24-hour urine samples of inpatients and morning fasting urine samples of outpatients. Left ventricular dimensions were assessed by echocardiography. The associations between urinary sodium excretion and the risks of cardiovascular events, LVH and left ventricular mass index (LVMI) were examined using linear regression analysis, logistic regression and restricted cubic splines (RCS).

**Results:** Urinary sodium excretion levels were positively associated with cardiometabolic risk factors, including systolic blood pressure, body mass index, waist circumference and LVMI (All $P<0.001$). Odds ratios of the highest quartile of urinary sodium excretion compared with the lowest quartile were 1.80 (95% CI, 1.28-2.54; $P=0.001$) for LVH and 1.77 (95% CI, 1.06-2.94; $P=0.028$) for CVD, after adjusted for demographics, lifestyle risk factors and cardiovascular risk factors. Multivariable-adjusted RCS analysis of the association between urinary sodium excretion and LVMI showed a significant association ($P=0.001$) and lacked evidence of a nonlinear association ($P=0.406$).

**Conclusion:** This study indicated that high urinary sodium excretion was independently associated with increased risk of LVH and CVD in patients with T2DM, suggesting that control of sodium intake may be valuable for the prevention of diabetic cardiovascular complications.

**Keywords: urinary sodium excretion, left ventricular hypertrophy, cardiovascular disease, type 2 diabetes mellitus, metabolism**

## INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in patients with type 2 diabetes mellitus (T2DM) (1, 2). It affects 15% to 41% of middle-aged people with diabetes in western countries (3). In China, 14.6% of patients with T2DM have CVD (4). It has been well documented that left ventricular (LV) hypertrophy (LVH), an increase in LV mass (LVM), is an important predictor of congestive heart failure and cardiovascular mortality (5, 6). Consequently, investigation of the origins of LVH and CVD in patients with T2DM may have implications for developing preventive strategies for cardiovascular complications in diabetes.

It has been proposed that increased sodium intake has a direct relationship with the risk of LVH (7–10). The proposed pathogenetic mechanisms linking sodium intake to LVH include elevated activity of the renin angiotensin system (11), increased reactivity of sympathetic nervous system (12) and an increase in the cardiac volume load (13, 14). Longitudinal studies have indicated that urinary sodium excretion was significant associated with the incidence of LVH in both normotensive (15, 16) and hypertensive individuals (9, 10, 17–19). However, the relationship between urinary sodium excretion levels and LVH in patients with T2DM remains largely unclear. The aim of current study was to explore the association between urinary sodium excretion levels and LVH in patients with T2DM.

## MATERIALS AND METHODS

### Study Participants

This cross-sectional study was derived from the NanFang Prospective Diabetes Study (NFPDS), a prospective cohort study designed to explore the associations of possible risk factors with T2DM complications in Chinese population. Individuals over aged 20 years old with T2DM were recruited from the Nanfang Hospital of Southern Medical University, Guangzhou, China. Individuals with any of the following conditions were excluded: 1) NYHA class III or IV congestive heart failure; 2) treatment with dialysis; 3) severe systemic infections; 4) females who were pregnant, or planning to become pregnant; 5) lack of signed informed consent. The study comprised 1,556 individuals with valid urine collections and LV dimension measurements were included from January 2018 to December 2020. All subjects completed a uniform questionnaire including social-demographic status, lifestyle habits (i.e., smoking status, alcohol consumption) and medical history. Hypertension was defined as mean blood pressure (BP) of 140/90 mmHg or greater or self-reported use of antihypertensive medication. Hyperlipidemia was defined as total cholesterol (TC) ≥ 6.22mmol/L, or low-density lipoprotein cholesterol (LDL-c) ≥ 4.14 mmol/L, or triglycerides (TG) ≥ 2.26 mmol/L. The presence of CVD was defined as a clinical history of coronary heart disease, myocardial infarction, unstable angina requiring hospitalization, heart failure, stroke and peripheral vascular disease.

Written informed consent was obtained from each individual. The study protocol was approved by the Institutional Review Board of Nanfang Hospital of Southern Medical University. The methods were carried out in accordance with the approved guidelines.

### Clinical and Biochemical Measurements

Anthropometric measurements included height, weight, waist circumference and BP. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters. Waist circumference was measured at the level of 1cm above the umbilicus. Three measurements were obtained with a non-stretchable tape, and the mean value was used for analysis. BP was assessed in triplicate using an electronic sphygmomanometer (OMRON Company).

Blood samples of all individuals were collected after 12-hour fasting and tested at the laboratory of Nanfang hospital. Fasting plasma glucose concentrations were measured using the hexokinase method. Glycated hemoglobin (HbA1c) was measured by high performance liquid chromatography. TG, TC, and LDL-c were measured by enzymatic methods using a fully automated biochemical analyzer.

For inpatients, 24-hour urine specimens were measured for urinary sodium excretion, which is generally considered the most reliable estimate of sodium intake (20). A fasting, morning spot urine sample was obtained from outpatients. For outpatients, the 24-hour urinary sodium excretion was estimated using the Kawasaki formula (21, 22). 24-hour and spot urinary sodium concentrations were measured using ion selective electrode method.

### Echocardiography

LV dimensions were assessed by 2-dimensional guided M-mode echocardiography with 2.25- and 3.5-MHz transducers according to American Society of Echocardiography recommendations (23). The echocardiography was performed in all participants by trained radiologist in ultrasound, who was blinded to the individuals' specific medical information/health status. LVM was calculate using the Penn-cube method, a necropsy-validated formula: LVM = 1.04 [(interventricular septal thickness + LV internal dimension + posterior wall thickness)$^3$ - LV internal dimension$^3$] - 13.6 (24). To reduce the confounding effects of body size, LVM was indexed for body height ($m^{2.7}$) as LV mass index (LVMI). The presence of left ventricular hypertrophy (LVH) was defined LVMI >46.7 g/$m^{2.7}$ in women and LVMI >49.2 g/$m^{2.7}$ in men (25). Relative wall thickness was calculated as interventricular septal thickness plus posterior wall thickness divided by LV end-diastolic diameter.

### Statistical Analysis

Continuous variables are presented as means ± standard deviation (SD) or median (interquartile range), and categorical variables are presents as frequencies and percentages. Data that were not normally distributed were logarithmically transformed before analysis. Baseline characteristics of study participants were compared across quartiles of urinary sodium excretion using general linear models (GLM) for continuous variables and $\chi^2$ -test for categorical variables. Multivariable linear regression analyses were used to investigate the association of the following variables with urinary sodium excretion: BMI, waist circumference, systolic BP (SBP), diastolic BP (DBP), duration of diabetes, HbA1c, fasting glucose, TC, TG, LDL-c and LVMI. Multivariable logistic

regression models were used to examine the association of urinary sodium excretion levels with risks of LVH and CVD. Forest plot was used to present the relationship between urinary sodium excretion levels and LVH in different subgroups. Interaction tests were done to assess whether the association between urinary sodium excretion level and LVH differed in subgroups. The relationship between urinary sodium excretion and LVMI was examined with restricted cubic splines (RCS) (26). Analyses were multivariable-adjusted and used 5 knots (located at the 5th, 25th, 50th, 75th, and 95th percentiles). The World Health Organization (WHO) has recommended that sodium intake should be below 2g/day, patients with 2g/day of urinary sodium excretion were chosen as the reference group (27). Statistical analyses were performed with SAS version 9.4 (SAS Institute Inc) and R version 4.1.0. Two-sided values of $P < 0.05$ were considered statistically significant.

## RESULTS

**Table 1** summarizes demographic and clinical characteristics of individuals categorized by quartiles of urinary sodium excretion levels. A total of 1,556 individuals (mean age, 55 years; 64% males) with T2DM were included in this study. Overall, the average duration of diabetes was 8 years and the mean ± SD of the estimated 24-hour urinary sodium excretion was 3.29 ± 1.43 g/day. Males had higher urinary sodium excretion than females. Individuals with higher urinary sodium excretion had longer duration of diabetes and higher proportion of smoking and take antidiabetic medications. Compared to individuals in the lowest quartile of urinary sodium excretion, those in the highest quartile had higher levels of BMI and waist circumference, and lower levels of HbA1c, TC, LDL-c. Of note, the level of LVMI and the proportion of LVH was significantly higher in individuals with highest levels of urinary sodium excretion than those with lowest values after adjusted for age and gender.

Clinical characteristics by gender and quartile of urinary sodium excretion levels are shown in **Table 2**. Both males and females with the highest quartile had higher levels of BMI and waist circumference, and lower levels of HbA1c, TC and LDL-c. Compared with the lowest quartiles of urinary sodium excretion, the level of LVMI and the proportion of LVH were significantly increased in the higher quartiles in males after adjusted for age.

Results of linear regression analysis of urinary sodium excretion on cardiovascular risk factors are shown in **Table 3**.

**TABLE 1** | Characteristics of individuals categorized by quartile of urinary sodium excretion.

| Variables | Estimated 24-hour urinary sodium excretion level | | | | *P*-value |
|---|---|---|---|---|---|
| | Quartile 1 (n = 389) | Quartile 2 (n = 389) | Quartile 3 (n = 389) | Quartile 4 (n = 389) | |
| Urinary sodium (g/day) | 1.72 ± 0.44 | 2.73 ± 0.25 | 3.52 ± 0.26 | 5.19 ± 1.19 | – |
| Outpatient (n, %) | 64 (17) | 136 (35) | 170 (44) | 176 (45) | <0.001 |
| Age (years) | 55 ± 12 | 56 ± 11 | 56 ± 11 | 54 ± 11 | 0.137 |
| Gender (Male n, %) | 217 (56) | 237 (61) | 257 (66) | 281 (72) | <0.001 |
| Smoking (n, %) | 160 (41) | 162 (42) | 193 (50) | 199 (51) | 0.004 |
| Alcohol use (n, %) | 118 (30) | 130 (34) | 152 (39) | 130 (34) | 0.066 |
| Duration of diabetes (years) | 4 (1-11) | 7 (2-13)‡ | 8 (3-13)‡ | 7 (3-13) ‡ | <0.001 |
| BMI (kg/m$^2$) | 24.0 ± 3.6 | 24.3 ± 3.2 | 24.6 ± 3.3† | 25.5 ± 3.7‡ | <0.001 |
| Waist circumference (cm) | 86.6 ± 9.7 | 87.6 ± 8.9 | 88.8 ± 8.9‡ | 90.6 ± 9.9‡ | <0.001 |
| SBP (mmHg) | 127 ± 18 | 127 ± 19 | 128 ± 18 | 129 ± 18† | 0.126 |
| DBP (mmHg) | 77 ± 11 | 77 ± 10 | 76 ± 11 | 78 ± 11 | 0.053 |
| Hypertension (n, %) | 155 (40) | 136 (35) | 130 (33) | 133 (34) | 0.234 |
| Hyperlipidemia (n, %) | 233 (60) | 232 (60) | 235 (60) | 219 (56) | 0.640 |
| CVD (n, %) | 38 (9.8) | 54 (14) | 46 (12) | 57 (15)† | 0.163 |
| Antidiabetic medication (n, %) | 245 (63) | 286 (74) | 313 (81) | 311 (80) | <0.001 |
| RAS blocking agents (n, %) | 66 (17) | 57 (15) | 72 (19) | 58 (15) | 0.416 |
| Diuretics (n, %) | 18 (4.6) | 8 (2.1) | 8 (2.1) | 11 (2.8) | 0.106 |
| Statin (n, %) | 50 (13) | 53 (14) | 58 (15) | 54 (14) | 0.872 |
| Aspirin (n, %) | 36 (9.3) | 29 (7.5) | 38 (9.8) | 38 (9.8) | 0.635 |
| HbA1c (%) | 10.1 ± 2.7 | 9.4 ± 2.5‡ | 8.9 ± 2.4‡ | 8.9 ± 2.2‡ | <0.001 |
| Fasting glucose (mmol/L) | 7.71 (5.56-10.37) | 7.42 (5.59-10.33) | 7.43 (5.55-9.85) | 7.44 (5.78-9.83) | 0.236 |
| TG (mmol/L) | 1.38 (0.96-2.32) | 1.52 (1.03-2.42) | 1.48 (1.03-2.36) | 1.58 (1.05-2.56) | 0.485 |
| TC (mmol/L) | 5.06 ± 1.51 | 5.14 ± 1.30 | 4.88 ± 1.31 | 4.97 ± 1.29 | 0.044 |
| LDL-c (mmol/L) | 3.23 ± 0.99 | 3.32 ± 0.97 | 3.14 ± 0.96 | 3.15 ± 0.84 | 0.025 |
| LVM (g) | 155.0 ± 40.7 | 163.5 ± 42.7† | 167.1 ± 44.7‡ | 171.2 ± 47.9‡ | <0.001* |
| LVMI (g/m$^{2.7}$) | 42.6 ± 11.4 | 44.7 ± 11.7† | 45.2 ± 12.3‡ | 45.1 ± 13.0‡ | 0.001* |
| RWT | 0.50 ± 0.09 | 0.50 ± 0.09 | 0.50 ± 0.09 | 0.50 ± 0.09 | 0.986 |
| LVH (n, %) | 111 (29) | 137 (35) † | 128 (33) | 137 (35)‡ | 0.004* |

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CVD, cardiovascular disease; RAS, renin-angiotensin system; HbA1c, glycated hemoglobin; TG, triglycerides; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; LVM, left ventricular mass; LVMI, left ventricular mass index; RWT, relative wall thickness; LVH, left ventricular hypertrophy.*

*†P < 0.05 compared with Quartile 1 of urinary sodium excretion.*

*‡P < 0.01 compared with Quartile 1 of urinary sodium excretion.*

**Adjusted for age and gender.*

**TABLE 2 |** Characteristics of individuals categorized by quartile of urinary sodium excretion and gender.

| Variables | Males (n = 992) | | | | *P*-value | Females (n = 564) | | | | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Estimated 24-hour urinary sodium excretion level | | | | | Estimated 24-hour urinary sodium excretion level | | | | |
| | Quartile 1 (n = 248) | Quartile 2 (n = 248) | Quartile 3 (n = 248) | Quartile 4 (n = 248) | | Quartile 1 (n = 141) | Quartile 2 (n = 141) | Quartile 3 (n = 141) | Quartile 4 (n = 141) | |
| Urinary sodium (g/day) | 1.83 ± 0.47 | 2.86 ± 0.25 | 3.67 ± 0.29 | 5.40 ± 1.26 | – | 1.57 ± 0.36 | 2.53 ± 0.24 | 3.29 ± 0.22 | 4.77 ± 1.03 | – |
| Outpatient (n, %) | 51 (21) | 88 (36) | 115 (46) | 100 (40) | <0.001 | 18 (13) | 45 (32) | 58 (41) | 71 (50) | <0.001 |
| Age (years) | 51 ± 11 | 54 ± 11‡ | 54 ± 11† | 53 ± 11 | 0.020 | 59 ± 11 | 59 ± 9 | 59 ± 10 | 57 ± 9 | 0.385 |
| Smoking (n, %) | 178 (72) | 168 (68) | 181 (73) | 170 (69) | 0.484 | 4 (2.8) | 0 (0.0) | 7 (5.0) | 6 (4.3) | 0.070 |
| Alcohol use (n, %) | 122 (49) | 122 (49) | 134 (54) | 109 (44) | 0.154 | 8 (5.7) | 13 (9.2) | 12 (8.6) | 10 (7.1) | 0.680 |
| Duration of diabetes (years) | 3 (1-8) | 6 (2-12)‡ | 8 (2-12)‡ | 7 (3-13)‡ | <0.001 | 6 (2-12) | 9 (3-14) | 9 (4-13) | 8 (3-15) | 0.521 |
| BMI (kg/m$^2$) | 23.9 ± 3.7 | 24.2 ± 2.9 | 24.7 ± 3.6† | 25.6 ± 3.6‡ | <0.001 | 23.6 ± 3.5 | 24.9 ± 3.2‡ | 24.6 ± 3.2† | 25.1 ± 3.9‡ | 0.002 |
| Waist circumference (cm) | 87.5 ± 10.0 | 88.0 ± 8.4 | 89.6 ± 9.6 | 91.6 ± 9.6 | <0.001 | 85.1 ± 9.2 | 87.4 ± 8.8 | 87.6 ± 8.5 | 87.9 ± 10.1 | 0.044 |
| SBP (mmHg) | 123 ± 18 | 126 ± 19 | 127 ± 18† | 129 ± 16‡ | 0.004 | 129 ± 18 | 131 ± 18 | 129 ± 18 | 131 ± 22 | 0.790 |
| DBP (mmHg) | 78 ± 12 | 77 ± 10 | 78 ± 11 | 80 ± 10† | 0.020 | 76 ± 11 | 77 ± 10 | 74 ± 10 | 76 ± 12 | 0.105 |
| Hypertension (n, %) | 82 (33) | 68 (27) | 79 (32) | 83 (27) | 0.448 | 67 (48) | 63 (45) | 59 (42) | 53 (38) | 0.377 |
| Hyperlipidemia (n, %) | 150 (61) | 147 (59) | 143 (58) | 145 (59) | 0.931 | 80 (57) | 87 (62) | 90 (64) | 77 (55) | 0.362 |
| CVD (n, %) | 19 (7.7) | 26 (11) | 32 (13) | 35 (14)† | 0.110 | 20 (14) | 23 (16) | 23 (16) | 17 (12) | 0.706 |
| Antidiabetic medication (n, %) | 140 (57) | 168 (68) | 197 (79) | 194 (78) | <0.001 | 101 (72) | 115 (82) | 123 (87) | 117 (83) | 0.008 |
| RAS blocking agents (n, %) | 33 (13) | 30 (12) | 45 (18) | 37 (15) | 0.251 | 31 (22) | 28 (20) | 30 (21) | 19 (14) | 0.249 |
| Diuretics (n, %) | 8 (3.2) | 5 (2.0) | 5 (2.0) | 6 (2.4) | 0.795 | 8 (5.7) | 5 (3.6) | 4 (2.8) | 4 (2.8) | 0.547 |
| Statin (n, %) | 22 (8.9) | 23 (9.3) | 41 (17) | 33 (13) | 0.026 | 29 (21) | 21 (15) | 31 (22) | 15 (11) | 0.041 |
| Aspirin (n, %) | 16 (6.5) | 20 (8.1) | 22 (8.9) | 24 (9.7) | 0.602 | 19 (14) | 7 (5.0) | 20 (14) | 13 (9.2) | 0.041 |
| HbA1c (%) | 10.5 ± 2.7 | 9.4 ± 2.7‡ | 8.8 ± 2.3‡ | 9.0 ± 2.4‡ | <0.001 | 9.7 ± 2.6 | 9.1 ± 2.4 | 8.9 ± 2.2‡ | 8.5 ± 2.1‡ | <0.001 |
| Fasting glucose (mmol/L) | 8.23 (5.90-11.10) | 7.67 (5.72-10.95) | 7.18 (5.58-9.44)‡ | 7.40 (5.65-9.82)† | 0.002 | 7.03 (5.19-9.79) | 7.14 (5.51-9.20) | 7.35 (5.61-9.94) | 7.69 (5.74-10.31) | 0.864 |
| TG (mmol/L) | 1.39 (0.92-2.58) | 1.49 (1.03-2.53) | 1.44 (0.97-2.39) | 1.61 (1.09-2.90) | 0.342 | 1.30 (0.97-1.99) | 1.63 (1.09-2.30) | 1.51 (1.19-2.19) | 1.54 (1.02-2.15) | 0.254 |
| TC (mmol/L) | 5.16 ± 1.66 | 5.08 ± 1.22 | 4.82 ± 1.43‡ | 4.86 ± 1.31† | 0.019 | 4.78 ± 1.16 | 5.32 ± 1.32‡ | 4.99 ± 1.26 | 5.17 ± 1.12‡ | 0.002 |
| LDL-c (mmol/L) | 3.28 ± 1.02 | 3.30 ± 0.91 | 3.09 ± 0.99† | 3.09 ± 0.81† | 0.009 | 3.05 ± 094 | 3.44 ± 0.99‡ | 3.20 ± 0.94 | 3.28 ± 0.90† | 0.005 |
| LVM (g) | 159.7 ± 44.2 | 166.7 ± 46.1 | 177.6 ± 46.1‡ | 176.5 ± 47.8‡ | <0.001* | 148.2 ± 33.8 | 155.2 ± 35.5 | 153.4 ± 39.5 | 158.5 ± 43.1† | 0.066* |
| LVMI (g/m$^{2.7}$) | 40.1 ± 11.6 | 42.1 ± 11.6 | 44.8 ± 12.1‡ | 44.1 ± 12.6‡ | <0.001* | 45.5 ± 10.8 | 47.9 ± 10.5‡ | 47.2 ± 12.0 | 48.1 ± 13.4† | 0.094* |
| RWT | 0.48 ± 0.09 | 0.49 ± 0.08 | 0.50 ± 0.09‡ | 0.50 ± 0.08‡ | 0.133 | 0.51 ± 0.10 | 0.51 ± 0.09 | 0.52 ± 0.10 | 0.50 ± 0.10 | 0.330 |
| LVH (n, %) | 46 (19) | 57 (23) | 69 (28)† | 72 (29)† | 0.005* | 55 (39) | 79 (56) | 62 (44) | 73 (52) | 0.073* |

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CVD, cardiovascular disease; RAS, renin-angiotensin system; HbA1c, glycated hemoglobin; TG, triglycerides; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; LVM, left ventricular mass; LVMI, left ventricular mass index; RWT, relative wall thickness; LVH, left ventricular hypertrophy.*

*†P < 0.05 compared with Quartile 1 of urinary sodium excretion.*

*‡P < 0.01 compared with Quartile 1 of urinary sodium excretion.*

**Adjusted for age.*

Urinary sodium excretion levels were significantly correlated with BMI, SBP, DBP, waist circumference, duration of diabetes, LVMI and HbA1c after adjusting age, gender, smoking, alcohol consumption and use of renin-angiotensin system (RAS) blocking agents, diuretics, statin and antidiabetic medication (all $P<0.05$). There were not significant associations between urinary sodium excretion with TG, TC and fasting glucose after adjusted for demographics, lifestyle risk factors and current medication use.

The odds ratios (ORs) with 95% confidence interval (CI) for LVH and CVD according to changes in urinary sodium excretion levels are showed in **Table 4**. Compared with individuals in quartile 1, the risk of LVH was significantly increased in quartile 2 [OR (95% CI), 1.59 (1.14-2.21; $P=0.007$], quartile 3 [OR (95% CI), 1.52 (1.08-2.11); $P=0.017$] and quartile 4 [OR (95% CI), 1.92 (1.37-2.68); $P<0.001$] after adjusted for age, gender, smoking status, alcohol consumption, history of hypertension and hyperlipidemia, and use of antihypertension and antihyperlipidemic medication. These relationships also remained significant when further adjusting for HbA1c and use of antidiabetic medication. Furthermore, individuals in the highest quartile had significantly higher risks of CVD than those in the lowest quartile, even after adjusting for demographics, lifestyle risk factors and cardiovascular risk factors (all $P<0.05$).

In addition, the subgroup analysis was performed to explore the association between LVH and urinary sodium excretion levels among different populations, according to the following variables: outpatients/inpatients, age (<60 years/≥60 years), gender (male/female), hypertension (yes/no), duration of diabetes (≤5 years/>5 years) (**Figure 1**). The association of

**TABLE 3 |** Clinical correlation of urinary sodium excretion levels with clinical and biochemical variables.

| Variables | Regression coefficient β | Standard error | *P*-value | Multiple adjusted *P*-value[§] |
|---|---|---|---|---|
| BMI (kg/m$^2$) | 0.549 | 0.088 | <0.001 | <0.001 |
| Waist circumference (cm) | 1.517 | 0.243 | <0.001 | <0.001 |
| SBP (mmHg) | 1.737 | 0.464 | <0.001 | <0.001 |
| DBP (mmHg) | 0.811 | 0.275 | 0.003 | 0.023 |
| Duration of diabetes (year) | 0.689 | 0.175 | <0.001 | 0.002 |
| HbA1c (%) | -0.420 | 0.063 | <0.001 | <0.001 |
| Fasting glucose (mmol/L) | 0.002 | 0.011 | 0.894 | 0.361 |
| TG (mmol/L) | 0.021 | 0.017 | 0.216 | 0.429 |
| TC (mmol/L) | -0.072 | 0.034 | 0.037 | 0.523 |
| LDL-c (mmol/L) | -0.043 | 0.024 | 0.074 | 0.050 |
| LVMI (g/m$^{2.7}$) | 0.877 | 0.308 | 0.005 | <0.001 |

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; TG, triglycerides; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; LVMI, left ventricular mass index; RAS, renin-angiotensin system.*

*[§]Adjusted for age, gender, smoking, alcohol consumption, RAS blocking agents, diuretics, statin antidiabetic medication and serum creatinine.*

urinary sodium excretion with the LVH was significant in the individuals aged under 60 years old (*P* for interaction=0.015). However, there were no significant differences in the associations between urinary sodium excretion and LVH among another subgroups (all *P* for interaction>0.05).

Multivariable-adjusted RCS analyses demonstrated that urinary sodium excretion was significantly associated with LVMI in overall patients (*P*=0.001) and males (*P*=0.013). However, the non-linear associations of urinary sodium excretion and LVMI were not significant (all *P*>0.05) (**Figure 2**). No significant relationship between urinary sodium excretion and LVMI was found in females (*P*=0.057).

## DISCUSSION

It has been documented that dietary sodium intake plays a role in modulating myocardial mass and cardiac structure (28, 29). Evidence from experimental studies indicated that dietary sodium loading promotes cellular hypertrophy in both arterial smooth muscle and cardiac myocytes, as well as interstitial fibrosis in the left ventricle and myocardial arteries (30, 31). Proposed mechanisms including fluid homeostasis, neurohormonal regulation and several novel pathways (29). In the present study, our data indicated that diabetic patients with high urinary sodium excretion had higher level of LVMI.

**TABLE 4 |** Odds ratios (ORs) of LVH and CVD according to urinary sodium excretion levels.

| Variables | LVH | | | CVD | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | *P*-value | OR | 95% CI | *P*-value |
| Crude model | | | | | | |
| urinary sodium (g/day) | | | | | | |
| (Quartile 2 *vs*. Quartile 1) | 1.36 | 1.01-1.84 | 0.046 | 1.49 | 0.96-2.31 | 0.077 |
| (Quartile 3 *vs*. Quartile 1) | 1.23 | 0.91-1.67 | 0.187 | 1.24 | 0.79-1.95 | 0.357 |
| (Quartile 4 *vs*. Quartile 1) | 1.36 | 1.01-1.84 | 0.046 | 1.59 | 1.02-2.45 | 0.039 |
| Model 1 | | | | | | |
| urinary sodium (g/day) | | | | | | |
| (Quartile 2 *vs*. Quartile 1) | 1.43 | 1.04-1.97 | 0.029 | 1.49 | 0.94-2.36 | 0.088 |
| (Quartile 3 *vs*. Quartile 1) | 1.33 | 0.96-1.85 | 0.082 | 1.18 | 0.73-1.90 | 0.504 |
| (Quartile 4 *vs*. Quartile 1) | 1.74 | 1.26-2.41 | 0.001 | 1.79 | 1.13-2.84 | 0.014 |
| Model 2 | | | | | | |
| urinary sodium (g/day) | | | | | | |
| (Quartile 2 *vs*. Quartile 1) | 1.59 | 1.14-2.21 | 0.007 | 1.65 | 1.01-2.69 | 0.047 |
| (Quartile 3 *vs*. Quartile 1) | 1.51 | 1.08-2.11 | 0.017 | 1.15 | 0.69-1.93 | 0.595 |
| (Quartile 4 *vs*. Quartile 1) | 1.92 | 1.37-2.68 | <0.001 | 1.93 | 1.17-3.17 | 0.010 |
| Model 3 | | | | | | |
| urinary sodium (g/day) | | | | | | |
| (Quartile 2 *vs*. Quartile 1) | 1.53 | 1.09-2.14 | 0.013 | 1.57 | 0.96-2.58 | 0.073 |
| (Quartile 3 *vs*. Quartile 1) | 1.41 | 1.00-2.00 | 0.048 | 1.07 | 0.64-1.81 | 0.794 |
| (Quartile 4 *vs*. Quartile 1) | 1.80 | 1.28-2.54 | 0.001 | 1.77 | 1.06-2.94 | 0.028 |

*LVH, left ventricular hypertrophy; CVD, cardiovascular disease; DBP, diastolic blood pressure; RAS, renin-angiotensin system; HbA1c, glycated hemoglobin.*

*Crude model: without adjustment.*

*Model 1: adjusted for age, gender, smoking and alcohol consumption.*

*Model 2: adjusted for model 1+ hypertension, RAS blocking agents, diuretics, hyperlipidemia and statin.*

*Model 3: adjusted for model 2+ HbA1c and antidiabetic medication.*

| Subgroup | No. of subjects | LVH (No. of events%) | Odds Ratio (95% CI) | *P*-Value | *P*-interaction |
|---|---|---|---|---|---|
| Overall | | | | | |
| Quartile 1 | 389 | 111 (29) | Reference | - | |
| Quartile 2 | 389 | 137 (35) | 1.53 (1.09-2.14) | 0.013 | |
| Quartile 3 | 389 | 128 (33) | 1.41 (1.00-2.00) | 0.048 | |
| Quartile 4 | 389 | 137 (35) | 1.80 (1.27-2.54) | 0.001 | |
| Outpatients | | | | | 0.719 |
| Quartile 1 | 136 | 42 (31) | Reference | - | |
| Quartile 2 | 137 | 44 (32) | 1.08 (0.61-1.92) | 0.785 | |
| Quartile 3 | 136 | 50 (37) | 1.69 (0.96-3.00) | 0.071 | |
| Quartile 4 | 137 | 43 (31) | 1.29 (0.73-2.29) | 0.382 | |
| Inpatients | | | | | |
| Quartile 1 | 252 | 72 (29) | Reference | - | |
| Quartile 2 | 253 | 87 (34) | 1.44 (0.95-2.19) | 0.089 | |
| Quartile 3 | 251 | 78 (31) | 1.13 (0.73-1.75) | 0.578 | |
| Quartile 4 | 254 | 97 (38) | 1.92 (1.26-2.95) | 0.003 | |
| Age | | | | | |
| <60 years old | | | | | 0.015 |
| Quartile 1 | 250 | 43 (17) | Reference | - | |
| Quartile 2 | 251 | 66 (26) | 2.10 (1.31-3.38) | 0.002 | |
| Quartile 3 | 251 | 72 (29) | 2.32 (1.44-3.74) | 0.001 | |
| Quartile 4 | 251 | 78 (31) | 2.63 (1.65-4.20) | <0.001 | |
| ≥60 years old | | | | | |
| Quartile 1 | 138 | 68 (49) | Reference | - | |
| Quartile 2 | 138 | 71 (52) | 1.17 (0.70-1.96) | 0.552 | |
| Quartile 3 | 138 | 53 (38) | 0.75 (0.44-1.27) | 0.281 | |
| Quartile 4 | 139 | 62 (45) | 1.12 (0.65-1.92) | 0.678 | |
| Gender | | | | | |
| Male | | | | | 0.718 |
| Quartile 1 | 248 | 46 (19) | Reference | - | |
| Quartile 2 | 248 | 57 (23) | 1.26 (0.79-2.01) | 0.330 | |
| Quartile 3 | 248 | 69 (28) | 1.54 (0.97-2.45) | 0.070 | |
| Quartile 4 | 248 | 72 (29) | 1.70 (1.08-2.67) | 0.023 | |
| Female | | | | | |
| Quartile 1 | 141 | 55 (39) | Reference | - | |
| Quartile 2 | 141 | 79 (56) | 2.34 (1.39-3.95) | 0.001 | |
| Quartile 3 | 141 | 62 (44) | 1.33 (0.78-2.25) | 0.291 | |
| Quartile 4 | 141 | 75 (52) | 2.14 (1.26-3.64) | 0.005 | |
| Hypertension | | | | | |
| Yes | | | | | 0.772 |
| Quartile 1 | 138 | 57 (41) | Reference | - | |
| Quartile 2 | 137 | 81 (59) | 2.07 (1.23-3.51) | 0.007 | |
| Quartile 3 | 140 | 70 (50) | 1.41 (0.83-2.40) | 0.206 | |
| Quartile 4 | 139 | 75 (54) | 2.21 (1.30-3.77) | 0.004 | |
| No | | | | | |
| Quartile 1 | 250 | 48 (19) | Reference | - | |
| Quartile 2 | 251 | 58 (23) | 1.33 (0.85-2.09) | 0.212 | |
| Quartile 3 | 250 | 60 (24) | 1.40 (0.89-2.21) | 0.145 | |
| Quartile 4 | 251 | 64 (26) | 1.65 (1.05-2.59) | 0.032 | |
| Duration of diabetes | | | | | |
| ≤5 years | | | | | 0.649 |
| Quartile 1 | 169 | 41 (24) | Reference | - | |
| Quartile 2 | 170 | 52 (31) | 1.39 (0.81-2.38) | 0.230 | |
| Quartile 3 | 169 | 43 (25) | 1.23 (0.70-2.14) | 0.474 | |
| Quartile 4 | 170 | 51 (30) | 1.61 (0.93-2.79) | 0.091 | |
| >5 years | | | | | |
| Quartile 1 | 219 | 73 (33) | Reference | - | |
| Quartile 2 | 220 | 88 (40) | 1.64 (1.06-2.52) | 0.026 | |
| Quartile 3 | 219 | 81 (37) | 1.49 (0.96-2.32) | 0.077 | |
| Quartile 4 | 220 | 84 (38) | 1.83 (1.18-2.84) | 0.007 | |

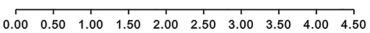


**FIGURE 1** | Odds ratios of LVH according to urinary sodium excretion levels in different subgroups. Adjusted for age, gender, smoking, alcohol consumption, hypertension, use of RAS blocking agents, use of diuretics, history of hyperlipidemia, use of statin, HbA1c and use of antidiabetic medication.

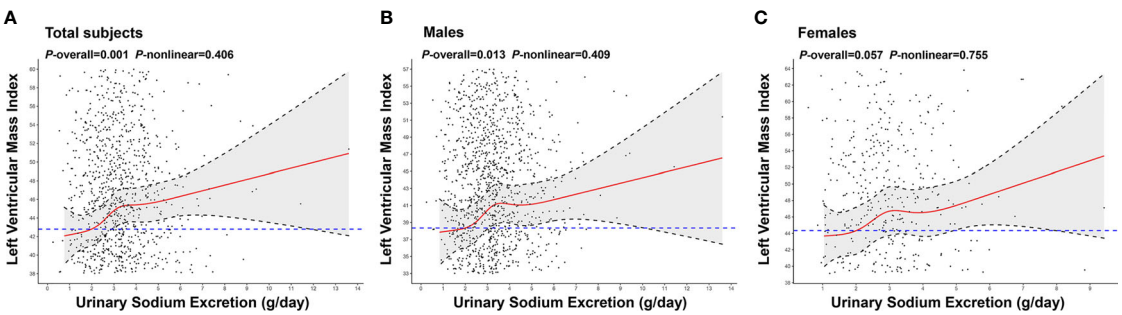


**FIGURE 2** | Association of urinary sodium excretion with LVMI. The restricted cubic spline (RCS) regression was used to analyze the relationship of urinary sodium excretion(g/day) with left ventricular mass index (LVMI) after adjusting for age, gender, smoking, alcohol consumption, hypertension, use of RAS blocking agents, use of diuretics, history of hyperlipidemia, use of statin, HbA1c and use of antidiabetic medication in total individuals **(A)**, males **(B)**, females **(C)**. Urinary sodium excretion was coded using an RCS function with five knots located at the 5th, 25th, 50th, 75th, 95th percentiles of the distribution of urinary sodium excretion. Y-axis represents the predicted value of LVMI. Individuals with 2g/day of urinary sodium excretion was the reference standard, indicated by the blue dashed lines. Black dots: "observed" data points. Black dashed lines: 95 percent confidence interval.

Additionally, we found that a J-shaped association between urinary sodium excretion and LVMI, instead of the direct linear correlation. Urinary sodium excretion was positively associated with cardiometabolic risk factors, including SBP, BMI and waist circumference. Of note, we provided the novel evidence that diabetic patients with higher urinary sodium excretion had a significantly increased risk of LVH and CVD independent of demographic factors, lifestyle risk factors and cardiovascular risk factors. These findings suggest that moderate sodium reduction among patients with T2DM may lower the risks of LVH and CVD.

It has been established that sodium intake influences the cardiovascular system profoundly in general population (14–18). Several studies have reported that increased urinary sodium excretion was significantly associated with greater LVM in healthy young adults and individuals with hypertension (8, 15, 19). In contrast, recent study indicated that dietary sodium restriction was associated with the decreased of LVM in hypertensive patients, even in those with proper BP control (32).However, information regarding the association of urinary sodium excretion with increased LVMI in individuals with T2DM is limited. In the present study, we found a positively association between urinary sodium excretion and LVMI in patients with T2DM. Of note, our data demonstrated that higher urinary sodium excretion concentrations were independently associated with increased risk of LVH in patients with T2DM. Our findings were consistent with prior studies in patients with essential hypertension (9, 10, 14). Additionally, several epidemiological studies reported that high sodium intake is strongly and independently associated with an increased risk of CVD and all-cause mortality (33, 34). These studies are congruent with our findings that high urinary sodium excretion was significantly associated with an increased risk of CVD in patients with T2DM. These findings have implications for monitoring sodium intake among T2DM patients.

In addition, several studies reported that the association between sodium consumption and CVD or mortality is J-shaped in general population (35–37). Evidence indicated that individuals with estimated sodium intake between 3 g/day and 6 g/day was associated with a lower risk of cardiovascular morbidity and mortality than those either with a higher or lower estimated level of intake (35, 36). In the present study, a possible J-shaped association was found between sodium excretion and LVMI in patients with T2DM. In addition, our data indicated that individuals with 2 g/day of urinary sodium excretion were associated with lower levels of LVMI, supporting that the sodium intake limitation of 2 g/day recommended by WHO and American Diabetes Association may also be suitable for patients with T2DM (27, 38).

Extensive observations have indicated that obesity and hypertension are the most important determinants of LVH in the general population (39–41). Elevated BP plays a driving role in the development of LVH through chronic hemodynamic overload and increased central pressure (42). Our data demonstrated that urinary sodium excretion was significantly and positively correlated with SBP after adjusted for demographics and lifestyle risk factors. Furthermore, our data showed that the association of sodium intake with LVH remained significant after adjusted for the history of hypertension. These data indicated that other mechanisms may play a role in the effect of dietary sodium on LVH in individuals with T2DM, which was consistent with previous findings of a positive association between dietary sodium intake and CVD independent of blood pressure (43, 44). Some possible mechanisms independent of blood pressure include circulating fluid volume (13), insulin resistance (45, 46) and obesity (41). Evidence from epidemiological studies have indicated that adiposity is one of the major predictors of LVH (41). Cross-sectional studies have demonstrated that high sodium intake is independently associated with elevated risk of obesity and central obesity (47, 48). In consist, our data also indicated that urinary sodium excretion was positively correlated with BMI and waist circumference. These results suggest that high sodium intake was significant associated with CVD risks, and may be an important risk factor for LVH and CVD risks among individuals with T2DM.

To our knowledge, this is the first analysis of the association between urinary sodium excretion levels and LVH in patients with T2DM, which can provide novel evidence for cardiovascular risk management in patients with T2DM. However, several limitations of this study must be considered. First, this was a cross-sectional study. Thus, it was not possible to determine a causal relationship between urinary sodium excretion and the development of LVH. Second, we used spot urine samples instead of 24-hour urine collection for the estimation of sodium excretion in outpatients. However, the Kawasaki formula is recognized as one of the most valid and least biased methods of estimating 24-h urinary sodium excretion (49). Third, it was not possible to determine clinical outcomes among individuals with urinary sodium excretion less than 2g/day due to the very small sample sizes among these subgroups.

## CONCLUSIONS

In summary, our study provides the clinical evidence revealing that high urinary sodium excretion levels were independently associated with increased risks of LVH and CVD in patients with T2DM. These findings suggests that it is critical to control sodium intake for the prevention of CVD in patients with T2DM. Further prospective studies need to determine the associations of urinary sodium excretion with cardiovascular complications in patients with T2DM.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Nanfang Hospital of Southern Medical University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JL, YX, and HZ contributed to conception and design of the study. YX and HZ supervised the study. DG, BX, and CH organized the database. JL, XY, and PZ performed the statistical analysis. JL wrote the first draft of the manuscript. JL, XY, PZ, and DG wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was supported by the National Key Research and Development Project (No.2018YFA0800404), Natural Science Foundation and Key-Area Research and Development Program of Guangdong Province (Nos.2018B030311031 and 2019B020227004), the National Natural Science Foundation of China (No.81970736), and Key-Area Clinical Research Program of Southern Medical University (No.LC2019ZD010). HZ was partially supported by Outstanding Youths Development Scheme of Nanfang Hospital, Southern Medical University (No. 2017J005).

## ACKNOWLEDGMENTS

The authors are grateful to all participants for their time and effort.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 02 November 2021
doi: 10.3389/fendo.2021.753039

# Ketogenic Diets and Cardio-Metabolic Diseases

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While the prevalence of cardio-metabolic diseases (CMDs) has become a worldwide epidemic, much attention is paid to managing CMDs effectively. A ketogenic diet (KD) constitutes a high-fat and low-carbohydrate diet with appropriate protein content and calories. KD has drawn the interests of clinicians and scientists regarding its application in the management of metabolic diseases and related disorders; thus, the current review aimed to examine the evidences surrounding KD and the CMDs to draw the clinical implications. Overall, KD appears to play a significant role in the therapy of various CMDs, which is manifested by the effects of KDs on cardio-metabolic outcomes. KD therapy is generally promising in obesity, heart failure, and hypertension, though different voices still exist. In diabetes and dyslipidemia, the performance of KD remains controversial. As for cardiovascular complications of metabolic diseases, current evidence suggests that KD is generally protective to obese related cardiovascular disease (CVD), while remaining contradictory to diabetes and other metabolic disorder related CVDs. Various factors might account for the controversies, including genetic background, duration of therapy, food composition, quality, and sources of KDs. Therefore, it's crucial to perform more rigorous researches to focus on clinical safety and appropriate treatment duration and plan of KDs.

**Keywords: ketogenic diets, metabolic diseases, obesity, diabetes mellitus, cardiovascular complications**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

***Received:*** *04 August 2021*
***Accepted:*** *13 October 2021*
***Published:*** *02 November 2021*

***Citation:***
*Zhang W, Guo X, Chen L, Chen T, Yu J, Wu C and Zheng J (2021) Ketogenic Diets and Cardio-Metabolic Diseases. Front. Endocrinol. 12:753039. doi: 10.3389/fendo.2021.753039*

## INTRODUCTION

Cardio-metabolic diseases (CMDs) have become a worldwide epidemic, as demonstrated by an increased prevalence of obesity, diabetes mellitus (DM), metabolic syndrome, cardiovascular disease (CVD), and chronic kidney disease (CKD), and culpable to a significant global financial burden (1). CVDs comprise a wide range of diseases detrimental to cardiac and vascular function (2, 3). To decrease cardiovascular (CV) mortality and related economic burden, it's important to reduce CV risk factors and employ appropriate therapy in developed countries (4). Various established risk factors such as age, gender, genetic heritage, smoking, high blood pressure, poor eating habits, type 2 diabetes mellitus, dyslipidemia and obesity had been accounted for the development and progression of cardiovascular diseases (CVD).

Dietary factors that profoundly influence human health are linked to cardiovascular disease and other chronic metabolic conditions such as obesity and type 2 diabetes (5); thus, dietary interventions have become an essential component in managing cardiovascular risks (6).

A ketogenic diet (KD) is a high-fat, low-carbohydrate diet with appropriate protein content and calories (7). A traditional KD consists of a 4:1 ratio of fats to carbohydrates and protein, with 90% of the calories from fat, 8% from protein, and only 2% from carbohydrate (8). In recent years, to improve compliance and imitate the effects of classic KD, alternative protocols with different formulations of KD have been proposed (9), including 3:1 KD, 2:1 KD, 1:1 KD, the modified Atkins diet (MAD), the medium-chain triglyceride ketogenic diet (MCTKD), the low glycemic index treatment (LGIT) (10, 11) (**Table 1**). With the implementation of KD therapy, a drastic decrease in dietary carbohydrates reduces glucose utilization. In the human body, KD treatment could imitate the metabolic changes of fasting. In addition, some of the beneficial effects of KDs could be attributable to the production of ketones, e.g., β-hydroxybutyrate (BHB), acetoacetate, and acetone in the liver (12).

KD was firstly used as a dietary treatment for epilepsy in the 1920s (8). However, with the progress in antiepileptic drugs (AEDs) development and application, the clinical use of KDs in epilepsy has dramatically decreased. Interestingly, about one-third of patients receiving epilepsy treatment couldn't gain significant relief from the disease, and the KD regained scientists' attention and became a choice for application in drug-resistant or difficult-to-treat epilepsies (13, 14). Apart from neurological diseases, KD has recently shown promising efficacy in a wide variety of diseases, including various cancers and metabolic diseases. Ovarian cancer, for instance, may reveal significantly better clinical outcomes under KD intervention, as revealed by a systematic review of randomized controlled trials (15). Furthermore, KD intervention has been found to inhibit tumor progression or mitigate cachexia symptoms (16).

KD has drawn more interest and gradually become an elective dietary intervention choice for CMDs (17). Moreover, it is significantly effective in mitigating various metabolic diseases, including obesity (18, 19), glucose transporter type 1 deficiency syndrome (GLUT1DS) (20), and pyruvate dehydrogenase (PDH) deficiency (21). Meanwhile, due to the uncertainty of dietary interventions, different voices have also occurred regarding the safety issues and drawbacks of employing KD. The clarity on how KD influences cardiovascular and metabolic diseases remains unclear. Therefore, the current review highlighted pertinent information concerning KD and CMDs (**Figure 1**). Qualified studies reflecting the advantages or disadvantages of KD in CMDs were all equally considered and incorporated without bias.

## EFFECTS OF KETOGENIC DIETS ON METABOLIC DISEASES

Various metabolic diseases have been recognized as cardiovascular risk factors, including diabetes mellitus, obesity and other metabolic diseases (22). The disrupted glucose and lipid metabolism lead to abnormal oxidative stress, inflammatory, vasoactive factors, cardiac and vascular function, and finally elevate the risk of CVD (23). KD can regulate metabolic profiles and may consequently regulate the risk of CVD.

As for the management of obesity, most studies indicated that KDs were efficient at weight loss (24, 25), especially in reducing food intake in humans and elevating energy consumption in animals (18, 19). Studies concerning the effects of KDs on body composition changes reported that KD-fed mice had an increased fat mass percentage than regular-chow fed mice (26, 27), and reduced (28) or no differences (26) in the percentage of lean body mass between diets; whereas in humans, weight loss affects both fat and lean mass (29). Obesity is commonly connected with insulin resistance and type 2 diabetes mellitus (T2DM), in which systemic ketone body metabolism is perturbed (30, 31). As such, the weight-loss effect of KDs is expected to be beneficial for diabetes; although it remains disputable that KD induces insulin resistance. There are studies indicating that KD led to ameliorated glucose homeostasis and reduced antidiabetic medications in T2DM subjects (32, 33), even with reduced baseline insulin levels and elevated insulin sensitivity in diabetic rats (34, 35). Moreover, the study by Farrés et al. (33) appeared to offer a plausible explanation of how KDs bring about the anti-diabetic effect, which might be attributable to the anti-inflammatory effect of KD itself and beneficial effects of the altered lipid metabolism on diabetes effector proteins. However, certain studies suggested that KD reduced glucose and

**TABLE 1** | Formulations of common ketogenic diets (KDs).

| Diet | Percent Total Daily Energy Intake | | |
|---|---|---|---|
| | Fat % | Carbohydrate % | Proteins % (g) |
| Classic KD (4:1 KD) | 90 | 2 | 8 |
| 3:1 KD | 87 | 4 | 9 |
| 2:1 KD | 82 | 8 | 10 |
| 1:1 KD | 70 | 10 | 20 |
| MAD | 60-65 | 5-10 | 30 |
| MCTKD | 70-75 | 15-19 | 10 |
| LGIT | 60 | 10 | 30 |

*MAD, the modified Atkins diet; MCTKD, the medium chain triglyceride ketogenic diet; LGIT, the low glycemic index treatment.*

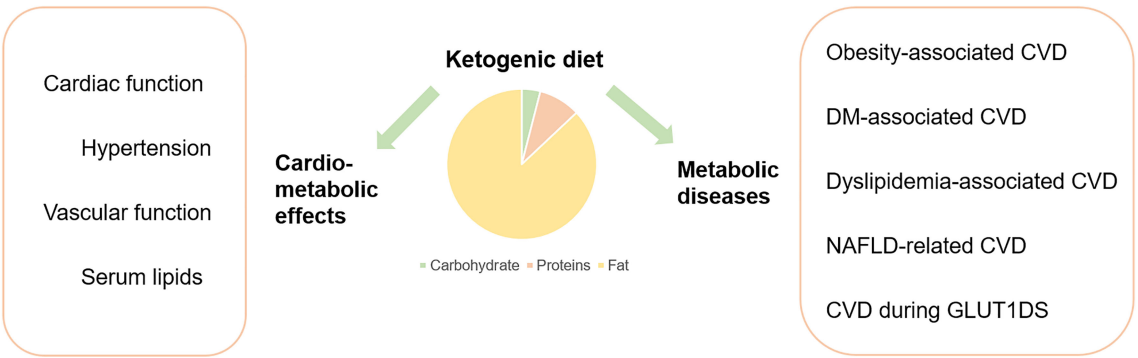


**FIGURE 1** | Graphic abstract.

insulin levels while inducing insulin resistance and glucose intolerance in rats (27, 36). Besides, utilizing KD in adults with type 1 diabetes mellitus (T1DM) is associated with dyslipidaemia and a high number of hypoglycaemic episodes apart from excellent HbA1c levels and little glycaemic variability (37). Thus, in T1DM, the safety issues are considerable; while in T2DM, more studies are needed to address how KD might impact on insulin resistance and other aspects.

In Polycystic ovarian syndrome (PCOS), KD appears to be a valuable non-pharmacological treatment. A 24-week low-carbohydrate KD (38) and a 12-week ketogenic Mediterranean diet with phyoextracts (39) were both reported to lead to remarkable improvement in body weight, percentages of free testosterone, LH/FSH ratios, and insulin levels in women with PCOS and obesity/overweight. Besides, KD has also been proven effective in other metabolic diseases including glucose transporter type 1 deficiency syndrome (GLUT1DS) (20), pyruvate dehydrogenase (PDH) deficiency (40), phosphofructokinase (PFK) deficiency and glycogenosis type V (McArdle disease) (41).

In summary, KDs are recommended in some inherited metabolic diseases and PCOS, while the effects of KDs on diabetes and some other metabolic diseases remain controversial. Rigorously-designed long-term studies are warranted to evaluate the effects and the safety problems of KDs and further evaluate whether the impact of KDs can be maintained.

## CARDIOMETABOLIC EFFECTS OF KETOGENIC DIETS

The occurrence and development of CMDs are closely related to systemic chronic low-grade inflammation characterized by the continuous increase of circulatory inflammatory factors (42). Dietary pattern is one of the important factors that affect chronic inflammatory states (43). Thus, the effects of dietary pattern on CMDs arouse scientists' interest and a large number of studies have focused on the cardiometabolic effects of KD. Apart from the above-mentioned metabolic diseases, the effects of KD on cardiac function, hypertension, vascular function and lipid profile have also been studied.

### Ketogenic Diets Regulate Cardiac Function

The effects of KD on cardiac health have been widely investigated, but researches concerning the effects of KD on cardiac functions provided a few relatively controversial data. Studies generally suggest that KD intake benefited cardiac metabolic efficiency and acted as a cardioprotective antioxidant. Selvaraj et al. (44) reviewed current evidence surrounding the use of therapeutic ketosis including KD in heart failure (HF) and pointed out its potential benefit in HF, particularly in HF with reduced ejection fraction. Further, Balietti et al. (45) found that an 8-week supplementation of medium-chain triglycerides KD (MCT-KD) to late-adult rats partly restored age-related decrease of succinic dehydrogenase (SDH) activity and metabolically active mitochondria, which might offset senescent alterations leading to apoptosis-induced myocardial atrophy and failure. Another study with a similar conclusion indicated that a 19-week low carbohydrate KD following global ischemic injury significantly increased the numbers of mitochondria in cardiac muscles and the reperfusion recovery of coronary flow (46). As such, the two studies demonstrated that KD was cardio-protective in terms of regulating cardiac energy metabolism including mitochondrial capability. However, some studies suggested that KD might be just not harmful to cardiac functions. A study utilizing KD for at least 12 months on cardiac functions in intractable epilepsy patients suggested that the KD used appeared to have no negative impact on ventricular functions in epileptic children in the midterm (47). Similarly, a 6-month KD therapy didn't affect electrocardiogram outcomes in the drug-resistant children with epilepsy (48). The subjects in these two studies are both epileptic children, which cannot represent all the patients who might use KD therapy. Thus, we can still stay optimistic about the effects of KD on cardiac functions.

Studies have also been conducted concerning the mechanism of how KD might affect cardiac health. Abnormal substrate metabolism is one of the major changes of insulin resistance

and diabetic myocardium (49). Given this, changes in the regulation of myocardial ketone body metabolism appear to be a novel diagnostic biomarker of altered ketolytic capacity. Wentz et al. (50) utilized ketogenic nutritional mouse models (24 h of fasting and a very low carbohydrate ketogenic diet) to demonstrate that cardiac muscle engages a metabolic response that limits ketone body utilization. Specifically, the results revealed that unmetabolized substrate concentrations were higher within the hearts of ketogenic diet-fed mice. Furthermore, a recent study suggested that a KD or a high-fat diet could reverse the structural, metabolic and functional remodeling of non-stressed cMPC1-/- (cardiomyocyte-restricted deletion of subunit 1 of mitochondrial pyruvate carrier) mouse hearts (51). A KD of 3 weeks before transverse aortic constriction was already enough to rescue cMPC1-/- hearts from rapid decompensation and early mortality after pressure overload. Another study also indicated that a high-fat, low-carbohydrate KD could completely reverse progressively developed cardiac dilation and contractile dysfunction in mice with cardiac-specific deletion of Mpc2 (CS-MPC2-/-) (52). Accordingly, KD therapy might be promising in improving cardiac fat metabolism to prevent or reverse cardiac dysfunction and remodeling in MPC deficiency.

As mentioned above, KDs are generally cardioprotective, which might be attributable to the effects of KDs on cardiac metabolism, such as ketone body metabolism and energy metabolism including mitochondrial capability (**Figure 2**). Despite the evidence supporting the cardioprotective effect of KDs, another study utilizing KD on cardiac remodeling in spontaneously hypertensive rats (SHRs) suggested that KD might deteriorate cardiac remodeling in the hypertensive heart and warranted fully evaluation of its reliability before clinical use (53). The different pathogenesis backgrounds of hypertension might account for the different results. More studies with larger samples, longer follow-up duration, and standardized basic health status can be conducted to further clarify the role of KDs in cardiac functions and other potential mechanisms.

## Ketogenic Diets and Hypertension

Attention has also been paid to the effects of KD in hypertension. Most studies showed positive effects of KD in hypertension. Castellana et al. (54) suggested that very-low-calorie ketogenic diet (VLCKD) manifested improvements in hypertension, type 2 diabetes and dyslipidemia, apart from being a promising lifestyle intervention for overweight and obesity. Another study incorporating 377 patients across Italy drew a similar conclusion that VLCKD could significantly lower SBP in three months (55). Even a short-term 4-week KD with micronutrient supplementation could result in improved hypertension control and in a reduction for the usage of hypertension medications in patients with preoperative T2DM and hypertension (56). Increasing ketone bodies by nutritional interventions of ketone bodies or their precursors, such as 1,3-Butanediol, was also reported to attenuate hypertension (57). However, Guo et al. (58) revealed that subjecting spontaneously hypertensive rats (SHRs) to KD for 4 weeks aggravated hypertension, increased the expression of IL1-β and TNF-α, impaired endothelium-dependent relaxation and decreased CD31 and eNOS expression in mesenteric arteries. This finding is opposite to the previous results; thus, it remind us to be cautious in treating

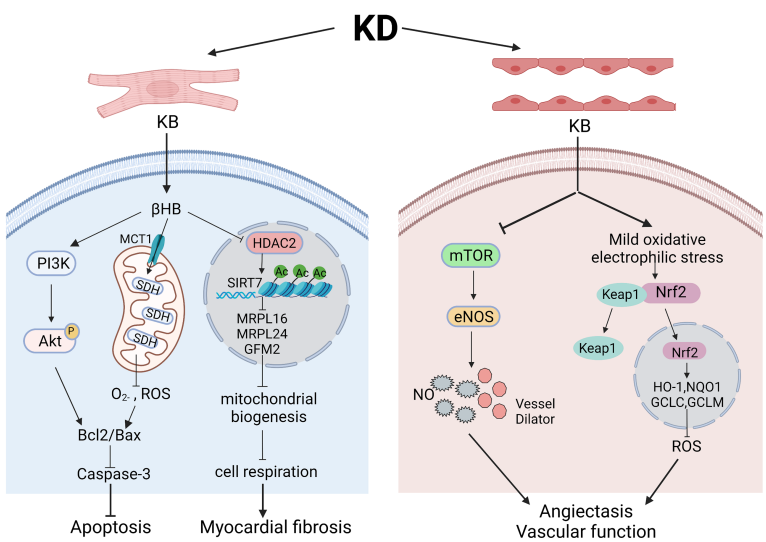


**FIGURE 2** | The role and mechanism of ketogenic diets in cardiac function and vascular function. Various pathways might underly the effects of ketogenic diets in cardiomyocytes and endothelial cells in different models. In cardiomyocytes, βHB regulates PI3K/Akt pathway and SDH in mitochondria to finally ameliorate cell apoptosis. However, elevated βHB might also acts through inhibiting HDAC2 and influencing mitochondrial biogenesis, leading to myocardial fibrosis. In endothelial cells, ketone bodies can functions through inhibiting mTOR pathway to regulate the level of eNOS, subsequently dilating blood vessels and enhancing vascular function. Besides, elevated ketone bodies can give rise to mild oxidative/electrophilic stress, activate Nrf2 in cytoplasm and enhance antioxidant gene expression, which lead to lowered ROS level and improved vascular functions. Created with BioRender.com.

hypertension with KD, and perform more studies to explore the effects of KD on hypertension in human studies and animal models.

The Dietary Approaches to Stop Hypertension (DASH) diet is a classic dietary approach that has been endorsed for patients with elevated blood pressure (BP). Besides, the addition of exercise and weight loss to the DASH diet resulted in even larger BP reductions, greater improvements in vascular and reduced left ventricular mass for obese people with elevated BP (59). The main drawback of DASH might be the difficulty in long-term adherence to this diet. Considering the different components of DASH and KD, KD might become another choice for those people who love a high-fat diet, although we suggest cautious application since the antihypertensive efficacy and side effects of KD under the background of hypertension remain unclear. Interestingly, a review suggested that intermittent fasting could lower both systolic and diastolic blood pressure in human studies and animal studies, possibly through reducing oxidative stress, syncing with circadian rhythm, and inducing a ketogenic state (60). The less consumption of fats is assumed as the reason why intermittent fasting appears to be more beneficial than KD in treating hypertension. Thus, the type of fats consumed in KD therapy is crucial to be considered both in treating hypertension and evaluating its effects.

## Ketogenic Diets Regulate Vascular Functions and Vascular Blood Flow

A study by Keogh et al. (61) has indicated that a very-low-carbohydrate, high-saturated-fat weight-loss diet did not impair FMD. How would the actual KD impact on vascular function and vascular blood flow?

In some studies, KD appears to play a protective role in vascular functions. Ischemic tolerance can reduce brain injury and neurological dysfunction after brain ischemia. Additional to the cardiovascular effects such as higher reperfusion recovery of coronary flow, KD also can enhance brain vascular function. As supported by the results from a study upon feeding a KD to young healthy mice, KD intervention enhanced neurovascular function through reducing mTOR protein expression and increasing eNOS levels (62). Yang et al. (63) discovered that feeding mice with KD-fed mice could remarkably decrease infarct volume and elevate regional cerebral blood flow in both ischemic and reperfusion phases. Besides, while investigating the effects of KB level on HMEC-1 endothelial cells, one study indicated that KB activated transcription factor Nrf2 and elevated the expression of cell antioxidant defending genes *via* inducing moderate oxidative stress (64). Thus, the increased KB level by KD might also lead to these protective effects.

However, as for big vessels such as carotid and aortic artery, the effects of KD remain controversial. For instance, after observing the effect of KD on the vascular structure and functions for at least one year, it was found that KD notably elevated the serum levels of lipids but didn't significantly affect carotid intima-media thickness, aortic and carotid strain, the stiffness index, distensibility, and elastic modulus (65). Another study by Doksoz et al. (66) also demonstrated that a 6-month KD didn't affect carotid intima-media thickness and elastic properties of the carotid artery and the aorta. In contrast, in the research of Coppola et al. (67), participants prescribed with KD had higher arterial stiffness parameters, including AIx and beta-index and higher serum levels of cholesterol or triglycerides. Another study revealed that a high-fat KD notably elevated atherogenic apolipoprotein B (apoB)-containing lipoproteins and decreased antiatherogenic HDL cholesterol and urged further researches to investigate whether this diet deteriorates endothelial function and facilitates inflammation and formation of atherosclerotic lesions (68). However, a clinical study involving 26 children after one year and 13 children after two years of KD suggested that the initial influences on arterial function observed within the first year of KD-treatment were reversible and were no longer significant after 2 years of the therapy (69). Therefore, the effects of KD on big vessels such as carotid and aortic artery were reversible and were no longer significant after 1-2 years, which might explain the above results.

## Ketogenic Diets Regulate Serum Lipids

Apart from the cardiometabolic effects mentioned above, the impact of KD on serum CVD biomarkers has also been investigated. Research on 20 normal-weight, normolipidemic men indicated that a 6-week KD notably decreased fasting serum triglyceride, postprandial lipemia, and fasting serum insulin concentrations, tended to increase HDL cholesterol, while not affecting fasting serum total and LDL cholesterol and oxidized LDL (70). These results revealed that short-term KD would not deteriorate CVD risk profile and, indeed, appeared to ameliorate lipid disorders that are characteristics of atherogenic dyslipidemia. Another research also indicated that changes in the ratio of protein to carbohydrate toward higher protein proportion could provide beneficial effects on serum lipids apart from lowering body weight (71).

However, Özdemir et al. (65) pointed out that prescribing patients with at least 12 months KD could significantly elevate serum total and LDL cholesterol and triglyceride at a median of 12.6 months while not affecting HDL level. Moreover, another research (72) found that a 6-month KD could notably increase median triglyceride, total cholesterol, LDL, and HDL. They suggested that classic KD was indeed efficient in treating refractory seizures in children but might give rise to hypercholesterolemia and hypertriglyceridemia.

As such, disputable voices concerning the impact of KDs on serum lipids remain to be settled by future work in this field.

# KETOGENIC DIETS AND CARDIOVASCULAR COMPLICATIONS OF METABOLIC DISEASES

The potential effects of KDs on the prevention or treatment of cardiovascular risk factors or diseases have been significantly studied over the past decades. Moreover, various animal and human studies have investigated the role of KDs in regulating

cardiovascular complications of obesity, insulin resistance and type 2 diabetes, dyslipidemia, NAFLD, and/or GLUT1DS. However, whether and how KDs influence the cardiovascular risk factors or complications in metabolic diseases remains undetermined.

## Obesity-Associated Cardiovascular Disease

Obesity is closely related to CVD, and complications of CVD are often witnessed in obese patients. Cicero et al. (55) evaluated the effect of a very low carbohydrate ketogenic diet (VLCKD) on overweight-related risk factors of CVD such as blood pressure, lipid levels, and glucose metabolism, and the study found that VLCKD intervention for 3 months was generally safe and found effective in inducing weight loss and improved CV risk factors levels.

Another study recruited a hundred obese patients and prescribed them a ketogenic diet for over six months showed significant improvement in patients' cardiovascular status in addition to weight reduction (73). Moreover, a meta-analysis study by Bueno et al. (74) assessed the long-term effects of VLCKD on body weight and cardiovascular risk factors. The results indicated that under VLCKD, the participants had a significant reduction in body weight, TAG, and diastolic blood pressure, while increased HDL-C and LDL-C levels were observed. Apart from long-term studies, a study by Ministrini et al. (75) that treated obese patients with VLCKD for 25 days also concluded that VLCKD had positive effects on cardiovascular risk factors, and such a beneficial outcome in the short term is remarkable.

Other studies investigated the effects of modified KDs on cardiovascular risks in obese participants. Perez-Guisado et al. (76) carried out a prospective evaluation in 31 obese participants with "Spanish Ketogenic Mediterranean Diet" (incorporating virgin olive oil as a principal source of fat, moderate red wine intake, green vegetables and salads as the primary source of carbohydrates and fish as the main source of protein, SKMD). The SKMD was found safe and effective interventional approach for improving non-atherogenic lipid profiles and lowering blood pressure while lowering body weight. Similarly, Paoli et al. (77) applied another modified KD that incorporated phytoextracts and ingredients imitating the taste of carbohydrates (ketogenic Mediterranean with phytoextracts, KEMEPHY). The study recruited 106 overweight Rome council employees and revealed a remarkable reduction in body weight, BMI, percentage of fat mass, total cholesterol, LDL-C, TAG and blood glucose while displaying a significant increase in HDL-C after the intervention with KEMEPHY. In addition to good compliance, extra beneficial effects on cardiovascular risk markers and waist circumference were also achieved by the KEMEPHY diet.

Since the beneficial effects of KDs on metabolism and cardiovascular risk factors are similar to those seen after n-3 polyunsaturated fatty acids (omega-3) supplementation, Paoli's team (78) modified the ketogenic Mediterranean diet with phytoextracts after their previous research through combining with omega-3 supplementation. The results suggested that this newly modified diet can further enhance the beneficial effects on cardiovascular risk factors and inflammation in overweight participants.

The influence of a multi-step dietary program including different dietary patterns has also been evaluated. In an open-label study by Castaldo et al. (79), 73 obese patients entered a rehabilitative multi-step dietary program: a 3-week protein-sparing, very low-calorie KD (<500 kcal/day; Oloproteic Diet) and a 6-week hypocaloric (25–30 kcal/kg of ideal body weight/day), low glycemic index, Mediterranean-like diet (hypo-MD). In both phases, improved glucose and lipid metabolism and blood pressure were observed. Based on this, it was concluded that a dietary program consisting of a KD and a subsequent MD could decrease cardiovascular risks efficaciously in obese patients.

## CVD During Diabetes Mellitus

Diabetes mellitus is often associated with obesity (80), and recent estimates showed that 87.5% of T2DM patients are overweight or obese (24). Moreover, obese subjects are prone to developing hypertension, CVD, and strokes, and the risk is even higher if it co-exists with T2DM (81).

In patients with both T2DM and obesity, LC diets not only cause weight loss but also improve postprandial plasma glucose levels, glucose variability, serum triglycerides, and HDL-C levels (82). Similar results were observed by a 2-year randomized clinical trial study (39) that investigated the effect of an LC diet with high unsaturated fat and low saturated fat on glycemic control and CVD risk factors in overweight or obese patients with T2DM. KD is a low-carbohydrate (LC) and high-fat (HF) diet, which sort of belongs to one type of LC diets. However, one of the potential concerns of KDs is postprandial hyperlipidemia, which leads to significant cardiovascular risks (83).

Studies have found that individuals with pre-diabetes or diabetes who received an earlier LCHF diet revealed several beneficial outcomes, including weight loss, improved insulin sensitivity, glucose homeostasis, and lower fasting blood glucose levels. These outcome improvements also decreased the risks of cardiovascular diseases development (38, 84). Mobbs et al. (28) analyzed the evidence concerning the treatment of diabetes and diabetic complications with a KD. They revealed that a classic KD significantly reduced blood glucose in animal models of type 1 and 2 diabetes and reversed diabetic nephropathy without producing significant cardiovascular risks. Moreover, a study on db/db mice revealed that KD ameliorates cardiac dysfunction by inhibiting apoptosis *via* activating the PI3K-Akt pathway in type 2 diabetic mice and suggested KD as a promising lifestyle intervention against diabetic cardiomyopathy (85).

However, there also are studies with conflicting or controversial findings and opinions. Westman et al. (86) stated that LCHF diets gave rise to decreased appetite, thus the improved surrogate markers of cardiovascular disease resulted from weight loss but not from low carbohydrate intake itself. In an animal study, Abdurrachim et al. (87) investigated the effects of long-term KD on cardiac metabolism and diabetic cardiomyopathy status in lean diabetic Goto-Kakizaki (GK)

rats. Upon KDs for 62 weeks, diabetic GK rats displayed decreased blood glucose, triglyceride, and insulin levels, revealing increased blood ketone body levels. Additionally, KDs decreased myocardial ketone body and glucose oxidation and induced cardiac hypertrophy. These results suggested that KDs might lead to maladaptive cardiac metabolic modulation and lipotoxicity and deteriorate diabetic cardiomyopathy in GK rats. Given this, the possible role of KDs in cardiovascular risks of DM remains controversial in rodent models and humans, which warrants more studies for elucidation.

### Dyslipidemia-Associated CVD

As for patients with dyslipidemia, Westman et al. (88) investigated the effect of KDs on serum lipoprotein subclasses to address the concern of KDs on cardiovascular risks. The study was a randomized, two-arm clinical trial involving overweight and hyperlipidemic participants motivated to lose weight. After 6 months, the KD group displayed more significant decreases in medium VLDL, small VLDL, and medium LDL, and more significant increases in VLDL particle size, large LDL, and HDL particle size than the control group. Although the KDs did not decrease total LDL cholesterol, they shifted from small, dense LDL to large, buoyant LDL, thus decreasing CVD risks in these participants.

### NAFLD-Related CVD

NAFLD contributes to CVD through various mechanisms. Weight loss has been commonly recommended for treating obesity-associated NAFLD; meanwhile, LCKD benefits weight loss. Recent studies have revealed an association between LC diets and NAFLD in both rodents and humans. In the study by Garbow et al. (28), mice fed a KD for 12 weeks were lean, euglycemic, ketotic, and hypo-insulinemic but were glucose intolerant and with NAFLD. Also, obese subjects on LC diets displayed enhanced weight loss, improved metabolic parameters and decreased intrahepatic triglyceride content. Nevertheless, long-term KDs led to NAFLD and systemic glucose intolerance in mice (89), negatively impacting CVD. As such, current evidence is insufficient to conclude, and more related studies are warranted to explore how KDs might influence NAFLD-related CVD in the long run.

### CVD During GLUT1DS

GLUT1DS is an inherited but treatable disease concerning cerebral energy metabolism (90). KDs are currently a treatment option for GLUT1DS from infancy into adulthood, raising concerns about long-term cardiovascular risks (43, 60, 69). To address this problem, Heussinger et al. (91) performed a 10-year follow-up study on cardiovascular risk of KDs in GLUT1DS and revealed that dyslipidemia caused by KDs might be transient; and carotid intimal wall thickness (CIMT), BMI and blood pressure parameters remained normal after 10 years. Because of this, Heussinger et al. suggested that cardiovascular risks of KDs in some previous studies appeared to be attributable to inadequate follow-up. Also, a period of at least five years appears to be necessary for evaluating the effect of KDs on lipid parameters. Moreover, the authors recommended KDs as a treatment of choice for GLUT1DS. Another study by Alter et al. (90) also characterized the long-term course of GLUT1DS and followed up for an average of 14.2 years (range = 8.9-23.6). The results indicated that earlier introduction of KDs correlated with better long-term outcomes and KDs seemed to be protective of vital organs. However, GLUT1DS is a rare disease, and therefore, the study cohort's size and external validity are limited. Long-term follow-up studies are warranted to confirm the above findings further.

## POTENTIAL SAFETY CONCERNS ON KD

The majority of the studies had found KD to be beneficial, but some studies had shown concerns regarding heart functions, liver inflammation and so on.

### The Effects of KD on Heart Functions in Rodents

One study found KD treatment ameliorates cardiac dysfunction by inhibiting apoptosis *via* activating the PI3K-Akt pathway in type 2 diabetic mice, suggesting that the KD is a promising lifestyle intervention offering protection against diabetic cardiomyopathy (85). In contrast, a ketogenic diet may lead to adverse effects on the remodeling in the hypertensive heart *via* mechanisms involving increased mTOR signaling, and they underscore the necessity to evaluate its reliability before clinical use (53). Preclinical studies results indicate that KD also has a potential safety concern, although much evidence suggests that KD is a promising approach for managing CVD.

### The Effects of KD on Hepatic Inflammation in Rodents

As a key factor that triggers or exacerbates CVD risk, liver inflammation is a potential safety concern related to KD. In support of this, a study observed that mice fed with KD sustained unimpaired insulin-induced hepatic Akt phosphorylation and whole-body insulin responsiveness but ultimately developed hepatic endoplasmic reticulum stress, steatosis, cellular injury, and macrophage accumulation (28).

### The Effects of KD on Lipid Profile

KD is enriched in lipid contents, and it's natural to speculate the potential risk of elevated levels of lipids. Apart from studies indicating the beneficial effects of KD, concerns regarding the elevated level of lipids, including serum total and LDL cholesterol and triglyceride, are subjective while prescribing KD (65, 72). It is reported that KDs are likely to deteriorate levels of total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides (27, 34, 92) in rodents while doing the opposite in humans (39). These contradictory results might be attributable to the different composition of diets since animal researches generally employ diets higher not only in total fat but also in saturated fat (93). Considering this, it is necessary to compare the fat composition, e.g., content of saturated fat versus unsaturated fat in KDs in long-term studies involving both rodents and humans.

As described above, while considering KD as an exciting approach for managing CMDs, it also is important to be cautious about the potential safety concern associated with KD. While future studies are warranted to confirm and elucidate whether and how KD causes potential safety concerns, it would also be important to consider to modifying KD or combining KD with other healthy diets for managing CMDs.

# DISCUSSION

Despite particular safety concerns, the beneficial and advantageous aspects of KD cannot be denied. Because multiple factors are affecting the results, including using different mouse strains, providing KD with different food compositions, short study duration, etc. In the future, studies essentially need to explore the possible factors influencing the responses to KD and improve KD dietary plan for utilizing KD as a dietary therapy to minimize safety concerns.

## Factors Affecting Ketogenic Diets Responses

### Genetic Control of the Responses to a Ketogenic Diet

Nutrigenetic research suggested that genetic markers critically regulate nutritional interactions that impact body weight and composition, which lays the foundation for personalized nutrition therapy (94, 95).

Barrington et al. (96) observed that mouse genetic backgrounds determined dietary outcomes on CVD risk. Specifically, the study included mice from four inbred strains (A, B6, FVB, and NOD), which accounted for genetic and phenotypic diversity and examined mice's metabolic responses to four human-comparable mice diets (American, Mediterranean, Japanese and ketogenic diets). The authors revealed that the effects of these diets on metabolic health were indeed dependent on genetic backgrounds. The outcomes of KD on body composition, glucose metabolism and liver health varied markedly among different strains.

The different diet responses could be partly attributable to the genetic background related to varying dietary therapy compliance. Parnell et al. (97) analyzed the interactions between single nucleotide polymorphisms (SNP) in various cardiometabolic pathways and the intake of different nutrients. Their results indicated that gene-environment (GxE) genes had better responses to plasma cholesterol-lowering or regression of atherosclerotic plaques, primarily through high-energy diets and fat intake.

As mentioned above, genetic background plays a vital role in individual responses to KDs and may consequently influence the effects of KDs. It is of great importance to take genetic background into account when initiating KD therapy.

### Food Composition, Quality and Sources of KDs Influence the Outcome

As KDs are a kind of macronutrient-focused diet, we should fully consider the food composition, quality and sources to avoid potential drawbacks when starting a KD. As indicated in the research by Seidelmann et al. (98), there was a U-shaped association between the percentage of energy consumed from carbohydrates and mortality: 50–55% carbohydrate intake was associated with minimal mortality risk. In comparison, a percentage of <40% or >70% led to greater mortality risk. Besides, different types of dietary fatty acids have different effects on CVD risk and replacing saturated fatty acid (SFA) with unsaturated fats especially polyunsaturated fatty acids can lead to a significant reduction in CVD risk (99). Moreover, diets that favored plant-derived protein and fat intake were associated with lower mortality than animal-derived protein and fat sources. Thus, the nutrient composition, types and sources should be taken into consideration when prescribing a KD therapy; the diversity in these nutrient details could affect the effects of KD and should be relatively standardized to compare the results.

### Duration of KD Therapy Affects the Responses

Interestingly, increasing the therapeutic duration of KDs appears to reduce some safety-related problems (34, 35). For instance, long-term follow-up research has demonstrated that dyslipidemia caused by KDs is transient. Moreover, over 10 years, KD therapy has ended with normal vascular function as indicated by carotid artery ultrasound (91).

## Modified KD Dietary Plan

Because of the irreconcilable options on the therapeutic use of KD, several studies concerning modified KD and cardiovascular risks have been performed. The "Spanish Ketogenic Mediterranean Diet" carried out by Perez-Guisado et al. (76) and two modified KDs (KEMEPHY (77) and KEMEPHY with omega-3 supplementation (78)) employed by Paoli et al. have all displayed beneficial effects on cardiovascular risk factors. A combined diet consisting of KD and a subsequent Mediterranean-like diet has been proven to decrease cardiovascular risks in patients (79). Therefore, a modified KD or multi-step dietary program including different diet patterns is promising in resolving the safety concerns associated with KDs.

# CONCLUSION

Based on the currently available evidence, KD appears to play a significant role in treating various cardio-metabolic diseases and reveals remarkable effects on cardiovascular function. KD therapy is generally promising in obesity, heart failure, and hypertension, though different voices still exist. In diabetes and dyslipidemia, the performance of KD remains controversial. As for cardiovascular complications of metabolic diseases, current evidence suggests that KD is generally protective to obese related cardiovascular disease (CVD), while remaining contradictory to diabetes and other metabolic disorder related CVDs. Various factors might account for the controversies, including genetic background, duration of therapy, food composition, quality and sources of KDs. Therefore, further studies are warranted to provide concrete and more conclusive opinions. Also, it is vital to monitor safety-related signs and biomarkers during the KD intervention, although most are reversible or transient. In addition, modified KD could be

adequately designed and utilized to enhance compliance as a therapeutic approach. Overall, there is a critical need to conduct more rigorous research focusing on the clinical implication and safety issues of KD.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication. WYZ: designing, conceptualization, writing, figure plotting, revising; JZ and CDW: designing, funding acquisition, review & editing; XG: funding acquisition, review & editing; LLC: supervision, editing, revising; TC and JYY: figure plotting.

## FUNDING

The development of this review was supported in whole or in part by grants from the National Natural Science Foundation of China (81770772 to JZ), the Hubei Province Natural Science Foundation (2019CFB701 to JZ), National Natural Science Foundation of China (81803224 to XG) and Young Scholars Program of Shandong University (2018WLJH33 to XG).

## ACKNOWLEDGMENTS

We especially express our appreciation to Mohammad Ishraq Zafar for his assistance with the English language of our review.

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 25 January 2022
doi: 10.3389/fendo.2021.828286

# Association of Brachial Ankle Pulse Wave Velocity With New Onset Stroke in Hypertensive Patients Aged Less Than 65 With Normal Fasting Glucose Among Chinese Community-Based Population

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**Background:** Previous studies have shown an association of stroke and brachial ankle pulse wave velocity (baPWV). However, due to limitations on total population size and small numbers of stroke cases, lack of power has prevented further detection among subgroups such as age and laboratory testing.

**Methods:** A total of 19217 participants including 660 incident stroke patients were pooled in the present study. Participants were divided to 2 groups, aged less than 65 years [56.0 (50.0, 61.0)] and aged 65 years or more [70.0 (67.0, 74.0)].

**Results:** After adjustment for demographic, anthropometric, and laboratory parameters, the incident stroke was positively associated to baPWV in the group aged less than 65 years (OR, 1.16; 95% CI, 1.05–1.28), but not in the older group aged 65 or more. When baPWV was assigned as quartiles, a significant, increased risk of new-onset stroke was found in quartiles 3-4 compared with quartile 1. In addition, the predictive value of baPWV for incident stroke was modified by fasting glucose in participants aged less than 65 years (*P*-interaction = 0.010). An increase in baPWV was strongly, positively associated to new-onset stroke in the subgroup of normal fasting glucose (< 5.6 mmol/L) (OR, 1.34; 95% CI, 1.15 - 1.57), but no effect was seen in the impaired fasting glucose (5.6-7.0 mmol/L) or diabetic fasting glucose (> 7.0 mmol/L) subgroups.

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**Edited by:**
*Si Jin,*
*Huazhong University of Science and Technology, China*

**Reviewed by:**
*Xingchun Wang,*
*Tongji University, China*
*Yanbo Shi,*
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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *03 December 2021*
**Accepted:** *30 December 2021*
**Published:** *25 January 2022*

**Citation:**
*Li C, Wang Z, Liu S, Guo S, Song Y, Liu L, Zhou Z, Wang B, Huang M, Wang R, Zhai L, Gao Y, Qin X, Wang X, Zheng H and Zhao Z (2022) Association of Brachial Ankle Pulse Wave Velocity With New Onset Stroke in Hypertensive Patients Aged Less Than 65 With Normal Fasting Glucose Among Chinese Community-Based Population. Front. Endocrinol. 12:828286. doi: 10.3389/fendo.2021.828286*

**Conclusions:** Increased baPWV was significantly associated with new-onset stroke in a hypertensive population aged less than 65 years. Particularly, it is of great importance to monitor baPWV for predicting incident stroke in "relatively healthy" hypertensive patients, i.e. aged less than 65 years with normal fasting glucose.

**Keywords: brachial ankle pulse wave velocity, new onset stroke, hypertensive patients, glucose, age**

## INTRODUCTION

Stroke ranks second among all causes of death, accounting for severe healthcare issues and huge financial burdens worldwide (1). The crude death rates of stroke have declined among patients over age 65, while the declines are modest among patients less than age 65; of note, from 2011 – 2016, the death rate flattened in patients between the ages of 45-54 and even increased in patients aged 55-64 (2). In addition, the incidence and prevalence of stroke have increased rapidly because of the ageing population in China, which accounts for approximately one-third of global stroke deaths (3). Thus, a predictive marker for determining those patients with a greater risk of stroke is of prominent clinical significance, for preventing new onset of stroke and enhancing patient welfare.

Arterial stiffness has been demonstrated to be a major risk factor for cardiovascular and neurological disorders such as diabetes, hypertension, atherosclerosis, cerebral small vessel disease, and stroke (4). Brachial ankle pulse wave velocity (baPWV) is a frequently used parameter for monitoring arterial stiffness due to its non-invasive measurement and reproducibility (5). Pulse wave velocity (PWV) largely increases with age, or in hypertension, even independent of blood pressure (6). Previous studies have demonstrated that PWV is positively associated with new onset stroke in hypertensive individuals (7, 8). However, due to study limitations of total population size and small numbers of stroke events, lack of power has prevented any further determination of differences among subgroups such as age or laboratory tests, i.e., glucose, cholesterol, and triglyceride levels, etc. Additionally, studies have focused on interactions with baPWV and the prognostic significance of stroke outcome (9–11), but researches of baPWV in predicting acute stroke are lacking.

In order to address the above-mentioned gap and illuminate new predictors of new-onset stroke, the present study aimed to (1) further investigate the role of baPWV in predicting stroke incidence, (2) evaluate whether risk predicting differs among sub-populations, and (3) find any potential effect modifiers on the predictive significance of baPWV, using data from a large-scale population study of 19217 hypertensive patients including 660 new-onset stroke patients.

## METHODS

### Study Population and Design

The study population was pooled from the China H-type Hypertension Registry Study (CHHRS; URL: http://www.chictr.org.cn; Unique identifier: ChiCTR1800017274), which is an ongoing community-based non-intervention, prospective, observational, multicenter, real-world registry study and was mainly conducted in Rongcheng County, Shandong Province, and Lianyungang, Jiangsu Province, China. Eligible participants were over 18 years of age with hypertension, which was defined as seated, resting systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg at both the screening and recruitment visit, or who were taking anti-hypertensive medications. Participants were excluded if they were unable to give informed consent or to participate in the follow-up according to the study protocol (**Supplementary Figure 1**). The investigators completed a standardized electronic medical record collection at baseline and at follow-up visits, which occurred every 3 months, for up to 3 years. At each visit, participants underwent a physical examination, and clinical outcomes were recorded. The present study was approved by the Ethics Committee of the Institute of Biomedicine, Anhui Medical University, Hefei, China. All participants signed an approved written consent form after the study protocol was thoroughly explained to them.

The primary outcome was an incident fatal or non-fatal symptomatic stroke, excluding subarachnoid hemorrhage or silent stroke (subclinical stroke); first ischemic stroke (fatal and nonfatal) and first hemorrhagic stroke (fatal and nonfatal) were secondary outcomes. Information on incident stroke for all participants was obtained from the Lianyungang CDC, and checked against a national health insurance system with electronic linkage to all hospitalizations, or was ascertained through active follow-up. In China, local public medical institutions are required to report all new onset stroke events to the local CDC. A report card including demographics, diagnosis, and date of stroke is required to be routinely submitted on the 28th of each month. Trained officials and the local CDC were responsible for quality control, such as characterizing and deleting repeated cases, error checking, and finding missed cases. Additionally, 5 percent of all cases were randomly selected for further validation by telephone or home-visit interviews (12).

### Laboratory Tests and Data Collection

Baseline characteristics were conducted by trained staff in accordance with standardized operating procedures. Participants were interviewed *via* a standard questionnaire for the current study, which included information on demographics, history of cigarette smoking, alcohol drinking, and medication usage. Anthropometric data, and physical and clinical characteristics were routinely measured by trained staff. Body

mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters ($kg/m^2$). Blood samples were collected from all participants after overnight fasting for clinical biomedical testing. Serum lipids, and fasting glucose were measured using automatic clinical analyzers (Beckman Coulter) at the Shenzhen Tailored Medical laboratory in Shenzhen, China.

## Measurements of baPWV

Level of baPWV was measured by a PWV/ABI instrument (form PWV/ABI, BP-203RPE; Omron-Colin, Japan) by trained technicians (7). After a minimum of 15 mins of rest, supine patients were fitted with oscillometric cuffs on ankles and bilateral brachia, with ECG electrodes on bilateral wrists. The semiconductor pressure sensor was used for recording pulse volume waveforms. Data of brachium and ankle volume waveforms were recorded in a 10s sampling interval with automatic adjustment of quality and gain. The time interval from brachium to ankle (ΔTba) was determined from the interval between the wave front of the brachial waveform and that of the ankle waveform. An automatic adjustment was applied to the distance between sampling points of baPWV by participant height. The path lengths from the suprasternal notch to the ankle (La) and from the suprasternal notch to the brachium (Lb) were determined by the following formula: La = 0.8129 × height (cm) + 12.328 and Lb = 0.2195 × height (cm) - 2.0734, respectively. Level of baPWV was calculated as: baPWV = (La−Lb)/Tba.

## Statistics

Data were presented as median ± interquartile range (IQR) for continuous variables and as frequency (%) for categorical variables. Population baseline characteristics of the different groups were analyzed using *t* tests, ANOVA tests, or $\chi 2$ tests, respectively. Baseline characteristics of participants were compared using 2-sample t tests, signed-rank tests, or $\chi 2$ tests between different groups, respectively. The relationship of baPWV and incident stroke was evaluated using multivariable logistic regression models and generalized linear regression models, with or without adjustment for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, smoking status, alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, homocysteine in model 1, and additionally, history of antihypertensive drug use in model 2. For further exploratory analysis, interaction testing and stratified analyses were used to detect possible modifications on the association between baPWV and incident stroke. A 2-tailed $P < 0.05$ was considered to be statistically significant in the present study. Data were analyzed by statistical package R (http://www.r-project.org) and Empower (R) (www.empowerstats.com; X&Y Solutions, Inc., Boston, MA).

# RESULTS

## Baseline Characteristics of the Study Participants

A total of 19217 participants including 660 new-onset stroke patients were registered in the present study. Characteristics of these participants stratified by age are illustrated in **Table 1**. Among those aged less than 65, compared with control participants [12061 patients aged 56.0 (50.0, 61.0)], new-onset stroke patients 320 subjects aged [59.0 (54.0, 62.0)] were older and had higher levels of SBP, DPB, baPWV, fasting glucose, triglycerides, and blood homocysteine. However, among participants aged 65 or more, no significant differences in SBP, DPB, baPWV, fasting glucose, and triglycerides between control participants [6496 subjects aged 70.0 (67.0, 74.0)] or stroke patients [340 subjects aged 71.0 (67.0, 76.0)] were found.

## baPWV Predicts New Onset Stroke in Hypertensive Subjects Aged Less Than 65 Years

To determine the association of baPWV and age in predicting incident stroke, participants were separated into 2 groups: those aged less than 65 years, and those aged 65 years or more. Each group was further divided into quartiles (Q1-Q4) according to level of baPWV (**Table 2**). Cox regression was performed to investigate the effect of age and baPWV on new onset stroke. Overall, increased baPWV was positively associated to incident stroke (per SD: OR, 1.37; 95% CI, 1.26-1.50) for those aged less than 65 years in the crude model. A similar positive association of baPWV with stroke incidence in the younger group was observed in both model 1 (per SD: OR, 1.17; 95% CI, 1.06-1.29) and in model 2 (per SD: OR, 1.16; 95% CI, 1.05-1.28) (**Figure 1A**, **Table 2**). In contrast, an increase in baPWV was not associated with stroke incidence in patients aged 65 or more (**Figure 1B**, **Table 2**).

In participants aged less than 65 years, compared with quartile 1 (Q1), the odds ratios of incident stroke increased along with increased baPWV in the crude model (Q3: OR, 2.68, 95% CI, 1.86 - 3.88, P < 0.001; Q4: OR, 3.23, 95% CI, 2.26 - 4.63, P < 0.001) and in the adjusted model 1 and model 2 (**Table 2**). However, these associations of baPWV with stroke were absent in patients aged 65 or more.

## Fasting Glucose Modifies the Association Between New-Onset Stroke and Elevated Arterial Stiffness in Subjects Aged Less Than 65 Years

To analyze potential modifiable risk factors in the association between incident stroke and baPWV, a stratified sub-analysis was further performed in participants stratified by age group (aged less than 65 or aged 65 or more), and the subgroups sex, body mass index, systolic blood pressure, diastolic blood pressure, smoking status, alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, homocysteine, and history of antihypertensive use (**Table 3**). Notably, only fasting glucose had a significant, modifiable effect on the association between new-onset stroke and baPWV in the younger age group (*P* for interaction, 0.010), but not in the older group (*P* for interaction, 0.233).

Particularly, the increase of baPWV was strongly, positively associated to new-onset stroke in the subgroup with normal fasting glucose (fasting glucose < 5.6 mmol/L) for those aged

**TABLE 1 |** Baseline characteristics of study participants stratified by age group[A].

| Characteristics | Age < 65 years | | | Age ≥ 65 years | | |
|---|---|---|---|---|---|---|
| | Stroke cases | Non-stroke | *P* | Stroke cases | Non-stroke | *P* |
| Participants, *n* | 320 (2.6) | 12061 | | 340 (5.0) | 6496 | <0.001[B] |
| Male | 121 (37.8) | 4115 (34.1) | 0.188 | 148 (43.5) | 2575 (39.6) | 0.170 |
| Age, y | 59.0 (54.0, 62.0) | 56.0 (50.0, 61.0) | <0.001 | 71.0 (67.0, 76.0) | 70.0 (67.0, 74.0) | <0.001 |
| BMI, kg/m$^2$ | 26.1 (23.5, 28.4) | 26.2 (23.9, 28.7) | 0.220 | 24.9 (22.4, 27.7) | 25.1 (22.7, 27.7) | 0.348 |
| SBP, mmHg | 148.3 (139.3, 154.0) | 145.3 (136.3, 152.0) | <0.001 | 147.3 (139.7,153.7) | 146.7 (140.3, 152.7) | 0.526 |
| DBP, mmHg | 97.0 (88.7, 105.7) | 93.7 (86.3, 101.7) | <0.001 | 91.0 (82.3, 99.3) | 89.7 (81.7, 97.7) | 0.110 |
| baPWV, cm/s | 1701.1 (1523.2, 1921.8) | 1588.0 (1403.0, 1759.0) | <0.001 | 1893.0 (1666.2, 2146.0) | 1849.5 (1644.8, 2109.0) | 0.048 |
| Baseline laboratory results | | | | | | |
| Fasting glucose, mmol/L | 5.4 (4.9, 6.3) | 5.3 (4.9, 5.9) | 0.032 | 5.4 (4.9, 6.3) | 5.4 (5.0, 6.1) | 0.747 |
| Total cholesterol, mmol/L | 4.7 (4.2, 5.6) | 4.7 (4.1, 5.4) | 0.163 | 4.6 (4.1, 5.3) | 4.8 (4.1, 5.5) | 0.121 |
| Triglycerides, mmol/L | 1.7 (1.2, 2.5) | 1.6 (1.1, 2.3) | 0.046 | 1.4 (1.0, 2.1) | 1.5 (1.0, 2.1) | 0.534 |
| Homocysteine, μmol/L | 12.6 (10.2, 14.8) | 12.0 (10.0,14.6) | 0.075 | 14.5 (12.1, 17.7) | 13.8 (11.4, 16.7) | 0.005 |
| Smoking status | | | 0.147 | | | 0.649 |
| Never | 257 (80.3) | 9822 (81.4) | | 246 (72.4) | 4813 (74.1) | |
| Former | 12 (3.8) | 666 (5.5) | | 33 (9.7) | 641 (9.9) | |
| Current | 51 (15.9) | 1573 (13.0) | | 61 (17.9) | 1042 (16.0) | |
| Alcohol drinking status | | | 0.623 | | | 0.661 |
| Never | 248 (77.5) | 9217 (76.4) | | 253 (74.4) | 4880 (75.1) | |
| Former | 15 (4.7) | 480 (4.0) | | 25 (7.4) | 399 (6.1) | |
| Current | 57 (17.8) | 2364 (19.6) | | 62 (18.2) | 1217 (18.7) | |
| Hypertensive | 319 (99.7) | 11704 (97.0) | 0.009 | 330 (97.1) | 6365 (98.0) | 0.330 |
| History of diseases | | | | | | |
| Hypertension | 275 (85.9) | 9665 (80.1) | 0.012 | 295 (86.8) | 5399 (83.1) | 0.092 |
| Diabetes | 31 (9.7) | 807 (6.7) | 0.046 | 31 (9.1) | 544 (8.4) | 0.703 |
| Hyperlipidemia | 39 (12.2) | 1357 (11.3) | 0.665 | 37 (10.9) | 575 (8.9) | 0.238 |
| History of drug treatments | | | | | | |
| Antihypertensive | 187 (58.4) | 5686 (47.1) | <0.001 | 204 (60.0) | 3639 (56.0) | 0.166 |

*BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; baPWV, brachial ankle pulse wave velocity. Data are presented as median (IQR)[A] or n (%), unless otherwise indicated. [B]Statistic differences was compared with rate of stroke cases in Age < 65 years or Age ≥ 65 years.*

**TABLE 2 |** The association between baseline brachial ankle pulse wave velocity (baPWV) and risk of first stroke in various age groups.

| Age < 65 years | | | | | | | | Age ≥ 65 years | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| baPWV, cm/s | Cases (%) | Crude model | | Adjusted model 1 | | Adjusted model 2 | | baPWV, cm/s | Cases (%) | Crude model | | Adjusted model 1 | | Adjusted model 2 | |
| | | OR (95% CI) | *P* | OR (95% CI) | *P* | OR (95% CI) | *P* | | | OR (95% CI) | *P* | OR (95% CI) | *P* | OR (95% CI) | *P* |
| per SD increment | 320 (2.6) | 1.37 (1.26,1.50) | <0.001 | 1.17 (1.06,1.29) | 0.002 | 1.16 (1.05,1.28) | 0.003 | per SD increment | 340 (5.0) | 1.13 (1.03,1.25) | 0.011 | 1.06 (0.94,1.19) | 0.354 | 1.05 (0.93,1.19) | 0.393 |
| Quartiles | | | | | | | | Quartiles | | | | | | | |
| Q1 (<1406) | 40 (1.3) | ref | | ref | | ref | | Q1 (<1643) | 80 (4.7) | ref | | ref | | ref | |
| Q2 (1406-1563) | 49 (1.6) | 1.23 (0.81,1.87) | 0.339 | 0.97 (0.63,1.51) | 0.900 | 0.96 (0.62,1.49) | 0.857 | Q2 (1643-1851) | 68 (4.0) | 0.84 (0.60,1.17) | 0.303 | 0.68 (0.48,0.97) | 0.033 | 0.68 (0.48,0.97) | 0.032 |
| Q3 (1563-1763) | 105 (3.4) | 2.68 (1.86,3.88) | <0.001 | 1.81 (1.21,2.70) | 0.004 | 1.79 (1.20,2.66) | 0.005 | Q3 (1851-2110) | 94 (5.5) | 1.18 (0.87,1.60) | 0.289 | 0.96 (0.69,1.34) | 0.816 | 0.96 (0.69,1.33) | 0.799 |
| Q4 (≥1763) | 126 (4.1) | 3.23 (2.26,4.63) | <0.001 | 1.85 (1.21,2.82) | 0.004 | 1.81 (1.19,2.77) | 0.006 | Q4 (≥2110) | 98 (5.7) | 1.23 (0.91,1.67) | 0.174 | 0.93 (0.65,1.33) | 0.693 | 0.93 (0.65,1.32) | 0.681 |
| *p* for trend | | | <0.001 | | <0.001 | | <0.001 | *p* for trend | | | 0.050 | | 0.809 | | 0.825 |

*Model 1 is adjusted for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, smoking status, alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, and homocysteine.*

*Model 2 is adjusted for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, smoking status, alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, homocysteine, and history of antihypertensive drug use.*

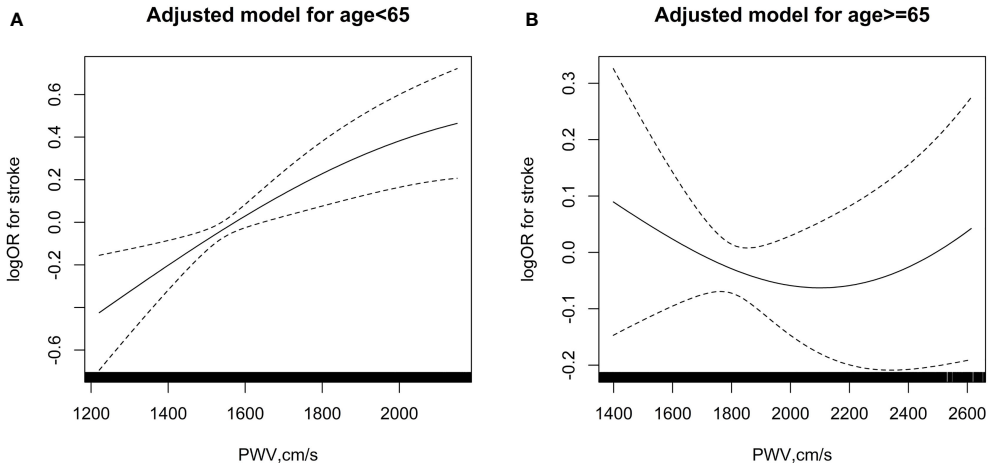


**FIGURE 1** | The association between baPWV and risk of first stroke in various age groups. **(A)** age < 65 years; **(B)** age≥ 65 years. The splines were adjusted for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, smoking status and alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, homocysteine and history of antihypertensive drug use.

**TABLE 3 |** The association between baPWV and risk of first stroke in various subgroups stratified by age.

| Subgroups | Age < 65 years | | | Age ≥ 65 years | | |
|---|---|---|---|---|---|---|
| | Cases (%) | OR (95% CI) | *P* for interaction | Cases (%) | OR (95% CI) | *P* for interaction |
| Sex | | | 0.917 | | | 0.780 |
| Male | 121 (2.9) | 1.16 (0.98,1.38) | | 148 (5.4) | 1.00 (0.83,1.20) | |
| Female | 199 (2.4) | 1.23 (1.03,1.48) | | 192 (4.7) | 1.08 (0.93,1.24) | |
| Body mass index, kg/m$^2$ | | | 0.566 | | | 0.527 |
| <24 | 96 (3.0) | 1.21 (0.94,1.55) | | 136 (5.3) | 0.99 (0.83,1.18) | |
| ≥24 | 221 (2.4) | 1.21 (1.05,1.39) | | 199 (4.8) | 1.10 (0.95,1.26) | |
| SBP, mmHg | | | 0.491 | | | 0.735 |
| <140 | 88 (2.2) | 1.25 (0.89,1.76) | | 86 (5.3) | 1.01 (0.77,1.32) | |
| ≥140 | 231 (2.7) | 1.20 (1.06,1.37) | | 253 (4.9) | 1.06 (0.94,1.19) | |
| DBP, mmHg | | | 0.152 | | | 0.376 |
| <90 | 88 (2.0) | 1.50 (1.09,2.08) | | 155 (4.6) | 0.94 (0.77,1.16) | |
| ≥90 | 232 (2.9) | 1.20 (1.05,1.36) | | 184 (5.4) | 1.13 (0.99,1.27) | |
| Smoking status | | | 0.989 | | | 0.125 |
| Never | 257 (2.5) | 1.22 (1.04,1.44) | | 246 (4.9) | 1.06 (0.94,1.20) | |
| Former | 12 (1.8) | 1.37 (0.71,2.66) | | 33 (4.9) | 0.70 (0.43,1.15) | |
| Current | 51 (3.1) | 1.16 (0.94,1.45) | | 61 (5.5) | 1.18 (0.89,1.56) | |
| Alcohol drinking status | | | 0.191 | | | 0.290 |
| Never | 248 (2.6) | 1.20 (1.02,1.42) | | 253 (4.9) | 0.98 (0.85,1.11) | |
| Former | 15 (3.0) | 2.20 (1.13,4.35) | | 25 (5.9) | 1.54 (1.03,2.33) | |
| Current | 57 (2.4) | 1.10 (0.88,1.39) | | 62 (4.8) | 1.25 (0.96,1.63) | |
| Fasting glucose, mmol/L | | | 0.010 | | | 0.233 |
| <5.6 | 188 (2.4) | 1.34 (1.15,1.57) | | 188 (4.8) | 0.92 (0.77,1.09) | |
| 5.6 to <7.0 | 74 (2.3) | 1.14 (0.83,1.56) | | 99 (5.1) | 1.11 (0.90,1.36) | |
| ≥7.0 or diabetes | 56 (4.2) | 0.93 (0.66,1.31) | | 48 (5.2) | 1.19 (0.96,1.36) | |
| Total cholesterol, mmol/L | | | 0.502 | | | 0.589 |
| <5.2 | 209 (2.5) | 1.26 (1.10,1.46) | | 232 (5.2) | 1.09 (0.95,1.25) | |
| ≥5.2 | 109 (2.8) | 1.05 (0.83,1.33) | | 101 (4.4) | 0.94 (0.77,1.15) | |
| Triglycerides, mmol/L | | | 0.408 | | | 0.320 |
| <1.7 | 155 (2.4) | 1.16 (0.97,1.39) | | 204 (5.1) | 1.01 (0.87,1.17) | |
| ≥1.7 | 155 (2.8) | 1.25 (1.05,1.50) | | 123 (4.7) | 1.09 (0.93,1.28) | |
| Homocysteine, $\mu$mol/L | | | 0.627 | | | 0.415 |
| <15 | 235 (2.5) | 1.19 (1.04,1.37) | | 181 (4.4) | 1.03 (0.87,1.19) | |
| ≥15 | 79 (2.9) | 1.24 (0.96,1.60) | | 153 (5.9) | 1.06 (0.90,1.25) | |

*Each subgroup analysis was adjusted, if not stratified for age, sex, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), smoking status, alcohol consumption, baseline fasting blood glucose, total cholesterol, triglycerides, homocysteine, and history of antihypertensive drug use. Data are presented as median (IQR) or n (%), unless otherwise indicated.*

less than 65 (OR, 1.34; 95% CI, 1.15–1.57), but the effect was not seen in those with either impaired fasting glucose (fasting glucose: 5.6-7.0 mmol/L) (OR, 1.14; 95% CI, 0.83-1.56) or diabetic fasting glucose (fasting glucose > 7.0 mmol/L) (OR, 0.93; 95% CI, 0.66-1.31) (**Table 3**). In addition, for those aged less than 65, the level of baPWV was significantly higher in stroke patients [baPWV: 1676.5 (1509.8, 1924.2) cm/s] compared with non-stroke control patients [baPWV: 1528.7 (1377.0, 1715.0)] ($P < 0.001$) in the normal glucose group; the level of baPWV was also higher in stroke patients [baPWV: 1724.0 (1580.2, 1854.5) cm/s] compared with controls [baPWV: 1588.5 (1432.0, 1797.0) cm/s] ($P < 0.001$) in the impaired fasting glucose group, while there was no statistical difference between cases and controls among diabetic fasting glucose patients ($P = 0.431$) (**Supplementary Table 1**).

## DISCUSSION

The present study revealed two new observations (1) new-onset stroke was positively associated to increased arterial stiffness in hypertensive patients aged less than 65, but not in those aged 65 or more, and (2) the predicting role of baPWV on incident stroke was modified by fasting glucose in younger patients (aged less than 65), but not in older patients; notably, a significant predictive value of baPWV for incident stroke was found in hypertensive patients with normal fasting glucose.

To the best of our knowledge, this cohort included the largest number of incident stroke cases for the assessment of the association between stroke and arterial stiffness. Both pioneer and recent studies have confirmed that arterial stiffness measured by carotid-femoral pulse wave velocity (cfPWV) independently predicts cardiovascular diseases, including first stroke in hypertensive patients (8, 13, 14). However, our current study indicates that the predictive value of arterial stiffness was modified by glucose in patients aged less than 65 years. There could be multiple potential mechanisms to explain these findings. First, although both cfPWV and baPWV are used for measurement of central artery stiffness and are significantly positively associated for predicting cardiovascular diseases (CVDs), baPWV is considered moderately associated to peripheral artery stiffness (15); in comparison to cfPWV, baPWV has shown a greater association with left ventricle mass, cardiovascular function, and coronary calcium (16, 17). The second possible reason might be due to characteristics of the study population. In our study, we divided study participants into two age groups: those under age 65 and those aged 65 or more, with age at 56.0 and 70.0 years, respectively (**Supplementary Table 2**); while patients from previous studies had age of 50 ± 13 (13), 51.05 ± 12.64 (8), and 51 ± 13 years (14). The base-line characteristics of stroke cases was shown in **Supplementary Table 3**. Interestingly, these results suggest a better prediction of PWV for first-ever stroke in younger compared with older individuals, which is consistent with recent findings from a meta-analysis of 17, 635 subjects (18).

Intriguingly, our current study clearly indicates a predictive value of baPWV in "relatively healthy" hypertensive patients, i.e., aged less than 65 years with normal fasting glucose. Arterial stiffness, a predictor of all-cause mortality and CVD events (18, 19), is positively related to increased age. Particularly, PWV levels significantly increase more steeply in hypertensive patients aged more than 50 years old compared to patients aged 50 or less (20, 21). As a result of a substantial increase of arterial stiffness in older patients aged 65 or more, we presumed that the predictive value of PWV among stroke and non-stroke patients might attenuate or even disappear. Multiple mechanisms could explain the association of PWV with first stroke. First, increased arterial stiffness relates to decreased regional cerebral blood flow and higher cerebrovascular reactivity (22), and these alterations of cerebrovascular hemodynamics and arteriole damage may contribute to injury of the central nervous system and, consequently, stroke. As level of PWV accumulates overtime, endothelial function is impaired in patients with acute stroke (23). Second, increased arterial stiffness also reflects stenosis of the peripheral arteries, which favors the likelihood of cardiovascular diseases (24).

Fasting blood glucose was a modifiable factor for the predictive value of baPWV in patients aged less than 65 years in our present study (**Table 3**). Increased arterial stiffness, measured by baPWV or cfPWV, has been shown to be positively associated with the risk of incident diabetes (25, 26); moreover, our findings suggest that arterial stiffness, in combination with fasting glucose, plays a role in predicting stroke: in hypertensive patients aged less than 65 years, level of baPWV was significantly higher in incident stroke patients compared to non-stroke controls with normal fasting glucose or impaired fasting glucose, while these effects were absent in older patients (**Supplementary Table 1**). In a large-scale study including 698782 participants, fasting glucose levels in individuals without diabetes had no significant improvement for predicting vascular disorders when status of conventional risk factors was provided (27). Our results from persons without diabetes provide a possibility to detect incident stroke, although the precise mechanism needs to be further determined.

Many studies indicate that the rise of incident stroke in young and middle-aged adults has become a critical problem in western countries and China (reviewed in (3, 28)). Of significance, the average age of Chinese stroke cases is about 10 years younger than that of western countries (29). Approximately one third of stroke patients are under age 60 in China, bringing significant losses to people of working age and their families (30). Our findings may provide evidence for improving upon the prevention of incident stroke in hypertensive middle-aged adults. In addition, the INTERSTROKE project suggests that hypertension still ranks as the number 1 substantial risk factor for acute stroke (31). In patients with hypertension, those with appropriate control of hypertension have lower baPWV levels and a decreased risk of first stroke.

Taken together, our study suggests that arterial stiffness is positively associated with new-onset stroke in hypertensive patients aged less than 65 years. Additionally, fasting glucose level is a modifiable factor of baPWV in predicting stroke, i.e., an independent predictive significance was found in middle-aged

hypertensive patients with normal fasting glucose. Our findings may provide evidence of clinical prevention and a potential therapeutic target for incident stroke. Targeted monitoring and intervention for decreasing baPWV might be an efficient strategy in the fight against incident stroke, to reduce a possible disability-adjusted life year in "relatively healthy" hypertensive patients, and ultimately, to achieve higher social and economic gains.

The present study has some limitations. First, the follow-up time was not long (3 years); further long-term studies should be performed. Second, the participants were from China which limits the generalizability of the results to other populations. Third, patients pooled in this study were taking different types of antihypertensive medications; since these medications might have different effects on arterial stiffness, further sub-group studies are required.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Anhui Medical University, Hefei, China. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

CL, ZhZ, HZ, and XW conceived and designed the study. CL wrote the manuscript. ZW, SL, YS, LL, ZiZ, MH, and LL performed statistics and generated the figure. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

The study was supported by MiaoPu Project of Beijing Tiantan Hospital (2020MP07); Yangfan Project of Beijing Hospitals Authority (ZYLX201827); Beijing Medical and Health Foundation (YWJKJJHKYJJ-B182875); China National Key Research and Development Program (2018YFC1311203), the National Key Research and Development Program (2016YFE0205400, 2018ZX09739010, 2018ZX09301034003); the Department of Science and Technology of Guangdong Province (2020B121202010); the Science and Technology Planning Project of Guangzhou, China (201707020010); the Science, Technology and Innovation Committee of Shenzhen (GJHS20170314114526143, JSGG20180703155802047); the Economic, Trade and Information Commission of Shenzhen Municipality (20170505161556110, 20170505160926390, 201705051617070).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.828286/full#supplementary-material

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 09 February 2022
doi: 10.3389/fendo.2022.828608

# Metformin Downregulates the Expression of Epidermal Growth Factor Receptor Independent of Lowering Blood Glucose in Oral Squamous Cell Carcinoma

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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *03 December 2021*
**Accepted:** *10 January 2022*
**Published:** *09 February 2022*

**Citation:**
*Wang W-M, Yang S-S, Shao S-H, Nie H-Q, Zhang J and Su T (2022) Metformin Downregulates the Expression of Epidermal Growth Factor Receptor Independent of Lowering Blood Glucose in Oral Squamous Cell Carcinoma. Front. Endocrinol. 13:828608. doi: 10.3389/fendo.2022.828608*

**Purpose:** Type 2 diabetes mellitus (T2DM) is among the risk factors for the occurrence and development of cancer. Metformin is a potential anticancer drug. Epidermal growth factor receptor (EGFR) plays an important role in the progression of oral squamous cell carcinoma(OSCC), but the relationship between metformin and EGFR expression in OSCC remains unclear.

**Methods:** This study involved the immunohistochemical detection of EGFR expression in cancer tissues of patients with T2DM and OSCC. The patients were divided into groups according to whether they were taking metformin for the treatment of T2DM, and the expression of EGFR in different groups was compared. Correlation analysis between the expression of EGFR and the fluctuation value of fasting blood glucose (FBG) was carried out. Immunohistochemistry was used to detect the expression of EGFR in cancer tissues of patients with recurrent OSCC. These patients had normal blood glucose and took metformin for a long time after the first operation.

**Results:** EGFR expression in T2DM patients with OSCC taking metformin was significantly lower than that in the non-metformin group. FBG fluctuations were positively correlated with the expression of EGFR in the OSCC tissues of the non-metformin group of T2DM patients. In patients with recurrent OSCC with normal blood glucose, metformin remarkably reduced the expression of EGFR in recurrent OSCC tissues.

**Conclusion:** Metformin may regulate the expression of EGFR in a way that does not rely on lowering blood glucose. These results may provide further evidence for metformin in the treatment of OSCC.

**Keywords: type 2 diabetes mellitus, EGFR, OSCC, metformin, blood glucose**

# INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the main malignant tumors of the head and neck, accounting for approximately 90% of all oral malignancies (1). It is a huge health problem facing the world, and its statistics has hardly changed over time (2). The five-year survival rate is less than 50%, in which 177,757 deaths out of the 377,713 new cases were recorded in 2020 (3).

Type 2 diabetes mellitus (T2DM) is an emerging systemic factor in the occurrence of OSCC (4, 5). In the United States, the prevalence of T2DM has increased remarkably in recent years and currently affects more than 34 million people (6). Diabetes-related hyperinsulinemia, insulin resistance, chronic inflammation, oxidative stress, and hyperglycemia may promote tumor progression in various ways (7, 8). Hyperglycemia is one of the most important factors that promote tumor transformation of potential oral malignant diseases (9, 10). Hyperglycemia stimulates cell proliferation, growth factor signaling, and chemoresistance in various cancer types (4, 5, 10).

Targeting epidermal growth factor receptor (EGFR) is one of the important treatments for OSCC, lung cancer and other tumors, and has shown good clinical effects (11). Cetuximab combined with radiotherapy and chemotherapy is one of the recommended therapies in the NCCN tumor treatment guidelines for the treatment of advanced tumors, including OSCC (12, 13). Metformin is a commonly used oral anti-diabetic drug with potential anti-cancer properties based on epidemiological studies (14). In comparison with T2DM patients treated with other anti-diabetic drugs, the risk of cancer in T2DM patients treated with metformin is reduced (15). When metformin is used in combination with anti-tumor drugs, such as traditional chemotherapy drugs, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), or immune checkpoint inhibitors (ICIs), it improves the anti-tumor effects of the drug (15, 16).

Hyperglycemia affects the proliferation, migration, and apoptosis of OSCC cell lines (4). In the present study, we compared FBG levels and EGFR expression in different OSCC populations to explore the effects of hyperglycemia and metformin on EGFR expression and found that the expression of EGFR in OSCC is related to FBG fluctuations. Metformin may reduce the expression of EGFR independent of lowering blood glucose. This study is expected to provide theoretical basis for the role of metformin and hyperglycemia in OSCC.

# MATERIALS AND METHOD

## Ethical Approval

The Institutional Review Board of Xiangya Hospital of Central South University approved this study, and all methods were carried out in accordance with relevant guidelines and regulations. All patients signed an informed consent form.

## Patients

This study involved a retrospective evaluation of the hospital archives of 83 patients with OSCC and T2DM in the Department of Stomatology, Xiangya Hospital, Central South University from January 2020 to August 2021. Among them, 29 patients with OSCC and T2DM were taking metformin (referred to hereafter OSCC-DM-M), and 54 patients with OSCC and T2DM were treated with other methods of hypoglycemic treatment (includes untreated cases, referred to hereafter OSCC-DM). This study also involved 22 cases of non-diabetic patients with OSCC who were admitted to the Department of Oral and Maxillofacial Surgery, Xiangya Hospital of Central South University from October 2019 to October 2021. These patients took metformin for a long time after the first surgery (referred to hereafter OSCC-N-M). The FBG of all T2DM patients was above normal.

## Immunohistochemistry

The expression of EGFR was determined by IHC. In short, tissue slides are sequentially rehydrated with xylene and graded alcohol. Then, the sections were blocked with 3% hydrogen peroxide, and the anti-EGFR antibody (9027T; Cell Signaling Technology, CST; 1:400 dilution) was kept at 4°C overnight. The next day, these sections were washed with PBS, and then incubated with goat anti-rabbit biotinylated secondary antibody for 15 min, horseradish peroxidase-conjugated streptavidin (SP-9001; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) incubate for 30 minutes at 37°C. DAB was used to visualize the primary antibody, while the nuclei were counterstained with hematoxylin. Image-Pro Plus 6.0 (Media Cybernetics, Inc.) was used to calculate the density determination as described earlier (4).

## Statistical Analysis

We used SPSS software (SPSS20.0; SPSS Inc., USA) for statistical analysis. All data are expressed as mean ± standard deviation. According to normality test, continuous variables were evaluated by Student's t test or Mann-Whitney U test. Chi-square test or Fisher's exact test was used to compare categorical variables as needed. The relationship between FBG fluctuations and the expression of EGFR was examined using two-tailed Pearson statistics. A P-value less than 0.05 is considered statistically significant.

# RESULTS

## Clinicopathological Characteristics of OSCC Patients With T2DM

A total of 83 newly diagnosed patients with OSCC and T2DM were included in this group. Among them, 29 patients with OSCC and T2DM were taking metformin, and 54 patients were treated with other methods of hypoglycemic treatment. The study involved 78 males and 5 females with a median age of 55 years (31–83 years). Tumors originated in 33 cases of the tongue, 29 cases of the cheek, 18 cases of other parts of the oral cavity, 48 cases of well-differentiated squamous cell carcinoma, 35 cases of medium and poorly differentiated squamous cell carcinoma, 57 cases of T1-T2 stage, and 26 cases of T3-T4 stage. TNM staging was conducted based on the AJCC 8th edition TNM staging standard. A total of 23 cases with lymph node metastasis and 60 cases without lymph node metastasis were recorded. The average

blood glucose fluctuations (FBG minus the highest value of the normal range) in the OSCC-DM-M group was 2.06. The average of the OSCC-DM group was 2.6. No significant difference was observed in the distribution of patients between the OSCC-DM-M and OSCC-DM group (**Table 1**). Detailed information on the patients is provided in **Supplementary Table 1** (OSCC-DM-M group) and **Supplementary Table 2** (OSCC-DM group).

## Expression of EGFR in the Cancer Tissues of OSCC-DM-M Group and OSCC-DM Group

We checked the expression of EGFR in the collected OSCC tissues through IHC analysis. As shown in **Figure 1A**, typical pictures of EGFR staining show that the staining intensity of EGFR in the OSCC-DM-M group is lower than that of the OSCC-DM group, and the number of stained cells in the OSCC-DM-M group is also lower than that of the OSCC-DM group. The staining positions on the cells were indistinguishable, all of which were staining of the cell membrane and part of the cytoplasm. Semi-quantitative analysis results show that EGFR in the OSCC-DM-M group was significantly lower than that in the OSCC-DM group (**Figure 1B**).

## Relationship Between EGFR Expression and Fluctuation of FBG in OSCC Patients

We tested the FBG of 83 patients and performed a correlation analysis with the expression of EGFR. Interestingly, no correlation was observed between the FBG of 83 patients and the expression of EGFR (**Figure 2A**). However, after eliminating the factor of metformin, we found that the expression of EGFR was positively correlated with the fluctuation of FBG (no matter what non-metformin hypoglycemic method the patient adopted), and the expression of EGFR in OSCC-DM-M group had no correlation with the fluctuation of FBG (**Supplementary Figure 1**). Therefore, the reduction of EGFR expression by metformin may not be related to the fluctuation of blood glucose (**Figure 2B**).

## Expression of EGFR in Recurrent OSCC Tissues of Patients With Normal Blood Glucose Taking Metformin

To verify whether the regulation of EGFR expression by metformin is related to blood glucose, we collected 22 patients with OSCC recurrence who started taking metformin after the first radical resection of OSCC. Interestingly, paired t-test analysis results show that the expression of EGFR was significantly reduced in the second cancer tissue (**Figure 3**). Detailed information on the patients is provided in **Supplementary Table 3**.

# DISCUSSION

Extensive epidemiological studies have shown that T2DM is an emergent contributing systemic factor in pancreatic and Liver (17, 18). Other cancers including breast, colorectal, endometrial, and kidney cancer are also related to T2DM, while prostate cancer is negatively related to T2DM (5). A recent meta-analysis showed that patients with T2DM have an increased risk of OSCC or precancerous lesions (10). Moreover, compared with patients

**TABLE 1** | Patient and tumor characteristics.

| Characteristics | Metformin (n = 29) | Other (n = 54) | *P* value |
|---|---|---|---|
| Age, mean (SEM), y | 53.34 (1.76) | 57.67 (1.39) | 0.1503 |
| Weight, mean (SEM), kg | 70.17 (1.78) | 71.10 (1.53) | 0.6908 |
| BMI, mean (SEM), kg/m2 | 24.85 (0.47) | 25.41 (0.43) | 0.4052 |
| Fasting blood glucose fluctuation, mean (SEM) mmol/L | 2.06 (0.51) | 2.67 (0.33) | 0.3131 |
| Gender No. (%) | | | |
| Male | 28 (33.73) | 50 (60.25) | |
| Female | 1 (1.20) | 4 (4.82) | 0.4698 |
| Tobacco smoking, No. (%) | | | |
| Yes | 27 (32.53) | 45 (54.22) | |
| No | 2 (2.41) | 9 (10.84) | 0.2107 |
| Alcohol drinking, No. (%) | | | |
| Yes | 16 (19.28) | 29 (34.94) | |
| No | 13 (15.66) | 25 (30.12) | 0.8981 |
| Tumor site, No. (%) | | | |
| Tongue | 10 (12.05) | 23 (27.71) | |
| Buccal | 10 (12.05) | 20 (24.10) | |
| Others (gingiva/lips/palates) | 9 (10.84) | 11 (13.25) | 0.5389 |
| T stage, No. (%) | | | |
| I+II | 18 (21.69) | 22 (26.51) | |
| III+IV | 11 (13.25) | 32 (38.55) | 0.0637 |
| Pathological grade, No. (%) | | | |
| Well differentiated | 19 (22.89) | 34 (40.96) | |
| Moderately/Poorly differentiated | 10 (12.05) | 20 (24.10) | 0.8174 |
| Lymph node metastasis | | | |
| No | 23 (27.71) | 37 (44.58) | |
| Yes | 6 (7.23) | 17 (20.48) | 0.2949 |

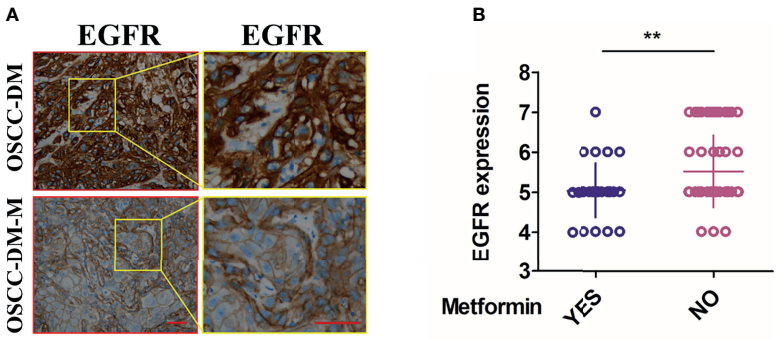


**FIGURE 1** | Expression of EGFR in the cancer tissues of OSCC-DM-M group and OSCC-DM group. **(A)** typical pictures of EGFR staining in the OSCC-DM-M and OSCC-DM group. **(B)** Semi-quantitative analysis results in the OSCC-DM-M and OSCC-DM group. Scale bar, 200 μm, $^{**}P < 0.01$.

without T2DM, OSCC patients coexisting with T2DM showed a higher risk of recurrence and a lower five-year OSCC-free survival rate (4). Therefore, T2DM is increasingly considered to be related to the development and progression of cancer.

In OSCC tissues, the relationship between EGFR overexpression and poor survival of patients has been repeatedly reported (19, 20). The expression of EGFR mediates systemic complications of T2DM that affect the heart, kidneys, and eyes (21). High-glucose culture of pancreatic cancer cell lines increases the expression of EGF, which then activates EGFR (18). The neuregulin-1 (Nrg1)-HER3 pathway is upregulated in tumors derived from hyperglycemic patients or rodents (22). In oral dysplastic keratinocytes, high glucose leads to EGFR activation in an FASN-dependent manner (10). In general, the mechanism by which high glucose promotes EGFR expression is still unclear. Our results show that in OSCC patients with T2DM, the expression level of EGFR has no correlation with blood glucose levels, but after excluding the factor of taking metformin, the expression of EGFR is positively correlated with FBG fluctuations. Therefore, in patients with OSCC, fluctuations in FBG may affect the expression of EGFR. After grouping and comparing, results show that taking metformin can significantly reduce the expression of EGFR possibly by lowering blood glucose. Alternately, metformin drugs may directly reduce the expression of EGFR without relying on lowering blood glucose.

Metformin is a commonly used drug for T2DM. In comparison with T2DM patients who are not taking metformin, it has been shown to reduce the incidence of cancer in T2DM patients (5). In breast and other cancers, metformin activates the AMPK signaling pathway and inhibits the mTOR pathway to trigger its anti-cancer effects (23, 24). In addition to the AMPK pathway, several non-AMPK pathways, such as RAS, AKT, and HIF-1α, may contribute to the anti-cancer effect of metformin (25–27). Moreover, metformin acts directly on mitochondria by reducing mitochondrial respiration and overall energy efficiency (28). To clarify the effect of metformin on the expression of EGFR in patients with T2DM and OSCC, we conducted a study on EGFR in cancer tissues of non-diabetic OSCC patients taking metformin. The results show that metformin can reduce the expression of EGFR in patients with non-diabetic recurrent OSCC. Generally, metformin does not lower blood glucose levels in patients with normoglycemia. Therefore, in OSCC, metformin may directly reduce the expression of EGFR independently of lowering blood glucose. The possible mechanism may be that metformin can directly weakly bind to EGFR, thereby inhibiting the expression or activity of EGFR (29).

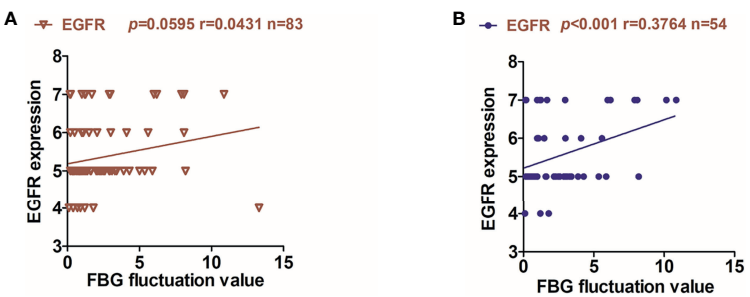


**FIGURE 2** | Relationship between EGFR expression and fluctuation of FBG in OSCC patients. **(A)** correlation between the FBG fluctuation of 83 patients and the expression of EGFR. **(B)** correlation between the FBG fluctuation of 54 patients and the expression of EGFR in OSCC-DM group.

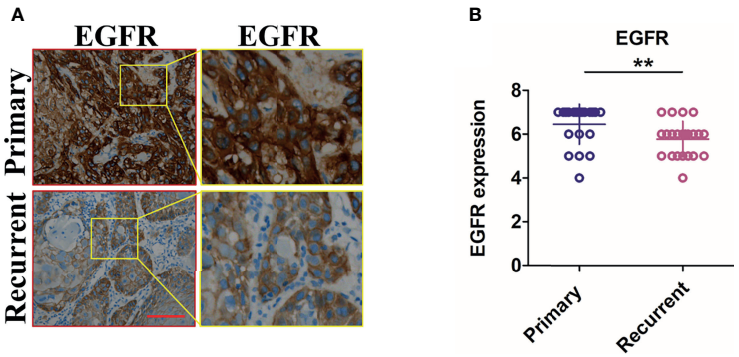


**FIGURE 3** | Expression of EGFR in recurrent OSCC tissues of patients with normal blood glucose taking metformin. **(A)** typical pictures of EGFR staining in the primary and recurrent OSCC in the same patient. **(B)** Semi-quantitative analysis results in the primary and recurrent OSCC in the same patient. Scale bar, 200 μm, $^{**}P < 0.01$.

In this study, we collected enough clinical specimens to study the expression level of EGFR in OSCC tissues with T2DM. In addition, we investigated the role of metformin in reducing EGFR expression in OSCC. However, our research still has some limitations. What we should realize is that due to the antibodies specificity and semi-quantitative accuracy of immunohistochemical assay, the experimental results need to be further proved by *in vivo* and *in vitro* experiments. T2DM is a complex metabolic disorder, and hyperglycemia is its only clinical symptom. Other potential factors such as hyperinsulinemia have not been covered in this context and need to be further studied. *In vitro* and *in vivo* experiments are needed to prove the possible role of T2DM and metformin in OSCC. Further animal models of T2DM are essential to confirm and explore the relationship between metformin, T2DM, and OSCC. Through our research, we hope to draw more attention from clinicians to OSCC patients with T2DM to improve their prognosis.

## CONCLUSION

FBG fluctuations in T2DM patients may remarkably affect the expression of EGFR in OSCC, and metformin can regulate the expression of EGFR in a way that does not rely on lowering blood glucose. This finding may partly clarify the connection between T2DM and metformin and OSCC, thus inspiring new strategies for the prevention and treatment of OSCC.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xiangya Hospital Central South University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

W-MW: concept/design, data analysis. S-SY, S-HS, H-QN, and JZ: data analysis. TS: critical revision, final approval. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.828608/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 02 March 2022
doi: 10.3389/fendo.2022.861760

# Triglyceride Glucose Index Is More Closely Related to Hyperuricemia Than Obesity Indices in the Medical Checkup Population in Xinjiang, China

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**Specialty section:**
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

**Received:** 25 January 2022
**Accepted:** 09 February 2022
**Published:** 02 March 2022

**Citation:**
Kahaer M, Zhang B, Chen W, Liang M, He Y, Chen M, Li R, Tian T, Hu C and Sun Y (2022) Triglyceride Glucose Index Is More Closely Related to Hyperuricemia Than Obesity Indices in the Medical Checkup Population in Xinjiang, China. Front. Endocrinol. 13:861760. doi: 10.3389/fendo.2022.861760

**Background:** Hyperuricemia (HUA) is a metabolic anomaly with an increased incidence rate, causing a global medical burden. Several studies have confirmed that obesity and insulin resistance (IR) are the risk factors for HUA. Reports on the predictive power of different obesity indices for HUA are limited. This study aimed to compare the association between different general, abdominal, and visceral obesity indices and markers of the IR-triglyceride glucose (TyG) index with serum uric acid (SUA) and to assess the ability of these indices to predict HUA.

**Methods:** A total of 2243 participants were recruited from Barkol County Hospital and surrounding township hospitals in Xinjiang. Obesity indices, including the atherogenic index of plasma, cardiometabolic index, visceral adiposity index, lipid accumulation product index, a body shape index, body roundness index, waist circumference, waist-to-height ratio, body mass index, and TyG index, were divided into four quartiles. Moreover, partial correlations and logistic regression were used to analyze the association between these indices and SUA. The area under the curve (AUC) and receiver operating characteristic curves were used to analyze the predictive value of these indices for HUA.

**Results:** After controlling for confounding variables, the association between the TyG index and HUA was stronger than that between the obesity indices in both males and females. The odds ratios (ORs) for HUA in the highest quartile of the TyG index were 2.098 (95% confidence interval, 1.555–2.831) in males and 7.788 (95% CI, 3.581–16.937) in females. For males, the AIP, CMI, VAI, LAP index, and TyG index were able to discriminate HUA, and the TyG index showed the highest AUC value of 0.586 (95% CI, 0.557–0.614; $P < 0.001$). For females, all indices, except BMI, can discriminate HUA. Moreover, the

visceral obesity index CMI showed the highest AUC value of 0.737 (95% CI, 0.691–0.782; $P < 0.001$). Meanwhile, the TyG index had a relatively high AUC value of 0.728 (95% CI, 0.682–0.773; $P < 0.001$).

**Conclusion:** The TyG index was significantly related to HUA and was superior to obesity indices in identifying HUA in the medical checkup population in Xinjiang, China.

**Keywords: Triglyceride glucose index, serum uric acid, obesity indices, hyperuricemia, medical checkup population**

## INTRODUCTION

Uric acid is the final product of purine metabolism, and hyperuricemia (HUA) is caused by the excessive production or insufficient excretion of uric acid in the body, the incidence rate of which is increasing annually worldwide. Data from a national survey have demonstrated that the prevalence of HUA among Chinese adults during 2010–2014 was 13.3% (1). According to the National Health and Nutrition Examination Survey, approximately 21% of American adults have HUA (2). The age-standardized prevalence of HUA in the general Korean population is 11.4% (3). Simultaneously, a number of epidemiological studies have reported that HUA causes gout and increases the risk of ischemic stroke, acute myocardial infarction, and other cardiovascular events (4, 5).

Both obesity and insulin resistance (IR) are associated with HUA (6, 7). The general obesity index body mass index (BMI) and abdominal obesity indices waist circumference (WC) and waist-to-height ratio (WHtR) have some implications in predicting the incidence of HUA. However, these indices cannot clearly distinguish between visceral and subcutaneous fats. Previous studies have confirmed a positive correlation between visceral fat deposition and increased uric acid production (8). Since the direct estimation of visceral fat requires diagnostic imaging, which is expensive and has low epidemiological availability, there has been increasing interest in finding simple and effective alternative markers of visceral obesity. In recent years, some lipid and visceral obesity-related indices, such as visceral adiposity index (VAI), lipid accumulation product (LAP) index, atherogenic index of plasma (AIP), cardiometabolic index (CMI), a body shape index (ABSI), and body roundness index (BRI), have also been proposed as supplementary indices to assess obesity and to predict the incidence of related metabolic diseases. The triglyceride glucose (TYG) index is a marker of IR and is associated with uric acid through obesity (9). A comparison with the gold standard method revealed that the TyG index was more suitable for the determination of IR than alternative indices, such as the homeostasis model assessment-estimated insulin resistance.

Many studies have investigated these indices in relation to cardiovascular diseases and diabetes, but few have predicted HUA (10, 11). Simultaneously, although many studies have pointed out that obesity indices can predict the risk of HUA, there was no conclusion on which indices were more suitable for predicting the risk of HUA in Xinjiang population, China. Therefore, this study aimed to use a cross-sectional survey to analyze and compare six visceral obesity indices (AIP, CMI, VAI, LAP index, ABSI, and BRI) with the general obesity index BMI, abdominal obesity indices WC and WHtR, and TyG index to predict the risk of HUA, determine more suitable risk predictors for HUA in our population, and provide a basis for early prevention of HUA.

## METHODS

### Study Participants

This was a cross-sectional, observational study. All participants were selected from the medical checkup population of Barkol County Hospital and the surrounding township hospitals from May 2016 to May 2021. A total of 2243 participants (age range, 20–68 years) were included in this study. Individuals with any of the following conditions were excluded: (1) chronic kidney disease or renal function impairment, (2) long-term use of uric acid-lowering drugs, (3) malignant tumors, and (4) autoimmune diseases that may affect SUA levels. Trained research interviewers administered standardized questionnaires through face-to-face interviews. The questionnaire included age, sex, medication history, and medical history. Height, weight, and WC were measured using standard methods. Blood pressure was measured using an electronic sphygmomanometer. Fasting blood samples (5 mL) were drawn from the participants under strict aseptic conditions and the serum was separated by centrifugation at 3000 rpm for 15 min after clotting. Myriad BS-800M fully automated biochemical analyzer and its matching reagents were used to assess the level of serum uric acid (SUA), fasting plasma glucose(FPG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triacylglycerol (TG), blood urea nitrogen (BUN), and creatinine (Cre) in Barkol County Hospital.

### Definition and Obesity Index Calculations

HUA was defined as an SUA level >7.0 mg/dL according to the guidelines for the diagnosis and management of HUA and gout in China (2019) (12). According to previous studies, obesity indices were calculated using the following formula (13, 14):

$$\mathrm{BMI} = \mathrm{weight\ (kg)}/\mathrm{height}^2(\mathrm{m}^2)$$

$$\mathrm{AIP} = \log\ (\mathrm{TG\,[mmol/L]}/\mathrm{HDL-C\,[mmol/L]})$$

$$CMI = TG/HDL - C \times WHtR$$

$$VAI\ (males) = (WC\ [cm]/39.68 + [1.88 \times BMI]) \times (TG\ [mmol/L]/1.03) \times (1.31/HDL - C\ [mmol/L])$$

$$VAI\ (females) = (WC\ [cm]/36.58 + [1.89 \times BMI]) \times (TG\ [mmol/L]/0.81) \times (1.52/HDL - C\ [mmol/L])$$

$$LAP\ index\ (females) = TG\ (mmol/L) \times (WC\ [cm] - 58)$$

$$LAP\ index\ (males) = TG\ (mmol/L) \times (WC\ [cm] - 65)$$

$$TyG\ index = \log\ (TG[mmol/L] \times fasting\ plasma\ glucose\ (FPG)[mmol/L]/2)$$

$$ABSI = WC\ (cm)/(height\ [cm])^{1/2} \times (BMI^2)^{1/3}$$

$$BRI = 364.2 - 365.5 \times [1 - (WC/2\pi)/(0.5 \times height)]^2]^{1/2}$$

## Statistical Analyses

The measures of normal distribution are expressed as means ± standard deviation, and the two groups were compared using an independent sample t-test. One-way analysis of variance was used to compare more than two groups. Non-normally distributed measures are expressed as M (P25, P75), the Mann–Whitney U-test was used for comparison between two groups, and the Kruskal–Wallis H-test was used for comparison between more than two groups. Partial correlation analysis was performed to assess the correlation between the different indices and SUA. Logistic analysis was performed to analyze the association between the different indices and HUA. A receiver operating characteristic (ROC) curve was used to analyze the predictive value of the different indices for HUA. All statistical analyses were performed using the Statistical Package for the Social Sciences version 21.0 (International Business Machines Corporation) and GraphPad Prism version 6.0. Statistical significance was defined as a two-tailed *P*-value < 0.05.

# RESULTS

A total of 2243 participants (1616 males and 627 females) were enrolled in the study, with an age range of 20–69 years (mean age, 41.55 ± 12.70 years). The basic characteristics of all participants are summarized in **Table 1**. Participants with HUA were older than those without HUA. Compared to participants with normal SUA, participants with HUA had significantly higher WC; BMI; WHtR; systolic blood pressure (SBP); diastolic blood pressure (DBP); TG, LDL-C, BUN, and Cre levels; AIP; CMI; VAI; LAP index; TyG index; ABSI; and BRI (all $P < 0.05$). No significant differences were found in FPG, TC, and HDL-C levels between the two groups.

The partial correlation coefficients between the different indices and SUA are shown in **Table 2**. SUA levels were significantly correlated with the TyG index, AIP, CMI, VAI, and LAP index after adjusting for age in both males and females (all $P < 0.05$). Among them, the SUA level had the strongest positive correlation with the TyG index in all participants (r = 0.332, 0.229, and 0.4, respectively; all $P < 0.05$), whereas the CMI, VAI, and LAP index had a relatively high correlation. SUA levels were significantly correlated with WC, WHtR, ABSI, and BRI in females only.

Multivariate logistic regression revealed the odds ratios (ORs) and 95% confidence intervals (CIs) for HUA according to sex-specific TyG index, BMI, WC, WHtR, AIP, CMI, VAI, LAP index, ABSI, and BRI quartiles. After full adjustment for age, SBP, DBP, and selected biochemical indices in model 3, compared with the first quartile, the other three quartiles of visceral obesity TyG index were strongly associated with HUA in both males and females (all $P < 0.05$). For males and females, the ORs for HUA in the upper quartile of the TyG index were 2.098 (95% CI, 1.555–2.831) and 7.788 (95% CI, 3.581–16.937), respectively. The adjusted relative risk of HUA increased with increasing TyG quartiles. The results are presented in **Tables 3**, **4**.

The ROC curves of the different indices for HUA are presented in **Table 5** and **Figure 1**. For males, the TyG index, AIP, CMI, VAI, and LAP index were able to discriminate HUA, and the TyG index showed the highest area under the curve (AUC) value of 0.586 (95% CI, 0.557–0.614; $P < 0.05$), with a cutoff value of 8.353 according to the maximum Youden index of 0.133. For females, all indices, except BMI, could discriminate HUA. The CMI showed the highest AUC value of 0.737 (95% CI, 0.691–0.782; $P < 0.05$), with a cutoff value of 0.595 according to the maximum Youden index of 0.384. Meanwhile, the TyG index, AIP, VAI, and LAP index had a relatively high AUC value.

# DISCUSSION

## Main Findings

This study investigated and compared the predictive strength of the TyG index and nine obesity indices reflecting general (BMI), abdominal (WC, WHtR), and visceral (AIP, CMI, VAI, LAP index, ABSI, BRI) obesity in the assessment of HUA in a medical checkup population in Xinjiang, China. The main findings of this study were as follows: (1) the TyG index was significantly associated with HUA, and the association remained significant after controlling for age, SBP, DBP, and selected biochemical indices. Further stratification showed that when the TyG index was higher (in the third and fourth quartiles), the risk of developing HUA was higher (2). Some visceral obesity indices were also associated with HUA, but were inferior to the TyG index (3). The TyG index was a better predictor of HUA, especially in females. Therefore, the TyG index may be the best choice for the HUA risk screening index for this regional population.

**TABLE 1** | Clinical characteristics of study participants with and without hyperuricemia.

| Variables | Non-hyperuricemia (n = 1471) | Hyperuricemia (n = 772) | $\chi^2$/t/z | *P*-value |
|---|---|---|---|---|
| Males, n (%) | 1001 (68.05) | 615 (79.66) | 33.91 | <0.001 |
| Age (years) | 42.44 ± 12.24 | 45.17 ± 12.28 | –5.014 | <0.001 |
| SBP (mmHg) | 121.36 ± 17.83 | 123.14 ± 17.87 | –2.252 | 0.024 |
| DBP (mmHg) | 78.95 ± 11.59 | 80.65 ± 13.33 | –3.129 | 0.002 |
| SUA (mg/dL) | 4.72 ± 1.28 | 8.20 ± 1.29 | –60.870 | <0.001 |
| FPG (mmol/L) | 5.13 ± 1.38 | 5.13 ± 1.80 | –0.020 | 0.984 |
| TC (mmol/L) | 4.50 ± 1.84 | 4.44 ± 1.56 | 0.749 | 0.454 |
| TG (mmol/L) | 1.28 (0.86–2.1) | 1.85 (1.2–3.18) | –11.603 | <0.001 |
| HDL-C (mmol/L) | 1.19 ± 0.51 | 1.17 ± 0.41 | 0.491 | 0.623 |
| LDL-C (mmol/L) | 2.67 (2.1–3.33) | 2.87 (2.3–3.49) | –4.030 | <0.001 |
| BUN (mmol/L) | 5.33 ± 3.32 | 5.73 ± 3.09 | –2.767 | 0.006 |
| Cre (mL/min) | 80.38 ± 41.72 | 86.08 ± 34.17 | –3.261 | 0.001 |
| TyG index | 8.61 ± 0.73 | 8.96 ± 0.71 | –10.802 | <0.001 |
| General obesity index | | | | |
| BMI (kg/m$^2$) | 25.01 (22.65–27.68) | 25.51 (23.18–27.85) | –2.739 | 0.017 |
| Abdominal obesity indices | | | | |
| WC (cm) | 89.48 ± 12.89 | 91.44 ± 13.70 | –3.339 | 0.001 |
| WHtR | 0.53 ± 0.08 | 0.54 ± 0.09 | –3.022 | 0.003 |
| Visceral obesity indices | | | | |
| AIP | 0.09 ± 0.34 | 0.24 ± 0.34 | –9.887 | <0.001 |
| CMI | 0.6 (0.36–1.18) | 0.9 (0.54–1.72) | –9.792 | <0.001 |
| VAI | 1.72 (1.07–3.1) | 2.38 (1.44–4.42) | –8.387 | <0.001 |
| LAP index | 32.19 (18.48–59.8) | 46.2 (27.39–87.48) | –9.146 | <0.001 |
| ABSI | 0.80 ± 0.07 | 0.81 ± 0.09 | –2.977 | 0.003 |
| BRI | 4.11 ± 1.57 | 4.34 ± 1.92 | –3.073 | 0.002 |

*SBP, systolic blood pressure; DBP, diastolic blood pressure; SUA, serum uric acid; FPG, fasting plasma glucose; TC, total cholesterol; TGs, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; BUN, blood urea nitrogen; Cre, creatinine; TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.*

## General and Abdominal Obesity Indices With Hyperuricemia (HUA)

In many previous studies, obesity was often reflected in BMI, WC, and WHtR, and some studies have shown that these indices are associated with HUA (15). However, BMI reflects only the degree of obesity in humans. Although WC and WHtR can reflect abdominal obesity, they cannot distinguish subcutaneous fat from visceral fat (16, 17), and the accumulation of visceral fat is more likely to lead to disorders of uric acid metabolism than subcutaneous fat (18). However, BMI, WC, and WHtR were not significantly associated with HUA in the present study. The general obesity indices for this population may not be the most appropriate way to assess HUA, which is characterized by excessive weight and fat accumulation. There were differences in body fat distribution between the ethnic groups (19). According to the World Health Organization guidelines, thresholds for BMI, WC, and WHtR are recommended for several different ethnicities and populations (20).

## Visceral Obesity Indices With HUA

Studies have shown that obesity, especially visceral obesity, is closely associated with HUA. However, there is no definitive

**TABLE 2** | Partial correlation coefficients between different indices and SUA.

| Variables | Total | | Males | | Females | |
|---|---|---|---|---|---|---|
| | *r* | *P*-value | *r* | *P*-value | *r* | *P*-value |
| TyG index | 0.332 | <0.001 | 0.229 | <0.001 | 0.4 | <0.001 |
| BMI | 0.049 | 0.021 | 0.014 | 0.563 | 0.051 | 0.2 |
| WC | 0.118 | <0.001 | 0.029 | 0.247 | 0.199 | <0.001 |
| WHtR | 0.079 | <0.001 | 0.034 | 0.178 | 0.188 | <0.001 |
| AIP | 0.31 | <0.001 | 0.198 | <0.001 | 0.41 | <0.001 |
| CMI | 0.226 | <0.001 | 0.151 | <0.001 | 0.321 | <0.001 |
| VAI | 0.198 | <0.001 | 0.152 | <0.001 | 0.316 | <0.001 |
| LAP index | 0.198 | <0.001 | 0.126 | <0.001 | 0.331 | <0.001 |
| ABSI | 0.081 | <0.001 | 0.014 | 0.573 | 0.243 | <0.001 |
| BRI | 0.055 | 0.009 | 0.018 | 0.467 | 0.161 | <0.001 |

*TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.*

**TABLE 3 |** Multivariate logistic regression of different indices for HUA (males).

| Variables | Quartile 1 | Quartile 2 | | Quartile 3 | | Quartile 4 | |
|---|---|---|---|---|---|---|---|
| | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| TyG index | ≤8.33 | 8.34–8.78 | | 8.79–9.34 | | ≥9.35 | |
| Model 1 | Reference | 1.573 (1.169–2.116) | 0.003 | 1.818 (1.355–2.441) | <0.001 | 2.202 (1.643–2.952) | <0.001 |
| Model 2 | Reference | 1.519 (1.127–2.047) | 0.006 | 1.748 (1.3–2.351) | <0.001 | 2.131 (1.585–2.867) | <0.001 |
| Model 3 | Reference | 1.501 (1.108–2.032) | 0.009 | 1.762 (1.303–2.383) | <0.001 | 2.098 (1.555–2.831) | <0.001 |
| BMI | ≤23 | 24–25 | | 26–28 | | ≥29 | |
| Model 1 | Reference | 1.277 (0.959–1.7) | 0.094 | 1.452 (1.089–1.935) | 0.011 | 1.15 (0.861–1.535) | 0.343 |
| Model 2 | Reference | 1.221 (0.911–1.635) | 0.182 | 1.351 (1.003–1.82) | 0.048 | 1.029 (0.757–1.4) | 0.855 |
| Model 3 | Reference | 1.211 (0.901–1.629) | 0.205 | 1.369 (1.013–1.85) | 0.041 | 1.045 (0.767–1.424) | 0.781 |
| WC | ≤82 | 83–90 | | 91–99 | | ≥100 | |
| Model 1 | Reference | 1.164 (0.88–1.539) | 0.287 | 1.189 (0.893–1.584) | 0.236 | 1.04 (0.78–1.387) | 0.79 |
| Model 2 | Reference | 1.116 (0.839–1.483) | 0.451 | 1.077 (0.8–1.45) | 0.626 | 0.914 (0.673–1.241) | 0.564 |
| Model 3 | Reference | 1.128 (0.846–1.506) | 0.412 | 1.091 (0.807–1.474) | 0.573 | 0.941 (0.691–1.281) | 0.697 |
| WHtR | ≤0.49 | 0.5–0.53 | | 0.54–0.59 | | ≥0.6 | |
| Model 1 | Reference | 1.1 (0.827–1.463) | 0.513 | 1.195 (0.9–1.588) | 0.219 | 1.055 (0.792–1.404) | 0.715 |
| Model 2 | Reference | 1.042 (0.779–1.394) | 0.78 | 1.093 (0.814–1.468) | 0.555 | 0.927 (0.683–1.258) | 0.627 |
| Model 3 | Reference | 1.03 (0.767–1.382) | 0.845 | 1.109 (0.823–1.495) | 0.496 | 0.945 (0.695–1.285) | 0.717 |
| AIP | ≤−0.04 | −0.03–0.18 | | 0.19–0.45 | | ≥0.46 | |
| Model 1 | Reference | 1.475 (1.102–1.974) | 0.009 | 1.531 (1.145–2.048) | 0.004 | 1.869 (1.399–2.497) | <0.001 |
| Model 2 | Reference | 1.444 (1.077–1.938) | 0.014 | 1.5 (1.119–2.011) | 0.007 | 1.819 (1.355–2.444) | <0.001 |
| Model 3 | Reference | 1.418 (1.054–1.908) | 0.021 | 1.502 (1.116–2.02) | 0.007 | 1.839 (1.364–2.48) | <0.001 |
| CMI | ≤0.47 | 0.48–0.8 | | 0.81–1.54 | | ≥1.55 | |
| Model 1 | Reference | 1.454 (1.083–1.953) | 0.013 | 1.896 (1.417–2.536) | <0.001 | 1.808 (1.35–2.421) | <0.001 |
| Model 2 | Reference | 1.407 (1.045–1.895) | 0.024 | 1.85 (1.379–2.483) | <0.001 | 1.739 (1.289–2.346) | <0.001 |
| Model 3 | Reference | 1.39 (1.029–1.877) | 0.032 | 1.854 (1.376–2.499) | <0.001 | 1.752 (1.292–2.374) | <0.001 |
| VAI | ≤1.19 | 1.2–1.98 | | 1.99–3.74 | | ≥3.75 | |
| Model 1 | Reference | 1.383 (1.031–1.855) | 0.031 | 1.883 (1.409–2.516) | <0.001 | 1.668 (1.246–2.231) | 0.001 |
| Model 2 | Reference | 1.349 (1.003–1.814) | 0.048 | 1.82 (1.358–2.44) | <0.001 | 1.608 (1.194–2.165) | 0.002 |
| Model 3 | Reference | 1.33 (0.986–1.794) | 0.062 | 1.822 (1.354–2.453) | <0.001 | 1.615 (1.194–2.186) | 0.002 |
| LAP index | ≤21.6 | 21.61–39.96 | | 39.97–77.14 | | ≥77.15 | |
| Model 1 | Reference | 1.821 (1.356–2.445) | <0.001 | 1.758 (1.309–2.362) | <0.001 | 2.091 (1.56–2.803) | <0.001 |
| Model 2 | Reference | 1.761 (1.303–2.38) | <0.001 | 1.709 (1.261–2.318) | 0.001 | 2.016 (1.48–2.747) | <0.001 |
| Model 3 | Reference | 1.787 (1.317–2.425) | <0.001 | 1.749 (1.283–2.383) | <0.001 | 2.043 (1.493–2.794) | <0.001 |
| ABSI | ≤0.76 | 0.77–0.8 | | 0.81–0.84 | | ≥0.85 | |
| Model 1 | Reference | 0.895 (0.669–1.197) | 0.454 | 1.351 (1.021–1.788) | 0.035 | 1.351 (1.012–1.788) | 0.827 |
| Model 2 | Reference | 0.872 (0.651–1.168) | 0.359 | 1.291 (0.973–1.713) | 0.077 | 0.968 (0.722–1.296) | 0.825 |

*(Continued)*

**TABLE 3** | Continued

| Variables | Quartile 1 | Quartile 2 | | Quartile 3 | | Quartile 4 | |
|---|---|---|---|---|---|---|---|
| | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| Model 3 | Reference | 0.867 (0.645–1.166) | 0.346 | 1.3 (0.976–1.73) | 0.073 | 0.981 (0.729–1.319) | 0.899 |
| BRI | ≤3.08 | 3.09–3.93 | | 3.94–5.11 | | ≥5.12 | |
| Model 1 | Reference | 1.1 (0.827–1.463) | 0.513 | 1.195 (0.9–1.588) | 0.219 | 1.055 (0.792–1.404) | 0.715 |
| Model 2 | Reference | 1.042 (0.779–1.394) | 0.78 | 1.093 (0.814–1.468) | 0.555 | 0.927 (0.683–1.258) | 0.627 |
| Model 3 | Reference | 1.03 (0.767–1.382) | 0.845 | 1.109 (0.823–1.495) | 0.496 | 0.945 (0.695–1.285) | 0.717 |

*Model 1: unadjusted; model 2: adjusted for age, SBP, and DBP; model 3: adjusted for all variables in model 2 plus BUN, Cre, TC, and LDL-C. TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.*

conclusion regarding which criterion is more valuable in reflecting visceral fat content and predicting HUA. One of the important reasons for this is that there are some limitations to the various criteria for measuring obesity. New indices, such as the ABSI and BRI, have been presented to distribute adipose tissue more precisely, and some studies have reported a close association between these indices and HUA (21, 22). In contrast to previous studies, no correlation between these indices and HUA was found in the present study. The reasons for this different conclusion may include the following: (1) participants from different areas had different lifestyles and variations in age distribution and sex composition. (2) The ABSI and BRI cannot distinguish between the distribution of subcutaneous and visceral adipose tissues (23). (3) The ABSI was originally established to predict mortality in a follow-up cohort (24), whereas this study utilized it in a cross-sectional manner. This may be the reason for the low screening value.

Previous studies have shown that indices consisting of TG were well suited to identify individuals with unhealthy metabolism (25). The CMI is a new index for evaluating visceral obesity using lipid parameters TG and HDL-C to waist height ratio, which has been shown to be associated with several metabolic syndromes (26, 27). The VAI integrates the traditional obesity indices WC and BMI with TG and HDL-C, which can better indicate visceral fat content and its distribution (28). The LAP index integrates WC, an indicator of abdominal obesity, and TG, which is closely related to visceral fat distribution (6). The present study showed that the CMI, VAI, and LAP index could affect uric acid levels and increase the risk of HUA, which is consistent with the results of previous studies (29). Compared with traditional anthropometric indices, the CMI, VAI, and LAP index have better predictive power for HUA in both males and females. In particular, the sensitivity of the ROC curve was higher in females than in males. This may be related to the fact that the CMI, LAP index, and VAI fully integrate WC, an indicator for assessing abdominal obesity, and TG, which is related to the visceral fat distribution. This suggests that higher visceral fat accumulation expressed by elevated LAP has a greater effect on uric acid metabolism than BMI values, which includes less specificity for subcutaneous fat accumulation. Visceral fat promotes the synthesis of phosphoribosyl pyrophosphate from very low-density lipoprotein and ribose 5-phosphate, which leads to the excessive production of uric acid. Meanwhile, the VAI was a better predictor of HUA than BMI, WC, and WHR independently in the Chinese population (30), probably because of the accumulation of visceral fat, which allows free fatty acids (FFAs) to enter the liver *via* the portal vein, increasing the synthesis of TGs and causing hypertriglyceridemia. Moreover, FFAs can increase the synthesis of purines *via* the pentose phosphate pathway. The correlation between the CMI, VAI, and LAP index and uric acid in this study further confirmed the association between visceral fat and HUA.

The AIP is a sensitive index of lipid metabolism disorders proposed by Dobiasova et al. (31), which uses log (TG/HDL-C) as an index that can be used as a predictor of the risk of plasma atherosclerosis development, and its value is negatively correlated with LDL particle size and LDL-C as a routine index of clinical testing if abnormally elevated, leading to a high risk of cardiovascular disease. The AIP has been significantly associated with HUA, hypercholesterolemia, hyperlipidemia, and metabolic syndrome, which are all risk factors for cardiovascular disease (32). Zhu et al. found a positive association between higher levels of AIP, a new biomarker associated with obesity (33). In the present study, the partial correlation analysis suggested that the AIP was positively associated with SUA and could be used as an independent risk factor for predicting HUA in both sexes. This is consistent with the results of a cross-sectional study by Chang et al. on the association between blood uric acid and AIP in a population from northeastern China (34). The mechanism may be due to the fact that changes in cholesterol and TG can cause disorders of lipid metabolism, whereas the association between dyslipidemia and HUA is bidirectional. On the one hand, due to the long and cold winter in Xinjiang, the local population has a high proportion of high-purine and high-fat diet intake, which easily causes fat accumulation and disorders of lipid and purine metabolism. On the other hand, high concentrations of uric acid can affect lipid peroxidation and LDL cholesterol oxidation, reducing the activity of the corresponding enzymes and decreasing cholesterol catabolism, causing changes in blood lipids (35).

**TABLE 4 |** Multivariate logistic regression of different indices for HUA (females).

| Variables | Quartile 1 | Quartile 2 | | Quartile 3 | | Quartile 4 | |
|---|---|---|---|---|---|---|---|
| | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| TyG index | ≤7.99 | 8–8.36 | | 8.37–8.81 | | ≥8.82 | |
| Model 1 | Reference | 3.619 (1.759–7.443) | <0.001 | 3.665 (1.787–7.52) | <0.001 | 12.923 (6.487–25.745) | <0.001 |
| Model 2 | Reference | 3.622 (1.756–7.472) | <0.001 | 3.718 (1.809–7.64) | <0.001 | 12.834 (6.434–25.603) | <0.001 |
| Model 3 | Reference | 3.125 (1.373–7.114) | 0.007 | 2.9 (1.275–6.593) | 0.011 | 7.788 (3.581–16.937) | <0.001 |
| BMI | ≤22 | 23–25 | | 26–27 | | ≥28 | |
| Model 1 | Reference | 0.657 (0.383-1.125) | 0.126 | 0.674 (0.395–1.149) | 0.148 | 1.627 (1.004–2.634) | 0.048 |
| Model 2 | Reference | 0.593 (0.342–1.028) | 0.063 | 0.544 (0.309–0.956) | 0.034 | 1.195 (0.698–2.047) | 0.516 |
| Model 3 | Reference | 0.528 (0.256–1.092) | 0.085 | 0.884 (0.449–1.74) | 0.721 | 1.192 (0.611–2.325) | 0.608 |
| WC | ≤79 | 80–87 | | 88–94 | | ≥95 | |
| Model 1 | Reference | 2.125 (1.224–3.688) | 0.007 | 1.729 (0.991–3.015) | 0.054 | 2.547 (1.473–4.407) | 0.001 |
| Model 2 | Reference | 1.846 (1.045–3.261) | 0.035 | 1.525 (0.852–2.729) | 0.155 | 1.946 (1.071–3.534) | 0.029 |
| Model 3 | Reference | 1.327 (0.647–2.722) | 0.44 | 1.722(0.853–3.475) | 0.129 | 1.727 (0.837–3.564) | 0.139 |
| WHtR | ≤0.49 | 0.5–0.53 | | 0.54–0.57 | | ≥0.58 | |
| Model 1 | Reference | 1.992 (1.146–3.462) | 0.015 | 1.527 (0.864–2.699) | 0.145 | 2.768 (1.614–4.748) | <0.001 |
| Model 2 | Reference | 1.808 (1.028–3.179) | 0.04 | 1.21 (0.661–2.212) | 0.537 | 2.094 (1.155–3.798) | 0.015 |
| Model 3 | Reference | 1.375 (0.672–2.813) | 0.383 | 1.52(0.727–3.177) | 0.266 | 1.88 (0.902–3.919) | 0.092 |
| AIP | ≤–0.22 | –0.21 to –0.22 | | –0.01–0.19 | | ≥0.2 | |
| Model 1 | Reference | 2.089 (1.051–4.155) | 0.036 | 3.887 (2.026–7.457) | <0.001 | 9.093 (4.828–17.125) | <0.001 |
| Model 2 | Reference | 1.924 (0.958–3.863) | 0.066 | 3.611 (1.868–6.983) | <0.001 | 8.004 (4.214–15.202) | <0.001 |
| Model 3 | Reference | 1.751 (0.709–4.323) | 0.224 | 4.815 (2.086–11.117) | <0.001 | 9.127 (4.008–20.784) | <0.001 |
| CMI | ≤0.32 | 0.33–0.49 | | 0.5–0.85 | | ≥0.86 | |
| Model 1 | Reference | 2.107 (1.059–4.191) | 0.034 | 3.212 (1.661–6.209) | 0.001 | 10.143 (5.389–19.09) | <0.001 |
| Model 2 | Reference | 1.909 (0.953–3.825) | 0.068 | 2.833 (1.452–5.527) | 0.002 | 8.708 (4.576–16.572) | <0.001 |
| Model 3 | Reference | 1.351 (0.551–3.31) | 0.511 | 3.52 (1.552–7.983) | 0.003 | 8.839 (3.976–19.654) | <0.001 |
| VAI | ≤1.15 | 1.16–1.81 | | 1.82–3.08 | | ≥3.09 | |
| Model 1 | Reference | 2.413 (1.225–4.753) | 0.011 | 3.376 (1.749–6.515) | <0.001 | 9.218 (4.897–17.35) | <0.001 |
| Model 2 | Reference | 2.207 (1.111–4.383) | 0.024 | 3.139 (1.614–6.107) | 0.001 | 8.065 (4.247–15.313) | <0.001 |
| Model 3 | Reference | 2.278 (0.948–5.472) | 0.066 | 3.834 (1.645–8.934) | 0.002 | 9.34 (4.119–21.18) | <0.001 |
| LAP index | ≤20.28 | 20.29–30.8 | | 30.81–51.7 | | ≥51.71 | |
| Model 1 | Reference | 1.755 (0.926–3.325) | 0.085 | 2.46 (1.335–4.533) | 0.004 | 6.89 (3.847–12.339) | <0.001 |
| Model 2 | Reference | 1.567 (0.814–3.015) | 0.179 | 2.181 (1.159–4.103) | 0.016 | 5.792 (3.126–10.729) | <0.001 |
| Model 3 | Reference | 1.123 (0.492–2.563) | 0.782 | 2.579 (1.214–5.48) | 0.014 | 4.843 (2.294–10.225) | <0.001 |
| ABSI | ≤0.76 | 0.77–0.79 | | 0.8–0.83 | | ≥0.84 | |
| Model 1 | Reference | 1.104 (0.663–1.838) | 0.704 | 1.234 (0.699–2.178) | 0.468 | 1.84 (1.106–3.061) | 0.019 |
| Model 2 | Reference | 1.034 (0.615–1.737) | 0.9 | 1.167 (0.653–2.085) | 0.602 | 1.638 (0.965–2.782) | 0.068 |
| Model 3 | Reference | 1.409 (0.747–2.658) | 0.29 | 1.054 (0.492–2.259) | 0.893 | 1.476 (0.764–2.852) | 0.247 |

*(Continued)*

**TABLE 4** | Continued

| Variables | Quartile 1 | Quartile 2 | | Quartile 3 | | Quartile 4 | |
|---|---|---|---|---|---|---|---|
| | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| BRI | ≤3.12 | 3.13–4.03 | | 4.04–4.82 | | ≥4.83 | |
| Model 1 | Reference | 1.992 (1.146–3.462) | 0.015 | 1.527 (0.864–2.699) | 0.145 | 2.768 (1.614–4.748) | <0.001 |
| Model 2 | Reference | 1.808 (1.028–3.179) | 0.04 | 1.21 (0.661–2.212) | 0.537 | 2.094 (1.155–3.798) | 0.015 |
| Model 3 | Reference | 1.375 (0.672–2.813) | 0.383 | 1.52 (0.727–3.177) | 0.266 | 1.88 (0.902–3.919) | 0.092 |

*Model 1: unadjusted; model 2: adjusted for age, SBP, and DBP; model 3: adjusted for all variables in model 2 plus BUN, Cre, TC, and LDL-C. TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.*

## The Triglyceride Glucose Index With HUA

Epidemiological studies have established a significant association between IR and SUA (36, 37), and compensatory hyperinsulinemia that occurs after IR can reduce uric acid excretion by renal tubular sodium reabsorption and cause HUA (38). Conversely, higher uric acid levels can reduce nitric oxide bioavailability and mitochondrial oxidative stress, leading to IR (39). Therefore, the evaluation of IR status in a normal physical examination population is beneficial not only for uric acid control but also for the prevention of other metabolic diseases. Traditional IR assessment tools, such as the high insulin glucose clamp test and steady-state mode assessment method, cannot be commonly used because of their complicated, invasive, and expensive characteristics. In recent years, several simple IR assessment tools have been developed and used clinically. The TyG index was developed in 2008 by Simental-Mendía et al. (40) and has better sensitivity and specificity compared to the gold standard for IR detection (41). Some studies have reported that the TyG index is associated with atherosclerosis, metabolic syndrome, and type 2 diabetes mellitus (42–44). Mazidi et al. (9) found a correlation between the TyG index and HUA in Caucasian populations. In the present study, logistic regression models revealed a significant correlation between the TyG index and the risk of HUA in both males and females compared to other obesity indices, even after multivariate adjustment. Moreover, this correlation was consistent across all subgroups after performing a stratified analysis of the TyG index, and the risk of HUA was higher in the high quartile than in the low quartile. The TyG index also had a significant value in identifying HUA in both sexes in the ROC analysis compared to other indices ($P < 0.001$), especially in females. This may be due to the fact that estrogen, as a uric acid producing agent, leads to more complex endocrine factors in its metabolism than in males, as well as differences in lipid metabolism between the sexes. This finding is consistent with the results of a cross-sectional study by Shi et al.

**TABLE 5** | Comparison of the ability of different indices to predict HUA.

| Variable | AUC (95% CI) | Cut-off | Sensitivity | Specificity | Youden index | *P*-value |
|---|---|---|---|---|---|---|
| Males | | | | | | |
| BMI | 0.515 (0.487–0.544) | 24.07 | 0.759 | 0.293 | 0.052 | 0.295 |
| WC | 0.507 (0.478–0.536) | 86.5 | 0.675 | 0.369 | 0.043 | 0.638 |
| WHtR | 0.513 (0.484–0.541) | 0.433 | 0.948 | 0.089 | 0.037 | 0.389 |
| AIP | 0.569 (0.54–0.597) | 0.121 | 0.636 | 0.486 | 0.122 | <0.001 |
| TyG index | 0.586 (0.557–0.614) | 8.353 | 0.815 | 0.318 | 0.133 | <0.001 |
| CMI | 0.569 (0.541–0.597) | 0.605 | 0.701 | 0.422 | 0.123 | <0.001 |
| VAI | 0.568 (0.54–0.596) | 1.444 | 0.728 | 0.392 | 0.12 | <0.001 |
| LAP index | 0.578 (0.55–0.606) | 27.1 | 0.748 | 0.399 | 0.147 | <0.001 |
| ABSI | 0.514 (0.485–0.543) | 0.803 | 0.514 | 0.547 | 0.061 | 0.343 |
| BRI | 0.513 (0.484–0.541) | 2.613 | 0.901 | 0.166 | 0.067 | 0.389 |
| Females | | | | | | |
| BMI | 0.55 (0.495–0.604) | 27.291 | 0.363 | 0.8 | 0.163 | 0.063 |
| WC | 0.59 (0.538–0.642) | 88.5 | 0.465 | 0.672 | 0.137 | 0.001 |
| WHtR | 0.591 (0.539–0.644) | 0.452 | 0.904 | 0.147 | 0.051 | 0.001 |
| AIP | 0.734 (0.688–0.779) | 0.029 | 0.688 | 0.672 | 0.36 | <0.001 |
| TyG index | 0.728 (0.682–0.773) | 8.575 | 0.631 | 0.743 | 0.373 | <0.001 |
| CMI | 0.737 (0.691–0.782) | 0.595 | 0.688 | 0.696 | 0.384 | <0.001 |
| VAI | 0.735 (0.689–0.78) | 2.196 | 0.662 | 0.704 | 0.367 | <0.001 |
| LAP index | 0.715 (0.668–0.762) | 45.04 | 0.573 | 0.781 | 0.354 | <0.001 |
| ABSI | 0.579 (0.525–0.632) | 0.788 | 0.682 | 0.464 | 0.145 | 0.003 |
| BRI | 0.591 (0.539–0.644) | 4.233 | 0.471 | 0.683 | 0.154 | 0.001 |

*TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.*

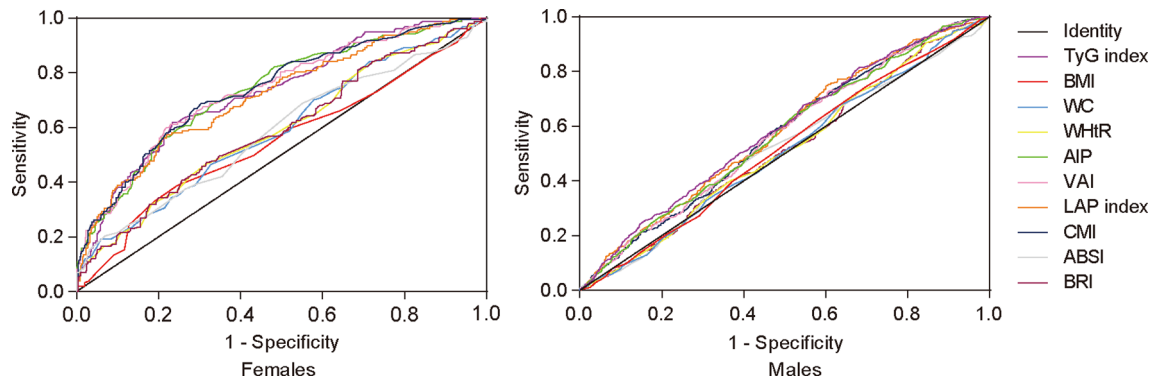


**FIGURE 1** | Receiver operating characteristic curve analysis by sex. TyG index, triglyceride glucose index; BMI, body mass index; WC, waist circumference; WHtR, waist-to-height ratio; AIP, atherogenic index of plasma; CMI, cardiometabolic index; VAI, visceral adiposity index; LAP index, lipid accumulation product index; ABSI, a body shape index; BRI, body roundness index.

(45) in northeastern China. One possible mechanism is that IR affects lipid metabolism by decreasing lipoprotein lipase activity and reducing lipocalin production (4). Second, high TG levels are degraded to FFAs, which are transported to other tissues and accelerate adenosine triphosphate breakdown. Abnormal lipid metabolism also impairs the kidney, decreases renal blood flow, reduces urinary uric acid excretion, and increases SUA levels (46).

This study systematically investigated the predictive value of the TyG index and nine obesity indices for HUA and included a comprehensive range of indices. All data in the study were obtained from the same regional population, which reduced the risk of bias in the sample source. However, this study has some limitations. First, it investigated a specific region, Barkol County, which may not best represent the overall status of ethnicity and related diseases in Xinjiang. Second, it has been previously reported that uric acid levels can be affected by diet, but information on dietary patterns, such as dairy and meat intake, was not analyzed in this study population, this may lead to biased results. Third, the cross-sectional survey could not clearly determine the causal association between the risk factors and the incidence of HUA. Systematic large-scale studies are required to further elucidate the changes in uric acid levels and their associated risk factors.

## CONCLUSIONS

In conclusion, different indices were differentially related to HUA and had different predictive abilities. In addition to generalized obesity, abdominal obesity and the resulting visceral fat accumulation also need to be considered. The TyG index was more closely related to HUA than the obesity indices in the medical checkup population in Xinjiang, China. Thus, it can be used as an important index for HUA risk screening and population health management.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

YS and CH contributed to conception and design of the study. BZ, WC, MC, and TT organized the database. ML, YH, RL performed the statistical analysis. MK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

## FUNDING

This study was funded by the National Natural Science Foundation of China (No.81960169,81760169, and 81900795), the Natural Science Foundation of the Xinjiang Uygur Autonomous Region (No. 2019D01C219 and 2021D01C275), the Project of Scientific Research Program for Universities in Xinjiang Uygur Autonomous Region(No.XJEDU2021Y054),the Postgraduate Research Innovation Project in Xinjiang Uygur Autonomous Region(XJ2021G228).

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fendo.2022.856331

# Mechanism of Endoplasmic Reticulum Stress Pathway in the Osteogenic Phenotypic Transformation of Aortic Valve Interstitial Cells

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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *17 January 2022*
**Accepted:** *07 February 2022*
**Published:** *08 March 2022*

**Citation:**
*Tao Y, Geng Y, Dang W, Xu X, Zhao H, Zou L and Li Y (2022) Mechanism of Endoplasmic Reticulum Stress Pathway in the Osteogenic Phenotypic Transformation of Aortic Valve Interstitial Cells. Front. Endocrinol. 13:856331. doi: 10.3389/fendo.2022.856331*

**Background and Purpose:** Calcific Aortic Valve Disease (CAVD) is a crucial component of degenerative valvular disease in old age and with the increasing prevalence of the aging population. we hope that by modeling valvular osteogenesis and intervening with endoplasmic reticulum stress inhibitor TUDCA to observe the effect of endoplasmic reticulum stress on valve osteogenesis

**Methods:** In this study, rabbit heart valvular interstitial cells (VICs) were isolated and cultured. They treated with ox-LDL (Oxidized Low Density Lipoprotein) stimulation to establish a model of valvular osteogenic transformation. BMP2 (Bone Morphogenetic Protein 2), PERK (Protein kinase R-like endoplasmic reticulum kinase), CHOP (CCAAT/enhancer-binding protein homologous protein) and transcriptional regulatory factor ATF4 (Activating Transcription Factor 4 )were recorded after intervention with ER stress inhibitor TUDCA. The effects of er stress on valvular osteogenic transformation were analyzed.

**Result:** After stimulation of VICs with ox-LDL, the expression levels of BMP2, PERK, CHOP, and ATF4 increased. However, TUDCA treatment can alleviate the increased expression levels of BMP2, PERK ATF4, and CHOP under ox-LDL stimulation to a certain extent.

**Conclusion:** The endoplasmic reticulum stress signaling pathway is involved in ox-LDL-induced calcification of rabbit valve interstitial cells. Inhibition of endoplasmic reticulum stress using TUDCA can improve the progression of rabbit aortic valve calcification.

**Keywords: aortic valve calcification, endoplasmic reticulum stress, valve interstitial cells, ox-LDL, TUDCA, BMP2**

## INTRODUCTION

Calcific Aortic Valve disease (CAVD), the most common form of valvular heart disease, according to data, the global death toll of aortic valve calcification was 102700 in 2017, an increase of 101% compared with 1990 (1). Aortic valve calcification disease terminal lesions such as, aortic valve sclerosis, stenosis, incomplete closure, etc., will lead to a significant increase in cardiac load, seriously affecting people's life and health and quality of life.

In the past, calcified valvular heart disease was often regarded as a degenerative disease of age, but in recent years, more and more studies have shown that it is an active regulation process involving multiple factors, and mechanical stimulation may be the initiating factor of this process (2), that is, early lesions of CAVD are like atherosclerosis. Changes in blood shear forces stimulate bone morphogenetic protein 2 (BMP2) and its downstream pathways and lead to injury of the valvular endothelium, followed by infiltration of lipids and inflammatory cells, the release of cytokines, calcium deposition, and valvular mineralization.

Unlike atherosclerosis, where macrophages phagocytose lipids and form foam cells, leading to calcium deposition, cardiac valve calcification involves valvular interstitial cells, resulting in osteogenic phenotype transformation and inducing calcification (3, 4).There are five main valve interstitial cells, namely embryonic endothelial/mesenchymal progenitor cells, qVICs, pVICs, aVICs and obVICs (3). AVICs play a significant role in valve calcification.

A high-fat diet can increase the serum ox-LDL level in rats, and it is often used to construct aortic valve calcification model (5), while inhibition of ER (endoplasmic reticulum) stress can protect aortic valve calcification in ApoE-/- mice fed with high-cholesterol diet (6). Therefore, we hypothesized that ox-LDL might induce calcification of valvular osteogenesis through ER stress, that is, ox-LDL is involved in osteogenesis of valvular interstitial cells through the classic ER stress pathway PERK-ATF4-CHOP, while ER stress inhibitors attenuated this effect. To this end, we isolated and cultured rabbit primary valve interstitial cells, administered ox-LDL-treated cells, observed the expression of PERK, ATF4, CHOP, and BMP2 in the ER stress pathway, and treated them with ER stress inhibitors (7). To further elucidate the role of PERK pathway in rabbit valve interstitial cell osteogenic differentiation, we hope to provide new therapeutic targets and ideas for the treatment and prevention of aortic valve calcification.

## MATERIALS AND METHODS

### Animals

Eight male New Zealand white rabbits were kept in a temperature-controlled room with a cycle of 12-hour light and 12-hour darkness and fed with regular laboratory chow and unrestricted access to water. Approval of all experiments and animal care was provided by the Animal Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

### Extract and Culture VICs

After the aortic valve of New Zealand White Rabbits were isolated, the interstitial cells of the aortic valve were digested with trypsin containing EDTA. The culture medium was composed of Hyclone high glucose DMEM medium and Gibco 20% fetal bovine serum, supplemented with 100 U/mL penicillin and 100μg/ml streptomycin (Gibco). After 24h and 48h, the culture medium was changed respectively until the cells reached 80%-90% fusion. After the first passage, the cells were cultured with DMEM/F12 of 10% FBS. Cells from 3 to 8 generations were cultured at 37°C in a moist atmosphere with 5% carbon dioxide in the air.

### Characterization of VICs

Immunofluorescence was used to identify VICs. After the first and second antibodies of α -smooth muscle protein (α-SMA), V-WF and VemIntent were incubated successively, the cell slivers were cleaned with 4°C precooled PBS solution for 3 times, 5 min each. 0.5 mL of 1*DAPI (ABCAM) was added to stain the nuclei for 10 minutes: the cell sliders after treatment were placed under a forward fluorescence microscope for observation, and the wavelength was adjusted to 520-530nm to stimulate red fluorescence. Cell morphology was observed under a microscope of 20 and 40 times, respectively, and photographed for preservation.

### Experimental Protocols

To investigate whether ox-LDL stimulation can promote osteogenic phenotype transformation of VICs, ox-LDL (100 μg/mL) was given to stimulate aortic valve interstitial cells on day 1, day 3, and day 7, and the cells were collected to detect BMP2 mRNA levels. And BMP2 protein expression levels on day 7. The BMP2 mRNA content of aortic valve interstitial cells without ox-LDL stimulation was taken as day 0; In order to investigate whether ox-LDL stimulation can cause endoplasmic reticulum stress of VICs, VICs without ox-LDL stimulation and VICs after ox-LDL stimulation for 7 days were collected. The protein expression levels of PERK/ATF4/CHOP protein pathway in endoplasmic reticulum were determined by Western blot analysis. To investigate whether endoplasmic reticulum stress is involved in osteoblastic phenotypic transformation of aortic valve interstimal cells, the cells were divided into four groups: CTL group, ox-LDL group (100 μg/mL), ox-LDL group (100 μg/ml) +TUDCA (100umol/L, HY-19696, MCE) group and TUDCA (1mmol/L) group, in which ox-LDL (100 μg/ml) +TUDCA group cells were treated with TUDCA for 2 h, and then ox-LDL was added for 7 days. ox-LDL group cells were stimulated with ox-LDL for 7 days, and then cells in each group were collected. Western blot analysis was used to detect the expression levels of proteins related to BMP2 and ER stress signaling pathway.

### Western Blot Analysis

After being washed by ice-cold PBS, VICs were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (HY-K1001, MCE) for approximately 30 min. Then, the samples

were centrifuged at 12,000 rpm at 4 ∘C for 15 min. Protein concentration was determined using the bicinchoninic acid (BCA) method (Beyotime, China). Subsequently, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (EpiZyme, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010 0.45 μm, Millipore, USA). Then, the membranes were blocked with 5% skim milk in Tris-buffered saline solution containing Tween-20 (Sigma-Aldrich, USA) for 1 h at room temperature and incubated in specific primary antibodies at 4 ∘C overnight. After detecting horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the proteins were visualized using enhanced chemiluminescence. The primary antibodies were as follows: Anti-CHOP antibody, Anti-BMP2 antibody, Anti-ATF4 antibody, Anti-PERK antibody, Rabbit anti-vimentin antibody, rabbit anti-vWF antibody, rabbit anti-α -SMA antibody (Wuhan Sanying Biotechnology Co., LTD).

## qRT-PCR

After the cells were treated according to the above experimental design, total RNA was extracted by Trizol method and the RNA was reversely transcribed into cDNA(reaction system: 20 dishes; Reaction conditions: 42°C2 rain, 37°C15 rain, 85°C5 S), using qRT-PCR method (reaction system: 20 anal L; Reaction conditions: "two-step reaction" was adopted, the first step was 94°C for 30 S; The second step is 94°C5 s, 60. C 30 s, 44 cycles) the mRNA levels of BMP2 and PERK were detected (see **Table 1** for primers).

# RESULT

## Cell Identification

Early studies have shown that activation of aortic valve interstitial cells increases the expression of α -smooth muscle actin (α-SMA) on its surface (8). Vimentin protein is not only expressed on the valve interstitial cells, but also the valve endothelial cells, to further remove the influence of the valve endothelial cells (9), endothelial cell marker vWF is also used to identify the aortic valve interstitial cells and aortic valve endothelial cells. Therefore, α-SMA (+)/Vimentin (+)/vWF (-) cells are the de sired aortic valve interstitial cells. Under a fluorescence microscope, using green light to excite red wavelengths, the aortic valve stromal cells appear red color α-SMA (+) and Vimentin (+), indicating positive expression of α-SMA and Vimentin, vWF uses blue light to stimulate the wavelength of green light, no green fluorescence is seen, only blue stained nucleus is seen, indicating negative expression of vWF (**Figure 1**).

## ox-LDL Stimulation Can Induce Osteoblastic Phenotypic Transformation of Aortic Valve Stromal Cells

ox-LDL was given on day 1 to stimulate the aortic valve interstitial cells, and the cells were collected on day 1, 3 and 7 to detect the BMP2 mRNA content. The BMP2 mRNA content of aortic valve interstitial cells not stimulated with ox-LDL was taken as day 0, and the results showed that compared with day 0, we found that the BMP2 mRNA content increased successively on day 1, 3 and 7, and reached the peak on day 7 (**Figure 2A**). VICs was collected after ox-LDL stimulation for 7 days, and the protein expression level of osteogenic protein BMP2 was detected by WB. The results showed that the expression level of osteoblastic protein BMP2, which causes VICs, was increased after ox-LDL stimulation compared with CTL group (**Figure 2B**). These results suggest that ox-LDL stimulation can induce osteoblastic phenotypic transformation of aortic valve stromal cells.

## ox-LDL Induced Endoplasmic Reticulum Stress of VICs

VICs was collected 7 days after ox-LDL stimulation, and the protein expression levels of PERK/ATF4/CHOP protein in endoplasmic reticulum were measured by WB. The results showed that compared with the CTL group, the expression levels of PERK, ATF4, and CHOP in the OX-LDL group were increased (**Figure 3**). These results suggest that ox-LDL can induce endoplasmic reticulum stress in VICs.

## Endoplasmic Reticulum Inhibitor TUDCA Can Inhibit the Osteoblastic Phenotypic Transformation Induced by ER Stress Induced by OX-LDL

VICs after 7 days of ox-LDL+TUDCA stimulation were collected, and the protein expression levels of PERK/ATF4/CHOP in ER stress signaling pathway were detected by WB. Compared with ox-LDL group, ox-LDL +TUDCA group reduced ox-LDL to VICs BMP2 and PERK mRNA levels to a certain extent (**Figure 4A, B**), and also alleviated ox-LDL to VICs PERK, ATF4, CHOP (**Figure 4C**), These results suggest that inhibition of ER stress may reduce osteoblastic phenotypic transformation of VICs.

# DISCUSSION

CAVD was considered as an age-related degenerative valvular disease in the early years, but more and more evidence indicates

**TABLE 1** | Primer Sequence.

| Gene symbol | Forward prime sequence (5'→3') | Reverse prime sequence (5'→3') |
|---|---|---|
| BMP2 | CCCAAGCTTACCACCATGGTGGCCGGGACCCGCTGTCTTC | CGCGGATCCCTAGCGACACCCACAACCCTCCAC |
| PERK | GCGGCAATGAGAAGTGGAAT | TCCCTCTGGGCTTAAAGGTG |
| GAPDH | CGCCTGGAGAAAGCTGCTA | ACGACCTGGTCCTCGGTGTA |

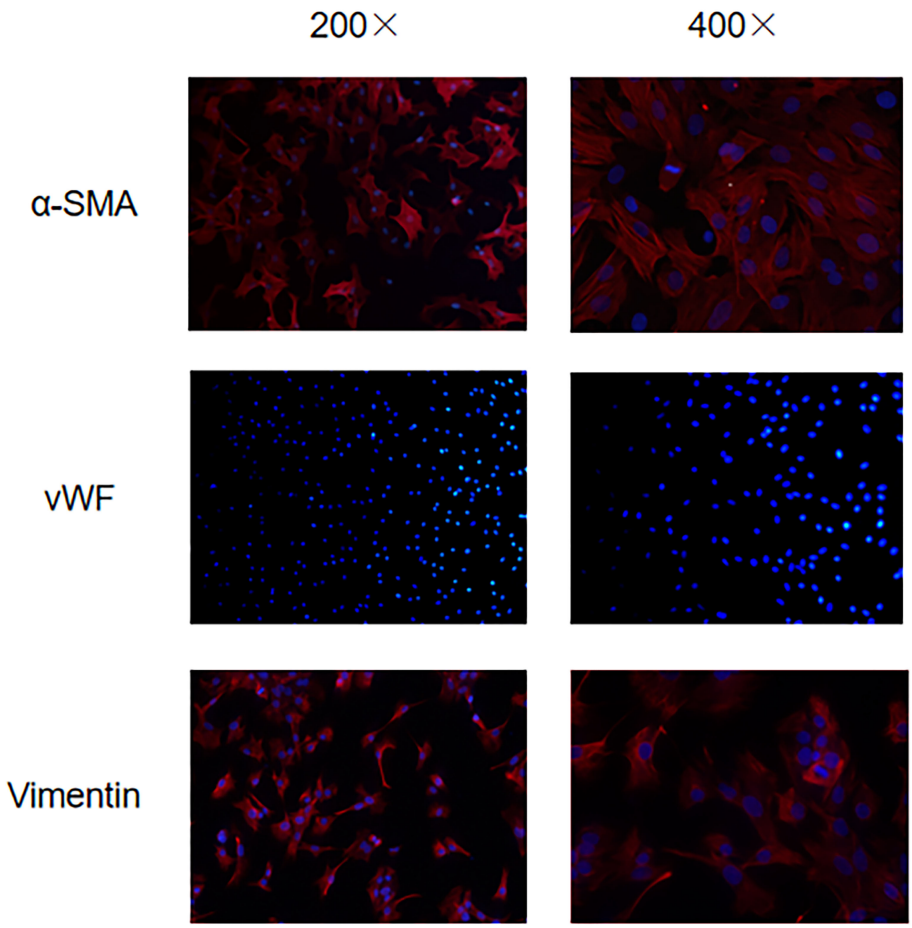


**FIGURE 1** | Immunofluorescence microscopy to identify the expression of α-SMA/vWF/Vimentin in VICs.

that CAVD is an active activation process, and damage of valve endothelial cells caused by mechanical stimulation may be the cause of such diseases (2). The aortic valve is mainly composed of three lobules, each of which is primary composed of valvular endothelial cells (VECs) and valvular interstitial cells (VICs), of which the valve endothelial cells (VECs) are arranged on the outer surface of the valve, and their main role is to regulate permeability and maintain valve homeostasis, and to limit the infiltration of inflammatory cells as a barrier (10). Studies have shown that VICs increase and VECs decrease in diseased valves, and VICs are more pleomorphic than VECs (11, 12). Therefore, VICs plays an important role in the process of valve calcification, participating in valve mineralization, and ultimately leading to severe CAVD.

Therefore, we selected rabbit aortic valve, which is easy to obtain, for the experiment, and used differential centrifugation to reduce the pollution of VECs and repeated passage for purification. The cells of α-SMA (+)/Vimentin (+)/vWF (-) are the rabbit aortic valve interstitial cells (VICs) required by us (13).

ox-LDL is a common inducer of oxidative stress (14). Atherosclerosis involved in blood vessels can lead to calcification of vascular smooth muscle. However, ox-LDL can also induce ER stress and promote osteogenic effect (15). Therefore, ox-LDL was used to treat purified rabbit aortic valve cells to stimulate endoplasmic reticulum stress and osteogenesis. The results showed that ox-LDL treated VICs and its BMP2 mRNA expression increased, which was consistent with ox-LDL treated human VICs (16). It was also found that ox-LDL can also induce the activation of human VICs Notch signal, and can significantly increase the activation of inflammatory pathway NF-KB when acting together with LPS (17, 18). Therefore, it is not difficult to understand the subsequent osteogenic phenotype transformation effect of VICs caused by inflammation. However, under the action of ox-LDL, alkaline phosphatase ALP, another preosteogenic factor of VICs, did not increase significantly (19). When costimulated with LPS, the expression of ALP increased, suggesting that LPS could lead to the increase of ALP, while ox-LDL did not have such an obvious effect on ALP. ox-LDL can not only promote cell apoptosis (20, 21), but also promote the proliferation of vascular smooth muscle cells (18). However, whether ox-LDL can promote AOV calcification by increasing VIC proliferation needs further research.

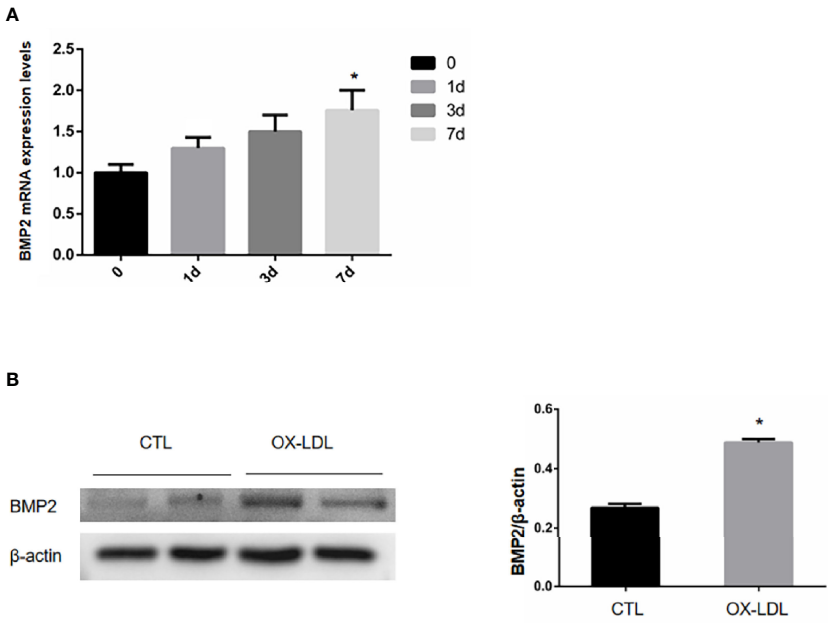


**FIGURE 2** | mRNA and protein expression levels of BMP2 in VIC after ox-LDL stimulation. **(A)** The relative expression of BMP2 mRNA under different time stimulation; **(B)** BMP2 protein expression in VICs after ox-LDL stimulation. * represents P value less than 0.05, indicating significant difference.

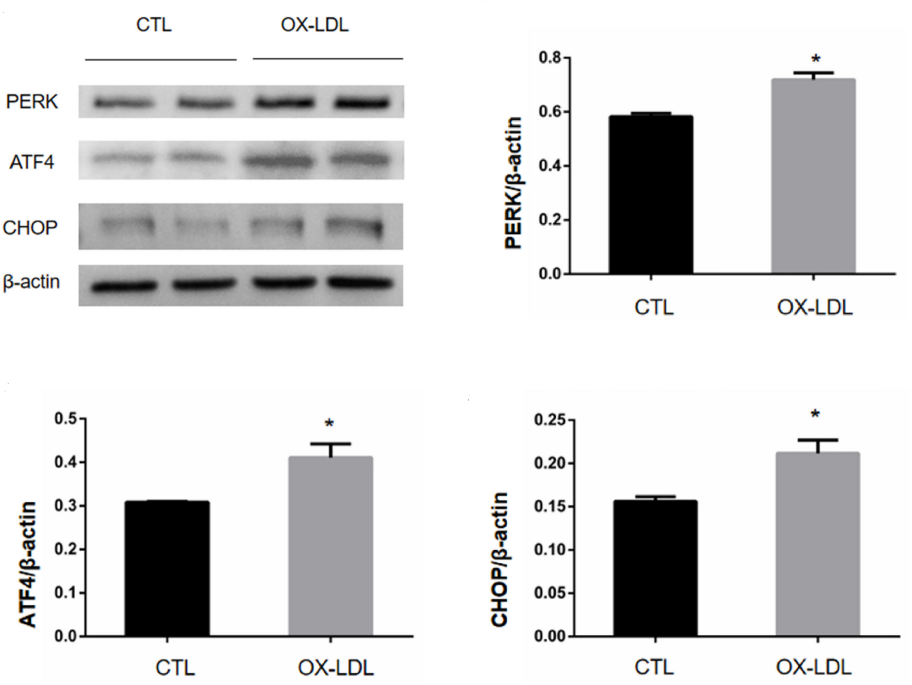


**FIGURE 3** | PERK/ATF4/CHOP protein expression of VICs after ox-LDL stimulation. * represents P value less than 0.05, indicating significant difference.

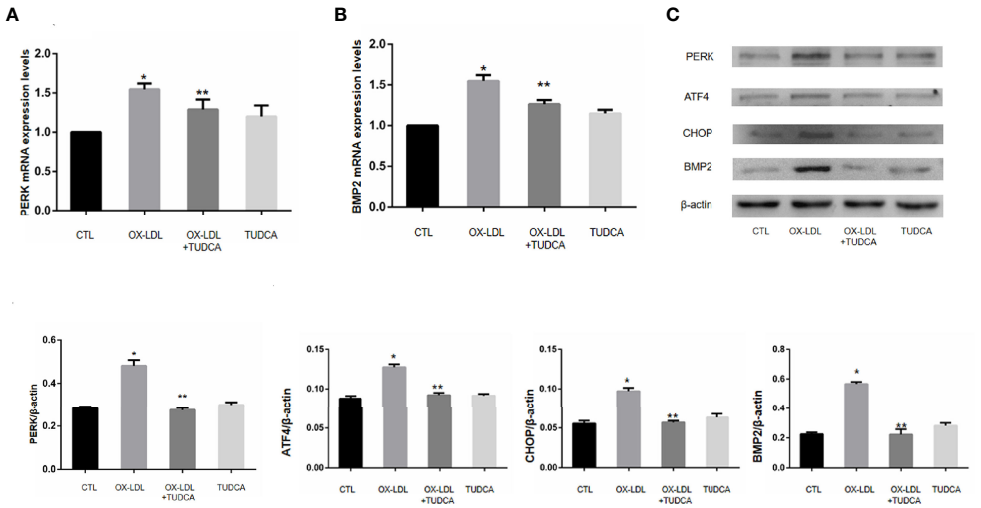


**FIGURE 4** | TUDCA can improve the osteogenic phenotype transformation of VICs. **(A)** PERK mRNA expression levels. **(B)**.BMP2 mRNA expression levels. **(C)** Immunoblot analysis of the pore-forming mediator of Er stress.* represents P value less than 0.05, indicating significant difference; ** means p value less than 0.01, indicating extremely significant difference.

Studies have confirmed that increased expression of BMP2 was found in calcified valves (22), indicating that BMP2 is involved in valve osteogenesis. We verified the osteogenic effect of ox-LDL-induced VICs at the protein level by experiments, and confirmed that the protein expression of BMP2 increased under ox-LDL stimulation. In addition, IL-37 can inhibit ox-LDL-induced valve calcification, and the increase of BMP2 expression is also inhibited (23), which also confirmed the osteogenic effect of OX-LDL-induced VICs and the regulatory effect of inflammatory factors on valvular osteogenesis.

The PERK/ATF4/CHOP pathway is the classic er pathway and the main source of CHOP protein, which is mainly involved in cell apoptosis. ox-LDL, as a common er stress inducer, can induce ER stress in cells, such as vascular endothelial cells (24) and macrophages (25). In ApoE-/- mice fed a high cholesterol diet, the deficiency of Receptor for Advanced Glycation end products (RAGE) slowed the process of valve calcification. RAGE deficiency works by inhibiting er stress. *In vitro*, HMGB1(High Molecular group Box 1 Protein) induces ER stress through RAGE to activate and promote the differentiation of AVICs osteoblasts (6). Therefore, endoplasmic reticulum stress is considered to be involved in valve osteogenesis and calcification.

Taurodeoxycholic acid (TUDCA), a hydrophilic bile acid derivative, is a classic er stress inhibitor (7). Studies have shown that TUDCA's use is no longer limited to hepatobiliary diseases. Studies have shown that TUDCA can down-regulate the activity of PERK during ER stress in the hypothalamus of obese mice and reduce ER stress, and its neuroprotective effect has also been observed in retinal diseases (26). Thus, it has been shown to show potential therapeutic benefits in various models of many diseases, including diabetes, obesity and neurodegenerative diseases, possibly due to its cellular protective effects. The possible mechanism lies in the reduction of er stress and the stabilization of unfolded protein response. In addition, TUDCA has been found to reduce oxidative stress, inhibit apoptosis and reduce inflammation in *in vitro* and *in vivo* models of many diseases (27).

Subsequently, TUDCA was selected as an ER stress inhibitor to verify whether the use of ER stress inhibitors could improve the osteogenesis of valves. The results showed that TUDCA could inhibit er stress and osteogenesis induced by ox-LDL at protein and gene levels. Studies have shown that the expression levels of VICs phosphorylated IRE-1,PERK,eIF2α, ATF4 and RUNX2 in CAVD patients were down-regulated to varying degrees after the addition of TUDCA (28), which confirmed that ER stress is involved in valvular osteogenesis, and er stress inhibitors can inhibit ER stress and valvular osteogenesis. Therefore, combined with the experimental results, we concluded that ox-LDL-mediated ER stress is involved in regulating the osteogenic phenotype transformation of rabbit aortic valve interstitial cells.

In this study, the gene and protein expression levels of BMP2, PERK, ATF4, and CHOP in ox-LDL-stimulated valvular interstitial cells showed differences between the experimental group and the control group, and the transcription factor ATF4 downstream of PERK was confirmed to be involved in osteogenesis (29). It can be speculated that ATF4 in the PERK pathway has a significant correlation with BMP2 expression, but the specific mechanism in valve interstitial cells remains to be further studied.

This study verified that ox-LDL-induced ER stress is involved in the phenotypic transformation of VICs, and inhibition of ER stress can reduce the osteogenic effect of VICs. However, the relationship between other ER stress pathways and CAVD, such as IRE1, ATF6 pathway and changes of related transcription factors and proteins, needs further verification. Although this

study is only a small finding, it is expected to provide some ideas for future treatment of CAVD.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Tongji Hospital Committee for Ethical Approval for Research Involving Animals.

## AUTHOR CONTRIBUTIONS

YT was the guarantor of the article, YG and YL drafted the manuscript, WD, XX, HZ, and LZ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This study is supported by the National Science Foundation of China (no. 81070190) And Hubei Chen Xiaoping Science and Technology Development Fund (CXPJJH12000005-07-40).

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/fendo.2022.861452

# Long-Term Oral Administration of Salidroside Alleviates Diabetic Retinopathy in db/db Mice

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**Specialty section:**
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

**Received:** 24 January 2022
**Accepted:** 21 February 2022
**Published:** 16 March 2022

**Citation:**
Yao F, Jiang X, Qiu L, Peng Z, Zheng W, Ding L and Xia X (2022) Long-Term Oral Administration of Salidroside Alleviates Diabetic Retinopathy in db/db Mice. Front. Endocrinol. 13:861452. doi: 10.3389/fendo.2022.861452

Diabetic retinopathy (DR), a microvascular complication of diabetes mellitus, is the leading cause of vision loss in the working-age population worldwide. Unfortunately, current clinical treatments cannot completely prevent the occurrence and development of DR. Salidroside (Sal) is a medicinal supplement that has antioxidative and cytoprotective properties. This study aimed to investigate the therapeutic effect of Sal on DR. Briefly, Sal treatment was applied to wide-type mice and db/db mice (a widely used diabetic mice) at 25 mg/kg by oral gavage once daily from 8 weeks to 20 weeks. Mice's bodyweight, blood glucose, total cholesterol, triglyceride, high density lipoprotein and low density lipoprotein were recorded and analyzed. Retinal trypsin digestion and evans blue dye assay were used to detect retinal microvessel changes and function. Retinal glutathione and malondialdehyde content measurements were applied to assess retinal oxidative stress. Full-length transcriptome analysis was performed to explore the underlying mechanisms of Sal protection. Our results found that Sal treatment could successfully relieve blood glucose and blood lipid abnormalities, and reduce retinal oxidative stress level in diabetic mice. Also, Sal treatment repaired the abnormal transcriptome caused by diabetes, alleviated the microvascular lesion of the fundus in diabetic mice, and protected retinal normal barrier function. This study enriches the indications of Sal in the treatment of diabetic diseases, providing practical research ideas for the comprehensive preventions and treatments of DR.

**Keywords: salidroside, diabetes, retinopathy, oxidative stress, vascular barrier, transcriptome**

## INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, insulin resistance, and impaired insulin secretion. According to the International Diabetes Federation (IDF) statistics, the number of diabetic patients globally is around 463 million people, and it is expected to achieve 700 million in 2045 (1). As one of the most common microvascular complications of DM,

diabetic retinopathy (DR) has affected 50% of type 1 diabetes and 30% of type 2 diabetes worldwide (2), and it is the leading cause of vision loss in the working-age population (3). Especially in the last 30 years, due to the aging population and the changes in lifestyles, the number of patients with type 2 diabetes has increased sharply, and the corresponding incidence rate has also shown a significantly increasing trend. The global age-standardized prevalence for blindness caused by DR has increased from 14.9% in 1990 to 18.5% in 2020 (4), indicating an urgent and severe need for DR prevention and treatment.

Retina is the only part of the body where the arteries, veins and capillaries can be directly observed with the naked eye. Retinal vascular system can effectively reflect the systemic blood circulation and microvascular changes under DM condition (5, 6). Damage to the retinal vascular system is an essential feature of the pathogenesis of DR. The typical morphological characteristics include loss of pericytes, thickening of the basement membrane, increased vascular permeability, vascular occlusion, and microaneurysms (7, 8). Retinal inflammation, oxidative stress, and glial cell dysfunction are the main pathogenic factors of DR injury (9, 10). These factors can induce damages such as destructive retinal vascular barrier, abnormal angiogenesis, and impaired retinal ganglion cell, ultimately leading to decreased vision and blindness in patients (8). So far, the clinical treatments for DR mainly include retinal laser photocoagulation, intravitreal anti-VEGF injection, sustained released dexamethasone (Ozurdex) implant, and vitreoretinal surgery (11–13). However, these treatments cannot completely prevent the occurrence and development of DR. Many patients still experience deteriorations and eventually develop blindness after clinical treatments (14). Therefore, in-depth studying the pathogenesis of DR, discovering new strategies and targets for DR treatment have crucial clinical and social significance for the prognosis of DR patients and the alleviation of medical burden (15).

Rhodiola Rosea is a precious functional medicinal plant. In China and other Asian countries, it is often used as a commercial dietary supplement to treat stress, fatigue, and altitude sickness (16–18). Salidroside (Sal) is a biologically active ingredient extracted from the Rhodiola plant, with potent antioxidant, anti-inflammatory and neuroprotective effects (19–21). This pharmacological feature can effectively target the retinal oxidative stress, retinal inflammation and nerve cell damage caused by DR. In addition, recent studies have shown that Sal can reduce the blood glucose level in diabetic mice by increasing insulin sensitivity and reducing ß-cell loss, effectively lowering diabetic deteriorations (22–24). However, the role of Sal in DR is still unclear.

In this study, we revealed that long-term application of Sal could both reduce the blood glucose levels and blood lipid levels in diabetic mice and significantly alleviate DR-induced vascular leakage and microangiopathy. Moreover, Sal treatment greatly reversed the changes in the retinal transcriptome caused by DM. All these results highlight the potential value of Sal as a dietary supplement for DR control.

# MATERIALS AND METHODS

## Animals

Male wide-type C57BLKS/J mice (WT, 20-30 g, 8 weeks) and C57BLKS/J db/db mice (40-60 g, 8 weeks, genetically diabetic leptin receptor-mutated mice charactered by spontaneous obesity and hyperglycemia) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and housed in a comfortable environment at 22 ± 2°C with 12 hours light/dark cycle. All the experimental procedures were approved by the Animal Ethical Committee of Xiangya hospital, Central South University (Changsha, China).

## Experimental Design

In total, 21 WT mice and 24 db/db mice were used in the study. Based on previous study (25), Sal treatment was administered at 25 mg/kg by oral gavage once daily, while the control group received water. Before the experiment, the mice were kept for 3 days to acclimate. Then WT mice and db/db mice were divided into four groups: WT group (WT mice treated with water; n = 12), WT + Sal group (WT mice treated with 25 mg/kg Sal; n = 9); db/db group (db/db mice treated with water; n = 12), db/db + Sal group (db/db mice treated with 25 mg/kg Sal; n = 12).

## Analysis of Mouse Blood Samples

Before blood samples measurement, mice were deprived of food and water for 6 hours to maintain a fasting state. Blood glucose levels in the caudal veins were measured every 4 weeks using an automatic blood glucose mete (Accu-Chek; Roche, Mannheim, Germany). Blood lipid levels, including total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL), were determined using an automatic blood lipid mete (CCM-111; On·call, Hangzhou, China) 12 weeks after Sal treatment. All measurements were performed and calculated according to the instructions.

## Retinal Trypsin Digestion Assay

Fresh dissected retinas were fixed in 4% paraformaldehyde for 1 hour and divided into four quadrants at the optic disk. Then retinas were digested in 3% Trypsin, 0.1 M Tris (pH 7.8) for 3 hours at 37°C until the medium became cloudy. After the incubation, the internal limiting membrane and disintegrated neuronal tissue were carefully removed, and then the remaining retinal vasculature was flattened on a glass slide for air dry. At last, air-dried retinal vasculature was treated with periodic acid solution and stained with Schiff's reagent and hematoxylin (C0142S; Solarbio, Beijing, China). A digital imaging system was used to observe the histological change of retinal vasculature. All measurements were conducted in a masked manner and observed under the bright field.

## Evans Blue Dye Assay

Retinal vascular barrier function was determined by evans blue dye assay. Briefly, 30 mg/kg evans blue was injected through the caudal vein. Two hours later, mouse was anesthetized and perfused with

10 ml PBS to flush blood out of circulation. The retinas were collected and fixed in 4% paraformaldehyde for 1 hours. The left retina was flattened on a glass slide and observed under 652 nm excitation using a fluorescence microscope (DM5000 B; Leica, Wetzlar, Germany), the right retina was incubated with 0.3 ml formamide overnight at 70°C and centrifuged at 12,000 g for 15 minutes to extract evans blue from the retina. The absorbance of extract was determined with a spectrophotometer at 620 nm, the concentration of evans blue dye in retinal extract was calculated from the standard curve of evans blue in formamide and normalized to the dry retinal weight.

## Measurement of Retinal Oxidative Stress

Malondialdehyde (MDA) and glutathione (GSH) are crucial biochemical indicators reflecting oxidative stress levels. The retinal MDA content was measured using a Lipid Peroxidation MDA Assay Kit (S0131S; Beyotime, Shanghai, China). The retinal GSH content was measured using a Micro Reduced GSH Assay Kit (BC1175; Solarbio, Beijing, China). The measured contents were calibrated using the samples' protein concentration.

## Full-Length Transcriptome Analysis

Mouse retinas were collected 12 weeks after Sal intervention. Two individual retinas were pooled and treated as one sample; each group contained 3 samples. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA, cDNA-PCR Sequencing Kit (SQK-PCS109, Oxford Nanopore Technologies Ltd, Oxford, UK) was applied to convert total RNA to cDNA. The final cDNA was then run on the PromethION platform at the Biomarker Technology Company (Beijing, China). Genes with a fold-change ≥ 1.5 identified by edgeR and a false discovery rate (FDR) < 0.05 were considered differentially expressed (BMKCloud, http://www.biocloud.net/). Gene functional annotation were based on the following databases: KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/) and GO (Gene Ontology, http://www.geneontology.org/).

## Statistical Analysis

SPSS version 22.0 (IBM, Armonk, NY, USA) was used for the statistical analyses. Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were used for comparisons between two sets. Statistical significance was set at $p < 0.05$.

# RESULTS

## Sal Improved Blood Glucose and Lipid Profiles in db/db Mice

Overweight and hyperglycemia are typical features in db/db mice. As shown in **Figures 1A, B** and **Supplementary Table 1**, db/db mice showed a significant increase in bodyweight and blood glucose level compared with those in WT mice, and these pathological changes were more obvious along with aging. At week 16 and week 20, the blood glucose levels of db/db mice were nearly five times higher than that of WT mice, but such an alteration was effectively alleviated by Sal intervention. In addition, dyslipidemia is also an important feature that

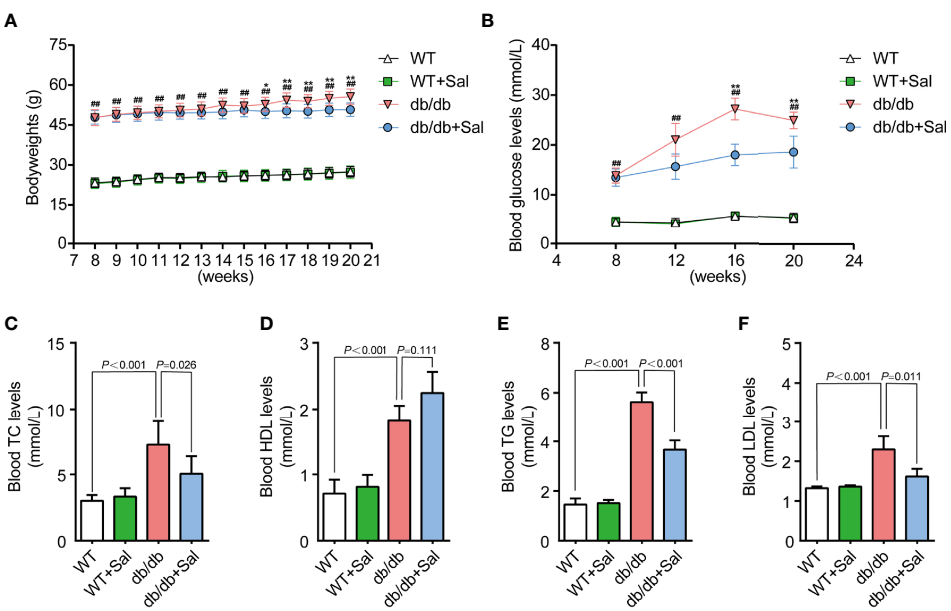


**FIGURE 1** | Sal improved blood glucose and lipid profiles in db/db mice. **(A)** The bodyweight of WT and db/db mice after Sal intervention. **(B)** The blood glucose of WT and db/db mice after Sal intervention. **(C-F)** The blood levels of total cholesterol (TC), high density lipoprotein (HDL), triglyceride (TG) and low density lipoprotein (LDL) in WT and db/db mice after Sal intervention. Data are the mean ± SD; $^{\#\#}p < 0.01$ (db/db mice compared with WT mice), $^{*}p < 0.05$, $^{**}p < 0.01$ (db/db + Sal mice compared with db/db mice).

distinguishes db/db mice from WT mice. At 20 weeks, the contents of TC, TG, HDL and LDL in the blood of db/db mice were all greatly higher than those of WT mice. However, Sal treatment effectively improved these lipid profiles (**Figures 1C–F** and **Supplementary Table 1**). Despite Sal's powerful function in regulating blood glucose and lipids in db/db mice, its therapeutic effect on normal WT mice was not found (**Figure 1** and **Supplementary Table 1**). Taken together, these data suggested that the long-term oral administration of Sal improved blood glucose and lipid profiles in db/db mice, indicating that the Sal is an effective and safe drug in treating dysglycemia and dyslipidemia caused by diabetes.

## Sal Alleviated Retinal Microvascular Changes in db/db Mice

Retinal microvascular changes are the early feature DR (26). To investigate the role of Sal on DR, we quantified the number of retinal pericyte ghosts and acellular capillaries in db/db mice after Sal administration. The results showed that retinal pericyte ghosts and acellular capillaries were greatly increased in db/db mice when compared with WT mice (**Figure 2** and **Supplementary Table 1**). However, Sal treatment significantly blunted the pericyte dropout and retinal acellular capillary formation in db/db mice without obvious influence on WT mice (**Figure 2** and **Supplementary Table 1**). These results demonstrated that Sal intervention alleviated retinal microvascular changes, suggesting a therapeutic effect of Sal on early DR.

## Sal Protected Retinal Vascular Barrier Function in db/db Mice

Pericyte loss and acellular capillaries formation could alter retinal vascular permeability and lead to vascular leakage (27). To further determine the therapeutic effect of Sal on early DR, evans blue assays were applied to assess retinal vascular barrier function in db/db mice. As shown in **Figure 3A**, db/db mice exhibited more evans blue leakage area in the retina compared with WT mice, and Sal treatment markedly alleviated this leakage extent. Evans blue quantitative results also revealed that Sal treatment greatly decreased retinal evans blue leakage levels in diabetic conditions (**Figure 3B** and **Supplementary Table 1**). All these results suggested that Sal protects retinal vascular barrier function in db/db mice, indicating a therapeutic effect on diabetes-induced retinal vascular dysfunction.

## Sal Relieved Retinal Oxidative Stress in db/db Mice

Previous studies have demonstrated that oxidative stress plays a crucial role in DR pathogenesis (28). Our results found that retinal

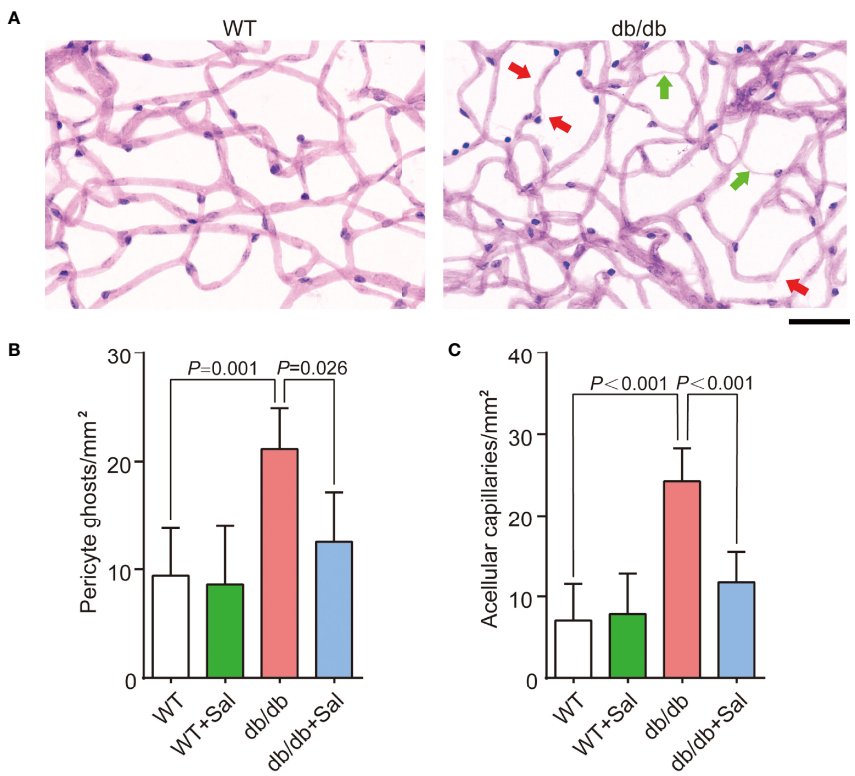


**FIGURE 2** | Sal alleviated retinal microvascular changes in db/db mice. **(A)** The representative photomicrographs of normal and diabetic microvessel stained by Schiff's reagent and hematoxylin; The green arrows pointed is acellular capillaries, and the red arrows pointed is pericyte ghost. **(B)** The number of pericyte ghost in WT and db/db mice after Sal intervention. **(C)** The number of acellular capillaries in WT and db/db mice after Sal intervention. Data are the mean ± SD. Scale bar = 50 μm.

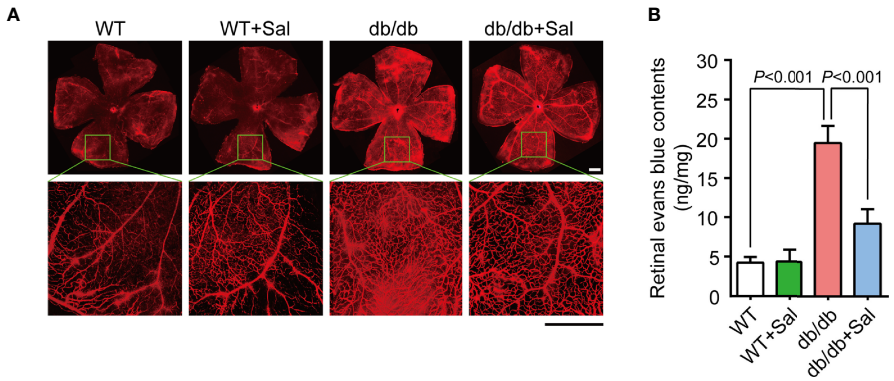


**FIGURE 3** | Sal protected retinal vascular barrier function in db/db mice. **(A)** The representative photomicrographs of retinal vessels stained by evans blue in WT mice and db/db mice with or without Sal intervention. **(B)** Retinal normalized evans blue contents in WT and db/db mice after Sal intervention. Data are the mean ± SD. Scale bar = 500 μm.

GSH levels (an important biochemical indicator reflecting cellular anti-oxidative stress ability) were decreased in db/db mice, while Sal intervention restored retinal GSH levels in diabetic conditions (**Figures 4A** and **Supplementary Table 1**). Moreover, retinal MDA contents, a metabolite of oxidative stress, were increased in diabetic mice and were recovered to normal levels after Sal treatment (**Figures 4B** and **Supplementary Table 1**). It was noteworthy that Sal had no effect on GSH and MDA contents in WT mice. All these results suggested that Sal relieves retinal oxidative stress in db/db mice, showing an excellent antioxidant property in DR.

## Sal Ameliorated Retinal Transcriptome Abnormalities in db/db Mice

To determine the underlying mechanism of Sal treatment on early DR, full-length transcriptome analysis was performed on the retina of WT, db/db and Sal-treated db/db mice at 12 weeks after intervention. As shown in **Figure 5A**, there were 208 up-regulated differentially expressed genes (DEGs) and 182 down-regulated DEGs between WT mice and db/db mice, and 144 up-regulated DEGs and 133 down-regulated DEGs between db/db mice and Sal-treated db/db mice. Among these diabetes-induced DEGs, 14 up-regulated DEGs and 21 down-regulated DEGs were completely restored by Sal treatment, and only one DEGs (*Tmem252*) was aggravated after Sal intervention (**Figures 5A, B**). Except for the completely restored DEGs, Sal treatment mitigated the expression of approximately 73.08% up-regulated DEGs and 66.35% down-regulated DEGs induced by diabetes, including 8 oxidation-related genes (*Aldh4a1*, *Acad12*, *Ado*, *Kdm4d*, *Acacb*, *Mical1*, *Th* and *Htatip2*) (**Supplementary Figure 1**). These results suggested that Sal exhibits its powerful

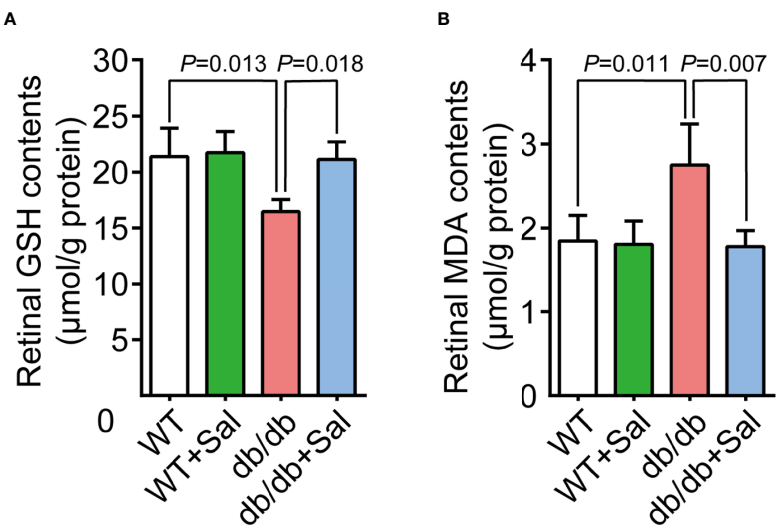


**FIGURE 4** | Sal relieved retinal oxidative stress in db/db mice. **(A)** Retinal GSH contents in WT and db/db mice after Sal intervention. **(B)** Retinal MDA contents in WT and db/db mice after Sal intervention. Data are the mean ± SD.

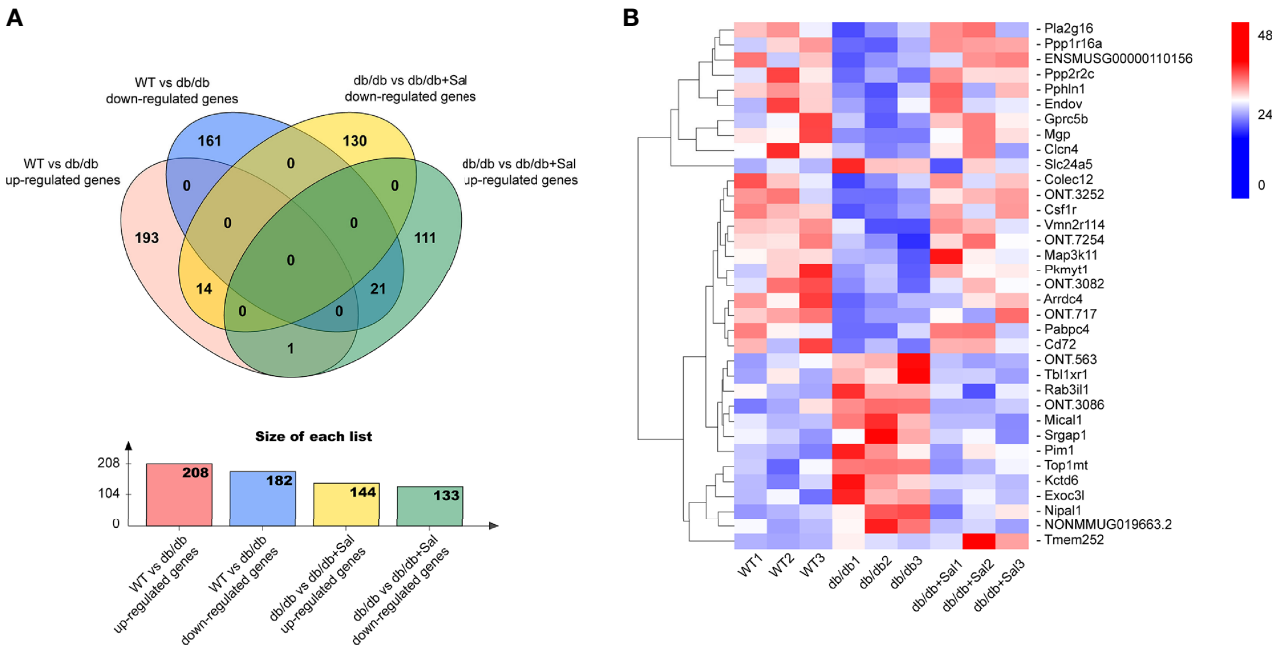


**FIGURE 5** | Sal ameliorates retinal transcriptome abnormalities in db/db mice. **(A)** The Venn diagram showing the differentially expressed genes (DEGs) among WT mice, db/db mice and Sal-treated db/db mice. **(B)** The heat map showing the Sal-restored DEGs (35 genes) and Sal-aggravated DEGs (1 gene) between WT and db/db mice.

DR therapeutic effect largely depending on ameliorating retinal transcriptome abnormalities.

## The Analysis of DEGs Between db/db Mice and Sal-Treated db/db Mice

To systematically identify the retinal biological process and pathways associated with Sal treatment, we performed GO and KEGG analysis on the up-regulated DEGs and down-regulated DEGs between db/db mice and Sal-treated db/db mice (**Figure 6).** KEGG analysis indicated that the AMPK signaling pathway and the PI3K-Akt signaling pathway were two crucial pathways after Sal treatment, as DEGs were mainly concentrated in these two pathways (**Figure 6A**).GO analysis results showed that the retinal biological process, cellular component, and molecular function were all altered by Sal intervention. The most DEGs were enriched in biological process, and the less DEGs were enriched in molecular function (**Figure 6B**).

# DISCUSSION

DR is one of the most destructive microvascular complications of diabetes, which can lead to irreversible retinal damage (1). Although DR is not a fatal disease, it significantly influences the patient's vision, inconveniences the patient's daily life, and affects people's quality of life. In addition, the cost of DR care and treatment has become an important source of medical burden for families and society. The prevention and treatment of DR has been unsatisfactory for many years. Existing clinical treatment methods have not benefited all patients, and there is yet a need to discover new therapeutic targets for DR (14, 15).

So far, DR is divided into two stages based on the degree of microvascular disease: early non-proliferative diabetic retinopathy (NPDR) and late proliferative diabetic retinopathy (PDR) (29). Patients with NPDR have no obvious symptoms in the early stage. Microaneurysm, capillary leakage and local bleeding points can be seen by fundus examination. These microvascular abnormalities increase the permeability of retinal blood vessels, causing retinal edema and the symptoms of blurred vision in patients. This stage is the best time to prevent and treat DR clinically (30). With the progression of DR, patients with NPDR may have abnormal neovascularization in the fundus while entering the PDR stage. Because of the greater fragility of neovascularization, vitreous hemorrhage is likely to be caused by easy rupture and leakage. In addition, secondary formation of retinal fibroproliferative membrane and tractional retinal detachment will induce permanent damage to patients' vision, leading to blindness (31). At this stage, the treatment cost is higher, while the treatment outcome is far inferior to the NPDR stage (32). Therefore, early intervention, prevention and control of DR is important.

In this study, we started by giving 25 mg/kg of Sal oral treatment for diabetic mice at the early stage of abnormal blood glucose to realize the effective prevention and the control of DR through the early intervention of drugs. The results demonstrated that Sal treatment can effectively reduce the blood glucose and the

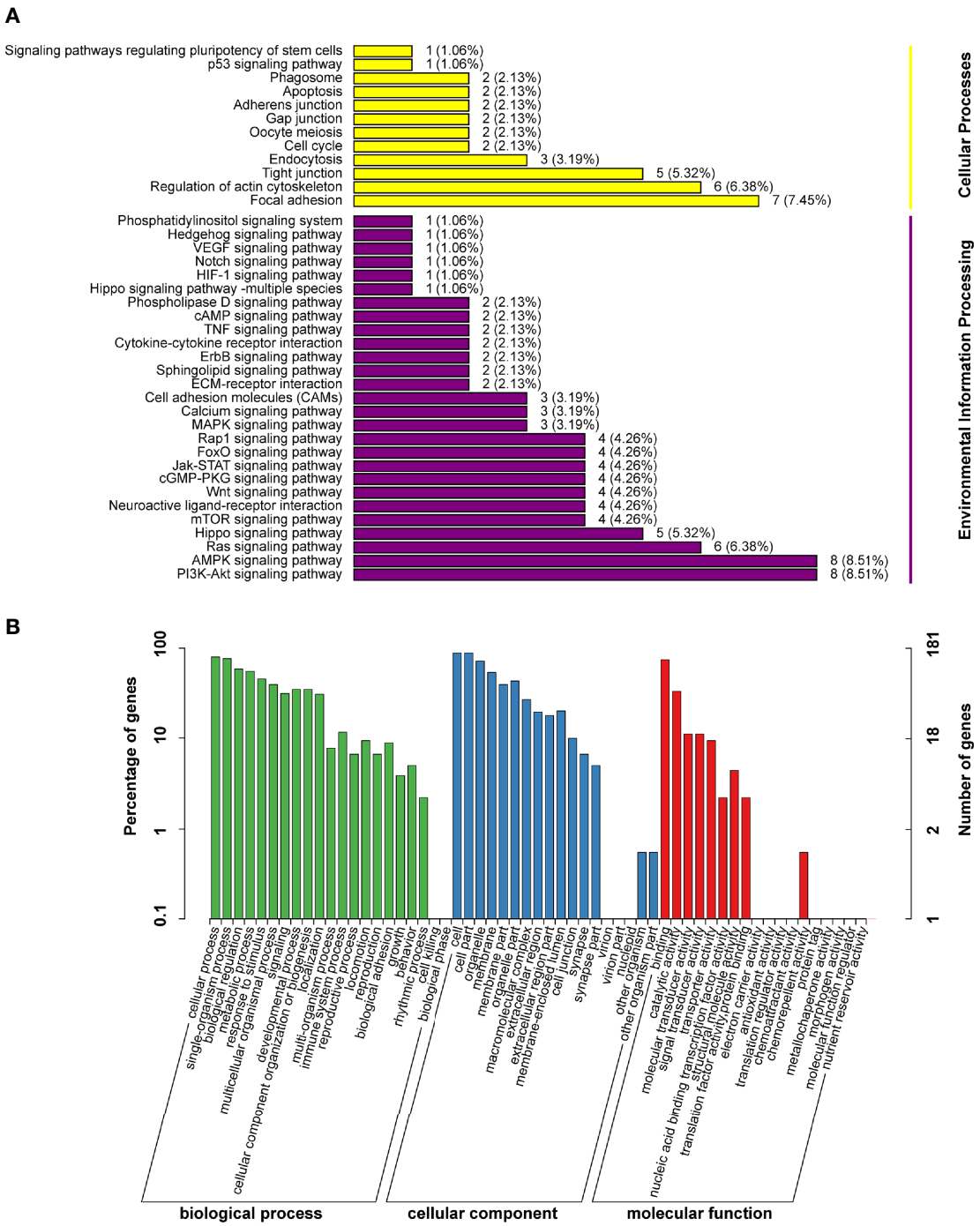


**FIGURE 6** | The analysis of DEGs between db/db mice and Sal-treated db/db mice. **(A)** The DEGs between db/db mice and Sal-treated db/db mice were analysed by KEGG databases. **(B)** The DEGs between db/db mice and Sal-treated db/db mice were analysed by GO databases.

blood lipid levels of diabetic mice, which is consistent with the results of some previous studies (22, 23, 25). The mechanism of its occurrence may be related to Sal improving the insulin resistance of mice and increasing the survival of pancreatic islet b cells (22). Our results also found that Sal treatment can effectively improve the fundus microvascular disease in diabetic mice, protect the blood-retinal barrier function and reduce microvascular leakage. This may benefit from regulating blood glucose and blood lipids by Sal. Many studies have shown that hyperglycemia and hyperlipidemia are substantial causes of DR

through various pathways, such as protein kinase C (PKC), polyol, and hexosamine (33–35). Also, the advanced glycation end products (AGEs) induce apoptosis of vascular endothelial cells, pericytes and nerve tissues, leading to the occurrence of DR (36). Thus, the application of hypoglycemic drugs and hypolipidemic drugs can reduce the incidence of DR.

Although hyperglycemia is an important cause of DR, the impact of simply lowering blood glucose in controlling the progression of DR is limited. A large sample of clinical studies has shown that the active control of blood glucose has not significantly affected the progression of DR in patients with type 2 diabetes (37). Further, intensive blood glucose control has no remarkable benefit in improving the prognosis of DR (38). In fact, abnormal glucose and lipid metabolism in diabetic patients can interfere with the normal redox reaction in the cell, leading to an increment in ROS and oxidative stress (26). Increased ROS further induces epigenetic changes in mitochondrial enzymes, forming a metabolic memory that is unable to alleviate the symptoms of DR even when blood glucose is controlled to a normal level (39, 40). Thus, in the early stage of DR, reducing the level of oxidative stress in the retina is the key to preventing the progression of DR. As a natural antioxidant, Sal has been widely concerned about its antioxidant function (19, 41) In the retina, Sal has been proved to prevent the pigment epithelial cell damage induced by hydrogen peroxide and the vascular endothelial cell damage caused by hypoxia through reducing oxidative stress (42–45). This statement is consistent with the result of the excellent antioxidant properties of Sal in DR in this study, illustrating that the improvement of fundus microvascular disease by Sal in diabetic mice is partially due to its antioxidant activity.

The sequencing results of this study found that Sal can significantly repair the abnormal transcriptome expression caused by diabetes. Among the 390 differential genes in db/db mice and normal wild-type mice, about 70% were improved to varying degrees after the Sal treatment, 9% of which completely repaired, and only less than 0.3% of which deteriorated. This result indicated the powerful role of Sal treating DR from another perspective. Previous studies have confirmed that the P13K-AKT and AMPK signalling pathways are crucial for Sal to exert therapeutic effects by activating the above pathways to reduce diabetic-induced damages in the heart, liver, and kidney (24, 25, 46, 47). In our study, the sequencing results displayed that the retina after Sal treatment was also accompanied by the enrichment of genes related to the P13K-AKT and AMPK signalling pathways, suggesting the DR protective effect of Sal is likely to depend on the above pathways. However, the specific mechanism of action needs further studies in the future.

## CONCLUSION

This study explored the effects of oral Sal administration on the prevention and treatment of early diabetes and DR. Our results depicted that a long-term and low-dose oral Sal treatment can successfully relieve blood glucose and blood lipid abnormalities and reduce the oxidative stress level in the retina in diabetic mice. Also, Sal treatment repairs the abnormal transcriptome caused by diabetes, alleviates the microvascular disease of the fundus in diabetic mice, and protects the normal retinal barrier function. This study enriches the indications of Sal in the treatment of diabetic diseases, providing practical research ideas for the comprehensive preventions and treatments of DR.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA, PRJNA800617.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethical Committee of Xiangya Hospital, Central South University.

## AUTHOR CONTRIBUTIONS

FY wrote the first draft of the paper. XJ and WZ edited the paper. FY, XX, and LD designed research. FY, WZ, LQ, and ZP performed research. FY analyzed data. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the National Natural Science Foundation of China (grant numbers 81974134 and 82171058 to XX; 82070966 to LD), the National key research and development program of China (grant numbers 2020YFC2008205 to XX), the Key R & D plan of Hunan Province of China (grant numbers 2020SK2076 to XX), the Natural Science Foundation of Hunan Province (grant numbers 2019JJ40474 to XX) and the Science and Technology Innovation Program of Hunan Province (grant numbers 2021RC3026 to LD).

## ACKNOWLEDGMENTS

We thank Mingfang Xia, Manjuan Peng, Jingning Long, Zhou Zeng, Yangzhou Zhang, Rong Rong, Mengling You and Zhaolin Gao for technical assistance.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.861452/full#supplementary-material

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REVIEW
published: 07 April 2022
doi: 10.3389/fendo.2022.851941

# Endothelial Dysfunction and Diabetic Cardiomyopathy

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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *10 January 2022*
**Accepted:** *14 March 2022*
**Published:** *07 April 2022*

**Citation:**
*Wang M, Li Y, Li S and Lv J (2022) Endothelial Dysfunction and Diabetic Cardiomyopathy. Front. Endocrinol. 13:851941. doi: 10.3389/fendo.2022.851941*

The cardiovascular complications contribute to a majority of diabetes associated morbidity and mortality, accounting for 44% of death in those patients with type 1 diabetes mellitus (DM) and 52% of deaths in type 2 DM. Diabetes elicits cardiovascular dysfunction through 2 major mechanisms: ischemic and non-ischemic. Non-ischemic injury is usually under-recognized although common in DM patients, and also a pathogenic factor of heart failure in those diabetic individuals complicated with ischemic heart disease. Diabetic cardiomyopathy (DCM) is defined as a heart disease in which the myocardium is structurally and functionally abnormal in the absence of coronary artery disease, hypertensive, valvular, or congenital heart disorders in diabetic patients, theoretically caused by non-ischemic injury solely. Current therapeutic strategies targeting DCM mainly address the increased blood glucose levels, however, the effects on heart function are disappointed. Accumulating data indicate endothelial dysfunction plays a critical role in the initiation and development of DCM. Hyperglycemia, hyperinsulinemia, and insulin resistance cause the damages of endothelial function, including barrier dysfunction, impaired nitric oxide (NO) activity, excessive reactive oxygen species (ROS) production, oxidative stress, and inflammatory dysregulation. In turn, endothelial dysfunction promotes impaired myocardial metabolism, intracellular $Ca^{2+}$ mishandling, endoplasmic reticulum (ER) stress, mitochondrial defect, accumulation of advanced glycation end products, and extracellular matrix (ECM) deposit, leads to cardiac stiffness, fibrosis, and remodeling, eventually results in cardiac diastolic dysfunction, systolic dysfunction, and heart failure. While endothelial dysfunction is closely related to cardiac dysfunction and heart failure seen in DCM, clinical strategies for restoring endothelial function are still missing. This review summarizes the timely findings related to the effects of endothelial dysfunction on the disorder of myocardium as well as cardiac function, provides mechanical insights in pathogenesis and pathophysiology of DCM developing, and highlights potential therapeutic targets.

**Keywords: diabetic cardiomyopathy, endothelial dysfunction, oxidative stress, metabolism, diabetes**

## INTRODUCTION

It was estimated that the global diabetes prevalence would rise to 10.2% (578 million) in 2030 (1). The prediction from the World Health Organization is that diabetes will be the seventh leading cause of death by 2030. Diabetes severely endangers public health, especially its cardiovascular complications, which are the largest challenge accounting for 50%-80% of death of diabetic patients (2). HbA1c, the important index to evaluate blood glucose levels, is not sufficient to predict the risk of macrovascular events (3, 4). Therapeutic management and monitoring of blood glucose are the basis of diabetes therapy. However, present therapeutic strategies do not reduce the fatality rate of cardiovascular complications in diabetes. This finding suggests that metabolic disturbance is not the exclusive cause for macrovascular events followed by diabetes and other metabolic disorders, highlighting the contribution of other factors. Diabetic cardiomyopathy is an outcome of intrinsic heart muscle malfunction, different from atherosclerotic vascular disease (5, 6). The etiology of diabetic cardiomyopathy is complex, with a change in cardiomyocyte metabolism, which has been considered as a pivot stimulus for cardiomyopathy dysfunction (7).

Diabetic cardiomyopathy is a significant entity characterized by diastolic impairment and left ventricular hypertrophy in the absence of vascular defects (7, 8). Furthermore, it has been identified as a microvascular complication, in which cardiac microvascular endothelial dysfunction in the first ring of diabetic cardiomyopathy and running throughout the entire process, suggested by many studies (9, 10). The development of diabetic cardiomyopathy has been reported correlated with several factors, including decreased cardiac compliance, insulin resistance (11, 12), endothelial dysfunction, increased oxidative stress (11), aberrant ion flux (13), and coronary microcirculation abnormalities (12). It has been widely documented that endothelial dysfunction occurs in diabetes patients and in individuals with insulin resistance or at high risk for developing type 2 diabetes.

Therapeutic methods targeting specific molecules modification in microvascular endothelial cells (MVECs) and cardiomyocytes exposed to high glucose stimuli have shown beneficial effects in clinical trials (14, 15). Hyperglycemia has been identified as an important contributor to endothelial dysfunction in both type 1 and type 2 diabetes (16, 17). Microvascular endothelial dysfunction is primarily characterized by decreased release of NO, enhanced oxidative stress, increased production of inflammatory factors, abnormal angiogenesis, and impaired endothelial repair (18). Endothelial cells and cardiomyocytes both are major components of the heart, and their interaction is complicated and crucial in the progression of diabetic cardiomyopathy. Cheang et al. reported endothelial nitric oxide synthase enhancer increased NO bioavailability and reduced oxidative stress, further ameliorating endothelial dysfunction in db/db mice (19).

The endothelium is the biggest organ of the body, maintains and regulates the normal function of vessels. The endothelium functions as a mechanical lining in vessels, it also plays a pivot role in the regulation of leucocyte-adhesion, platelet aggravation, and blood vessel patency. Endothelium functions by regulating the release of secretory factors in response to mechanical stimuli. The major role of endothelium is to ensure adequate blood flow, which is dependent on the counterbalance between vasodilators and vasoconstrictors. Vasodilators, including prostacyclin I2 (PGI2) and nitric oxide (NO), aim to maintain adequate blood through dilating the vessels; vasoconstrictors, including endothelin-1 (ET1) and thromboxane A2 (TXA2), address to counterbalance the excessive vasodilation and maintain the vascular tone. Insulin resistance and diabetes have both been reported initiated or associated with endothelial dysfunction. In addition, endothelial dysfunction is correlated with obesity, sedentary lifestyle, and smoking. These observations suggest the complex pathophysiology of endothelial dysfunction involves multiple mechanisms and take part in various diseases. Therefore, we will discuss the role of endothelial dysfunction in the development of diabetic cardiomyopathy in followed sections.

## ENDOTHELIAL DYSFUNCTION IN DIABETES

### Altered Vascular Endothelial Barrier Function

Chronic hyperglycemia results in metabolic derangements in endothelial cells and organs damage. It has been indicated that hyperglycemic conditions not only impair endothelial cells by increasing ROS and inhibiting NO synthase, but also promote increased permeability of the endothelial cells layer (20). The activation of diacylglycerol (DAG) - Protein Kinase C (PKC) signaling pathway contributes to the increased permeability of endothelial cells (21, 22). It was reported that the activation of cPKC and nPKC is DAG dependent, and associated with increased vascular permeability and leukocyte adhesion in diabetes, involving the heart and kidney (23). In human umbilical vein endothelial cells (HUVECs), PKC leads to the phosphorylation of the myosin light chain (MLC), further inducing phosphorylation modification of VE-cadherin on tyrosine and the disruption of adherence junctions (24). There are several protein families taking part in the formation and regulating tight junction in endothelial cells, including transmembrane, scaffolding, and signaling proteins (25). Diabetic condition induced decreased expression of occluding elevated contents of glycated-occludin, and the loss of transendothelial electrical resistance (TEER) in endothelial cells. The disruption of the endothelial barrier is closely associated with excessive oxidative stress, N-acetylcysteine (NAC) prevented endothelial cell dysfunction induced by high glucose exposure (26). The precise mechanisms of regulating these proteins are still uncertain and deserved further investigation. In addition, it has been well documented that oxidative stress impaired endothelial barrier function *via* promoting $Ca^{2+}$ influx into endothelial cells to disrupt inter-endothelial tight junctions. Overactivated TRPM2-mediated

Ca2+ signaling leads to the internalization of VE-Cadherin and degradation of ZO-1, further increasing trans-endothelial migration of neutrophils in response to various pathological factors induced by ROS (27).

## Abnormal Nitric Oxide Synthase Activity

Impaired NO signaling is closely correlated with myocardial damage in diabetes mellitus, characterized by the dysregulation of NO generation and bioavailability (28). Felaco et al. (29) reported the reduced deposition of eNOS in endothelial cells in diabetic rat hearts, compared with non-diabetic controls, without influence on the protein level or the mRNA level of eNOS in the heart. This observation highlights the correlation between the diabetes pathogenesis and expression of eNOS in cardiac ECs of diabetic rats, suggesting the important role of cardiac ECs. Further studies reported by Sampaio et al. (30) indicated the cardiac abnormalities observed in diabetic rats similar to the counterparts in L-NAME-induced cardiomyopathy, suggesting the pivot role of NO in the pathophysiology of DCM. In addition, inhibition of NOS reduced the levels of NO, nitrotyrosine, and reactive oxygen species (ROS), indicating eNOS uncoupling in diabetic hearts (31, 32). The oxidation of tetrahydrobiopterin induced by reactive oxygen species (ROS) and increased content of asymmetric dimethylarginine (ADMA) contribute to eNOS uncoupling in DM.

iNOS is an inductive isoform of NOS, whose expression increases while addressing cytokines and other agents (33). The altered expression and activity of iNOS have been reported to contribute to diabetes-associated cardiovascular complications. The expression of iNOS has been reported to increase in mesenteric arteries and hearts of diabetic rats (34–36). Puthanveetil et al. (37) reported iNOS caused cardiomyocyte cell death by mediating the nitrosylation of GAPDH and caspase-3. Excessive iNOS formation causes contractile dysfunction and heart failure when iNOS uncouples, produces ROS, and contributes to oxidative stress due to limited substrates. Moreover, oral sepiapterin improved left ventricular function in diabetic mice by inhibiting iNOS uncoupling (38).

Besides eNOS and iNOS, there are some studies about the role of nNOS in diabetic cardiomyopathy. However, the alterations of iNOS expression are still controversial (36, 39, 40). Previous studies suggested that PKC-$\beta_2$ induced iNOS upregulation in DCM may be mediated by RhoA/ROCK pathway. And a recent study by Lei et al. reported that the inhibition of PKC-$\beta_2$ caused increased expression of caveolin-3, augmented the phosphorylation of eNOS, and decreased iNOS expression, further improving diastolic dysfunction in diabetic rats. In addition, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, has elevated in the plasma of patients with type 2 diabetes (41). Further studies suggest that hyperglycemia induces the impaired activity of dimethylarginine dimethylaminohydrolase (DDAH), leading to ADMA accumulation and may cause a reduction of NO expression and endothelial vasodilator dysfunction in diabetes (42). Moreover, AGEs quench nitric oxide activity *in vitro* and *in vivo* and mediate impaired vasodilation (43).

## Reactive Oxygen Species (ROS) Generation and Oxidative Stress

It has been widely reported oxidative stress resulting from an imbalance between pro-oxidative and anti-oxidative compounds, significantly augments in long-standing diabetic cardiomyopathy (44, 45). Increased glucose flux through activating NAD(P)H oxidase (NOX) and following augmented the superoxide anion ($O_2^-$) production results in the production of oxidative stress (46). Increased $O_2^-$ anion acts with hydroxyl radicals and hydrogen peroxide, which may lead to oxidant injury (47). Superoxide may react with NO to produce peroxynitrite ($ONOO^-$), further leading to an increase of lipid peroxidation, protein nitration, and oxidizing low-density lipoproteins (LDLs) (48). Peroxynitrite causes eNOS uncoupling by oxidation of $BH_4$ and oxidation of the zinc-thiolate center of eNOS (49, 50). In addition, excessive ADMA contributes to an imbalance between NO and ROS, *via* stimulating superoxide release from endothelial cells (51).

Abnormal metabolism and dysfunction of mitochondria are related to aberrant metabolism in diabetic cardiomyopathy. It has been reported that hyperglycemia causes oxidative damage of mitochondrial DNA in endothelial cells, by increasing mitochondrial reactive oxygen species (ROS) (52). Damaged mitochondria DNA activates the (poly ADP-ribose) PARP-1 pathway in the nucleus of endothelial cells, further leading to inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), further impaired glycolysis process The inhibition of glycolysis causes the accumulation of glycolytic intermediates, resulting in relatively enhanced branching pathways of glucose metabolism including the polyol pathway, the hexosamine biosynthesis pathway, or the glycation pathway (53). Importantly, these pathways all contribute to endothelial dysfunction. Further experimental evidence suggested that inhibition of PARP-1 in endothelial cells prevent endothelial dysfunction induced by diabetes (54).

Other mechanisms are involved in endothelial dysfunction induced by excessive ROS exposed to high glucose. It was reported that induction of sphingosine-1-phosphate receptor 1 (S1PR1) or reduction in S1PR2 both improve endothelial dysfunction (55). In addition, activation of AMPK shows an inhibitory effect on overproduction of mitochondrial ROS (mtROS) in endothelial cells. And it has been shown the upregulation of AMPK may prevent endothelial dysfunction in diabetic mice (56). Taken together, these findings suggest the important role of endothelial dysfunction in diabetic cardiomyopathy, and ROS inhibition may be a therapeutic target for diabetic cardiomyopathy.

## Inflammation

To address damage factors, endothelial cells are activated and produce interleukins, chemokines, interferons, monocyte chemoattractant protein-1 (MCP-1), and other inflammatory factors (57). Monocytes and neutrophils are recruited to the activated endothelium following the release of these substances, and initiate inflammation. Elevated proinflammatory factors stimulate endothelial cells to secrete other proinflammatory

factors, which induce the secretion of diverse acute-phase reactants and modulate chronic inflammation. It has been reported that insulin inhibits NO bioavailability *via* the p38 MAPK-cFOS pathway and increases inflammation in hyperinsulinemic insulin-resistant subjects. Furthermore, the inflammatory levels are elevated when in endothelial cells exposed to hyperinsulinemic serum. In addition, mitochondrial dysfunction induces endothelial dysfunction and promotes inflammation by producing excessive ROS (58, 59). The inflammatory state triggered by endothelial activation may be the consequence of an imbalance of excessive ROS and insufficient antioxidants, resulting in oxidative stress and cell damage.

### Hemodynamic Alterations

Vascular endothelial cells are prone to be damaged by hyperglycemia owing to its characteristic and location. Damaged endothelial cells cause permeability increase, barrier dysfunction, and vasodilatation impairment (10, 46, 60). Many studies on hemodynamic studies all suggested that endothelium-dependent vasodilatory response in diabetes is impaired (61, 62). The balance between the vasoconstrictors and vasodilators is impaired, which are released by endothelial cells to help maintain coronary vascular structure and normal blood flow. Diabetic cardiomyopathy causes the over-release of various vasoconstrictors.

These alterations are associated with increased vasoconstriction and impaired vasodilatation, caused by aberrant levels of vasodilators, with NO and PGI2 as representatives, as well as vasoconstrictors, represented by ET-1 and TAX2. Moreover, ET-1 is upregulated in the target organs of diabetic complications, including the heart, and kidney (63, 64). It was reported that ET-1 is predominantly expressed in cardiac endothelial cells compared with cardiomyocytes in normal adult heart tissue, highlighting the important role of endothelial cells in diabetic cardiomyopathy (65). Moreover, increased endothelin production in the diabetic heart may lead to vessel hypertrophy and increased myocardial fibrosis, which both are the characteristic of diabetic cardiomyopathy (66, 67). Excessive vasoconstrictors prostanoids increased ROS production by upregulating NADPH oxidase and type 4 and type 5 phosphodiesterases (PDE4 and PDE5) (67, 68).

A crucial component involved in endothelium-dependent relaxation is NO. NO is synthesized by nitric oxide synthase (NOS) catalyzed by L-arginine and NADPH. in the presence of oxygen. However, in diabetic vessels, NO synthesis damages. Some studies suggested that it may be the inactivation of NO that causes NO deficiency due to an increase in free radicals, instead of the downregulation of activity or expression of eNOS (66, 69, 70).

### Aberrant Endothelial Cell Metabolism

In ECs, GLUT1 is the major isoform among diverse glucose transporters. In the view of the insulin-independent characteristic of GLUT1 and the "first response" location of ECs while in the condition of hyperglycemia, ECs are susceptible to high blood glucose. The expression of GLUT1 was previously thought of as unresponsive to hyperglycemia (71, 72). However, some studies indicated that the expression of GLUT-1 and the rate of glucose transport is downregulated, in response to extended exposure to high glucose concentrations (73). Thioredoxin-interacting protein (TXNIP), which is upregulated in response to low insulin and HG, reduces the level of GLUT1 mRNA (74, 75). These adaptive changes of GLUT1 expression may protect ECs against the damage of excessive glucose influx by reducing the uptake of glucose. On the other hand, the downregulation of GLUT1 would lead to decreased glucose efflux across the abluminal border, further reducing glucose expulsion to the cardiomyocyte. Hypoxic and inflammatory conditions occurring in diabetic cardiomyopathy promote endothelial cells to revascularizing tissue to address scarce oxygen and nutrients. During the period of vessel sprouting and migration, the glycolytic flux is enhanced (76). Enhanced glycolytic flux facilitates endothelial cells to migrate into the hypoxic area and proliferate in metabolism-impaired cardiac tissue.

In addition, insulin-independent glucose uptake of GLUT1 in endothelial cells results in an increase of glucose concentrations in endothelial cells. Excessive glucose in endothelial cells would be shunted into side branches of glycolysis, such as the pentose phosphate pathway, the hexosamine biosynthesis pathway, the polyol pathway, and the glycation pathway, leading to angiogenesis impairment, mitochondrial dysfunction, and protein kinase C activation (77). These alterations ultimately cause excess ROS and reactive nitrogen species production, and advanced glycation end product (AGE) synthesis, ECs, furthermore the impaired function of cardiomyocytes (71, 77–79). During diabetes, excessive AGEs cause ECs dysfunction by binding to the receptor for advanced glycation end products (RAGE), resulting in endothelial cell permeability increase (80), eNOS activity inhibition (81), and the coagulation system impairment (82, 83), NADPH oxidative (NOX) and NF-kB activating (84–86).

## PATHOPHYSIOLOGIC ALTERATION OF CARDIOMYOCYTES

Diabetic cardiomyopathy has two phenotypes: hyperglycemia, lipotoxicity, and insulin resistance are more important factors for DCM with HFpEF phenotype, autoimmunity is particularly related with DCM with HFrEF phenotype, and AGEs deposition and microvascular rarefaction seem to contribute to both phenotypes (87). Type 2 diabetes mellitus (T2DM) comprises 90–95% of all people with diabetes. Mounting evidence points to endothelial dysfunction of the coronary microvessels as a pivotal contributor to restrictive left ventricular (LV) remodeling and diastolic dysfunction, and subsequent heart failure with preserved ejection fraction (HFpEF), the most common form of HF in DM (88, 89). DM-related metabolic derangements favor the development of the HFpEF phenotype, which is more prevalent in obese type 2 DM patients.

Endothelial cells are the third most abundant cell type in the mammalian heart, accounting for about 12% of atrial and 8% of ventricular cell numbers respectively (90). The cardiac vasculature consists of abundant capillaries for the large requirement of cardiac muscle which is the most aerobic organ in the body.

The ratio of muscle to capillary in the heart is about 1:1, and the distance between a capillary endothelial cell and a neighboring close cardiomyocyte is approximately 1 μm, suggesting the intimate relationship and intercellular dependence between endothelial cells and cardiomyocytes. Endothelial cell is a critical component in cardiac tissue, which regulates cardiac constriction and blood flow which provides oxygen and nutrients for cardiomyocytes. Impaired NO bioactivity and oxidation of BH4 which functions as a NOS cofactor, have been well documented in diabetes and diabetes-associated complications. Recently, Carnicer et al. reported that increased concentration of BH4 in cardiomyocytes prevented and attenuated LV dysfunction through improving NO activity and further leading to enhance insulin-independent glucose uptake and utilization. Importantly, there is experimental evidence supporting that endocardial and endothelium regulate cardiomyocyte development and maturation by secreting endocrine.

It has been well documented that endothelial-derived cardio-active factors including nitric oxide (NO), endothelin-1, neuregulin-1(NRG-1), and Prostaglandin I2, regulate cardiomyocyte activity. Recently angiopoietins, angiotensin II, prostaglandins, connective tissue growth factor, fibroblast growth factor, vascular endothelial growth factor, Dickkopf-3, apelin, and endothelial miRNAs have been added into the panel of endothelial-derived cardio-active factors (91).. A large amount of endothelial-derived cardio-active factors and the specific modulation of each one emphasizes the precise regulation net of endothelial cells on cardiomyocytes. Cardiomyocyte aberrant metabolism and coronary microvascular dysfunction (CMD) are major pathogenesis of diabetic cardiomyopathy. Coronary microvascular dysfunction is characterized by endothelial cell damage, closely correlated with the incidence of heart failure in diabetic patients.

## Regulation on Cardiomyocyte Metabolism

Heparanase secreted from endothelial cells binds Heparan sulfate proteoglycans (HSPGs) which are located in the membrane of cardiomyocytes, triggering the release of LPL and VEGF from cardiomyocytes. LPL is released onto HSPG-binding sites on the plasma membrane, then captured by glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) and transferred across to the vascular lumen. In hyperglycemia conditions, above-mentioned process is intensified, resulting in a rapid increase of LPL and FA at the vascular lumen. This process provides increased FA for diabetic cardiomyocytes to generate ATP and maintain normal function as a compromised method to address acutely diabetic cardiomyopathy. Abnormal cardiac mitochondrial metabolism causes decreased ATP synthesis and a lower myocardial creatine phosphate/ATP ratio.

## Mitochondrial Defect

Mitochondrial dysfunction promotes the development of diabetic cardiomyopathy. Excessive FFA, exceeding the capacity of mitochondrial β-oxidation, promotes the accumulation of toxic metabolic intermediates and further mitochondrial dysfunction and cell death. This process is referred to as lipotoxicity and has been reported in numerous DM animal models. Moreover, Excessive mitochondrial fatty acid uptake and β-oxidation cause a large number of consumptions of ATP, resulting in mitochondrial dysfunction. During the advanced stage of diabetic cardiomyopathy, mitochondrial biogenesis and respiratory function is impaired severely owing to the abnormality of adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, leading to mitochondrial dysfunction (11). Intracellular $Ca^{2+}$ mishandling further results in mitochondrial respiratory dysfunction leading to cell death. Opening of the mitochondrial permeability transition pores induced by overload $Ca^{2+}$ results in cardiomyocyte autophagy and cardiac necrosis. Impaired mitochondrial function further exacerbates aberrant redox imbalance and leads to reduction of mitochondrial calcium concentration [mito-($Ca^{2+}$)]. Experimental evidence indicated increased mito-$Ca^{2+}$ uptake by pharmacological or genetic facilitation, improving cardiac systolic dysfunction. Reticulum-mitochondria $Ca^{2+}$ miscoupling disrupted mitochondrial bioenergetics and organelle $Ca^{2+}$ exchange, further damaging cell contraction (92). On the other hand, sustaining lipid accumulation and mitochondrial dysfunction induces ROS generation, which in turn leads to further impaired mitochondrial function and decreased FAO capacity, resulting in lipid accumulation, diastolic dysfunction and eventually heart failure.

## Intracellular $Ca^{2+}$ Mishandling and Endoplasmic Reticulum Stress

In DCM, impaired insulin signaling results in decreased glucose uptake into cardiac myocytes and activity of efflux pumps, leading to the increase of intracellular calcium and subsequently affecting the dynamic balance of the contraction-relaxation cycle of the cardiomyocytes (11). In diabetes patients, abnormal insulin metabolic signaling causes the production of reactive oxygen species (ROS) and subsequently induces oxidative damage. Oxidative damage impairs the function of RyR, ATPase pumps and exchange channels. The depression of insulin-stimulated coronary endothelial nitric oxide synthase (eNOS) activity and increased NO production, are the results of abnormal insulin metabolic signaling, leading to the decrease of $Ca^{2+}$ sensitization and reducing sarcoplasmic $Ca^{2+}$ uptake in cardiomyocytes.

Cardiac oxidative stress, lipotoxicity, inflammation, and the accumulation of misfolded proteins lead to cardiac endoplasmic reticulum (ER) dysfunction, induce the unfolded protein response (UPR) and promote ER stress. ER stress and the unfolded protein response led to decreased cellular protein synthesis and accumulation of damaged proteins, ultimately inducing cell apoptosis and autophagy. Increased apoptosis further endangers diabetic cardiomyopathy. It was reported that diabetic cardiac tissue showed cardiomyocyte apoptosis 85-fold increase than the counterpart in control hearts. ER stress also induces autophagy through a $Ca^{2+}$-dependent pathway. The activation of autophagic response is compensatory feedback to protect the cell from apoptosis (8). Autophagy function is impaired in diabetic cardiomyopathy (93). Yang et al. reported inflammation to induce UPR

dysfunction through iNOS-mediated S-nitrosylation of IRE1α, which causes impaired IRE1α activity, ER dysfunction, and prolonged ER stress in obesity (94). Zhou and colleagues showed S-nitroso-coenzyme A (SNO-CoA) delivers nitric oxide to the enzyme pyruvate kinase M2 (PKM2), which is modified with S-nitrosylation, thereby inhibiting glycolysis (95). Glucose turns to produce NADPH, a cofactor used by antioxidants, further generates the antioxidants to inhibit excessive oxidative stress (95). Growing evidence indicates the great influence of S-nitrosylation in UPR and cellular metabolism, suggesting the pivot role of nitric oxide signaling pathway and endothelial function in metabolic disorders. Abnormal endothelial function induces the imbalance between components derived from endothelial cells. ET-1 stimulates the entry of extracellular $Ca^{2+}$ and activates the intracellular PLC/IP3/$Ca^{2+}$ pathway *via* cGMP-dependent pathway (92).

## Accumulation of Advanced Glycation End Products

Abundant myocardial microvascular AGEs deposition in diabetic cardiomyopathy has been reported by mounting evidence. Further, the deposition of AGEs triggers vascular inflammation and quenches endothelially produced NO, resulting in decreased myocardial NO bioavailability and the predisposition to restrictive LV remodeling. AGEs deposition also occurs in the myocardial interstitium between cardiomyocytes. Interstitial AGEs deposition triggers ROS production in cardiomyocytes by NADPH oxidase, further leading to the activation of cell death pathways and eccentric LV remodeling (96). Hyperglycemia induces a protein glycation reaction of non-enzymatic glycosylation of lipids, lipoproteins, and amino acids, leading to the increase of AGEs. Elevated AGE deposition leads to increased connective tissue crosslinking, fibrosis, cardiac stiffness, thereby impairing diastolic relaxation. A RAGE antagonist ameliorated myocardial collagen deposition, fibrosis, stiffness, and diastolic dysfunction (97).

## ECM Deposition

Diabetic cardiomyopathy is characterized by cardiac interstitial and perivascular fibrosis, which contribute to the development of diastolic dysfunction correlated with a high prevalence of heart failure with preserved ejection fraction (HFpEF) in patients with diabetes. The pathogenesis of diastolic dysfunction is closely related to extracellular matrix (ECM) deposition. Sustaining hyperglycemia damaged endothelial cells, ultimately leading to cell loss and reduced coronary microcirculation blood flow (98). In addition, elevated factors including transforming growth factor-beta (TGF-β) and ET-1 both promote the expression of fibronectin, playing an important role in regulating ECM composition (99). In the diabetic hearts of rats, the expression of fibronectin and collagen IV are increased. Interestingly, Bosentan, an ET-1 receptor antagonist, exhibits prevention the increased expression of fibronectin and collagen IV in diabetic hearts of rats (66). Moreover, ET-mediated BM thickening and myocardial fibrosis resemble those in diabetic rats (100).

Sustained activation of inflammatory pathways ultimately would lead to excessive deposition of ECM. In the period of ECM accumulation, myofibroblasts are the crucial mediators (101). In normal conditions, myofibroblasts are usually removed by apoptosis at the end of the repair. In pathological situations, ECM deposition occurs under the uncontrolled activation of myofibroblasts. Endothelial cells transform to fibroblasts, through a process known as endothelial to mesenchymal transition (EndMT), which is triggered by high glucose concentrations, inflammation, and vascular complications (102, 103). It was thought that EndMT is the key link between inflammation and endothelial dysfunction in diabetic complications (104). In the process of EndMT, endothelial cells lose their typical cobblestone morphology, tight junctions, and typical markers, they acquire increased motility, secretion function of ECM proteins, and begin to express several mesenchymal markers (105). Several pathways are involved in EndMT regulation, including transforming growth factor-beta (TGF-β) signaling, Notch signaling, fibroblast growth factor/fibroblast growth factor receptor 1 (FGF/FGFR1) signaling pathway, Smad2/3-mediated pathways, Wnt-β/Catenin pathway, and pro-inflammatory signaling cascades (106, 107). Single-cell RNA sequencing data revealed that chronic exposure to high glucose and inflammation initiates ECM deposition, eventually perpetuating TGF-β signaling and EndMT (108).

ECM deposition and cardiac fibrosis increase the distance between capillaries and myocytes, leading to oxygen diffusion slowing down and exposing the myocardium to the risk of hypoxia. The expression of VEGF and its receptor have been shown downregulated in cardiac tissues of diabetic animals and humans (109). This change in the heart would further exacerbate hypoxic conditions and result in severe damage. Angiogenesis increases blood flow and reduces vasoconstriction, which may be an important therapeutic intervention for diabetic cardiac tissues. Moreover, VEGF gene therapy in animal studies has shown amelioration in diabetic cardiomyopathy (44). In the future, therapeutic administration of VEGF in clinical trials targeting diabetic cardiomyopathy would provide further evidence.

## Left Ventricle Remodeling and Cardiac Stiffness

Diabetes mellitus leads to Left ventricle (LV) diastolic dysfunction, restrictive LV remodeling, and HFpEF, by inducing a proinflammatory state accompanied by impaired coronary microvessel endothelial function. In HFpEF patients, coronary microvascular endothelial inflammation and cardiomyocytes exposed to altered paracrine endothelial signaling mainly contribute to concentric LV remodeling (87). The association between depressed NO bioavailability in coronary microvessels and impaired cardiac diastolic function is complex, involving multiple pathways. Troponin I and titin protein kinase G-dependent hypophosphorylation is responsible for delayed LV active relaxation and lower passive LV distensibility. Depressed coronary flow reserve (CFR) is also an important contributor. Microvascular endothelial dysfunction leads to impaired NO-dependent braking addressing pro-

hypertrophic stimuli, resulting in LV hypertrophy with enlarged cardiomyocytes (110). In addition, abnormal endothelial function is accompanied by increased adhesion molecules and local infiltration, inducing the transformation of myocardial fibroblasts into myofibroblasts and the consequent occurrence of reactive interstitial fibrosis. The HFpEF phenotype of DCM showed worse clinical manifestations, with more frequent hospitalizations and less exercise capacity, characterized by LV hypertrophy and higher LV stiffness. Previous studies reported that these hemodynamic features attributed to microvascular advanced glycation end-products (AGEs) deposition and stiff cardiomyocytes.

## THE REGULATION OF ENDOTHELIAL CELLS ON CARDIOMYOCYTES

Similar to the vascular endothelium regulating vascular smooth muscle contraction addressing to shear stress of flowing blood, the contractile state of cardiomyocytes is regulated by the endocardial endothelial cells and the endothelial cells of intramyocardial capillaries. Endothelial cells within the heart regulate myocardial contractile function by releasing bioactive substances, including nitric oxide, endothelin, prostanoids, adenylpurines, and other agents. Exosomes derived from ECs may regulate cardiac remolding in DCM and the development of DCM. The precise regulation of cardiomyocytes by endothelial exosomes needs further investigation, exosome cargoes delivering siRNAs and drugs is a potential strategy in diabetic cardiomyopathy (111). These agents mainly function by modifying the properties of cardiac myofilament, rather than altering cytosolic $Ca^{2+}$ transients, thus leading to different modulation effects on myocardial relaxation and diastolic tone (112). Yao et al (113). demonstrated in endothelial cells hyperglycemia induces a series of changes, which are all memorized by endothelial cells and not erased when switched to a low glucose condition, leading to perivascular fibrosis and cardiac dysfunction. Moreover, the production of NO, generation of ROS, and the mitochondrial oxygen consumption rate are found similar to the metabolic memory in endothelial cells. *In vivo*, the disruption of endothelial cell metabolic memory restores cardiovascular function by regulating corresponding signaling pathways in diabetic mice, whereas insulin alone does not improve cardiac function (113). This regulation on cardiac myocyte function by paracrine of endothelial cells is worth investigating both physiologically and in pathological states (112).

### ET1

Endothelin (ET)-1 is an amino acid peptide with vasoconstrictive effects, which is produced and released by endothelial cells. ET1 binds to ETB receptors on cardiac endothelial cells, triggering the release of NO and PGI2, rather than vasoconstriction. While binding to the ETA and ETB receptors on cardiomyocytes, Gq protein is activated which increases the release of sarcoplasmic reticulum calcium, resulting in the contraction of cardiomyocytes. Moreover, it has been reported the activation of ETA and ETB receptors on cardiomyocytes is associated with the development of cardiac myocyte hypertrophy. Importantly, Zhao et al. demonstrated that endogenous endothelin-1 is necessary to maintain normal cardiac function and cardiomyocyte survival in mice and ET1 upregulates NF-kappaB signaling to diminish TNF-related apoptosis (114).

### NO

Coronary microvessel-derived NO activates soluble guanylate cyclases, further promoting the production of cyclic guanosine monophosphate, which regulates the onset of ventricular relaxation and maintains normal pump function. Impaired NO availability results in low cGMP levels and decreased PKG activity, leading to hypo-phosphorylation of titin and an increase in cardiomyocyte stiffness (115).

### Prostaglandin I2

PGI2 is a physiologically active lipid compound synthesized from arachidonic acid. PGI2 has been reported to regulate cardiomyocyte morphology and survival. Treatment with PGI2 demonstrates improvement on cardiomyocyte hypertrophy induced by ET1, which may be mediated by activating IP prostanoid receptor and the cyclic adenosine monophosphate–dependent signaling in cardiomyocytes. Shinmura et al. reported PGI2 analog. Alleviates oxidative injury of myocyte through opening mitochondrial ATP-sensitive $K^+$ channels *via* the EP3 receptor (116). PGI2 analog showed protective effects on cardiac function through decreasing cardiomyocyte apoptosis.

### Neuregulin-1

NRG-1 derived from coronary endothelial cells, is a growth factor regulating the size, structure, and survival of cardiomyocytes. NRG-1 regulates downstream signal pathways by binding to the tyrosine kinase receptor erythroblastic leukemia viral oncogene homolog 4 on the cellular membranes of the myocytes. The activation of downstream pathways includes extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways, which both take part in cardiomyocyte hypertrophy. In addition, NRG-1 is documented to improve cardiomyocyte apoptosis through an Akt-dependent pathway. Moreover, NRG and recombinant NRG-1β protect cardiomyocytes against the cardiomyocyte death induced by anthracycline, β-adrenergic receptor, and $H_2O_2$. Activated NRG-1/ErbB4 signaling or ErbB4 expression causes cardiomyocyte proliferation and promotes myocardial regeneration after cardiac injury (117).

### Apelin

Endothelium in heart secrets Apelin to oppose the effects of the rennin-angiotensin system. The expression of Apelin increases in response to hypoxia in the ischemic heart, further promoting vasodilation *via* nitric-oxide dependent mechanism. Moreover, Apelin regulates cardiomyocytes *via* binding its GPCR receptor APJ (118).

## CLINICAL ASSESSMENT OF ENDOTHELIAL FUNCTION AND STRATEGY FOR ENDOTHELIAL PROTECTION

Endothelium function assessment includes evaluating the responsiveness of endothelial cells to the stimulus of vasodilation or vasoconstriction. With the ongoing understanding of endothelial dysfunction, the evaluation of endothelial function is considered as an important way of assessing disease development, with potential clinical applicability as a risk factor in cardiovascular complications, even in asymptomatic patients.

And invasive assessment is a direct method to evaluate vasodilation in response to endothelium-dependent and -independent stimuli. The occurrence and development of pathogenic events in cardiovascular complications are always accompanied by the alterations of circulating biomarkers, including inflammation-related cytokines (IL-1β and IL-6), cell surface induced adhesion molecules (E-selectin and VCAM-1), and systemic indicators of inflammation (high-sensitivity c-reactive protein), endothelin-1, NRG-1, and so on. The measurement of circulating biomarkers is a practicable way to assess endothelial function. Moreover, noninvasive evaluations including echocardiography, magnetic resonance imaging, and positron emission tomography also provide a potential risk of future cardiometabolic events, classified as low, medium, or high risk, which is conducive to the prevention and early treatment of diseases.

Furthermore, imaging technology is a relatively visualized way to evaluate the vascular injury and endothelial dysfunction (119). Currently, there are some methods to evaluate endothelial dysfunction, but endothelial dysfunction fails to meet diagnostic criteria or therapeutic targets. Furthermore, new noninvasive methods for assessing endothelial function from experimental studies to human patients are required, precise elevation of endothelial function would provide the possibility for endothelial dysfunction to become the standard of clinical diagnosis and treatment, further becoming a therapeutic target.

Currently available cardiovascular drugs, including angiotensin-converting enzyme (ACE) inhibitors, AT1 receptor antagonists, statins and many antioxidant and anti-inflammatory cardiovascular drugs, are therapeutic strategies. In addition, repair therapy with pharmacological agents, epigenetic approaches, endothelium-specific antioxidant and anti-inflammatory drug-delivery are promising candidates for the future. Moreover, exercise and dietary changes are highly potent "natural" alternatives (120).

## CONCLUSIONS AND FUTURE PERSPECTIVES

Patients with early diabetic cardiomyopathy are asymptomatic, which brings difficulties to early diagnosis and treatment (11). Abnormal myocardial metabolism including enhanced FA uptake and insulin signaling cascade has been present in these patients (121). With the progression of diabetic cardiomyopathy, there is a decrease in insulin secretion, leading to increased blood glucose levels and increased FA oxidation. However, excessive fatty acids are provided to cardiomyocytes, leading to the damage of cardiomyocytes (109). As diabetic cardiomyopathy is characterized by decreased capillary density, enhanced requirement for oxygen in FA mitochondrial metabolism presents a challenge to cardiac metabolism. Moreover, scarce oxygen delivery cannot match augmented FA uptake, leading to increased FA storage as TG in cardiomyocytes, which may induce cardiomyocyte death by lipotoxic damage (122).

Endothelial dysfunction has been regarded as a crucial link in diabetic cardiomyopathy. The role of endothelial dysfunction in the development of diabetic cardiomyopathy involves many aspects, including impaired NO activity, disturbed metabolism, aberrant ROS production, and oxidative stress. Direct evidence provided by scRNA-seq demonstrated the character of EC dysfunction *in vitro* from a single-cell transcriptome level. Substantial changes occur in ECs, including gene expression and phenotypic change, when exposed to chronic high glucose and inflammation. As endothelial dysfunction has been identified as a key factor in diabetic cardiomyopathy, it is urgent to evaluate the precise role of ECs in the development and progression of diabetic cardiomyopathy. On the one hand, endothelial dysfunction contributes to the development of diabetes and indirect cardiac function damage; on the other hand, endothelial dysfunction also leads to direct damage of heart. Moreover, ECs are heterogeneous across tissues, which remains further investigation. The single-cell transcriptome atlas of endothelial is important tools, which provide an access to explore the difference among ECs in diverse organs and the precise role of ECs from different vascular beds. Different phenotypes and percentages of ECs have been shown, LECs take part 3.7% in ECs in the heart, and LECs from hearts are major in LECs (123). In decades, a large amount of evidence has indicated the critical role of LECs in regulation of heart physiology and pathology (124–126). Lymphatic vessels not only regulate fluid drainage but also function as antigen-presenting. Currently, studies on the relationship between lymphatic vessels and diabetic cardiomyopathy are still lacking. Considering the important role of lymphatic vessels, the role of lymphatic vessels in DCM may become a new research direction in the future.

The crosstalk between ECs and cardiomyocytes is critical in cardiac development, and also plays a key role during the onset and progression of cardiac disease. ECs and cardiomyocytes are in close proximity and communicate through paracrine signals, as well as direct cell-to-cell contact (91). However, the specific transport molecules or interactions between ECs and cardiomyocytes during normal heart development and/or disease progression remain unclear. Further investigation is required to understand the crosstalk between endothelial-cardiomyocyte signals and cardiomyocyte-endothelial signals. Furthermore, it is necessary to establish more complex *in vitro* models to integrate ECs, cardiomyocytes, and other cell types into a three-dimensional structure, mimicking *in vivo* conditions.

The measurement of circulating biomarkers is another means to assess endothelial dysfunction. The occurrence and development of pathogenic events in cardiovascular complications are always accompanied by the alterations of circulating biomarkers, including inflammation-related cytokines (IL-1β and IL-6), cell surface induced adhesion molecules (E-selectin and VCAM-1), and systemic indicators of inflammation (high-sensitivity c-reactive protein), endothelin-1, NRG-1, von Willebrand factor, thrombospondin-1, endothelial miRNAs (miR-146a), and endothelial microparticles. Convenient and effective evaluation of endothelial function, promotes the widespread application of endothelial dysfunction evaluation in clinical practice, and timely monitoring of endothelial dysfunction and the progression of diabetic cardiomyopathy.

Current therapeutic strategies for diabetic cardiomyopathy mainly focus on decreasing blood glucose, ignorant of the continuous progress of cardiac remodeling and heart failure. Novel DM medications significantly improve the prognosis of diabetic cardiomyopathy, which may be one of the reasons for saving endothelial function. SGLT2i improves endothelial function by increasing NO production, endothelium-dependent vasodilation, reducing vascular inflammation and oxidative stress, and attenuating endothelial cell senescence. Importantly, the improvement of SGLT2i is associated with increased Flow-mediated dilation (FMD). Novel DM medications show better clinical outcomes, which suggests their multiple effects are critical. It is unclear about the further mechanism of SGLT2i, more studies are needed.

Taken together, with the deepening understanding of the role of endothelial dysfunction in diabetic cardiomyopathy and the increase of methods for assessing endothelial dysfunction, endothelial dysfunction may become a new target for the clinical treatment of DCM in the future. Clinical therapies need to consider the extent of endothelial damage. Considering the population with severe endothelial dysfunction are more prone to be influenced by endothelial function, it is urgent to identify this population and give clinical interventions for endothelial dysfunction.

## AUTHOR CONTRIBUTIONS

MW prepared the initial draft of the manuscript. SL supervised the study. YL and JL edited the manuscript for the intellectual content. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the National Natural Science Foundation of China to SL [81974032] and JL [82070396].

## ACKNOWLEDGMENTS

The National Natural Science Foundation of China is gratefully acknowledged.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 14 April 2022
doi: 10.3389/fendo.2022.864008

# The Predictive Value of Serum Calcium on Heart Rate Variability and Cardiac Function in Type 2 Diabetes Patients

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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *27 January 2022*
**Accepted:** *03 March 2022*
**Published:** *14 April 2022*

**Citation:**
*Wang J, Xu Z, Lv K, Ye Y, Luo D, Wan L, Zhou F, Yu A, Wang S, Liu J and Gao L (2022) The Predictive Value of Serum Calcium on Heart Rate Variability and Cardiac Function in Type 2 Diabetes Patients. Front. Endocrinol. 13:864008. doi: 10.3389/fendo.2022.864008*

**Background:** Cardiovascular autonomic neuropathy (CAN) is common in patients with type 2 diabetes mellitus (T2DM), mainly presented as decreased heart rate variability (HRV) which often leads to cardiac death. However, HRV measurement is not convenient in most clinics. Therefore, identifying high-risk patients for CAN in diabetes with easier measurements is crucial for the early intervention and prevention of catastrophic consequences.

**Methods:** In this cross-sectional study, 675 T2DM patients with normocalcemia were selected. Of these, they were divided into two groups: normal HRV group (n = 425, 100 ms≤ SDNN ≤180 ms) *vs*. declined HRV group (n = 250, SDNN <100 ms). All patients' clinical data were collected and the correlation of clinical variables with HRV were analyzed by correlation and logistic regression analysis. The area below the ROC curve was used to evaluate the predictive performance of serum calcium on HRV.

**Results:** In this study, declines in HRV were present in 37.0% of T2DM patients. Significant differences in albumin-adjusted serum calcium levels (CaA) (8.86 ± 0.27 *vs.* 9.13 ± 0.39 mg/dl, $p$ <0.001) and E/A (0.78 ± 0.22 *vs.* 0.83 ± 0.26, $p$ = 0.029) were observed between declined HRV and normal HRV groups. Bivariate linear correlation analysis showed that CaA and E/A were positively correlated with HRV parameters including SDNN ($p$ < 0.001), SDNN index ($p$ < 0.001), and Triangle index ($p$ < 0.05). The AUC in the ROC curve for the prediction of CaA on HRV was 0.730 (95% CI (0.750–0.815), $p$ < 0.001). The cutoff value of CaA was 8.87 mg/dl (sensitivity 0.644, specificity 0.814). The T2DM patients with CaA <8.87 mg/dl had significantly lower HRV parameters (SDNN, SDNN index, rMSSD, and triangle index) than those with CaA ≥8.87 mg/dl ($p$ < 0.01, respectively). Multivariate logistic regression analysis showed a significantly

increased risk of declined HRV in subjects with CaA level <8.87 mg/dl [OR (95% CI), 0.049 (0.024–0.099), $p < 0.001$].

**Conclusions:** Declined HRV is associated with a lower CaA level and worse cardiac function. The serum calcium level can be used for risk evaluation of declined HRV in T2DM patients even within the normocalcemic range.

**Keywords: calcium, heart rate variability, type 2 diabetes mellitus, SDNN, cardiovascular autonomic neuropathy**

## BACKGROUND

Type 2 diabetes mellitus (T2DM) has become one of the major chronic diseases in the world. Besides macrovascular and microvascular complications, T2DM is also commonly complicated with cardiovascular autonomic neuropathy (CAN) (1, 2). CAN, characterized by increased sympathetic activity and decreased parasympathetic activity, can be evaluated by heart rate variability (HRV) obtained from ambulatory electrocardiogram (3–5). Moreover, reduced HRV is considered to be a risk marker of cardiovascular death and also a leading cause of T2DM death (6, 7). Although HRV has been suggested to be highly associated with the onset and development of CAN in diabetes (8–10), more convenient measures to identify high-risk CAN patients in T2DM for early prevention are needed.

It is well known that serum calcium affects cardiac electrical activity and cardiac contraction. Evidence demonstrated that hypocalcemia led to an increase in ventricular action potential duration and prolongation of the QTc interval, which is associated with increased risk of arrhythmias (11–13). On the other hand, patients with hypercalcemia had a shortened QTc interval (12, 13). Moreover, a decrease in serum calcium level was associated with increased risk of sudden cardiac arrest (14) and an increase in serum calcium level was associated with an increased risk of T2DM (15–18). However, the relationship between serum calcium and CAN in diabetes is not certain. The aim of this study was to assess the predictive value of serum calcium (within normal range) on CAN in T2DM patients.

## METHODS

### Participants

675 T2DM patients who visited the Renmin Hospital of Wuhan University for evaluation or treatment from January 2019 to June 2021 with normocalcemia (serum calcium in the reference range of 8.46–10.10 mg/dl) were recruited. The exclusion criteria for patients were (1) dilated cardiomyopathy or hypertrophic cardiomyopathy; (2) pacemaker implantation; (3) degree II and above atrioventricular block, atrial fibrillation, sick sinus syndrome, and frequent premature contraction; (4) glomerular filtration rate <60 ml/min or urine albumin per gram urine creatinine (Alb/Cr) >300 mg/g; (5) alanine aminotransferase >120 U/l; (6) parathyroid disease or vitamin D-related disorders; (7) medication history including bisphosphonate, vitamin D, and diuretics which may influence calcium within the past 1 month; (8) serum calcium out of reference range (8.46–10.10 mg/dl); and (9) malignant tumor.

The patients' smoking history, drinking history, and current and past medical history were collected. T2DM was diagnosed according to the World Health Organization criteria (19). This study was approved by the ethical review board of Renmin Hospital of Wuhan University and complied with the Helsinki declaration.

### Biochemical Measurements

A 12-h overnight fasting venous blood sample was collected in all subjects. The serum electrolytes (calcium, sodium, potassium, chlorine, phosphate, and magnesium), uric acid, creatinine, albumin (ALB), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), high-sensitivity C-reactive protein (hs-CRP), NT-proBNP, and fasting plasma glucose (FPG) were measured by a biochemical autoanalyzer (Abbott C8000, Chicago, IL, USA). HbA1c and homocysteine (HCY) were measured by high-performance liquid chromatography (HPLC; Bio-Rad, Hercules, CA, USA).

Serum calcium $CV_w$ (within-subject coefficient of variation) = 2.1 mmol/l; $CV_b$ (between-subject coefficient of variation) =2.5 mmol/l; imprecision = 1.1%; bias = 0.8%; TEa (total allowable error, $p < 0.05$) = 2.5%. Albumin-adjusted serum calcium levels (CaA) (mg/dl) were derived from formula (serum total calcium concentration (mg/dl) + 0.8 × [4 − serum albumin concentration (g/dl)] (17) to adjust total calcium for hypoalbuminemia to permit approximation of the ionized calcium, which is physiologically active and under homeostatic control. The fasting triglyceride-glucose index (TyG), which was sensitive for recognizing insulin resistance, was calculated using the formula: Ln [TG (mg/dl) × FPG (mg/dl)/2] (20).

### Ambulatory Electrocardiogram Monitoring

24-hour ambulatory electrocardiogram (ECG) was recorded using a wearable 12-lead digital Holter device (JincoMed, Beijing, China) with a data acquisition speed of 4,000 Hz. Subjects were told to follow their daily routines but to avoid intense physical activities or shower. ECG data were processed using a professional Holter analysis system developed by JincoMed, including algorithms for QRS labeling, arrhythmia detection, artifact identification, and data correction, followed by manual review by Holter technicians in Renmin Hospital of Wuhan University. Both time- and frequency-domain HRV parameters were derived from ambulatory ECG data. Time-

domain HRV parameters quantify the variability of successive heartbeat interval, including standard deviation of normal R–R intervals (SDNN), mean standard deviation of normal R–R intervals for 5-min segments within 24 h (SDNN index), root mean square of successive RR interval differences (rMSSD), and the integral of the density of the RR interval differences (triangular index). Frequency-domain parameters estimate the distribution of power into different frequency bands, including high-frequency (HF, 0.15–0.40 Hz), low-frequency (LF, 0.04–0.15 Hz), and LF/HF ratio. 24-hour and hourly averages of time-domain HRV measures and hourly averages of frequency domain measures were obtained.

## Grouping

SDNN is widely used to evaluate autonomic function and is considered to be a sensitive indicator in T2DM patients (21). In this study, T2DM subjects were divided into two groups, declined HRV (SDNN <100 ms) and normal HRV (100 ms ≤ SDNN ≤180 ms), according to heart rate variability standards recommended by the European Society of Cardiology and the North American Society of Pacing and Electrophysiology Task Force (22).

## Echocardiography

Echocardiographic examinations were performed using commercially available ultrasound diagnostic instruments (GE Vingmed Ultrasound, Horten, Norway) in accordance with the guidelines issued by the American Society of Echocardiography (23). We measured cardiac structure indicators, which included left atrial diameter (LAD), aortic root dimension (AOD), left ventricular end diastolic dimension (LVDd), diastolic interventricular septum thickness (IVSd), main pulmonary artery diameter (MPAD), right ventricular end diastolic diameter (RVDd), right atrium diastolic transverse diameter (RADd), and diastolic left ventricular posterior wall thickness (LVPWd).

The Doppler spectrum of the mitral valve pulse was recorded in an apical four-chamber view. The peak velocity of the filling peak in the early diastolic period (E) and late diastolic period (A) and the early diastolic mitral annulus velocity (e′) were measured. Cardiac diastolic function was assessed by E/A ratio and E/e′ ratio. Three cardiac cycles were measured, and the average value was used. Diastolic dysfunction was defined as E/A ratio <1.0 or E/e′ ratio >15 (24). Left ventricular systolic function was assessed by left ventricular ejection fraction (LVEF).

## Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD), as well as frequencies and percentages for categorical variables. Continuous variables with non-normal distribution are represented by median (M) and quartile range (QR). Differences in normally distributed variables were determined by independent-sample T test or one-way ANOVA. Chi-square tests were applied for categorical variables. Bivariate linear correlation (Pearson correlation) analysis was carried out to evaluate the associations between CaA and HRV parameters.

Logistic regression analysis was performed using HRV as the dependent variable to analyze the association between CaA and HRV after adjusting for potential confounders. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for the relative risk of lower serum calcium level with declined HRV. The ability to predict declined HRV of CaA was evaluated using the area under the curve (AUC) in the receiver operating characteristic (ROC) curve. All statistical analysis were performed using SPSS version 22.0. All tests were two-sided, and $p$ <0.05 was considered statistically significant.

# RESULTS

## Clinical Characteristics

In this study, 675 T2DM subjects (399 men and 276 women) were included, with a mean age of 62.49 ± 11.36 years (from 23 to 95 years old). Declined HRV (SDNN <100 ms), hypertension, coronary heart disease, and carotid atherosclerosis were present in 250 (37.0%), 501 (74.2%), 370 (54.8%), and 132 (71.4%) patients, respectively. There were no individuals of SDNN >180 ms in the enrolled T2DM patients.

Significant differences in CaA (8.86 ± 0.27 *vs.* 9.13 ± 0.39 mg/dl, $p$ <0.001) were observed between declined HRV and normal HRV groups (**Table 1**). The patients with declined HRV were older and had higher levels of HbA1c, NT-proBNP, and hs-CRP, as well as

**TABLE 1** | Baseline characteristics of subjects categorized by SDNN.

| Characteristics | HRV | | *p* |
|---|---|---|---|
| | SDNN <100 ms (n = 250) | 100 ms≤ SDNN ≤180 ms (n = 425) | |
| Age (years) | 64.36 ± 11.05 | 61.39 ± 11.41 | 0.001 |
| Male, n (%) | 140 (56.0) | 259 (60.9) | 0.207 |
| Smoking, n (%) | 73 (29.2) | 97 (22.8) | 0.065 |
| Drinking, n (%) | 60 (24.0) | 84 (19.8) | 0.195 |
| Hypertension, n (%) | 178 (71.2) | 323 (76.0) | 0.148 |
| Coronary heart disease, n (%) | 145 (58.0) | 225 (52.9) | 0.182 |
| Carotid atherosclerosis, n (%) | 56 (75.7) | 76 (68.5) | 0.288 |
| FPG [M(QR), mmol/L] | 8.09 (3.59) | 7.52 (3.01) | 0.073 |
| TyG index | 9.17 ± 0.74 | 9.07 ± 0.71 | 0.079 |
| HbA1c (%) | 7.99 ± 1.42 | 7.58 ± 1.50 | 0.001 |
| Uric acid (μmol/L) | 343 ± 99 | 349 ± 96 | 0.483 |
| Creatinine (μmol/L) | 65.9 ± 16.7 | 64.8 ± 15.5 | 0.400 |
| ALB (g/L) | 40.89 ± 3.69 | 41.05 ± 3.68 | 0.581 |
| TG (mmol/L) | 1.87 ± 1.22 | 1.80 ± 1.17 | 0.460 |
| TC (mmol/L) | 4.03 ± 1.04 | 4.20 ± 1.14 | 0.065 |
| HDL-C (mmol/L) | 0.96 ± 0.26 | 1.03 ± 0.27 | 0.002 |
| LDL-C (mmol/L) | 2.31 ± 0.92 | 2.40 ± 0.88 | 0.030 |
| K [M(QR),mg/dl] | 15.56 (2.02) | 15.48 (1.70) | 0.560 |
| Na (mg/dl) | 324.83 ± 8.11 | 325.59 ± 7.66 | 0.223 |
| Cl (mg/dl) | 375.26 ± 10.78 | 376.44 ± 10.78 | 0.192 |
| P (mg/dl) | 3.58 ± 0.58 | 3.56 ± 0.60 | 0.708 |
| Mg (mg/dl) | 1.93 ± 0.21 | 1.95 ± 0.20 | 0.229 |
| Ca (mg/dl) | 9.00 ± 0.64 | 9.30 ± 0.67 | <0.001 |
| CaA (mg/dl) | 8.86 ± 0.27 | 9.13 ± 0.39 | <0.001 |
| Ln(NT-proBNP, pg/ml) | 4.66 ± 1.08 | 4.38 ± 0.90 | 0.001 |
| hs-CRP [M(QR), mg/L] | 2.15 (4.42) | 0.88 (1.85) | <0.001 |
| HCY (μmol/L) | 14.97 ± 4.82 | 15.09 ± 4.46 | 0.866 |

lower levels of HDL-C and LDL-C than those with normal HRV ($p < 0.05$, respectively). There was no significant difference in prevalence of smoking or drinking history, hypertension, coronary heart disease, and carotid atherosclerosis between the two groups (**Table 1**).

## Echocardiography and HRV

We analyzed echocardiographic indicators between the declined HRV and normal HRV groups. The data showed that echocardiographic cardiac structure indicator LAD (35.40 ± 4.56 mm *vs.* 34.47 ± 4.56 mm, $p = 0.015$) was significantly increased, and cardiac function index E/A (0.78 ± 0.22 *vs.* 0.83 ± 0.26, $p = 0.029$) was significantly decreased in the declined HRV group (**Table 2**). However, LVEF, E/e′, and most cardiac structure indicators did not differ between groups.

Furthermore, bivariate linear correlation analysis showed that the E/A level was significantly and positively correlated with HRV parameters including SDNN ($r = 0.148$, $p < 0.001$), SDNN index ($r = 0.164$, $p < 0.001$), and Triangle index ($r = 0.088$, $p = 0.030$), but not with rMSSD and LF/HF (**Table 3**).

## Serum Calcium and HRV

To determine the variables associated with SDNN, logistic regression analysis was developed to include CaA, age, HbA1c, HDL-C, LDL-C, Ln(NT-proBNP), and hs-CRP. Declined HRV (SDNN <100 ms) was significantly associated with CaA [OR (95% CI), 0.056 (0.028–0.110), $p < 0.001$], age [OR (95% CI), 1.018 (1.001–1.035), $p = 0.037$], HbA1c [OR (95% CI), 1.253 (1.109–1.416), $p < 0.001$], Ln(NT-proBNP) [OR (95% CI), 1.230 (1.017–1.488), $p = 0.033$], and hs-CRP [OR (95% CI), 1.031 (1.013–1.049), $p = 0.001$] (**Table 4**). Bivariate linear correlation analysis showed that the CaA level was significantly and positively correlated with HRV parameters including SDNN ($r = 0.413$, $p < 0.001$), SDNN index ($r = 0.209$, $p < 0.001$), rMSSD ($r = 0.121$, $p = 0.002$), and Triangle index ($r = 0.218$, $p < 0.001$), but not with the LF/HF (**Table 3**).

To evaluate the predictive performance of CaA on HRV, the AUC in the ROC curve was calculated, which was 0.730 [95% CI (0.750–0.815), $p < 0.001$]. The cutoff value of CaA was 8.87 mg/dl (sensitivity 0.644, specificity 0.814) (**Figure 1**). We divided these patients into two groups (CaA level <8.87 mg/dl and ≥8.87 mg/dl) based on the cutoff value. HRV parameters, including SDNN ($p < 0.001$), SDNN index ($p < 0.001$), rMSSD ($p = 0.006$), and Triangle index ($p < 0.001$), were significantly decreased in the group of lower calcium level (**Table 5**). However, LF/HF did not differ between the two groups.

The multivariate logistic regression analysis shows the ORs (95% CI) for declined HRV according to the two groups of CaA

**TABLE 2** | Echocardiographic examinations of subjects categorized by SDNN.

| Echocardiographic examinations | HRV | | *p* |
|---|---|---|---|
| | SDNN <100 ms (n = 250) | 100 ms≤ SDNN ≤180 ms (n = 425) | |
| AOD (mm) | 32.89 ± 3.57 | 32.56 ± 3.85 | 0.301 |
| LAD (mm) | 35.40 ± 4.56 | 34.47 ± 4.56 | 0.015 |
| LVDd (mm) | 44.69 ± 4.95 | 44.73 ± 3.63 | 0.354 |
| IVSd (mm) | 9.89 ± 1.45 | 9.74 ± 1.21 | 0.160 |
| MPAD (mm) | 20.90 ± 2.33 | 20.89 ± 2.26 | 0.960 |
| RADd (mm) | 33.56 ± 3.40 | 33.95 ± 3.70 | 0.202 |
| RVDd (mm) | 20.30 ± 2.09 | 20.43 ± 2.16 | 0.471 |
| LVPWd (mm) | 9.72 ± 1.14 | 9.64 ± 1.11 | 0.403 |
| LVEF (%) | 58.56 ± 3.99 | 59.25 ± 2.42 | 0.072 |
| E/e' | 11.10 ± 3.45 | 10.80 ± 2.69 | 0.528 |
| E/A | 0.78 ± 0.22 | 0.83 ± 0.26 | 0.029 |

**TABLE 3** | Correlation coefficients between CaA, E/A, and HRV variables.

| HRV | CaA | | E/A | |
|---|---|---|---|---|
| | *r* | *p* | *r* | *p* |
| SDNN | 0.413 | <0.001 | 0.148 | <0.001 |
| SDNN Index | 0.209 | <0.001 | 0.164 | <0.001 |
| rMSSD | 0.121 | 0.002 | 0.028 | 0.493 |
| Triangle index | 0.218 | <0.001 | 0.088 | 0.030 |
| LF/HF | -0.018 | 0.645 | 0.033 | 0.415 |

**TABLE 4** | Logistic regression analysis of risk factors for SDNN.

| | B | S.E. | Wald | df | p value | OR | 95% CI |
|---|---|---|---|---|---|---|---|
| Age | 0.018 | 0.009 | 4.350 | 1 | 0.037 | 1.018 | 1.001–1.035 |
| HbA1c | 0.226 | 0.062 | 13.076 | 1 | <0.001 | 1.253 | 1.109–1.416 |
| HDL-C | -0.396 | 0.366 | 1.174 | 1 | 0.279 | 0.673 | 0.328–1.378 |
| LDL-C | -0.154 | 0.111 | 1.920 | 1 | 0.166 | 0.858 | 0.690–1.066 |
| CaA | -2.884 | 0.346 | 69.532 | 1 | <0.001 | 0.056 | 0.028–0.110 |
| Ln(NT-proBNP) | 0.207 | 0.097 | 4.538 | 1 | 0.033 | 1.230 | 1.017–1.488 |
| hs-CRP | 0.031 | 0.009 | 12.021 | 1 | 0.001 | 1.031 | 1.013–1.049 |

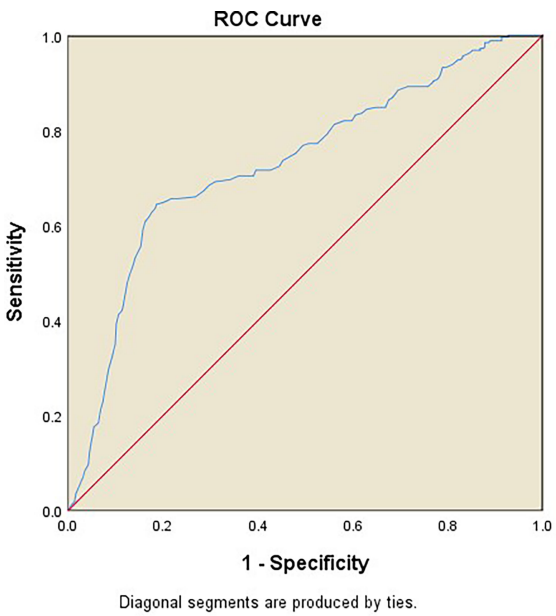


**FIGURE 1** | ROC curves of CaA on prediction of HRV. The AUC in the ROC curve was 0.730 [95% CI (0.750–0.815), $p < 0.001$]. The cutoff value of CaA was 8.87 mg/dl (sensitivity 0.644, specificity 0.814).

**TABLE 5** | Relationship of serum calcium with HRV and cardiac function in T2DM patients.

| | CaA (mg/dL) | | *p* |
|---|---|---|---|
| | <8.87 (n = 242) | ≥8.87 (n = 433) | |
| SDNN [M(QR), ms] | 92 (34) | 120 (37) | <0.001 |
| SDNN index [M(QR)] | 38 (17) | 46 (22) | <0.001 |
| rMSSD [M(QR), ms] | 21 (14) | 23 (15) | 0.006 |
| Triangle index [M(QR)] | 23 (11) | 26 (12) | <0.001 |
| LF/HF | 1.81 ± 1.37 | 1.92 ± 1.21 | 0.506 |
| AOD (mm) | 33.07 ± 3.65 | 32.46 ± 3.79 | 0.054 |
| LAD (mm) | 35.10 ± 4.51 | 34.66 ± 4.62 | 0.250 |
| LVDd (mm) | 45.22 ± 4.04 | 44.44 ± 4.21 | 0.026 |
| IVSd (mm) | 9.77 ± 1.22 | 9.81 ± 1.36 | 0.721 |
| MPAD (mm) | 20.89 ± 2.52 | 20.89 ± 2.15 | 0.663 |
| RADd (mm) | 34.19 ± 3.23 | 33.60 ± 3.77 | 0.050 |
| RVDd (mm) | 20.22 ± 1.97 | 20.47 ± 2.22 | 0.155 |
| LVPWd (mm) | 9.66 ± 1.10 | 9.68 ± 1.13 | 0.768 |
| LVEF (%) | 58.67 ± 3.67 | 59.17 ± 2.73 | 0.373 |
| E/e′ | 10.94 ± 2.90 | 10.90 ± 3.07 | 0.940 |
| E/A | 0.79 ± 0.24 | 0.82 ± 0.26 | 0.251 |
| Ln(NT-proBNP, pg/ml) | 4.57 ± 0.95 | 4.44 ± 0.10 | 0.113 |
| FPG (mmol/L) | 7.77 ± 3.38 | 7.71 ± 3.17 | 0.827 |
| HbA1c (%) | 7.86 ± 1.36 | 7.66 ± 1.54 | 0.093 |
| TyG index | 9.06 ± 0.71 | 9.13 ± 0.74 | 0.195 |

levels. In contrast to subjects with CaA level ≥8.87 mg/dl, there was a significantly increased risk of declined HRV in subjects with CaA level <8.87 mg/dl [OR (95% CI), 0.049 (0.024–0.099), $p < 0.001$] after adjusting for possible confounding factors including creatinine, serum phosphate, age, gender, smoking, drinking, HbA1c, Ln(NT-proBNP), hs-CRP, TyG index, dyslipidemia, hypertension, coronary heart disease, carotid atherosclerosis, and the use of hypertension medication.

## Serum Calcium and Cardiac Function

We analyzed echocardiographic indicators and cardiac function between the two groups (CaA level <8.87 and ≥8.87 mg/dl). LVDd (45.22 ± 4.04 mm *vs.* 44.44 ± 4.21 mm, $p = 0.026$) was significantly increased in the group of lower calcium level. However, cardiac function indexes (LVEF, E/e′, E/A, and NT-proBNP) and most cardiac structure indicators (AOD, LAD, IVSDd, MPAD, RADd, RVDd, and LVPWd) did not differ between the two groups (**Table 5**). There were no significant differences in FBG, HbA1c, and TyG index between the two groups.

# DISCUSSION

Previous studies confirmed that the independent predictors of CAN in T2DM were age, HbA1c, BMI, and triglycerides (8, 25, 26). However, these features as determinants of the autonomic dysfunction are insufficient. In this study, we showed that T2DM patients with CAN (declined HRV) had a significantly lower CaA level, and poorer cardiac function, than those with normal HRV. On the other hand, T2DM patients with a lower CaA level had significantly decreased HRV parameters, but cardiac function did not differ between the two groups. Therefore, for the first time we showed that CaA level, even within the normal range, was independently associated with declined HRV in T2DM after adjustment for other confounding factors, which suggests that decreased serum calcium level may be an effective predictor of CAN in T2DM.

Early studies found that decreased HRV was associated with an increased risk of cardiovascular death (27). HRV is now considered to be a reliable method to evaluate CAN in diabetes (8, 25, 26). A large proportion of diabetic patients have autonomic dysfunction, which is an independent predictor of vascular dysfunction. The prevalence of CAN in diabetic patients varies from 7.7% in newly diagnosed diabetic patients to 90% in patients planning pancreas transplant (28). In our study, 37.0% of the T2DM patients were accompanied by declined HRV, which is less than the incidence rate of CAN in the DCCT/EDIC (25). The reason of this may be the different exclusion criteria and different diabetes population between these two studies.

Among HRV parameters, SDNN reflects the whole 24-h HRV and can be used to be a marker of overall autonomic modulation (29). Our study showed that age was negatively correlated with SDNN, which matches previous studies that age was an important prognostic factor for HRV (30). Hs-CRP was also a risk factor for declined HRV in T2DM, indicating that inflammation may be involved in the initiation and progression of CAN in T2DM (31, 32). Our data also indicated that HbA1c was the important risk factor of declined HRV, indicating that glycemic control may be an important means of therapy for reducing CAN risk in diabetes. From the ACCORD Study (33), it showed that CAN patients with decreased SDNN values had an increased risk of mortality during follow-up. However, the increased mortality of T2DM was independent of glycemia control. T2DM is associated with abnormal cardiovascular autonomic function (34). Our study showed that T2DM patients with declined HRV had a significantly poorer cardiac function than those with normal HRV, suggesting that declined HRV in T2DM increases the risk of heart failure. We further analyze the linear relationship between E/A and HRV indicators. It showed that E/A was significantly and positively correlated with time-domain HRV indices including SDNN, SDNN index, and triangle index, which suggested that declined HRV in T2DM was associated with cardiac diastolic dysfunction.

In our study, only T2DM patients with normocalcemia were included. We showed that the cutoff value of CaA to predict risk of declined HRV was 8.87 mg/dl. T2DM patients with CaA level <8.87 mg/dl had a significant declined HRV compared to patients with CaA level ≥8.87 mg/dl. It indicates that the fluctuation of serum calcium in the normal range still affects HRV. However, cardiac function was not significantly different between the two groups. A similar study showed that albumin-adjusted serum calcium was positively associated with an increased risk of left ventricular hypertrophy in T2DM patients (35). The different results may be due to different inclusion and exclusion criteria.

Our study provided not only a convenient alternative method to assess early CAN patients in diabetes but also a basis for

selection of suitable anti-glycemic agents. For example, sodium-glucose cotransporter 2 (SGLT2) inhibitors are a class of glucose-lowering agents for the treatment of type 2 diabetes by selectively inhibiting renal glucose reabsorption and increasing urinary glucose excretion. Clinical studies have found that SGLT2 inhibitors improve heart failure and cardiovascular outcomes in patients with T2DM (36). Evidence also showed that SGLT2 inhibitors may slightly increase calcium levels by reducing urinary calcium excretion (37, 38). This evidence suggests that SGLT2 inhibitors may be beneficiary for T2DM patients with CAN, but further studies are needed.

Although there have been no studies that focused on the direct mechanism responsible for the effects of calcium on HRV and cardiac function in diabetic patients, we infer possible mechanisms including reduced vascular reactiveness, increased intima-media thickness, and endothelium dysfunction (39). Besides, calcium could be involved in the sympathetic nervous activation *via* insulin resistance and its associated compensatory hyperinsulinemia, contributing to diminished HRV in diabetic patients, resulting in cardiac function impairment such as left ventricular hypertrophy or diastolic dysfunction (40). However, our study did not show a correlation between serum calcium levels and glycemic control (data not shown).

Several limitations of this study should be noted. First, the enrolled patients with diabetes are relatively mild owing to strict exclusion criteria. The conclusions of this study may be inconsistent with other studies which included more severe patients. Second, most of the patients are unavailable for serum parathyroid hormone and vitamin D levels. Although no hypercalcemia or hypocalcemia individuals were excluded, it is impossible to completely exclude potential confounding factors. Third, these results were based on serum calcium at admission, which may not be representative for the patients.

## CONCLUSIONS

Declined HRV is associated with a lower CaA level and worse cardiac function in patients with T2DM. A lower serum calcium level in the normal range was independently associated with decreased HRV in T2DM patients. Routinely monitoring calcium level may help to identify/screen high-risk CAN patients in T2DM and facilitate its early intervention.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical review board of Renmin Hospital of Wuhan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

JW concepted and designed the research. JW, KL, YY, DL, LW, FZ, AY, SW, and JL contributed to the acquisition of data. JW, ZX, and LG contributed to the analysis, interpretation of data, and drafting of the article. ZX and LG revised the manuscript critically for important intellectual content. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was supported by the National Natural Science Foundation of China (Project #81571376 to LG), Diabetes Study Fund from the Chinese Medical Association (Project # 13060906481 to LG), the Fundamental Research Funds for the Central Universities (Project # 2042020kf1079 to LG), and Hubei Province Natural Science Foundation (Project # 2021CFB448 to ZX).

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ORIGINAL RESEARCH
published: 20 April 2022
doi: 10.3389/fendo.2022.865863

# Residual Risk of Coronary Atherosclerotic Heart Disease and Severity of Coronary Atherosclerosis Assessed by ApoB and LDL-C in Participants With Statin Treatment: A Retrospective Cohort Study

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**Background:** Low-density lipoprotein cholesterol (LDL-C) is the primary target of lipid-lowering therapy on the management of hypercholesterolemia in the United States and European guidelines, while apolipoprotein B (apoB) is the secondary target. The objective was to determine if elevated levels of apoB is superior to LDL-C in assessing residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in participants with statin treatment.

**Methods:** This study included 131 participants with statin treatment. The generalized linear model and relative risk regression (generalized linear Poisson model with robust error variance) were used to analyze the association of the levels of apoB and LDL-C with the severity of coronary atherosclerosis and residual risk of coronary atherosclerotic heart disease.

**Results:** Categorizing apoB and LDL-C based on tertiles, higher levels of apoB were significantly associated with the severity of coronary atherosclerosis ($P_{trend}$ = 0.012), whereas no such associations were found for elevated levels of LDL-C ($P_{trend}$ = 0.585). After multivariate adjustment, higher levels of apoB were significantly associated with residual risk of coronary atherosclerotic heart disease. When compared with low-level apoB (≤0.66 g/L), the multivariate adjusted RR and 95% CI of intermediate-level apoB (0.67–0.89 g/L) and high-level apoB (≥0.90 g/L) were 1.16 (1.01, 1.33) and 1.31 (1.08, 1.60), respectively ($P_{trend}$ = 0.011). There was a 45% increased residual risk of coronary atherosclerotic heart disease per unit increment in natural log-transformed apoB ($P_{trend}$ <0.05). However, higher levels of LDL-C were not significantly associated with residual risk of coronary atherosclerotic heart disease. When compared with low-level LDL-C (≤1.56 mmol/L), the multivariate adjusted RR and 95% CI of intermediate-level LDL-C (1.57–2.30 mmol/L) and high-level LDL-C (≥2.31 mmol/L) were 0.99 (0.84, 1.15) and 1.10 (0.86, 1.42), respectively ($P_{trend}$ =

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**Edited by:**
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*Huazhong University of Science and Technology, China*

**Reviewed by:**
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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *30 January 2022*
**Accepted:** *08 March 2022*
**Published:** *20 April 2022*

**Citation:**
*Yao T, Lu W, Ke J, Zhang H, Zhao X, Song B, Liu T, Ke Q and Liu C (2022) Residual Risk of Coronary Atherosclerotic Heart Disease and Severity of Coronary Atherosclerosis Assessed by ApoB and LDL-C in Participants With Statin Treatment: A Retrospective Cohort Study. Front. Endocrinol. 13:865863. doi: 10.3389/fendo.2022.865863*

0.437). Similar results were observed in the stratified analyses and sensitivity analyses. No significant interactions were detected for both apoB and LDL-C (all $P_{interaction}$ >0.05).

**Conclusions:** Elevated apoB are superior in assessing the residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in participants with statin treatment.

**Keywords: statin, ApoB, LDL-C, coronary atherosclerotic heart disease, residual risk, coronary atherosclerosis, syntax scores**

## INTRODUCTION

With the improvement of living standards and the accelerated aging of the population in China, the incidence of coronary atherosclerotic heart disease has increased dramatically. Dyslipidemia as an independent risk factor for coronary artery disease has drawn widespread attention. A previous study predicted that elevated serum cholesterol levels will lead to an additional 9.2 million coronary atherosclerotic heart disease in China between 2010 and 2030 (1). Studies showed that lowering levels of serum low-density lipoprotein cholesterol (LDL-C) can significantly reduce the risk of coronary artery disease (2), and LDL-C is the primary target of lipid-lowering therapy in the United States and European guidelines in hypercholesterolemia management (3, 4). Guidelines focus on lowering LDL-C concentration to reduce atherosclerotic cardiovascular disease (ASCVD) risk. However, numerous clinical trials of statin and non-statin therapy showed persistent residual ASCVD risk despite aggressive LDL-C lowering (5–7), suggesting other atherosclerotic lipoproteins need to be considered. Identifying residual risk of coronary atherosclerotic heart disease in populations with low levels of LDL-C is essential for the prevention of ASCVD.

At present, more factors are increasingly considered to be associated with residual risk of ASCVD after LDL-C lowering. For example, Triglyceride-rich lipoproteins (TGRLs) and lipoprotein(a) have been shown to be associated with residual risk in patients treated to low concentrations of LDL-C (8, 9). Apolipoprotein B (ApoB) is the main protein constituent of atherogenic lipoproteins, namely, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), lipoprotein(a) (Lp(a)), and LDL-C, and each atherogenic lipoprotein particle contains one molecule of apoB, so the concentration of apoB is proportional to the total number of atherogenic lipoprotein particles (10, 11). The accumulation of apoB under the endothelium was confirmed to be a key initiating event of atherosclerosis (12, 13). A study found that elevated levels of apoB, but not LDL-C, were associated with an increased risk of myocardial infarction in participants with statin treatment (14). However, this study only included people of white ancestry. To explore whether apoB is superior to LDL-C in assessing residual risk of coronary atherosclerotic heart disease in the Chinese population with statin treatment, and to further investigate whether the concentrations of apoB and LDL-C are associated with the severity of coronary atherosclerosis in participants with statin treatment, we conducted a retrospective cohort study. We calculated the syntax scores according to the invasive coronary angiography, and the syntax scores was used to evaluate the severity of coronary atherosclerosis.

## MATERIALS AND METHODS

### Study Population

Our study included 1,280 statin-treated participants with measurements for apoB and LDL-C at baseline. We excluded participants with acute coronary syndrome, hyperthyroidism, tumors, abnormal liver function or surgery of PCI and CABG at baseline, those with missing lipid data at baseline, and persons with missing results of coronary angiography during follow-up (2014 to 2018). The final cohort for analysis included 131 participants (**Figure 1**).

### Measurements of Lipid

We measured the concentration of total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol using colorimetric assays. When triglycerides were below 4 mmol/l, Friedewald equation was used to estimate the LDL-C concentration (LDL cholesterol = total cholesterol − HDL cholesterol − triglycerides/2.2 in mmol/l), and otherwise it was measured directly. ApoB, apolipoprotein A1 (apoA1), and lipoprotein(a) were measured using turbidimetric assays.

### Assessment of Covariates

Information on age, sex, smoking status, disease status, and medication use were collected from the hospital inpatient system. Hypertension was defined as taking antihypertensive medication, systolic BP ≥140 mmHg, or diastolic BP ≥90 mmHg (15). Diabetes was defined as taking antidiabetes drugs, injecting insulin, fasting blood glucose ≥7.0 mmol/L, 2-h plasma glucose (PG) ≥200 mg/dl (11.1 mmol/L) during OGTT or glycated hemoglobin (HbA1c) ≥6.5% (16). Venous blood samples were collected in the morning after an overnight fast and were processed within 2 h. Invasive coronary angiography was performed by experienced interventional doctor blinded to the data of subjects.

### Outcomes

The primary outcome of the study was new-onset coronary atherosclerotic heart disease between January 1, 2014, and December 31, 2018. In patients receiving lipid-lowering

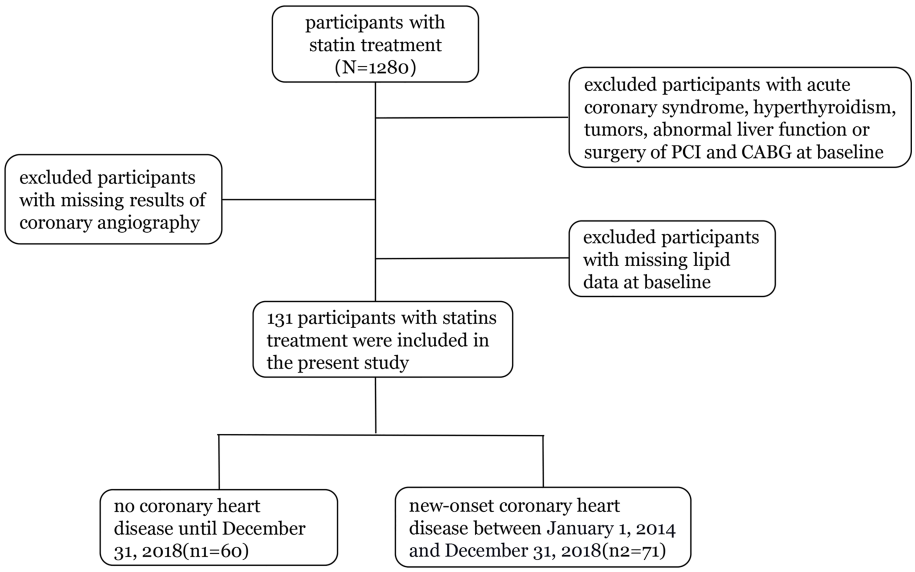


**FIGURE 1** | Flow diagram of participants enrolled.

therapy, we assessed residual risk of coronary atherosclerotic heart disease based on new-onset coronary atherosclerotic heart disease. Invasive coronary angiography showed more than 50% luminal diameter narrowing in at least one major coronary artery by two experienced interventional cardiologists, and coronary atherosclerotic heart disease can be diagnosed. The secondary outcome was the severity of coronary atherosclerosis, also based on invasive coronary angiography. The syntax scores were used to quantitatively evaluate the severity of coronary atherosclerosis (17). The syntax scores can comprehensively and quantitatively evaluate the complex anatomical characteristics of the coronary arteries, namely, the location, severity, bifurcation, and calcification of the coronary arteries (18, 19). According to the invasive coronary angiography, we calculated the syntax scores on the website (www.syntaxscore.com), and another researcher calculated the score again a week later. The results of the two calculations were basically the same.

## Statistical Analysis

Categorizing concentrations of apoB and LDL-C were based on tertiles. The baseline characteristics of the study population were described according to apoB and LDL-C concentrations. The differences among groups were analyzed using the Chi-squared test for categorical variables, expressed as absolute frequency (%). For continuous variables, one-way analysis of variance or Kruskal–Wallis test were used to analyze the differences among groups, expressed as mean ± standard deviation (SD).

The generalized linear model was also used to detect the association of the levels of apoB and LDL-C with the severity of coronary atherosclerosis (evaluated by the syntax scores), covariates, namely, age, sex, diabetes, hypertension, smoking status, total cholesterol, triglycerides, apolipoprotein A1, and lipoprotein(a) were adjusted. There was no multicollinearity (defined as a correlation r ≥0.8 between variables) between apoB or LDL-C and adjusted covariates. Relative risk regression (generalized linear Poisson model with robust error variance) was used to estimate relative risk (RR) and 95% CIs for the association of the level of apoB and LDL-C with the residual risk of coronary atherosclerotic heart disease. According to tertiles, the levels of apoB and LDL-C were categorized into three groups: low-level apoB (≤0.66 g/L), moderate-level apoB (0.67–0.89 g/L), and high-level apoB (≥0.90 g/L); low-level LDL-C (≤1.56 mmol/L), moderate-level LDL-C (1.57-2.30mmol/L), and high-level LDL-C (≥2.31 mmol/L). The levels of apoB and LDL-C were also analyzed as a continuous variable after natural log transformation. In the multivariate models, we did not adjust any variables in model 1. In model 2, we adjusted for age (years), sex (female or male), smoking status (never smoker or smoker), diabetes (no or yes), and hypertention (no or yes). In model 3, we further adjusted for total cholesterol, triglycerides, apolipoprotein A1, and lipoprotein (a). Testing for linear trends was by assigning a median value to each category as a continuous variable.

Stratified analyses were also conducted by age (<65 or ≥65 years), sex (female or male), smoking status (never smoker or smoker), diabetes (no or yes), and hypertention (no or yes). The *P*-values for the product terms between level of apoB or LDL-C and stratification variables were used to estimate the significance of interactions. In order to further test the robustness of the research findings, we conducted several sensitivity analyses. First, logistic regression was used to estimate odds ratios (OR) and 95% CIs for the association of levels of apoB or LDL-C with the residual risk of coronary atherosclerotic heart disease. Second, the concentration of lipids may be affected by renal function; renal function assessed by estimated glomerular filtration rate (calculated by using the Chronic Kidney Disease Epidemiology Collaboration formula) was further adjusted (20). Third, we

analyzed whether the association would change if only individuals with higher syntax scores were selected. All of the analyses were conducted using SPSS (version 26.0). Two-sided $P$ <0.05 was considered statistically significant.

# RESULTS

## Baseline Characteristics of Participants With Statins Treatment

A total of 131 participants (mean age, 64.98 years; 57.25% male) with statins treatment were included in the present study. The mean (95% CI) concentration of apoB and LDL-C at baseline was 0.81 (0.76, 0.85) g/L and 2.04 (1.90, 2.18) mmol/L. The baseline characteristics of the study participants according to apoB concentration are shown in **Table 1**. No significant differences were found in baseline characteristics, namely, age, sex, diabetes, hypertension, and smoking status. Serum lipids, namely, total cholesterol, triglycerides, and LDL-C differed significantly according to concentration of apoB. **Supplementary Table 1** shows the baseline characteristics of study participants based on LDL concentration.

## Association of ApoB and LDL-C With Severity of Coronary Atherosclerosis

Least squares means of syntax scores was estimated according to the levels of apoB and LDL-C. The least squares means and 95% CI of syntax scores from lowest to highest apoB categories (≤0.66, 0.67–0.89, and ≥0.90 g/L) were 8.53 (4.04, 13.02), 13.90 (9.84, 17.97), and 17.75 (13.06, 22.44), respectively (**Figure 2A**). Higher levels of apoB were significantly associated with the severity of coronary atherosclerosis ($P_{trend}$ = 0.012) (**Table 2**). However, higher levels of LDL-C were not significantly associated with the severity of coronary atherosclerosis ($P_{trend}$ = 0.585) (**Table 2**). The least squares means and 95% CI of syntax scores from lowest to highest LDL-C categories (≤1.56, 1.57–2.30, and ≥2.31 mmol/L) were 12.12 (6.61, 17.62), 13.18 (8.94, 17.41), and 14.74 (9.07, 20.40), respectively (**Figure 2B**).

## Association of ApoB and LDL-C With Residual Risk of Coronary Atherosclerotic Heart Disease

In total, 71 new coronary atherosclerotic heart disease cases were identified. After multivariate adjustment, higher apoB levels were significantly associated with residual risk of coronary atherosclerotic heart disease. When compared with low-level apoB (≤0.66 g/L), the multivariate adjusted RR and 95% CI of intermediate-level apoB (0.67–0.89 g/L) and high-level apoB (≥0.90 g/L) were 1.16 (1.01, 1.33) and 1.31 (1.08, 1.60), respectively, for the residual risk of coronary atherosclerotic heart disease ($P_{trend}$ = 0.011) (**Table 3**). There was a 45% increased residual risk of coronary atherosclerotic heart disease per unit increment in natural log-transformed apoB ($P_{trend}$ <0.05) (**Table 3**). However, higher LDL-C levels were not significantly associated with residual risk of coronary atherosclerotic heart disease. When compared with low-level LDL-C (≤1.56 mmol/L), the multivariate adjusted RR and 95% CI of intermediate-level LDL-C (1.57–2.30mmol/L) and high-level LDL-C (≥2.31 mmol/L) were 0.99 (0.84, 1.15) and 1.10 (0.86, 1.42), respectively, for the residual risk of coronary atherosclerotic heart disease ($P_{trend}$ = 0.437) (**Table 3**).

**TABLE 1** | Baseline characteristics of participants with statins treatment according to ApoB concentrations.

| | ApoB concentrations (g/L) | | | | |
|---|---|---|---|---|---|
| | **Total** | **≤0.66** | **0.67–0.89** | **≥0.90** | $P_{trend}$ |
| Baseline characteristics | | | | | |
| Age(years) | 64.98 ± 7.68 | 65.66 ± 7.44 | 64.36 ± 8.13 | 64.91 ± 7.55 | 0.647 |
| Sex | | | | | |
| Female | 56 (42.7%) | 21 (47.7%) | 16 (36.4%) | 19 (44.2%) | 0.734 |
| Male | 75 (57.3%) | 23 (52.3%) | 28 (63.6%) | 24 (55.8%) | |
| Diabetes | | | | | |
| No | 83 (63.4%) | 25 (56.8%) | 27 (61.4%) | 31 (72.1%) | 0.141 |
| Yes | 48 (36.6%) | 19 (43.2%) | 17 (38.6%) | 12 (27.9%) | |
| Hypertention | | | | | |
| No | 40 (30.5%) | 13 (29.5%) | 11 (25.0%) | 16 (37.2%) | 0.443 |
| Yes | 91 (69.5%) | 31 (70.5%) | 33 (75%) | 27 (62.8%) | |
| Smoking status | | | | | |
| Never smoker | 85 (64.9%) | 32 (72.7%) | 29 (65.9%) | 24 (55.8%) | 0.100 |
| Smoker | 46 (35.1%) | 12 (27.3%) | 15 (34.1%) | 19 (44.2%) | |
| Lipids | | | | | |
| TC (mmol/L) | 4.18 ± 1.18 | 3.45 ± 1.08 | 3.94 ± 0.53 | 5.16 ± 1.11 | <0.001 |
| TG (mmol/L) | 1.73 ± 2.46 | 1.71 ± 3.62 | 1.42 ± 0.58 | 2.07 ± 2.16 | <0.001 |
| HDL-C (mmol/L) | 1.18 ± 0.31 | 1.18 ± 0.37 | 1.15 ± 0.31 | 1.21 ± 0.23 | 0.143 |
| ApoA1 (g/L) | 1.26 ± 0.25 | 1.26 ± 0.25 | 1.25 ± 0.25 | 1.27 ± 0.24 | 0.945 |
| Lp(a) (mg/dl) | 23.94 ± 26.69 | 20.58 ± 23.09 | 20.97 ± 21.21 | 30.41 ± 33.68 | 0.159 |
| LDL-C (mmol/L) | 2.04 ± 0.81 | 1.39 ± 0.53 | 1.95 ± 0.49 | 2.79 ± 0.68 | <0.001 |

*Data expressed as absolute frequency (%) and mean ± SD.*
*ApoB was categorized into three groups: low-level (≤0.66 g/L), moderate-level (0.67–0.89 g/L), high-level (≥0.90 g/L).*

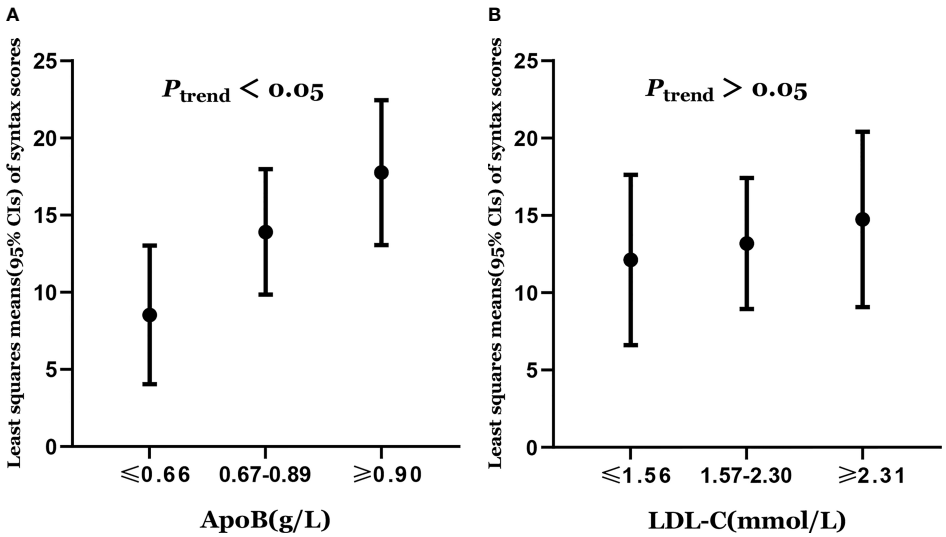


**FIGURE 2** | **(A)** Least squares means of syntax scores was estimated according to the levels of apoB. **(B)** Least squares means of syntax scores was estimated according to the levels of LDL-C. Dots represents the least squares means of syntax scores, up and down lines respectively represent the upper and lower limits of the 95% confidence interval for the least squares means. The least squares means of syntax scores was estimated using generalized linear model with adjustment of age (years), sex (female or male), smoking status (never smoker or smoker), diabetes (no or yes), hypertention (no or yes), total cholesterol, triglycerides, apolipoprotein A1, and lipoprotein (a).

**TABLE 2** | B (95%CIs) for the levels of apoB and LDL-C.

| **ApoB (g/L)** | **≤0.66** | **0.67-0.89** | **≥0.90** | $P_{trend}$ |
|---|---|---|---|---|
| | 1,00 | 5.37 (0.12, 10.63) | 9.22 (2.14, 16.31) | 0.012 |
| **LDL-C (mmol/L)** | **≤1.56** | **1.57-2.30** | **≥2.31** | $P_{trend}$ |
| | 1.00 | 1.06 (–4.87, 6.98) | 2.62 (–6.78, 12.02) | 0.585 |

*B (95%CIs) for the levels of apoB and LDL-C was estimated using generalized linear model with adjustment of age (years), sex (female or male), smoking status (never smoker or smoker), diabetes (no or yes), hypertention (no or yes), total cholesterol, triglycerides, apolipoprotein A1, and lipoprotein (a).*

Stratified analyses by age, sex, smoking status, diabetes, and hypertension observed consistent results (**Supplementary Tables 2** and **3**). No significant interactions were detected for both apoB and LDL-C (all $P_{interaction}$ >0.05). In the sensitivity analyses, consistent results were demonstrated when logistic regression was used to estimate odds ratios (OR) and 95% CIs for the association of the level of apoB and LDL-C with the residual risk of coronary atherosclerotic heart disease (**Supplementary Table 4**). Similar results were detected after further adjusting for estimated glomerular filtration rate (**Supplementary Table 5**). The results were similar when we selected individuals with higher syntax scores (**Supplementary Table 6**).

## DISCUSSION

Our study provides insights into the value of apoB versus LDL cholesterol in participants with statin treatment for identifying residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis. Higher levels of apoB were significantly associated with residual risk of coronary atherosclerotic heart disease and severity of coronary

**TABLE 3** | RR (95% CIs) for residual risk of coronary atherosclerotic heart disease according to levels of apoB and LDL-C.

| **ApoB (g/L)** | **≤0.66** | **0.67–0.89** | **≥0.90** | $P_{trend}$ | **Per one-unit increment in natural log-transformed apoB** |
|---|---|---|---|---|---|
| Model 1 | 1.00 | 1.11 (0.96, 1.29) | 1.20 (1.04, 1.38) | 0.017 | 1.25 (1.04, 1.50) |
| Model 2 | 1.00 | 1.11 (0.97, 1.27) | 1.21 (1.06, 1.39) | 0.008 | 1.29 (1.06, 1.56) |
| Model 3 | 1.00 | 1.16 (1.01, 1.33) | 1.31 (1.08, 1.60) | 0.011 | 1.45 (1.06, 1.97) |
| **LDL-C (mmol/L)** | **≤1.56** | **1.57–2.30** | **≥2.31** | $P_{trend}$ | **Per one-unit increment in natural log-transformed LDL-C** |
| Model 1 | 1.00 | 0.95 (0.82, 1.10) | 1.08 (0.93, 1.25) | 0.265 | 1.10 (0.95, 1.28) |
| Model 2 | 1.00 | 0.96 (0.84, 1.11) | 1.08 (0.93, 1.26) | 0.294 | 1.11 (0.96, 1.28) |
| Model 3 | 1.00 | 0.99 (0.84, 1.15) | 1.10 (0.86, 1.42) | 0.437 | 1.26 (0.95, 1.67) |

*Model 1: no variables are adjusted.*
*Model 2: adjusted for age (years), sex (female or male), smoking status (never smoker or smoker), diabetes (no or yes), and hypertention (no or yes).*
*Model 3: further adjusted for total cholesterol, triglycerides, apolipoprotein A1 and lipoprotein (a).*

atherosclerosis in statin-treated participants, whereas no such associations were found for elevated levels of LDL-C. Therefore, elevated apoB are superior in assessing residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in patients with statin treatment. Identifying residual risk of coronary atherosclerotic heart disease in statin-treated patients with low levels of LDL-C is essential for the prevention of coronary atherosclerotic heart disease. Our results suggest that apoB can be used to guide further treatment in statin-treated patients with low levels of LDL-C.

The most likely explanation for our results is that apoB includes the atherogenic risk due to the TG-rich VLDL apoB particles and the cholesterol-rich LDL apoB particles (21), but LDL-C ignores the atherogenic potential of TG-rich lipoproteins. The concentration of apoB is proportional to the total number of atherogenic lipoprotein particles. Studies found that the trends of apoB and LDL-C differs in more than one quarter of people, especially in people with metabolic risk factors (such as obesity or type 2 diabetes) (22) and those taking statins (23). The reason for the inconsistent trends in the levels of apoB and LDL-C is that LDL-C is lowered relatively more than cholesterol of other apoB-containing lipoprotein (24).

Similar results have been reported in previous studies on participants with statin treatment. In the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPs), apoB concentration was significantly associated with future cardiovascular events after one year of treatment, but not LDL-C (25). Kastelein et al. pooled the TNT and IDEAL studies, and found that apoB were more closely associated with cardiovascular events than levels of LDL-C in patients with statin treatment (26). Ference et al. found that the risk of cardiovascular events was proportional to the attenuated reduction in apoB, but significantly less than per unit change in LDL-C. The clinical benefit of lowering LDL-C levels may depend on the corresponding reduction in apoB-containing lipoprotein (27). In another study of Ference, the association of the levels of triglycerides and LDL-C with the risk of coronary heart disease was proportional to the absolute change in apoB. In multivariable Mendelian randomization analyses, the associations of levels of triglyceride and LDL-C with the risk of coronary heart disease became null after adjusting for apoB (28). However, while none of these studies included Chinese, our study shows that apoB is also superior to LDL-C in assessing residual risk of coronary atherosclerotic heart disease in the Chinese population with statin treatment.

In multiple previous studies, apob and LDL-C were associated with the severity of coronary atherosclerosis, the severity of coronary atherosclerosis was estimated based on the number of coronary artery lesions or Gensini score, and the study population was patients with suspected coronary heart disease or untreated patients (29–31). We conducted this research to determine whether the concentrations of apoB and LDL-C are still associated with the severity of coronary atherosclerosis in participants with statin treatment. Both Gensini score and syntax score are used to quantitatively evaluate the severity of coronary atherosclerosis based on the results of invasive coronary angiography. The Gensini score calculates the lesion score according to the lesion location and the degree of stenosis. Syntax score not only includes the lesion location and the degree of stenosis, but also further considers the bifurcation, calcification, and thrombus of coronary. Studies have confirmed that the syntax score was superior to the Gensini score in assessing the severity of coronary atherosclerosis (32). Our study is the first to show that concentrations of apoB are associated with the severity of coronary atherosclerosis (evaluated by syntax scores) in participants with statin treatment, but not in LDL-C.

Traditional epidemiological methods such as prospective studies and randomized controlled trials have confirmed that cholesterol (especially LDL cholesterol) and triglycerides are risk factors of cardiovascular disease, and elevated levels of cholesterol and triglyceride can significantly increase the risk of cardiovascular disease (33–35). However, many participants still had the residual risk of cardiovascular disease after LDL-C lowering (5–7), cardiovascular disease may be caused by a series of complex factors. For example, statins tend to increase Lp(a) levels, possibly contributing to the residual risk of cardiovascular disease, and lowering plasma Lp(a) levels can significantly decrease the residual risk of cardiovascular disease (36). Residual risk of cardiovascular disease was also associated with elevated plasma triglycerides and abnormal metabolism of triglyceride-rich lipoproteins (TRLs) (9).

At present, LDL-C is still the primary target of lipid-lowering therapy on the management of hypercholesterolemia in the United States guidelines and the European guidelines (3, 4). The 2019 European Society of Cardiology/European Atherosclerosis Association guidelines emphasize routine measurement of apoB, whereas the US guidelines do not. Compared with the US guidelines, the European guidelines highlight the status of apoB. Analyzing the reasons for this, there is ample evidence that lowering apoB levels significantly reduces the risk of coronary heart disease. However, the current evidence on the threshold for apoB as a risk modifier in patients with statin treatment is relatively insufficient, and further research is required (14). Our results suggest that elevated apoB is superior in assessing the residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in patients with statin treatment. Thus, in patients receiving lipid-lowering therapy, apoB may be considered for guiding further treatment intensification even if LDL cholesterol is low. Routine measurement of apoB is recommended.

## CONCLUSIONS

We observed significant associations of higher levels of apoB with residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in statin-treated participants, but not in LDL-C. Our results suggested that elevated apoB are superior in assessing residual risk of coronary atherosclerotic heart disease and severity of coronary atherosclerosis in participants with statin treatment.

## STUDY LIMITATIONS

First of all, this is a single-center retrospective cohort study, so it is necessary to design a multi-center prospective study to further verify our conclusions. Then it is possible that lacking information on types and doses of statins will induce bias, and future studies with information on types and doses of statins are encouraged.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this study will be available from the corresponding author on reasonable requests.

## ETHICS STATEMENT

The study protocol was reviewed and approved by the Institutional Review Committee of Union Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, and conforms to the concept of the Declaration of Helsinki and its amendments. We verbally informed the participants that the data will be used for medical research anonymously. No informed consent was signed for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

TY, QK and CL conceived and designed the study. TY and WL jointly responded to the editor and reviewers and revised manuscript. TY and JK analyzed the data and wrote the first draft of the manuscript. WL, HZ, XZ, BS and TL collected data. QK and CL revised this manuscript. All authors had access to study data and approved the decision to submit the manuscript.

## FUNDING

This research was supported by the National Natural Science Foundation of China (No. 81974222).

## ACKNOWLEDGMENTS

We appreciates all the patients who took part in this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.865863/full#supplementary-material

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 April 2022
doi: 10.3389/fendo.2022.827107

# Effect of Washed Microbiota Transplantation on Patients With Dyslipidemia in South China

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**Specialty section:**
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

**Received:** 01 December 2021
**Accepted:** 10 January 2022
**Published:** 22 April 2022

**Citation:**
Liang F, Lu X, Deng Z, Zhong H-J, Zhang W, Li Q, Zhang H-H, Liou Y-L and He X-X (2022) Effect of Washed Microbiota Transplantation on Patients With Dyslipidemia in South China. Front. Endocrinol. 13:827107. doi: 10.3389/fendo.2022.827107

**Background and Aims:** Although the manual crude fecal microbiota transplantation (FMT) reduces blood lipids in animal models of hyperlipidemia, its clinical effect on blood lipid metabolism in patients with hyperlipidemia and hypolipidemia remains unclear, especially in the Chinese population. It was reported that washed microbiota transplantation (WMT) was safer, more precise, and more quality-controllable than the crude FMT by manual. This study aimed to investigate the feasibility and effectiveness of WMT on lipid metabolism in the Chinese population.

**Methods:** Clinical data of patients with various indications who received WMT for 1–3 treatment procedures were collected. Changes in blood lipids before and after WMT, namely, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), homeostasis model assessment of insulin resistance (HOMA-IR), liver fat attenuation, and liver stiffness measurement, were compared.

**Results:** A total of 177 patients (40 cases of hyperlipidemia, 87 cases with normal blood lipids, and 50 cases of hypolipidemia) were enrolled in the First Affiliated Hospital of Guangdong Pharmaceutical University. WMT has a significant therapeutic effect in reducing blood lipid levels (TC and TG) in the short- and medium term in patients with hyperlipidemia ($p < 0.05$). Hyper blood lipid decreased to normal in the short-term (35.14%; $p < 0.001$), and LDL-C changed to normal in the medium term (33.33%; $p = 0.013$). In the hypolipidemia group, 36.36% and 47.06% changed to normal in the short-term ($p = 0.006$) and medium term ($p = 0.005$) of therapeutic effects based on blood lipid levels. In the normal blood lipid group and the low-risk group of atherosclerotic

cardiovascular disease (ASCVD), the change was not statistically significant, indicating that WMT does not increase the risk of blood lipid and ASCVD in the long-term.

**Conclusions:** WMT treatment changes blood lipids in patients with hyperlipidemia and hypolipidemia without serious adverse events, with no risk for increasing blood lipids and ASCVD in the long-term. There were significant decreased TC, TG, and LDL-C levels in the medium term of WMT treatment for hyperlipidemia. Therefore, the regulation of gut microbiota by WMT may indicate a new clinical method for the treatment of dyslipidemia.

**Keywords: fecal microbiota transplantation, washed microbiota transplantation (WMT), hyperlipidemia, hypolipidemia, blood lipid**

## INTRODUCTION

Cardiometabolic syndrome (CS), also known as metabolic syndrome (MS), is caused by a series of metabolic diseases related to pathogenesis, namely, hyperlipidemia, hypertension, diabetes, and obesity. The presence of the combined effects of these metabolic diseases can significantly increase the risk of developing cardiovascular disease. We are entering an era of chronic metabolic and cardiovascular multi-comorbidities. It is estimated that 22% of the adult population in the US suffers from CS, and its prevalence is on the rise (1). Previous studies have proven that hyperlipidemia is a severe risk factor for coronary artery disease (CAD), which accounts for approximately 30% of deaths worldwide (2). The treatment strategy for hyperlipidemia is mainly lifestyle intervention and drug therapy. However, pharmacologic therapies are associated with significant side effects with long-term use (3).

In China, atherosclerotic cardiovascular disease (ASCVD) has become the first cause of death, and dyslipidemia is the most important pathogenic risk factor in the occurrence and development of ASCVD (4). According to the data of 29,678 Chinese adults aged ≥35 years from 2012 to 2015 (5), the prevalence of dyslipidemia was 34.7% higher in men than in women (40.0% vs. 29.3%; $p < 0.001$), and there was no significant difference between urban and rural residents (35.7% vs. 34.1%). In recent years, the mortality rate of CAD in China has been increasing, and the first reason is the increase in cholesterol (77%). Hyperlipidemia has become an important public health problem. In addition, high-density lipoprotein cholesterol (HDL-C) hypolipidemia is associated with many kinds of CS, and has displayed an increasing prevalence in China (6). Therefore, it is of great significance to comprehensively analyze the related factors of hyperlipidemia and find a treatment method with less side effects.

Gut microbiota disorder is a risk factor for CS (1, 7). Fecal microbiota transplantation (FMT) is a new therapeutic method, which uses "healthy" microbial configuration to replace the indigenous microbiome of patients. To date, FMT has been used in the clinical application of recurrent *Clostridium difficile* disease (CDI) infection and has been studied in other microbiome-associated diseases, such as ulcerative colitis (UC), inflammatory bowel disease (IBD), diabetes, and cardiac metabolic syndrome (8). Whether FMT can improve cardiac metabolic syndrome is a subject to be discussed in clinical medicine.

Given the safety, quality-control, and effective profile of washed microbiota transplantation (WMT) on microbiota disorder disease, we attempted to investigate the patients receiving WMT to observe whether WMT can improve human metabolism and blood lipid profiles (9, 10). We hypothesized that WMT could safely and continuously affect patients with various indications and change dyslipidemia without side effects. Therefore, we conducted a retrospective trial to collect the medical data of WMT-treated patients with dyslipidemia (hyperlipidemia and hypolipidemia).

## MATERIALS AND METHODS

### Patients

This study was conducted and approved by the Ethics Committee (no. 2017-98), in accordance with the Declaration of Helsinki at the First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, China. Written informed consent from all patients was obtained and reviewed. Consecutive patients who underwent WMT (9) for various indications with 1–3 courses at our hospital from January 1, 2017 to June 31, 2020 were enrolled in the study. The inclusion criteria were as follows: patients who were >18 years old, those who provided informed consent, and those who received WMT. The exclusion criteria were as follows: patients who were pregnant, those who had antibiotics and probiotics 3 months before and during the study, and those who had a change in their antihyperlipidemia medication regimen after WMT. The minimum sample size was 12 allogeneic patients based on the individual patient data from Vrieze et al. (11) and the calculation method from Craven et al. (12).

### WMT Procedure

The procedure of WMT was consistent with the Nanjing Consensus on Methodology of Washed Microbiota Transplantation (10). All healthy stool donors, aged between 18 and 25 years old, strictly underwent interviews, psychological, and physical examinations, biochemical testing, and infectious disease screening. Briefly, the microbiota for WMT was extracted from donated feces followed by centrifugation and suspension for three times using the intelligent microbial separation system (GenFMTer; FMT Medical, Nanjing, China), and five stages of filtration according to the recommendations of the manufacturer. Thereafter, a fecal

suspension is injected into the body of the patient through a nasojejunal tube (upper digestive tract) or an endoscopic intestinal tube (lower digestive tract) once a day for 3 days of one WMT course (13). The standard treatment timelines of the three WMT courses in our center were 0, 1, and 2 months respectively, and the end-point return visit time was 4 months. All patients underwent at least one WMT procedure and completed follow-up.

## Data Collection

Data were collected from the medical records of patients at prior treatment (baseline), before the second procedure (short-term effect), before the third procedure (medium term effect) and the clinical visit at the 4 months (long-term effect). Data included were age, sex, body mass index (BMI), smoking status, blood pressure (at admission), diseases or indications for WMT, and laboratory test results, namely, serum lipids (total cholesterol [TC], triglyceride [TG], high-density lipoprotein [HDL-C], and low-density lipoprotein [LDL-C]), fasting glucose (FG) and fasting insulin (FI), liver fat attenuation, and liver stiffness measurement (LSM). Homeostasis model assessment of insulin resistance (HOMA-IR) value was calculated as previously described (14). Hyperlipidemia was diagnosed based on the guideline for dyslipidemias (4, 15). All eligible patients are divided into three groups: the hyperlipidemia (TC ≥6.2 mmol/L or TG ≥2.3 mmol/L or LDL-C ≥4.1 mmol/L), hypolipidemia (TC <3.6mmol/L or TG <0.33 mmol/L or LDL-C <2.07 mmol/L or HDL <0.91) by hospital definition, and the normal groups according to their baseline, blood lipid status, and risk stratification of ASCVD. The diagnostic criterion of abnormal risk factors for HOMA-IR is ≥2.5 (16). The diagnostic threshold of fat attenuation is mild (≥240 db/m), moderate (≥266 db/m), and severe (≥295 db/m). LSM diagnostic threshold is >14.1 kPa (17). Diabetes (≥7.0), impaired glucose tolerance (<7.0), impaired fasting blood glucose (6.1–7.0), and normal (4.4–7.0) are defined by fasting blood glucose level (mmol/L) (18). Obesity (≥28.0), overweight (24.0–28.0), normal weight (18.5–24.0), and underweight (<18.5) are classified according to the definition of BMI ($kg/m^2$) (19).

All patients were divided into three groups according to the above definition. After all patients received 1–3 times of WMT treatment procedure and completed follow-up, the results of blood lipid changes were statistically analyzed and evaluated.

## Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Results were expressed as frequencies and percentages for categorical variables, means and standard deviations for normally distributed continuous variables, and medians and interquartile ranges for non-normally distributed continuous variables. Categorical variables were analyzed using the Chi-square or Fisher exact test. For comparisons of continuous variables between two independent groups, either unpaired Student's t-test (for normally distributed variables) or Mann–Whitney U test (for non-normally distributed variables) was applied. While comparing paired data, paired Student's t-test (for normally distributed variables) or Mann–Whitney U test (for non-normally distributed variables) was used. A two-tailed P-value <0.05 was considered statistically significant.

# RESULTS

## Clinical Characteristics of Patients Who Underwent WMT

A total of 177 patients (40 cases of hyperlipidemia, 50 cases of hyperlipidemia, and 87 cases with normal lipid) were treated with WMT, of which 52.0% were male, with an average age of 52.77 ± 15.79 years (**Figure 1**). **Table 1** shows the main indications for WMT, and the top three diseases were functional bowel disease (n = 91; 51.4%), followed by gastroesophageal reflux disease (n = 22; 12.4%) and ulcerative colitis (n = 18; 10.2%). The interval time of 3 treatment procedures were 37 days (33–47) for short-term, 41days (34–63) for medium term, and 96 days (77–119) for long-term. Blood lipid characteristics of patients are illustrated in **Table 2**. The average BMI of the hyperlipidemia group was classified as overweight, while the other two groups were normal. The average TG of the hyperlipidemia group was 2.66 (1.37–3.24) mmol/L higher, but TC and LDL-C were lower than the definition described in *Materials and Methods*. The average TC and LDL-C of the hypolipidemia group were 3.59 (3.34–3.83) mmol/L and 1.97 ± 0.65 mmol/L, which was lower than the definition of hypolipidemia. The average TC, TG, LDL of the normal group was 4.64 (4.29–5.15), 0.97 (0.72–1.19), and 2.93 ± 0.528 mmol/L. The average of HOMA-IR of the hyperlipidemia group is 2.71 (1.51–3.76), which was higher than the diagnostic threshold, and the others were in the normal range. The average of liver fat attenuation of hyperlipidemia and hypolipidemia groups were mild (251.00 [242.00–279.25]) and moderate (282.00 [259.50–287.75] db/m). There are no serious adverse events (AEs), and only a few of AEs include abdominal pain, abdominal distention, diarrhea, and dizziness.

## Effect of WMT on Lipid Profiles

**Table 3** and **Figure 2** show the effect of WMT on hyperlipidemic, normal, and hypolipidemic lipid profiles. In the short-term, WMT showed a significantly lower effect on TC (from 5.81 ± 1.13 to 5.26 ± 1.26 mmol/L) and TG (from 2.71 [1.45–3.46] to 1.76 [1.26–2.78] mmol/L) in the hyperlipidemia group ($p < 0.05$), and a significantly higher effect on TC (from 3.58 [3.21–3.82] to 3.74 [3.32–4.44]) in the hypolipidemia group ($p < 0.05$). In the medium term, WMT also showed a significantly lower effect on TC ($p = 0.01$), TG ($p = 0.048$), and LDL-C ($p = 0.013$) in the hyperlipidemia group ($p < 0.05$). In the hyperlipidemia group, WMT explained the short-term blood lipid reduction effect on TC and TG, and mid-term blood lipid reduction effect on TC, TG, and LDL-C. In hyperlipidemia group, TC, TG, and LDL-C except HDL-C ($p = 0.024$) had no significant change in the long-term effect of WMT. Since the number of long-term monitoring is only 9, it cannot be confirmed whether WMT treatment has long-term effects in the study. In the hypolipidemia group, the WMT treatment caused an increase in TC, LDL-C, and HDL-C levels from short to long-term, although all blood lipids results were not found significant in the long-term. WMT does not affect the changes in blood lipids of the normal group from short to long-term. There was no significant change in BMI before and after treatment in the three groups.

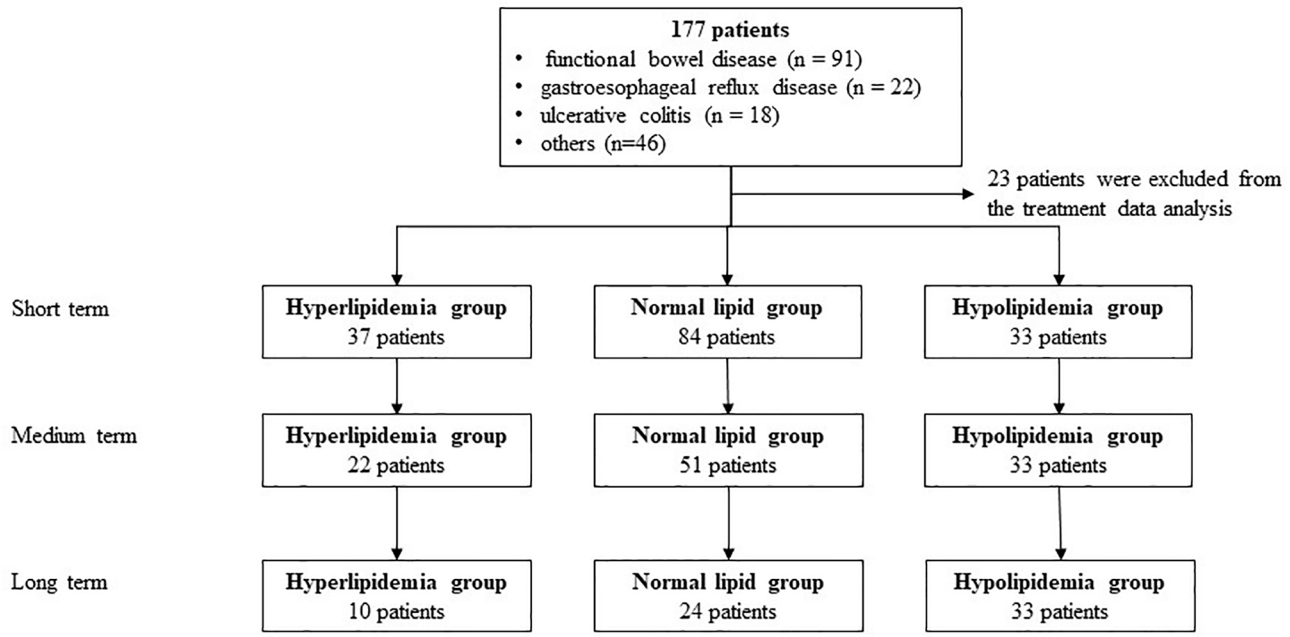


**FIGURE 1** | The Flow Chart of the Study.

**TABLE 1** | Main diagnoses of patients receiving washed microbiota transplantation.

| Diagnoses | Number (n) | Percentage (%) |
|---|---|---|
| Functional bowel disorders | 91 | 51.4% |
| Gastroesophageal reflux disease | 22 | 12.4% |
| Ulcerative colitis | 18 | 10.2% |
| Nonalcoholic fatty liver disease | 17 | 9.6% |
| Hyperuricemia | 8 | 4.5% |
| HBV-related liver cirrhosis | 8 | 4.5% |
| Radioactive enteritis | 2 | 1.1% |
| Hepatic encephalopathy. | 2 | 1.1% |
| Intestinal tuberculosis | 1 | 0.6% |
| Epilepsy | 1 | 0.6% |
| Colon carcinoma | 1 | 0.6% |
| Crohn's disease | 1 | 0.6% |
| Senile tremor | 1 | 0.6% |
| Parkinson's disease | 1 | 0.6% |
| Bipolar disorder | 1 | 0.6% |
| Gastric malignant tumor | 1 | 0.6% |
| Autoimmune hepatitis | 1 | 0.6% |
| Total | 177 | |

*HBV, Hepatitis B virus.*

## Effect of WMT on HOMA-IR, Liver Fat Attenuation, and LSM

Observation of WMT treatment effects from short-, medium- and long-term changes on FG, FI, HOMA-IR, liver fat attenuation, and LSM are shown in **Table 4**. After WMT treatment, abnormal HOMA-IR did not improve, and other indexes did not change significantly in the three groups. Only liver fat attenuation decreased significantly by WMT treatment in the long-term ($p = 0.043$).

## Evaluation of WMT Clinical Therapeutic Effect on Lipid Levels

All enrolled patients are divided into hyperlipidemia, normal lipid, and hypolipidemia groups based on the based line of blood lipid. Patients were regrouped according to the changes of blood lipid levels after 1–3 WMT treatment procedures (**Table 5**). The three groups showed significant changes in blood lipid levels in short and medium terms. In the hyperlipidemia group, 35.14% recovered to normal in the short-term, and 33.33% recovered to normal in the medium term ($p < 0.005$), and 40.00% in the long-term ($p = 0.094$). In the hypolipidemia group, 36.36% recovered to normal in the short-term, 47.06% recovered to normal in the medium term ($p < 0.005$), and 12.5% in the long-term ($p = 0.302$). Our data also demonstrated that WMT could significantly alter the higher and lower lipid levels of patients to normal in the short and medium term of treatments; however, the efficacy of WMT remained to be explored. Patients with normal lipid levels changed to abnormal in the short and medium terms (25.86 and 20.00%, respectively; $p < 0.05$) and in the long-term (27.78%; $p = 0.054$). Our data showed that WMT might have changed the blood lipid levels after one procedure treatment, while the lipid levels became steady in the medium and long-term treatments.

## Evaluation of the Therapeutic Effect of WMT on the Risk for ASCVD

Patients were divided into extremely-high-risk, high-risk, medium-risk, and low-risk groups according to the ASCVD risk stratification. Patients were regrouped into the no risk changed group and the risk-ascended group after WMT

**TABLE 2** | Demographics and clinical characteristics of patients at baseline.

| | Hyperlipidemia group (n = 40) | Normal lipid group (n = 87) | Hypolipidemia group (n = 34) |
|---|---|---|---|
| Age (years) | 53.20 ± 12.66 | 53.44 ± 16.36 | 52.76 ± 17.47 |
| Male n (%) | 18 (45) | 37 (42.5) | 28 (82.4) |
| BMI | 25.07 (21.82–28.34) (n = 37) | 22.32 (19.53–25.07) (n = 87) | 21.62 (19.19–23.38) (n = 34) |
| FG (mmol/L) | 4.69 (4.34–5.52) (n = 37) | 4.58 (4.18–5.03) (n = 80) | 4.56 (4.04–5.00) (n = 32) |
| FI (mmol/L) | 9.79 (6.78–16.56) (n = 28) | 7.81 (5.15–11.63) (n = 65) | 6.93 (4.99–9.47) (n = 22) |
| TC (mmol/L) | 5.91 (5.26–6.36) | 4.64 (4.29–5.15) | 3.59 (3.34–3.83) |
| TG (mmol/L) | 2.66 (1.37–3.24) | 0.97 (0.72–1.19) | 0.92 (0.57–1.17) |
| LDL-C(mmol/L) | 3.36 ± 1.13 | 2.93 ± 0.53 | 1.97 ± 0.65 |
| HDL-C(mmol/L) | 1.22 (1.03–1.39) | 1.14 (1.37–1.60) | 1.11(0.88–1.41) |
| HOMA-IR | 2.71 (1.51–3.76) (n = 27) | 1.62 (1.08–12.59) (n = 60) | 1.57(1.01–2.53) (n=22) |
| liver fat attenuation | 251.00 (242.00–279.25) (n = 20) | 237.00 (215.00–298.50) (n = 22) | 282.00(259.50–287.75) (n=6) |
| liver stiffness measurement | 6.55 (5.28–8.50) (n = 20) | 7.15 (5.25–8.65) (n = 22) | 8.40(6.63–9.63) (n=6) |

*Data presented as mean ± standard deviation, median (interquartile range), or n (%).*
*BMI, body mass index; FG, fasting glucose; FI, fasting insulin; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA–IR, homeostasis model assessment of insulin resistance.*

treatment procedures (**Table 5**). Patients with an acute coronary syndrome, stable coronary heart disease, postoperative revascularization, ischemic cardiomyopathy, ischemic stroke, transient ischemic attack, and peripheral atherosclerosis were included in the extremely high-risk group, and this group of patients will not be reassigned after WMT treatment.

No significant WMT therapeutic effect was found in short, medium, and long terms in the high-risk group. In the medium-risk group, 33.33% in the short-term and 55.56% in the medium term were regrouped to the low-risk group ($p < 0.05$). In the low-risk group of ASCVD, the change was not statistically significant, indicating that WMT does not increase the risk for ASCVD.

## DISCUSSION

To the best of our knowledge, this is the first clinical study to investigate the effect of WMT on dyslipidemia in Chinese patients, especially hyperlipidemia, indicating that the manipulation of intestinal microbiota may be a new method for the treatment of dyslipidemia. These data indicate that WMT has a lipid balance effect on patients with hyperlipidemia or hypolipidemia in the short and medium terms but has no improvement effect on the main outcomes of HOMA-RI, BMI, liver fat attenuation, and liver stiffness from short to long-term, which are the primary outcomes of this study. There was no significant difference between the low-risk and non-dyslipidemia groups, indicating that WMT would not increase the long-term risk for ASCVD and dyslipidemia, which are the secondary outcomes.

Many scientific evidences indicated the potential role of microbiota in influencing human health (20). FMT was not only limited in digestive system diseases, such as the *C. difficile* infection (CDI) (8, 21), irritable bowel syndrome (IBS) (22), and IBD (22) but also had improvement in CS (1, 23), neurologic, and psychiatric disorders (24). MS is often associated with an imbalance of the host microbiota and inflammation (25). Although increasing evidence from animal disease models has described the potential causal relationship between changes in gut microbiota and metabolic syndrome (26), there were limited data that supported the role of FMT in humans suffering from features of CS (27, 28).

Randomized controlled trials involving lipid status reported mixed results of metabolic parameters. For example, Kootte et al. (29) and Vrieze et al. (11) showed that FMT from lean donors to patients with obesity with metabolic syndrome could improve insulin sensitivity in the short to medium term (6 weeks), but no significant difference in the long-term (after 18 weeks). A total of three randomized controlled trials (3RCTs) with 76 patients were met the definition of hyperlipidemia (see *Data Collection* section) (11, 29–31). In the patients with hyperlipidemia in our study, the mean baseline of TC and TG were 11.3 and 90.0% higher than those of three RCTs, respectively, while the baseline of BMI and LDL-C were 28.1 and 4% lower, respectively. In conclusion, the study showed that the Chinese population have a lower BMI but higher TC and TG results than Westerners.

Fu et al. studied 893 patients from the LifeLines-DEEP population cohort (LL-D) to study the relationship between the microbiota and metabolic risk factors for cardiovascular disease. Studies have found that the intestinal microbiota, independent of age, gender, and host genetics, might support the potential of therapies altering the gut microbiome to control BMI and TG and HDL-C levels (32). In the three RCTs, FMT treatment was no benefit to reducing BMI, TG, HDL-C, and LDL-C levels of patients with obesity and MS (31). Our study showed that TC, TG, and LDL-C responded well to WMT treatment in the medium term. It is worth considering the similarities and differences of lipid profiles between the three RCTs, LL-D, and our study. First, our data provided real clinical

**TABLE 3 |** The lipid profiles of short-, medium-, and long-term washed microbiota transplantation treatment procedures in the study.

| Items | Baseline | Short-term | p-Value | Baseline | Medium-term | p-Value | Baseline | Long-term | p-Value |
|---|---|---|---|---|---|---|---|---|---|
| **Hyperlipidemia group** | | | | | | | | | |
| BMI | 25.01 ± 3.52 (n = 37) | 24.50 ± 3.68 (n = 37) | 0.070 | 24.68 ± 3.51 (n = 22) | 23.95 ± 3.72 (n = 22) | 0.233 | 25.31±4.48 (n=10) | 25.31 ± 4.98 (n = 10) | 0.999 |
| TC | 5.81 ± 1.13 (n = 37) | 5.26 ± 1.26 (n = 37) | 0.027 | 6.03 ± 1.10 (n = 21) | 5.09 ± 1.37 (n = 21) | 0.010 | 5.56±0.82 (n=9) | 5.27 ± 0.78 (n = 9) | 0.255 |
| TG | 2.71 (1.45–3.46) (n = 37) | 1.76 (1.26–2.78) (n = 37) | 0.004 | 2.6 (1.49–3.13) (n = 21) | 1.85 (1.12–2.82) (n = 21) | 0.048 | 2.52 ±0.73 (n=9) | 2.30 ± 1.1 (n = 9) | 0.391 |
| LDL-C | 3.32 (2.24–4.25) (n = 37) | 3.04 (2.26–3.92) (n = 37) | 0.225 | 3.48 ± 1.10 (n = 21) | 2.89 ± 1.33 (n = 21) | 0.013 | 3.17±0.95 (n=9) | 3.07 ± 0.84 (n = 9) | 0.623 |
| HDL-C | 1.17 ± 0.29 (n = 37) | 1.23 ± 0.31 (n = 37) | 0.161 | 1.22 (1.11–1.40) (n = 21) | 1.15 (1.03–1.43) (n = 21) | 0.985 | 1.24±0.16 (n=9) | 1.15 ± 0.18 (n = 9) | 0.024 |
| **Normal group** | | | | | | | | | |
| BMI | 22.40 (19.53–25.10) (n = 84) | 22.22 (19.63–25.38) (n = 84) | 0.737 | 22.48 (19.56–25.71) (n = 51) | 22.06 (19.40–25.65) (n = 51) | 0.447 | 22.25 (18.81–26.67) (n=24) | 22.91 (19.75–25.42) (n = 24) | 0.648 |
| TC | 4.62 (4.28–5.11) (n = 58) | 4.65 (4.23–5.09) (n = 58) | 0.367 | 4.44 (4.26–5.00) (n = 37) | 4.57 (4.03–4.91) (n = 37) | 0.956 | 4.87±0.68 (n=19) | 4.86 ± 1.10 (n = 19) | 0.904 |
| TG | 1.00 (0.78–1.21) (n = 58) | 0.99 (0.73–1.25) (n = 58) | 0.430 | 0.97 (0.78–1.16) (n = 37) | 0.89 (0.73–1.33) (n = 37) | 0.771 | 0.94 (0.76–1.16) (n=19) | 0.91 (0.69–1.16) (n = 19) | 0.687 |
| LDL-C | 2.70 (2.52–3.13) (n = 58) | 2.79 (2.24–3.15) (n = 58) | 0.470 | 2.69 (2.49–3.10) (n = 37) | 2.67(2.35–3.05) (n = 37) | 0.734 | 3.14±0.56 (n=19) | 3.08 ± 0.96 (n = 19) | 0.778 |
| HDL-C | 1.32(1.13–1.61) (n = 58) | 1.29 1.11–1.63) (n = 58) | 0.151 | 1.30 (1.13–1.59) (n = 37) | 1.27 (1.11–1.53) (n = 37) | 0.142 | 1.36±0.32 (n=19) | 1.33 ± 0.32 (n = 19) | 1.000 |
| **Hypolipidemia group** | | | | | | | | | |
| BMI | 21.65 ± 3.73 (n = 33) | 21.48 ± 3.51 (n = 33) | 0.559 | 21.22 ± 3.93 (n = 22) | 21.48 ± 3.05 (n = 22) | 0.434 | 20.34±3.63 (n=11) | 20.95 ± 0.99 (n = 11) | 0.249 |
| TC | 3.58 (3.21–3.82) (n = 22) | 3.74 (3.32–4.44) (n = 22) | 0.028 | 3.63 (3.36–3.88) (n = 17) | 3.73 (3.28–4.12) (n = 17) | 0.320 | 3.30 (2.78–3.61) (n=8) | 3.52 (2.79–3.87) (n = 8) | 0.093 |
| TG | 0.84 (0.50–1.14) (n = 22) | 0.69 (0.55–1.19) (n = 22) | 0.592 | 0.90 ± 0.40 (n = 17) | 0.83 ± 0.36 (n = 17) | 0.298 | 0.80±0.40 (n=8) | 0.94 ± 0.37 (n = 8) | 0.186 |
| LDL-C | 1.91 ± 0.68 (n = 22) | 2.06 ± 0.67 (n = 22) | 0.173 | 1.82 (1.79–2.10) (n = 17) | 2.07 (1.65–2.49) (n = 17) | 0.210 | 1.79(1.53–1.90) (n=8) | 1.84 (1.73–2.10) (n = 8) | 0.123 |
| HDL-C | 1.15 ± 0.37 (n = 22) | 1.21 ± 0.35 (n = 22) | 0.054 | 1.20 ± 0.44 (n = 17) | 1.24 ± 0.33 (n = 17) | 0.602 | 1.05±0.35 (n=8) | 1.20 ± 0.48 (n = 8) | 0.779 |

*presented as mean ± standard deviation, median (interquartile range), or n (%).*

*BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.*

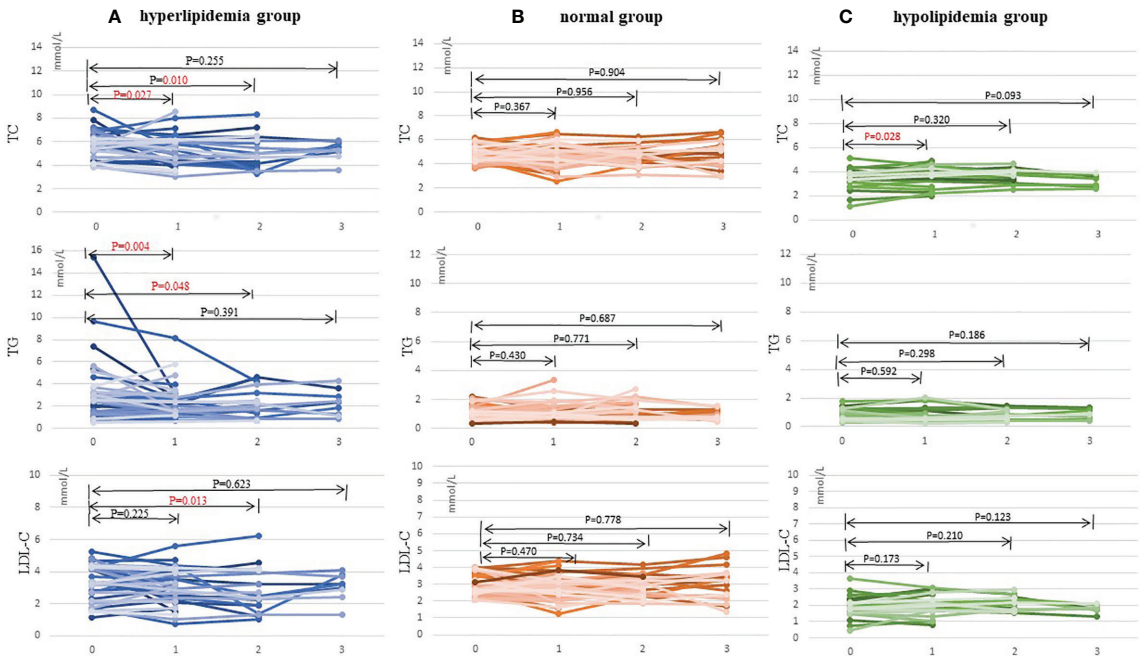


**FIGURE 2** | Changes in TC, TG, and LDL-C levels after 1–3 times of washing microbial transplantation. **(A)** Changes in TC, TG, and LDL-C levels in the hyperlipidemia group; **(B)** Changes in TC, TG, and LDL-C levels in the normal group; and **(C)** Changes in TC, TG, and LDL-C levels in the hypolipidemia group. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol.

evidence that supported the LL-D study, which showed that FMT has a better effect on reducing TC and TG in individuals with lower BMI. Second, pool-multiple feces and multiple treatments in a short time can improve the effect of FMT in our study and the study in recurrent CDI (33, 34). The diversity and richness of the gut microbiota supplied to the patients in the short-term may be an important factor in reducing blood lipids. Third, WMT is more effective in improving lipid metabolism disorder than manual FMT and oral capsule administration (35, 36). In addition, WMT was used to treat patients with dyslipidemia for three courses (each course was treated for three consecutive times) in our study. Different biological factors include a low BMI of 25 (similar to the Craven standard), as compared to other studies, and most of our patients had functional intestinal diseases and were Chinese.

Our results showed that WMT treatment could not only significantly reduce the blood lipid level of patients with hyperlipidemia but also increase the blood lipid levels of hypolipidemia to normal, significantly in the TC levels in the short and medium terms. The efficacy of WMT remained to be explored for long-term because of the small samples size. As to our data, there was no increased risk of blood lipid and AEs in people with normal blood lipid after WMT (37). We also found the same conclusion as Xi et al. that people with functional gastrointestinal diseases and dyslipidemia had no significant changes in BMI after WMT treatment (38).

The in-depth studies of intestinal function show that intestinal microbiota plays a key role in affecting systemic metabolism and other cardiac metabolic diseases (1, 39, 40). In Type 2 Diabetes Mellitus (T2DM) animal models, FMT can improve HOMA-IR and insulin sensitivity and repair damaged islets, providing a potential strategy for the treatment of T2DM (41–43). FMT has short-term or no benefit in improving insulin sensitivity and HOMA-IR in patients with obesity and MS (12, 29, 31, 36). In our hyperlipidemia data, HOMA-IR increased after WMT treatment from short- to long-term, but there was no significant change in HOMA-IR in the normal and hypolipidemic groups. We found no significant difference in liver fat attenuation and measurement of liver stiffness. The improvement of liver function cannot be concluded because the technical factors include the accuracy of the instrument and the limited number of patients for liver fat attenuation and measurement of liver stiffness. The relationship between FMT and hyperlipidemia on HOMA-IR needs to be further explored.

The increased risk for cardiovascular events is not only associated with dyslipidemia but also with abnormal glucose metabolism and liver function (44). Patients with T2DM have an increased risk for cardiovascular disease, and combination therapy consisting of metformin and statin are the commonly used treatments. However, both drugs act on glucose and lipid metabolism, which could lead to AEs when used in combination as compared to monotherapy. The percentage changes of lipid variables of patients during the combination therapy of metformin and atorvastatin/simvastatin were 10% to −31% in TC, 12% to −33% in TG, 1% to −36% in LDL-C, and 9% to −65% in HDL-C (45). Our data showed the percentage changes of lipid variables of patients after WMT treatment were TC (−5.2% to −9.5%), TG (−8.7% to −35.1%), LDL-C (−3.2% to −17%),

**TABLE 4 |** The glucose metabolism and liver profiles of short-, medium-, and long-term washed microbiota transplantation treatment in the study.

| Items | Baseline | Short-term | p-Value | Baseline | Medium-term | p-Value | Baseline | Long-term | p Value |
|---|---|---|---|---|---|---|---|---|---|
| **Hyperlipidemia group** | | | | | | | | | |
| FG | 4.66 (4.34–5.84 (n = 34) | 4.80 (4.34–6.05) (n = 34) | 0.412 | 4.61 (4.24–5.24) (n = 17) | 5.04 (4.25–6.04) (n = 17) | 0.117 | 5.28 (4.61–8.28) (n=7) | 4.95 (4.85–1023) (n=7) | 0.672 |
| FI | 11.76 (6.78–16.60) (n = 20) | 12.45 (8.02–18.29) (n = 20) | 0.247 | 12.91 (7.44–17.34) (n = 10) | 15.21 (13.47–22.72) (n = 10) | 0.074 | 16.57 (7.81–20.92) (n=5) | 14.63 (9.84–23.08) (n=5) | 0.686 |
| HOMA-IR | 2.71 (1.51–3.76) (n = 19) | 2.29 (1.61–4.15) (n = 19) | 0.212 | 3.20 ± 1.44 (n = 10) | 5.31 ± 3.77 (n = 10) | 0.059 | 3.90±1.70 (n=5) | 4.28±2.12 (n=5) | 0.500 |
| Liver fat attenuation | 255.00 (242.00–281.00) (n = 15) | 241.00 (226.00–282.00) (n = 15) | 0.532 | 249.00 (235.50–284.50) (n = 9) | 240.00 (226.50–287.0) (n = 9) | 0.761 | 275.75±28.93 (n=4) | 284.50±36.79 (n=4) | 0.743 |
| Liver stiffness measurement | 6.80 (5.80–8.80) (n = 15) | 6.60 (5.20–7.50) (n = 15) | 0.256 | 5.80 (3.80–7.90) (n = 9) | 6.30 (5.80–7.90) (n = 9) | 0.192 | 5.40 (3.75–7.43) (n=4) | 6.20 (5.98–7.55) (n=4) | 0.465 |
| **Normal group** | | | | | | | | | |
| FG | 4.59 (4.18–5.07) (n = 69) | 4.49 (4.24–4.87) (n = 69) | 0.204 | 4.52 (4.068–5.05) (n = 42) | 4.41 (3.91–4.90) (n = 42) | 0.129 | 4.37 (3.99–5.17) (n=19) | 4.39 (4.04–5.11) (n=19) | 0.387 |
| FI | 8.41 (5.16–12.49) (n = 39) | 8.23 (5.79–13.68) (n = 39) | 0.596 | 8.56 (4.29–14.54) (n = 22) | 9.04 (5.68–12.43) (n = 22) | 0.673 | 9.71 (6.52–16.06) (n=12) | 7.81 (6.14–15.94) (n=12) | 0.347 |
| HOMA-IR | 1.65 (1.08–2.87) (n = 36) | 1.96 (1.10–2.85) (n = 36) | 0.530 | 1.70 (0.81–3.99) (n = 19) | 1.98 (1.10–2.84) (n = 19) | 0.809 | 1.85 (1.23–4.30) (n=10) | 1.49 (1.25–2.71) (n=10) | 0.114 |
| Liver fat attenuation | 260.39 ± 64.48 (n = 18) | 255.30 ± 57.77 (n = 18) | 0.372 | 281.23 ± 68.12 (n = 13) | 262.23 ± 50.48 (n = 13) | 0.100 | 240.40±28.90 (n=5) | 229.60±38.53 (n=5) | 0.043 |
| Liver stiffness measurement | 7.2 (5.63–8.65) (n = 18) | 7.00 (5.90–10.43) (n = 18) | 0.248 | 8.42 ± 5.03 (n = 13) | 8.25 ± 4.28 (n = 13) | 0.916 | 8.60(5.55–12.95) (n=5) | 8.10(6.50–13.20) (n=5) | 0.893 |
| **Hypolipidemia group** | | | | | | | | | |
| FG | 4.52 (4.00–4.96) (n = 29) | 4.39 (3.86–5.25) (n = 29) | 0.552 | 4.51 (3.97–5.20) (n = 18) | 4.49 (4.11–5.30) (n = 18) | 0.845 | 4.43(3.84–4.77) (n=9) | 4.51(3.73–5.08) (n=9) | 0.515 |
| FI | 6.77 (3.20–10.15) (n = 18) | 8.26 (4.86–11.11) (n = 18) | 0.372 | 7.71 (6.53–11.06) (n=8) | 6.07 (4.58–18.06) (n=8) | 0.889 | 9.43±6.75 (n=4) | 8.48±2.17 (n=4) | 0.809 |
| HOMA-IR | 1.57 (0.85–2.69) (n = 18) | 1.73 (1.07–4.39) (n = 18) | 0.122 | 2.42 (1.53–3.10) (n=8) | 1.65 (0.87–6.20) (n = 8) | 0.674 | 1.90±1.37 (n=4) | 1.97±1.49 (n=4) | 0.918 |
| Liver fat attenuation | 281.20 ± 11.14 (n = 5) | 263.60 ± 31.90 (n = 5) | 0.290 | 283.00 (n=1) | 261.00 (n = 1) | – | 249.00 (n=1) | 266.00 (n=1) | – |
| Liver stiffness measurement | 8.40 ± 2.02 (n = 5) | 11.32 ± 6.45 (n = 5) | 0.236 | 6.10 (n=1) | 6.20 (n = 1) | – | 8.00 (n=1) | 6.10 (n=1) | – |

*Data presented as mean ± standard deviation, median (interquartile range), or n (%).*

*FG, fasting glucose; FI, fasting insulin; HOMA–IR, homeostasis model assessment of insulin resistance.*

**TABLE 5 |** Short-, medium-, and long-term therapeutic effects on blood lipid levels and risk stratification of atherosclerotic cardiovascular disease risk stratification.

| Data periods | n | Therapeutic effect base on blood lipid levels | | | | n | Therapeutic effect base on ASCVD risk stratification | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Unchanged group (n) | Changed group (n) | $X^2$ | p-Value | | Unchanged group (n) | Risk descended (n) | $X^2$ | p-Value |
| | **Hyperlipidemia group** | | | | | **High-risk group** | | | | |
| Short-term | 37 | 24 | 13 | 15.770 | <0.001 | 10 | 6 | 4 | 2.813 | 0.094 |
| Medium-term | 21 | 14 | 7 | 6.171 | 0.013 | 4 | 2 | 2 | 0.667 | 0.414 |
| Long-term | 10 | 6 | 4 | 2.813 | 0.094 | 2 | 1 | 1 | 1.726 | 1.789 |
| | **Hypolipidemia group** | | | | | **Medium-risk group** | | | | |
| Short-term | 22 | 14 | 8 | 7.486 | 0.006 | 15 | 10 | 5 | 3.840 | 0.050 |
| Medium-term | 17 | 9 | 8 | 8.010 | 0.005 | 9 | 4 | 5 | 4.431 | 0.035 |
| Long-term | 8 | 7 | 1 | 1.067 | 0.302 | 4 | 3 | 1 | 1.143 | 0.285 |
| | **Normal group** | | | | | **Low-risk group** | | | | |
| Short-term | 58 | 43 | 15 | 17.228 | <0.001 | 74 | 71 | 3 | 1.334 | 0.248 |
| Medium-term | 37 | 31 | 6 | 4.534 | 0.033 | 49 | 47 | 2 | 0.510 | 0.475 |
| Long-term | 18 | 13 | 5 | 3.716 | 0.054 | 21 | 21 | 4 | 2.446 | 0.118 |

*The definition of unchanged and changed of hyperlipidemia group were still hyperlipidemia and changed to normal.*
*The definition of unchanged and changed of hypolipidemia group were still hypolipidemia and changed to normal.*
*The definition of unchanged and changed of normal group were still normal and changed to abnormal, including hyperlipidemia and hypolipidemia.*
*The definition of unchanged of high-, medium-, and low-risk groups were still risk, medium-, and low-risk after WMT procedures, respectively.*

and HDL-C (5.1% to −7.3%), respectively. The comparison of drug treatment and WMT treatment shows that WMT has a reasonable therapeutic effect on hyperlipidemia, especially in reducing TC.

Many factors affect the outcome of FMT, namely, donor selection and preparation, sample handling, mode of administration, and colonization resistance (1). FMT-related AEs are challenges in the application of FMT. In most cases, FMT was well tolerated with mild gastrointestinal AEs (46). Zhang et al. showed washed microbiota preparation by repeated centrifugation plus suspension for three times based on an automatic purification system that significantly reduced AEs (9). Our WMT program is based on Zhang's standard. No serious AEs were found during or after WMT. Craven indicated that the improvement in small intestinal permeability was associated with allogenic WMT in patients with NAFLD and MS after WMT (12). In our study, patients who mainly received WMT had the characteristics of functional gastrointestinal disease. The results showed that the improvement of dyslipidemia was due to the improvement of intestinal permeability and gut microbiota after WMT. The results of clinical studies suggest that functional gastrointestinal diseases, WMT frequency, and BMI range are keys to the efficacy of WMT in the treatment of dyslipidemia. However, understanding the influence of the intestinal microbiota on metabolic diseases is in the initial stage, and data regarding the function of WMT on dyslipidemia are lacking. This is the first large-scale retrospective trial of dyslipidemia in China, which included hyperlipidemia, hypolipidemia, and normal blood lipid groups. We have established clinical evidence on the effect of WMT on lipid metabolism, which lays a foundation for the follow-up study of the effect of intestinal flora, metabolism, and human genome regulation on dyslipidemia. Furthermore, WMT may have potential bidirectional adjustment with lipid metabolism and may be a potential treatment for malnutrition. Taken together, these results suggest the beneficial effect of the microbiota in lipid metabolism; however, its mechanistic explanations need additional investigation.

This study has several limitations. Firstly, this study mainly focused on the analysis of clinical lipid and carbohydrate metabolism. Intestinal microbiota and metabolomics before and after WMT have not been evaluated. Therefore, the lowering effect of WMT on lipids and related microbiota remains unclear. Secondly, this is a single center study. A small number of patients returned to the hospital for long-term benefit evaluation of WMT treatment one month after the third treatment. Therefore, more data are needed to confirm the long-term efficacy of WMT in the treatment of dyslipidemia. Third, the risk factors of WMT on dyslipidemia, especially hyperlipidemia-related risk factors, may be insufficient. Fourth, we did not consider the potential confounding factors between the main symptoms for WMT treatment and blood lipids. Although the data show that WMT can improve dyslipidemia in the short and medium term, we should carefully interpret the research results of WMT and need large-scale prospective studies to further verify our conclusions. In the future, we plan to conduct a large sample prospective study to verify the effect of WMT on blood lipids.

## CONCLUSION

The retrospective analysis showed that WMT treatment changes blood lipids in patients with hyperlipidemia and hypolipidemia without serious AEs, whereas no risk was found in increasing blood lipids and ASCVD in the long-term. WMT treatment significantly decreased TC, TG, and LDL-C levels in the medium term for hyperlipidemia, but no significant difference in the long-term treatment. Therefore, the regulation of gut microbiota by WMT may indicate a new clinical method for the treatment of dyslipidemia.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

This study was conducted and approved by the Ethics Committee (No. 2017-98) in accordance with the Declaration of Helsinki at the First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, China. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

X-XH and FL designed the concept of the study. XL, ZD, and H-JZ collected and analyzed the data. H-HZ, WZ, and QL were the consultant for cardiology and pharmacology. FL and Y-LL wrote the draft manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## FUNDING

This study was supported by the Natural Science Foundation of Guangdong Province (2019A1515010125), the Department of Education of Guangdong Province (2019-GDXK-0013 and 2020KZDZX1132), and the Traditional Chinese Medicine Bureau of Guangdong Province (20201193).

## ACKNOWLEDGMENTS

We sincerely thank Dr. Ho-Shun Cheng (Department of Cardiology, Wanfang Hospital, Taipei Medical University) for serving as a cardiology consultant for the manuscript. We would like to thank all patients in the study and all funding agencies that supported the study.

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frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 29 April 2022
doi: 10.3389/fendo.2022.867600

# Identification and Validation of Autophagy-Related Genes in Diabetic Retinopathy

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**Specialty section:**
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

**Received:** 01 February 2022
**Accepted:** 14 March 2022
**Published:** 29 April 2022

**Citation:**
Wang N, Wei LF, Liu D, Zhang QY, Xia XB, Ding LX and Xiong SQ (2022) Identification and Validation of Autophagy-Related Genes in Diabetic Retinopathy. Front. Endocrinol. 13:867600. doi: 10.3389/fendo.2022.867600

**Background:** Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes, which is associated with damage of blood-retinal barrier and ischemia of retinal vasculature. It devastates visual acuity due to leakage of retinal vessels and aberrant pathological angiogenesis in diabetic patients. The etiology of DR is complex, accumulated studies have shown that autophagy plays an important role in the pathogenesis of DR, but its specific mechanism needs to be further studied.

**Methods:** This study chose the online Gene Expression Omnibus (GEO) microarray expression profiling dataset GSE146615 to carry on the research. Autophagy-related genes that were potentially differentially expressed in DR were screened by R software. Then, the differentially expressed autophagy-related genes were analyzed by correlation analysis, tissue-specific gene expression, gene-ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and protein-protein interaction (PPI) network analysis. Finally, retinal pigment epithelial cell line (ARPE-19) incubated with high glucose (HG) was used to mimic the DR model, and the mRNA level of key genes was verified by quantitative real-time polymerase chain reaction (qRT-PCR) *in vitro*.

**Results:** A total of 23 differentially expressed autophagy-related genes (9 up-regulated genes and 14 down-regulated genes) were identified by differential expression analysis. The analysis of tissue-specific gene expression showed that these differentially expressed autophagy-related genes were enriched in the retina. GO and KEGG enrichment analysis showed that differentially expressed autophagy-related genes were significantly enriched in autophagy-related pathways such as regulation of autophagy and macroautophagy. Then 10 hub genes were identified by PPI network analysis and construction of key modules. Finally, qRT-PCR confirmed that the expression of MAPK3 in the DR model was consistent with the results of bioinformatics analysis of mRNA chip.

**Conclusion:** Through bioinformatics analysis, we identified 23 potential DR autophagy-related genes, among which the down-regulated expression of MAPK3 may affect the occurrence and development of DR by regulating autophagy. It provides a novel insight into the pathogenesis of DR.

**Keywords: diabetic retinopathy, autophagy, differentially expressed genes, protein-protein interaction network, MAPK3**

## HIGHLIGHTS

- Differentially expressed genes (DEGs) related to autophagy in DR patients were identified.
- Major enrichment pathways of autophagy-related differential genes identified by bioinformatics, and the top 10 hub genes were identified.
- Experimental validation showed that down-regulation of MAPK3 gene might associated with DR by regulating autophagy.

## INTRODUCTION

Diabetic retinopathy (DR) is one of the most common and harmful microvascular complications of diabetes, and it is also a common eye disease that causes blindness (1). Most patients progress into DR after 20 years of diabetes (2, 3), and about half of the patients with untreated proliferative retinopathy will go blind within 5 years (4). It imposes a heavy economic burden on the families, health systems and societies. Previous studies have shown that oxidative stress, endoplasmic reticulum stress, apoptosis and autophagy (5–8) can induce mild and chronic retinal inflammation in retinal tissues (9), resulting in retinal vessel hyperpermeability (10), retinal angiogenesis and retinal neuron injury (11).

Autophagy is a process in which autophagy engulfs its own cytoplasmic proteins or organelles and encapsulates them into vesicles, fuses with lysosomes to form autophagy lysosomes, and degrades the contents of autophagy. It meets the demand of cellular metabolic needs and is involved in the renewal of some organelles. It is highly conserved in evolution and crucial for the degradation and circulation of cellular substances (12). Autophagy disorders may have fatal consequences to the cells and result in some ocular diseases (13), such as age-associated macular degeneration (AMD), glaucoma and other eye diseases (14–21). For example, glucosamine (GlcN) can induce autophagy to reduce photoreceptor outer segment (POS)-derived lipofuscin-like autofluorescence (LLAF) in retinal pigment epithelial (RPE) cells through the AMPK-mTOR pathway, which provides a novel insight for AMD (19). In the glaucoma model, the neurosteroid allopregnanolone (AlloP) can reduce the apoptosis of retinal ganglion cells (RGC) by activating autophagy (21).

Recently, the role of autophagy in DR has been gradually uncovered. Damage of outer blood-retina barrier due to diabetes is key to the pathogenesis of diabetic macular edema. It leads to decrease of visual acuity in patients with DR. RPE cells are the main components of the outer blood-retina barrier. It has been demonstrated that the outer blood-retina barrier was destroyed in diabetes by modulation of autophagy in RPE cells. However, the underlying mechanism of autophagy in devastating RPE cells under diabetes stress is still unclear. In this study, we analyzed the previously published dataset containing samples from DR and non-diabetic individuals to identify the differentially expressed genes (DEGs) related to DR. It was further analyzed to figure out the correlation between the differentially expressed autophagy-related genes in DR. Then, functional enrichment and protein-protein interaction (PPI) network analysis were used to clarify the interaction and biological function of these genes. 10 hub genes were identified by PPI network analysis, and it was further verified in the DR model by quantitative real-time polymerase chain reaction (qRT-PCR) *in vitro*. We found that the expression of MAPK3 was consistent with the results of bioinformatics analysis of mRNA chip in the DR model, suggesting it was involved in the development of DR by regulating autophagy (**Figure 1**).

## MATERIALS AND METHODS

### Microarray Data and Autophagy-Related Genes Datasets

The mRNA expression profile dataset GSE146615 was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). 232 autophagy-related genes were obtained from The Human Autophagy Database (http://www.autophagy.lu/index.html). GSE146615 was in GPL10558 platform. The dataset included 7 individuals without diabetes, 7 patients with type 1 diabetes (T1D) without complications from DR and 8 patients with DR. Lymphoblastoid cell lines (LCLs) were extracted from the peripheral blood of 22 individuals and processed with standard glucose (SG) and high glucose (HG), respectively. In this study, the data of LCLs of 7 non-diabetic individuals treated with SG and LCLs of 8 patients with DR treated with HG were extracted for follow-up analysis.

### Differential Expression Analysis of Autophagy-Related Genes

The DEGs related to autophagy were screened by the "limma" package in R software. The genes with P value < 0.05 were considered to be DEGs. Then, the "heatmap" and "ggplot2" packages in R software are used to draw heatmap and volcano plot and box plot respectively to visualize the differential genes.

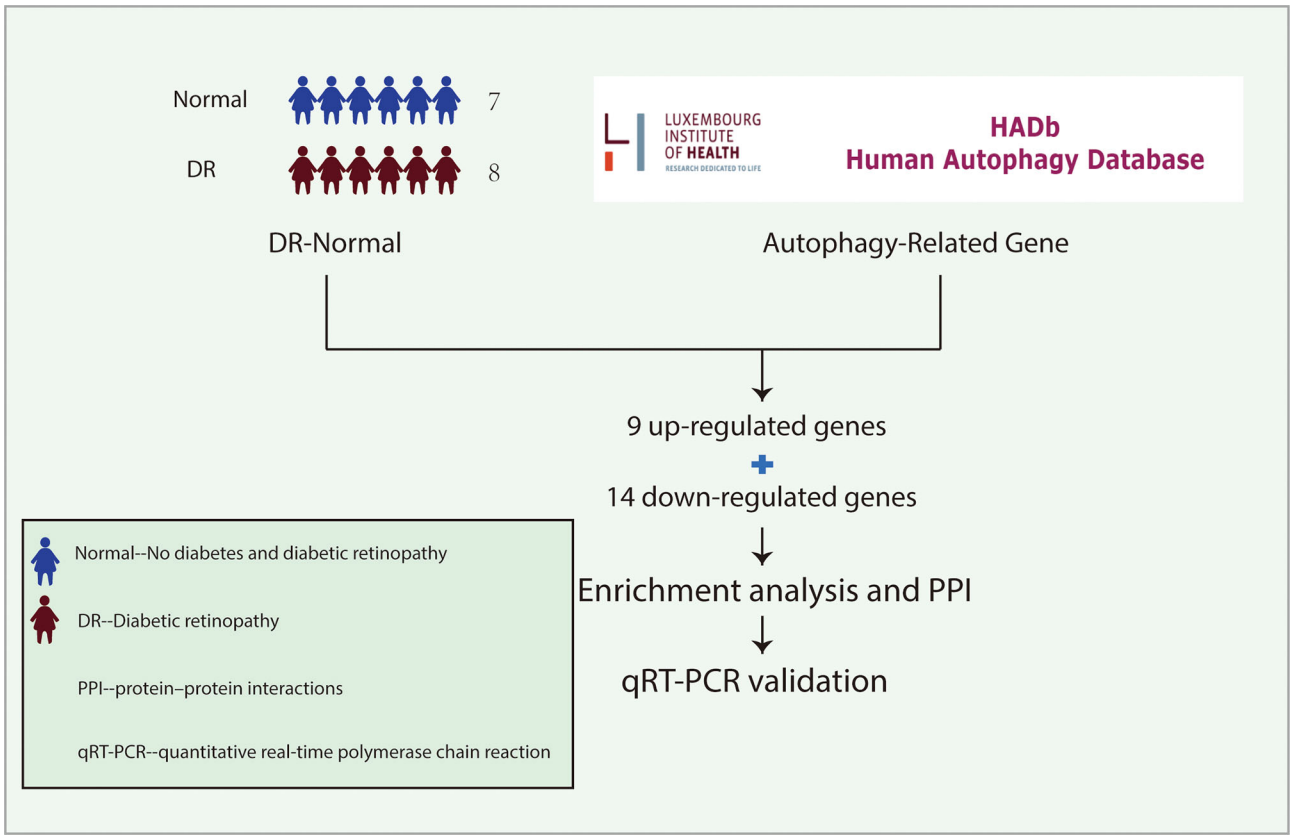



**FIGURE 1** | The idea of experimental design. The gene expression profiles of LCLs extracted from the peripheral blood of 7 non-diabetic individuals and 8 patients with DR were cultured under SG and HG conditions respectively in the GSE146615 dataset. 232 autophagy-related genes were collected from The Human Autophagy Database. Then, 9 up-regulated genes and 14 down-regulated genes were screened by differential analysis. After enrichment analysis and PPI network construction, 10 hub genes were identified. Finally, qRT-PCR was used to verify *in vitro* DR model. LCLs, lymphoblastoid cell lines; DR, diabetic retinopathy; SG, standard glucose; HG, high glucose; PPI, protein-protein interaction; qRT-PCR, quantitative real-time polymerase chain reaction.

## Correlation Analysis of DEGs and Tissue-Specific Gene Expression Analysis

The correlation analysis of differentially expressed autophagy-related genes was carried out by using Spearman correlation in the "corrplot" package of R software. The tissue-specific expression of differentially expressed autophagy-related genes was analyzed on the BioGPS website (http://biogps.org).

## GO and KEGG Pathway Enrichment Analysis of Differentially Expressed Autophagy-Related Genes

We used the "GO plot" package in R software to analyze the functional enrichment of differentially expressed autophagy-related genes, including Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. In the GO analysis, we evaluated the enriched biological processes (BPs), molecular functions (MFs) and cellular components (CCs).

## PPI Network

The STRING database (https://string-db.org/) of known and predicted protein-protein interactions was used to analyze the PPI network of differentially expressed autophagy-related genes, and then the Cytoscape v3.9.0 software was used to visualize and construct the PPI network, and finally 10 hub genes were identified by cytoHubba plugin.

## Cell Culture and Cell Grouping

Human retinal pigment epithelial cell line (ARPE-19) was purchased from the Procell Life Science & Technology in China (CL-0026) and cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% Antibiotic-Antimycotic (Gibco, USA) at 37°C with 5% carbon dioxide. When the cell density reached 80%, it was washed with PBS (Gibco, USA) and treated with 0.05% trypsin (Gibco, USA) for passage at the proportion of 1:3.

The logarithmic growth phase cells with good growth condition were inserted into 6-well plates with $1.2\times10^6$ cells per well. The cells were divided into two groups: the HG treated group and the normal group. The HG treated group was cultured in the medium containing 30mmol/L D-glucose, and the normal group was cultured in the SG medium. The 2.5mL medium was added to each well and cultured at 37°C for 48 hours.

## RNA Extraction and qRT-PCR

RNA was extracted from ARPE-19 cells using TRIzol kit (Invitrogen, USA) according to the manufacturer's plan, and 2 × SYBR Green qPCR Master Mix and UEIris II RT-PCR System using a First-Strand cDNA, as well as Synthesis Kit (Suzhou Yuheng Biological Co., Ltd.) were used for reverse transcription and qRT-PCR. The primers were designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China), and the sequence was listed in **Supplementary Table S1**. Configure the reaction mixture according to the system in **Supplementary Table S2**, gently swirl and centrifuge the reaction mixture, transfer it to the PCR plate, and carry out the experimental reaction according to the procedure in **Supplementary Table S3**. Finally, the results were analyzed by real-time PCR detection system (ABI). The expression level of mRNA was calculated by $2^{-\Delta\Delta Ct}$ method, and the relative expression level of gene mRNA was normalized by β-actin. Sterilized deionized water was used instead of nucleic acid template as negative control to ensure the quality of primers and no pollution of the system.

## Statistical Analysis

All the experimental data were statistically analyzed by GraphpadPrism software (version3.6.2), and 3 independent experiments were carried out. The gene expression level of the sample was compared by Student's t-test, and the difference was considered to be statistically significant when $P < 0.05$.

# RESULTS

## Identification of Differentially Expressed Autophagy-Related Genes

We downloaded the Expression profiling by array dataset GSE146615 from the GEO database, and selected LCLs cultured in SG from 7 non-diabetic individuals (Normal group) and 8 patients with T1D and proliferative diabetic retinopathy (PDR) cultured in HG (DR group). Next, we analyzed the expression of 232 autophagy-related genes in the sample by R software, identified 9 up-regulated genes and 14 down-regulated genes, and then plotted 23 differentially expressed autophagy-related genes between the DR group and the normal group (**Figures 2A, B**). In addition, the box chart showed the expression patterns of 23 autophagy-related genes differentially expressed between DR patients and normal samples (**Figure 2C**). The first five up-regulated genes include HGS, BAX, RAF1, TSC1 and ITPR1, and the first five down-regulated genes include CHMP4B, FKBP1A, CDKN1B, GABARAPL2 and RAB33B (**Table 1**).

## Correlation and Tissue-Specific Expression of Differentially Expressed Autophagy-Related Genes

In order to explore the expression correlation of these 23 autophagy-related genes, the correlation analysis has been performed by bioinformatics methods. The results showed that there was a high correlation between up-regulated genes and down-regulated genes, respectively (**Figures 3A, B**). At the same time, we identified the expression of these 23 genes in human retina by BioGPS. Except for CAMKK2, the expression levels of the other 22 genes in the retina were higher than the average levels in the tissues or organ systems of the whole body. Among them, the expression levels of 6 genes such as RAF1 in the human retina were more than three times the median, indicating that these autophagy-related genes were enriched in the human retina (**Table 2**).

## Functional and Pathway Enrichment of The Differentially Expressed Autophagy-Related Genes

In order to explore the potential function of differential genes more deeply from the level of biological function, we used R software for GO and KEGG enrichment analysis (**Table 3**). GO enrichment analysis showed that the differential genes were significantly enriched in 441 BPs, 20 MFs and 19 CCs. Among them, the most prominent projects involved regulation of autophagy, positive regulation of catabolic process, macroautophagy, positive regulation of autophagy (BPs); late endosome, mitochondrial outer membrane, organelle outer membrane (CCs); ubiquitin-like protein ligase binding, chaperone binding, BH domain binding (MFs) (**Figures 4A–D**). The relationship between these pathways was shown in the (**Figure 5A**). There were 12 common genes in the three most prominent pathways, namely CDKN1B, BAX, DAPK1, FOXO3, BAG3, MAPK3, CHMP4B, DRAM1, NPC1, CAMKK2, ITPR1 and TSC1 (**Figure 5B**). Besides, we analyzed the expression of differential genes in the significantly enriched pathway and showed the results in the Heatmap-like functional classification map (**Figure 5C**). In addition, the KEGG results showed that the enrichment was mainly in the process of autophagy, human cytomegalovirus infection and so on (**Figures 6A, B**).

## PPI Network Analysis and Hub Gene Identification

Toward a deeper understanding of the interaction between differentially expressed autophagy-related genes, we introduced these genes into the Search Tool for the Retrieval of Interacting Genes (STRING) to construct a PPI network (**Figure 7A**). The first 10 hub genes with the highest value were screened by Cytoscape (v3.9.0) (**Table 4**). Among them, TSC1, RAF1, RB1, ITPR1 is up-regulated and CDKN1B, MAPK1, FOXO3, DAPK1, MAPK3, BCL2L1 was down-regulated. The disorders of these autophagy motifs may be closely related to the occurrence and development of DR (**Figure 7B**).

## Validation the Differentially Expressed Autophagy-Related Genes in Diabetic Model

It was found that autophagy in RPE cells was related to damage of outer blood retinal barrier in diabetic retinopathy. Meanwhile, autolysosomes and autophagy associated markers were increased in RPE cells under HG condition (22–24). In this study, we incubated ARPE-19 cells with HG (30mM) to simulate an DR model *in vitro*. Interestingly, the expression of

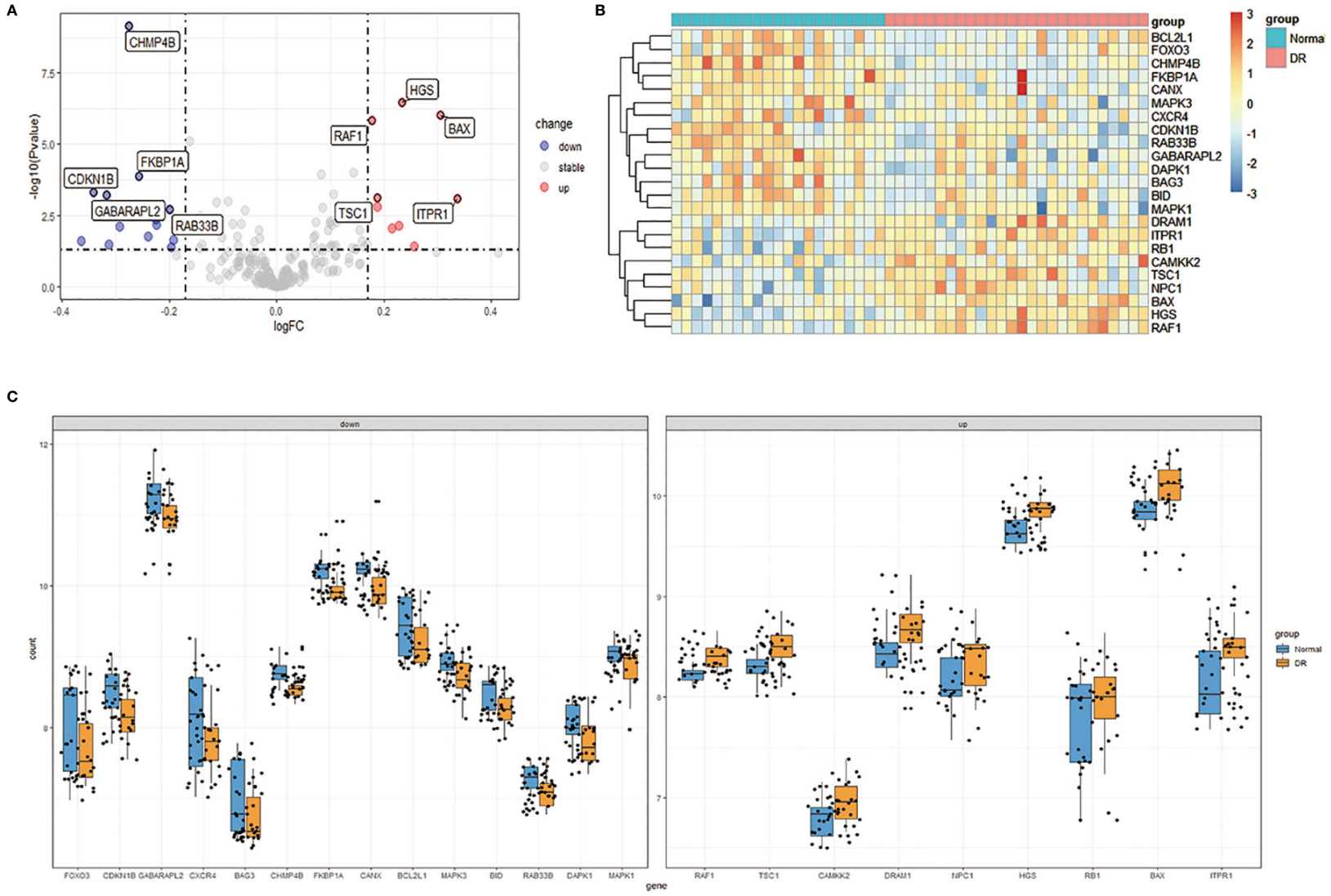



**FIGURE 2** | Differentially expressed autophagy-related genes in DR patients (DR group) and non-diabetic individuals (Normal group). **(A)**, Volcano plot of 232 differentially expressed autophagy-related genes. The red dots in the picture represent significantly up-regulated genes, blue dots represent significantly down-regulated genes, black dots represent genes that are not differentially expressed, and the five genes that are most significantly up-regulated or down-regulated are marked. **(B)**, The heatmap of 232 differentially expressed autophagy-related genes. Red represents up-regulated genes and blue represents downregulated genes. **(C)**, The boxplot of 23 differentially expressed autophagy-related genes in DR and normal samples. It includes 9 up-regulated genes and 14 down-regulated genes. DR, diabetic retinopathy.

**TABLE 1** | The 23 differentially expressed autophagy-related genes in DR samples compared to healthy samples.

| Gene Symbol | logFC | Changes | P-value | Adj. P-value | probe_id |
|---|---|---|---|---|---|
| HGS | 0.2329999 | Up | 3.340629E-07 | 3.965281E-05 | ILMN_1715994 |
| BAX | 0.3046402 | Up | 9.690973E-07 | 9.408659E-05 | ILMN_2321064 |
| RAF1 | 0.1775394 | Up | 1.512913E-06 | 1.344553E-04 | ILMN_1813489 |
| TSC1 | 0.1869165 | Up | 7.643098E-04 | 1.695813E-02 | ILMN_2246510 |
| ITPR1 | 0.3363179 | Up | 7.942211E-04 | 1.751126E-02 | ILMN_1789505 |
| CAMKK2 | 0.1882823 | Up | 1.550804E-03 | 2.798067E-02 | ILMN_1743021 |
| NPC1 | 0.2273093 | Up | 7.180077E-03 | 7.976330E-02 | ILMN_1713505 |
| DRAM1 | 0.2153663 | Up | 8.943841E-03 | 9.250777E-02 | ILMN_1669376 |
| RB1 | 0.2570772 | Up | 3.879564E-02 | 2.349566E-01 | ILMN_1696591 |
| CHMP4B | -0.2763448 | Down | 7.283285E-10 | 3.658531E-07 | ILMN_1771233 |
| FKBP1A | -0.2572678 | Down | 1.309686E-04 | 4.539371E-03 | ILMN_1757072 |
| CDKN1B | -0.3413209 | Down | 5.099296E-04 | 1.251711E-02 | IILMN_2196347 |
| GABARAPL2 | -0.3170168 | Down | 6.248193E-04 | 1.457351E-03 | ILMN_1796458 |
| RAB33B | -0.1992503 | Down | 2.001880E-03 | 3.376344E-02 | ILMN_1727738 |
| CANX | -0.2521261 | Down | 3.244758E-03 | 4.777541E-02 | ILMN_2401057 |
| MAPK3 | -0.2250177 | Down | 4.241640E-03 | 5.711548E-02 | ILMN_1667260 |
| BID | -0.2237670 | Down | 6.619973E-03 | 7.562566E-02 | ILMN_1763386 |
| BAG3 | -0.2929996 | Down | 7790892E-03 | 8451716E-02 | ILMN_1659766 |
| BCL2L1 | -0.2402096 | Down | 1.767939E-02 | 1.446571E-01 | ILMN_1654118 |
| MAPK1 | -0.1921206 | Down | 2.328004E-02 | 1.711489E-01 | ILMN_2235283 |
| FOXO3 | -0.3645971 | Down | 2.510350E-02 | 1.798444E-01 | ILMN_1681703 |
| CXCR4 | -0.3131656 | Down | 3.347568E-02 | 2.145289E-01 | ILMN_1801584 |
| DAPK1 | -0.1962781 | Down | 4.186049E-02 | 2.453443E-01 | ILMN_1708340 |

RAF1, RB1 and TPR1 were decreased and the level of TSC1 remained unchanged in HG treated ARPE-19 cells compared with the normal control cells. These genes were predicted to be upregulated according to aforementioned bioinformatics analysis. The level of CDKN1B, MAPK1, FOXO3, DAPK1 and BCL2L1 expected to be downregulated were found to be similar in both SG and HG treated ARPE-19 cells. The level of MAPK3 was decreased in ARPE-19 cells under HG condition, indicating MAPK3 and downstream signaling pathway might participate in the progression of DR by regulating autophagy in RPE cells (**Figure 8**).

## DISCUSSION

DR is a chronic progressive complication of patients with diabetes, which is an important cause of blindness in patients with diabetes (1, 25, 26). The pathogenesis of DR is complex. Current studies have

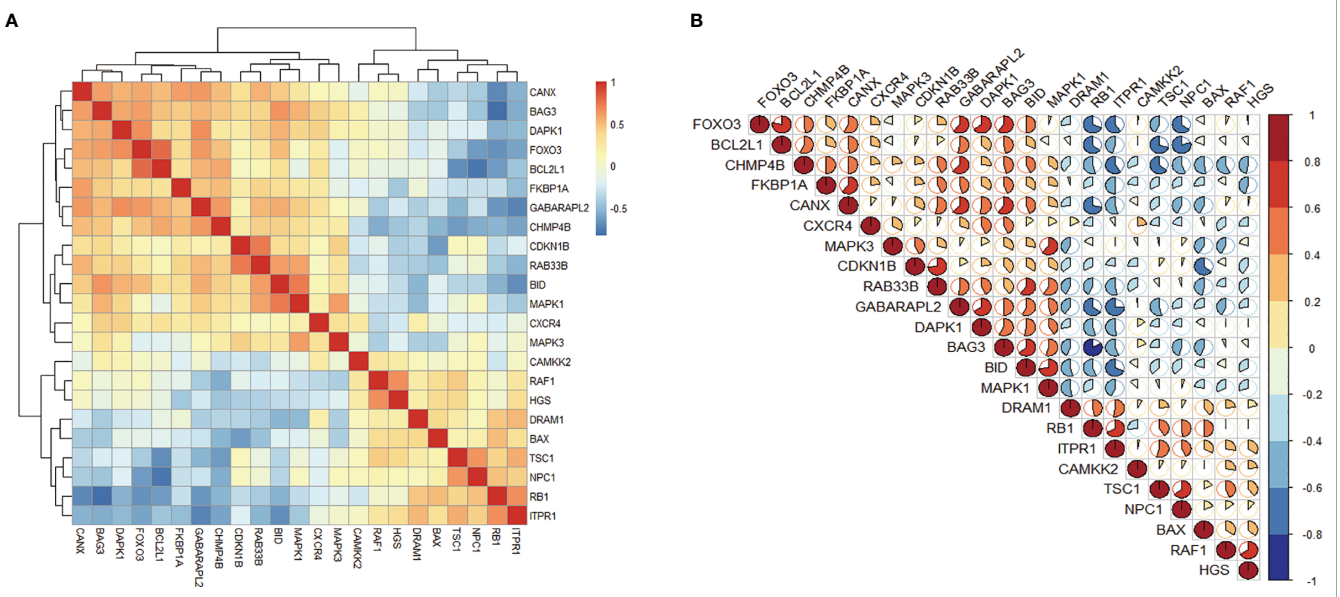



**FIGURE 3** | Correlation analysis of 23 differentially expressed autophagy-related genes. **(A, B)**, Correlation heatmap.

**TABLE 2 |** Expression levels of dierentially expressed genes identified by BioGPS in retinal tissues.

| Gene | Expression level | Median | Gene | Expression level | Median |
|---|---|---|---|---|---|
| >3×M | | | | | |
| RAF1 | 258.83±49.09 | 82.7 | CDKN1B | 456.62±99.99 | 120.3 |
| ITPR1 | 86.50±26.05 | 11 | GABARAPL2 | 2019.33±343.13 | 551.6 |
| | | | BAG3 | 86.45±2.13 | 23.5 |
| | | | CXCR4 | 25.30±2.50 | 4.3 |
| >1×M | | | CHMP4B | 687.08±74.31 | 376.3 |
| HGS | 130.57±16.11 | 63.5 | FKBP1A | 68.02±3.06 | 31.3 |
| BAX | 7.55±0.475 | 6.4 | RAB33B | 23.38±2.89 | 16.4 |
| TSC1 | 174.60±24.85 | 92.5 | CANX | 960.05±86.58 | 458.4 |
| NPC1 | 7.75±0.375 | 6.43 | MAPK3 | 152.32±45.69 | 77 |
| DRAM1 | 4.50±0.300 | 3.9 | BID | 11.25±0.675 | 10.2 |
| RB1 | 26.77±1.36 | 20.3 | BCL2L1 | 15.18±0.590 | 10.2 |
| | | | MAPK1 | 21.25±0.725 | 15.5 |
| | | | FOXO3 | 12.50±0.250 | 4.7 |
| | | | DAPK1 | 9.20±0.500 | 7.9 |
| <1×M | | | | | |
| CAMKK2 | 27.10±10.80 | | | | |

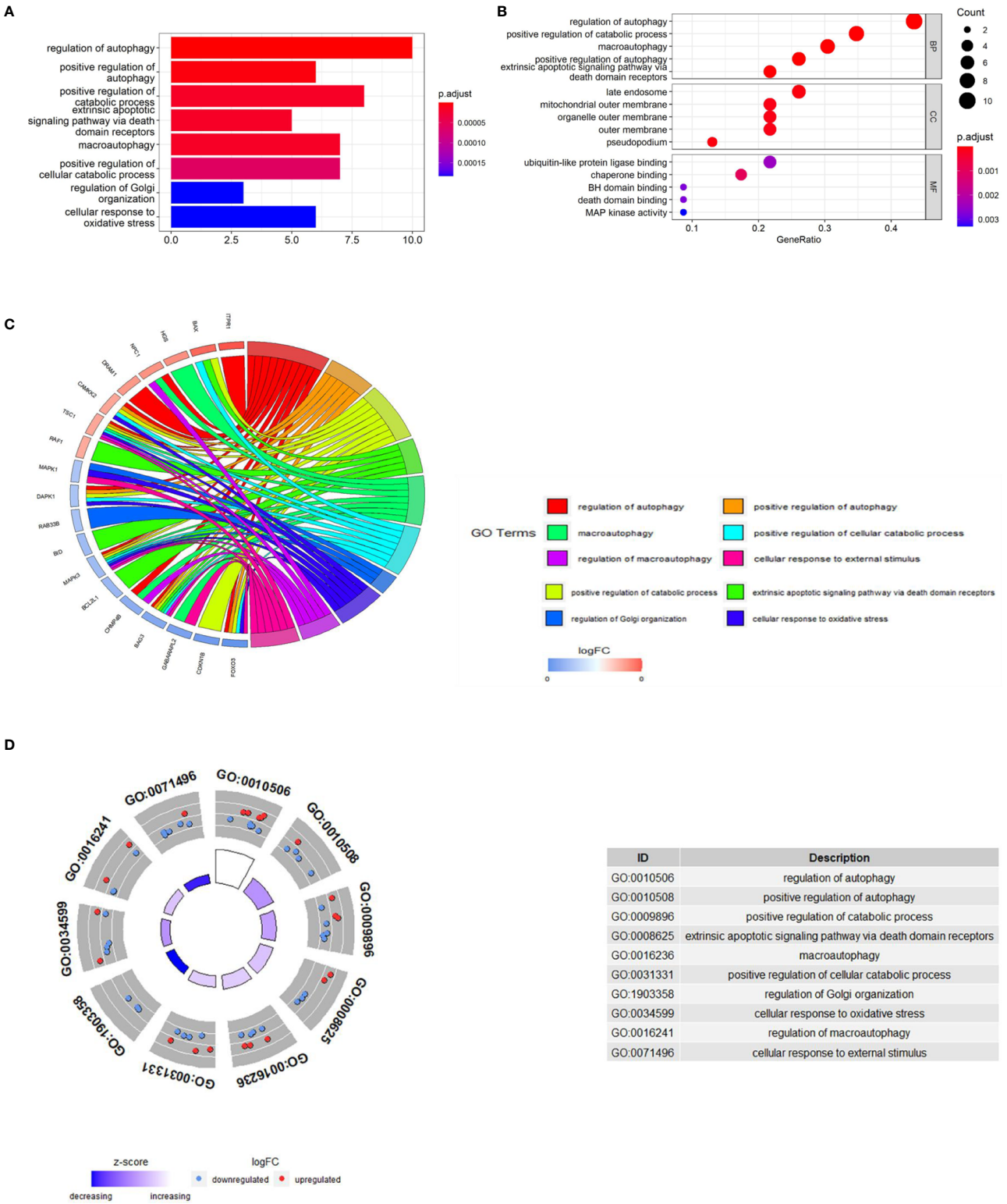
shown that a variety of metabolic pathways are involved in the formation of DR, such as the increase of polyol pathway, the activation of protein kinase C, oxidative stress and endoplasmic reticulum stress (5, 6). The abnormality of these pathways can not only cause microvascular complications such as the destruction of blood-retinal barrier (27, 28), but also lead to neurodegeneration and neuroinflammation (29). However, the exact pathogenic mechanism of DR has not been fully elucidated. Accumulating evidence show that autophagy, as the main catabolic pathway for the degradation and recycling of damaged proteins or organelles, may be involved in the pathogenesis of DR. Long-term hyperglycemia can cause autophagy disorder by inhibiting mTOR, resulting in the loss of retinal ganglion cells (30). In addition, low glucose (15mM) could induce mitochondrial autophagy in RPE cells, while under the stimulation of HG (50mM) or hydrogen peroxide, ROS could mediate the inactivation of mitochondrial autophagy-related proteins PINK1 and Parkin, and inhibited the occurrence of mitochondrial autophagy, indicating that glucose affected the occurrence of mitochondrial autophagy in RPE cells in a dose-dependent manner (31). Other studies have shown that knockout of high mobility group box1 (HMGB1) gene in RPE cells in the early stage of DR could save lysosome membrane permeabilization (LMP) through cathepsin B (CTSB)-dependent pathway. It restored the degradation ability of autophagy and thus protected RPE cells from apoptosis (32). The above results show that many forms of autophagy participate in the occurrence and development of DR, but its specific mechanism remains unclear. Further studies are required to broaden our knowledge of autophagy in the pathogenesis of DR.

In this study, we identified 23 potential autophagy-related genes in DR for the first time through bioinformatics analysis. GO and KEGG enrichment analysis showed that these genes

**TABLE 3 |** Functional and pathway enrichment analyses for module genes.

| Term | Description | Count | P-value | Adj. P-value | Genes |
|---|---|---|---|---|---|
| Biological processes | | | | | |
| GO:0010506 | Regulation of autophagy | 10 | 2.07E-12 | 2.88E-09 | TSC1, ITPR1, CAMKK2, NPC1, DRAM1, CHMP4B, MAPK3, BAG3, FOXO3, DAPK1 |
| GO:0010508 | Positive regulation of autophagy | 6 | 5.40E-09 | 3.76E-06 | TSC1, CAMKK2, MAPK3, BAG3, FOXO3, DAPK1 |
| GO:0009896 | Positive regulation of catabolic process | 8 | 3.53E-08 | 1.64E-05 | |
| Cellular component | | | | | |
| GO:0005770 | Late endosome | 6 | 6.11E-07 | 7.78E-05 | HGS, NPC1, CHMP4B, MAPK3, MAPK1, CXCR4 |
| GO:0005741 | Mitochondrial outer membrane | 5 | 2.75E-06 | 1.24E-04 | BAX, RAF1, BID, BCL2L1, FOXO3 |
| GO:0031968 | Organelle outer membrane | 5 | 4.96E-06 | 1.40E-04 | |
| Molecular functions | | | | | |
| GO:0051087 | Chaperone binding | 4 | 7.08E-06 | 9.56E-04 | BAX, TSC1, CDKN1B, BAG3 |
| GO:0044389 | Ubiquitin-like protein ligase binding | 5 | 3.61E-05 | 2.44E-03 | HGS, RB1, GABARAPL2, BID, CXCR4 |
| GO:0004674 | Protein serine/threonine kinase activity | 5 | 1.64E-04 | 3.29E-03 | RAF1, CAMKK2, MAPK3, MAPK1, DAPK1 |
| KEGG pathway | | | | | |
| hsa04140 | Autophagy – animal | 6 | 2.11E-08 | 3.12E-06 | GABARAPL2, RAB33B, MAPK3, BCL2L1, MAPK1, DAPK1 |
| hsa04068 | FoxO signaling pathway | 5 | 7.37E-07 | 5.46E-05 | CDKN1B, GABARAPL2, MAPK3, MAPK1, FOXO3 |
| hsa05163 | Human cytomegalovirus infection | 5 | 8.23E-07 | 1.12E-04 | BAX, RAF1, TSC1, ITPR1, RB1 |

*The top 3 terms were selected based upon Adj. P-value rankings when>3 terms were enriched for a given category.*




| ID | Description |
|---|---|
| GO:0010506 | regulation of autophagy |
| GO:0010508 | positive regulation of autophagy |
| GO:0009896 | positive regulation of catabolic process |
| GO:0008625 | extrinsic apoptotic signaling pathway via death domain receptors |
| GO:0016236 | macroautophagy |
| GO:0031331 | positive regulation of cellular catabolic process |
| GO:1903358 | regulation of Golgi organization |
| GO:0034599 | cellular response to oxidative stress |
| GO:0016241 | regulation of macroautophagy |
| GO:0071496 | cellular response to external stimulus |

**FIGURE 4** | GO enrichment analysis of 23 differentially expressed autophagy-related genes, including BPs, CCs and MFs. **(A)**, Bar plot of enriched GO terms. **(B)**, Bubble plot of enriched GO terms. **(C)**, Chordal graph of enriched GO terms. It shows the relationship between DEGs and the first 10 enriched GO pathways. **(D)**, Eight Diagrams of enriched GO terms. GO, Gene Ontology; BPs, biological processes; CCs, cellular components; MFs, molecular functions; DEGs, differentially expressed genes.

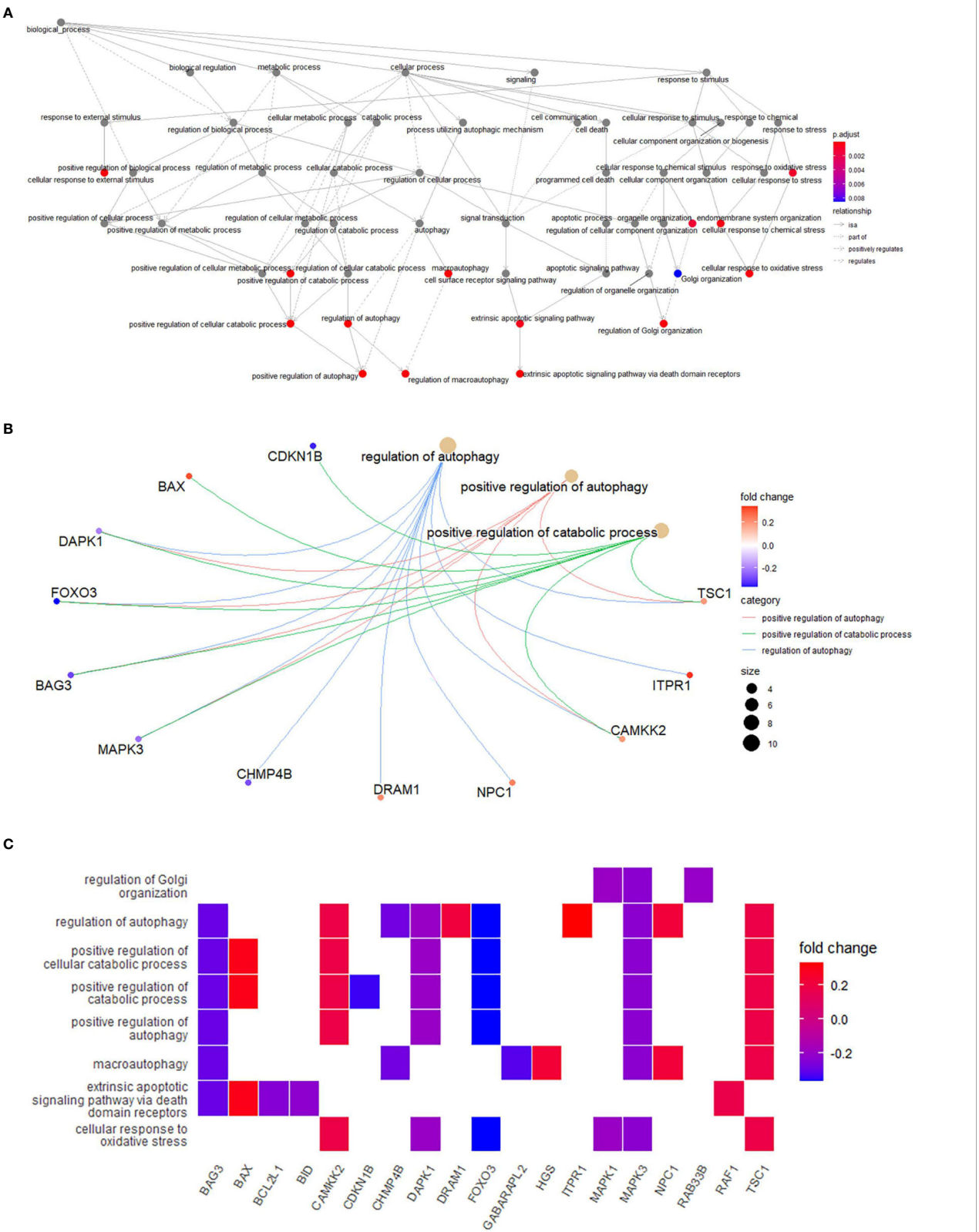



**FIGURE 5** | **(A)**, Relationships between enriched pathways. **(B)**, Common genes in the most top pathways. **(C)**, Heatmap-like functional classification.

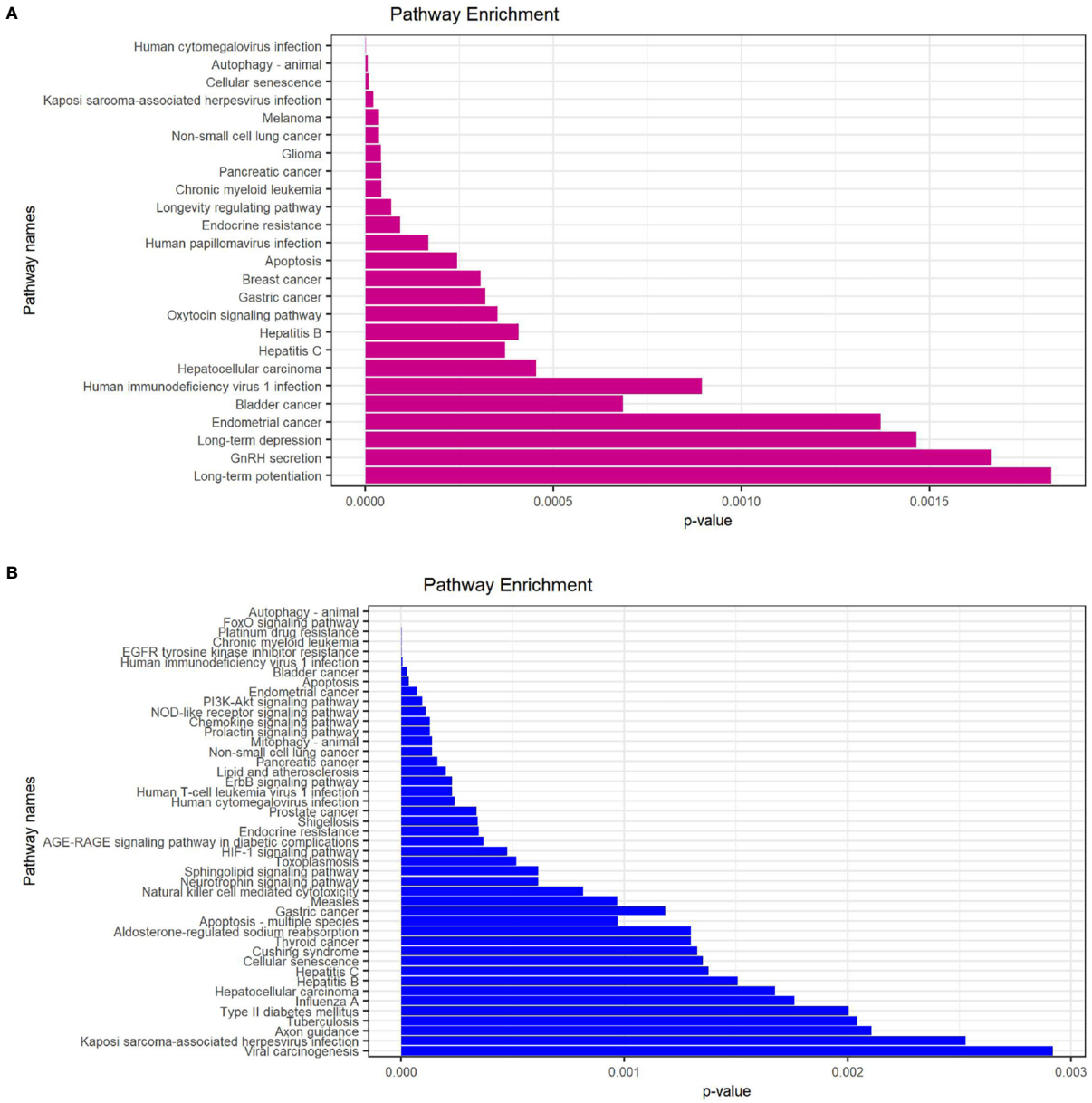



**FIGURE 6** | KEGG enrichment analysis of 23 differentially expressed autophagy-related genes. **(A)**, KEGG analysis of 9 up-regulated expressed autophagy-related genes. **(B)**, KEGG analysis of 14 down-regulated expressed autophagy-related genes. KEGG, Kyoto Encyclopedia of Genes and Genomes.

were closely related to regulation of autophagy, positive regulation of catabolic process, macroautophagy and other signal pathways. Next, we further identified 10 hub genes related to DR, including TSC1, RAF1, RB1, ITPR1, CDKN1B, MAPK1, FOXO3, DAPK1, MAPK3 and BCL2L1 by using PPI network and key module analysis. The function of these genes in the occurrence of DR has been extensively studied. For example, Ras/Raf-1/MEK/ERK signal cascade can promote the activation of MMP9, and Raf kinase can also interact with VEGF to promote the loss of retinal capillary cells, which eventually leads to the development of DR (33–36). Overexpression of Raf-1 Kinase Inhibitory Protein (RKIP) can prevent the occurrence of diabetic retinal neurodegeneration by inhibiting p38-MAPK pathway (37). Hu-zhang-qing-mai-yin (HZQMY) can regulate P38 and NF-κB pathways by targeting MAPK3 and inhibit the release of ROS under HG exposure in a dose-dependent manner, thus inhibiting the proliferation of human retinal capillary endothelial cells (HRCECs) and having a certain effect on DR (38). Studies have shown that these autophagy-related genes regulate autophagy activity in tumor, cerebral

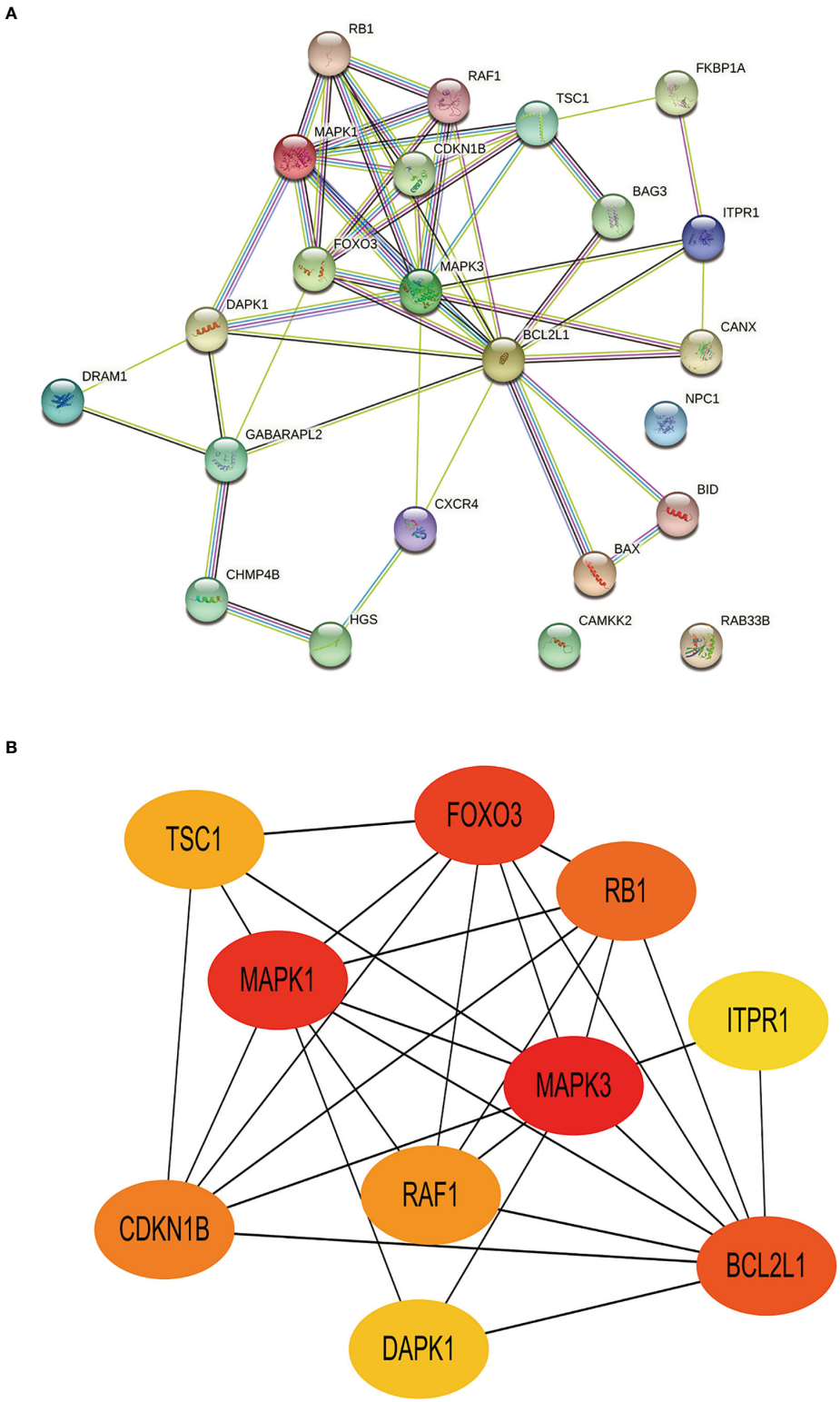



**FIGURE 7** | Construction of PPI network and identification of hub genes. **(A)**, The PPI between 23 differentially expressed autophagy-related genes was constructed by using the STRING database. The node represents the gene, and the edge represents the relationship between the genes. **(B)**, The top 10 key genes were screened through the PPI network map. Different colors in the image only represent different genes and have no other substantive meaning. PPI, protein-protein interaction.

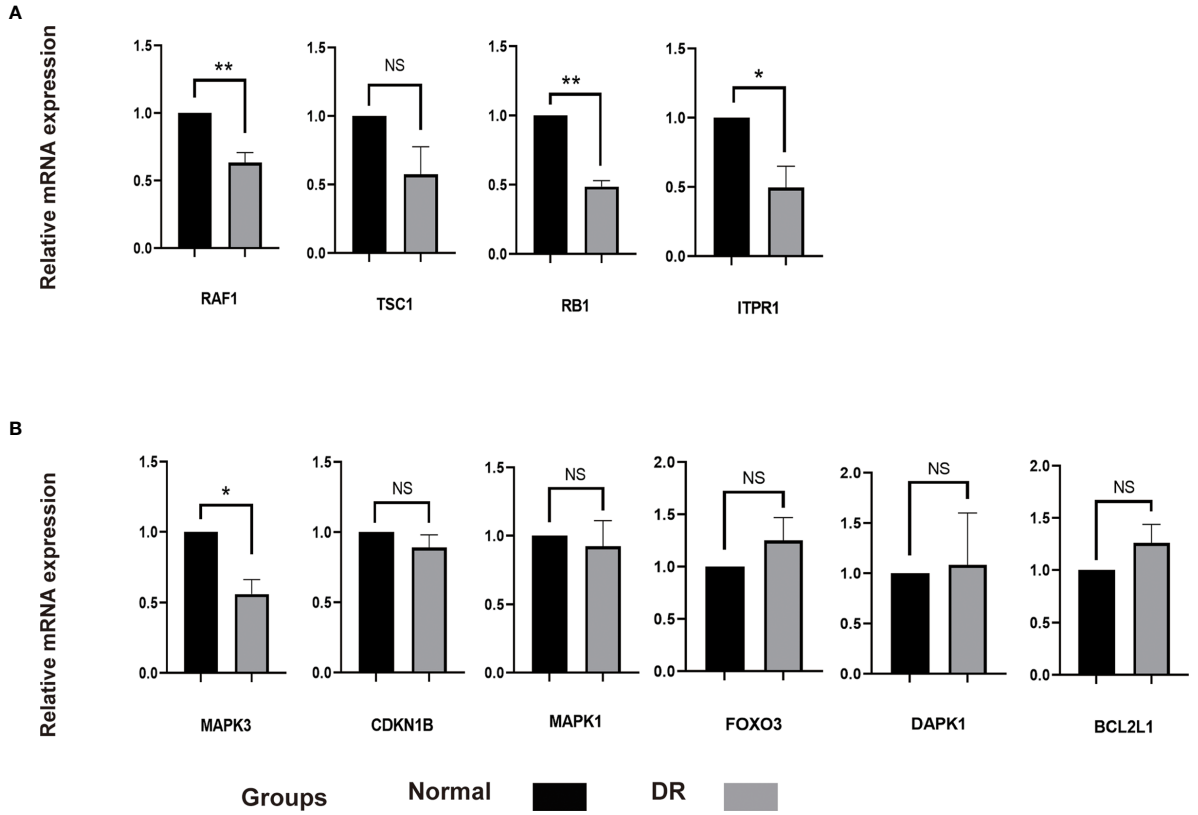



**FIGURE 8** | The mRNA level of 10 hub genes were measured in ARPE-19 cells. **(A)**, The mRNA level of RAF1, TSC1, RB1 and ITPR1 were evaluated in cell samples by qRT-PCR. **(B)**, The mRNA level of MAPK3, CDKN1B, MAPK1, FOXO3, DAPK1 and BCL2L1 were measured in cell samples by qRT-PCR. P-values were calculated using a two-sided unpaired Student's t-test. *P < 0.05; **P < 0.01; ns, non-significant. ARPE-19, retinal pigment epithelial cell line; qRT-PCR, quantitative real-time polymerase chain reaction.

**TABLE 4** | Top 10 in network ranked by MCC method.

| Rank | Gene ID | Gene name | Score | Changes |
|---|---|---|---|---|
| 1 | MAPK3 | Mitogen-Activated Protein Kinase 3 | 278 | Down |
| 2 | MAPK1 | Mitogen-Activated Protein Kinase 1 | 270 | Down |
| 3 | FOXO3 | Forkhead Box O3 | 266 | Down |
| 4 | BCL2L1 | BCL2-like 1 | 261 | Down |
| 5 | RB1 | Retinoblastoma 1 | 240 | Up |
| 6 | CDKN1 | Cyclin-Dependent Kinase Inhibitor 1B Raf-1 | 144 | Down |
| 7 | B RAF1 | Proto-Oncogene | 120 | Up |
| 8 | TSC1 | Tuberous Sclerosis 1 | 26 | Up |
| 9 | DAPK1 | Death-Associated Protein Kinase 1 | 10 | Down |
| 10 | ITPR1 | Inositol 1,4,5-Triphosphate Receptor, Type 1 | 7 | Up |

ischemic stroke and osteoporosis (39–42). However, the role of these genes in modulating autophagy in DR has not been fully explored.

In current study, HG treated ARPE-19 cells were used as DR model to testify the function of potential autophagy-related genes. It is due to following reasons. Firstly, the dysfunction and loss of RPE cells has been found in diabetic model. It is associated with macular edema arising from diabetes-induced disruption of outer blood-retinal barrier. Therefore, RPE cells have been widely utilized as *in vitro* model for DR research (22). Secondly, both autophagy associated markers and autolysosomes are obviously detected in HG treated RPE cells, indicating this *in vitro* DR model is suitable for autophagy investigation (23, 24). Among 10 predicted DR-related hub genes, we showed that only the expression of MAPK3 was consistent with that of bioinformatics analysis of mRNA chip. We speculate that because the bioinformatics results were originated from peripheral blood lymphocytes of DR patients and non-diabetic individuals, the differences in cell type and culture condition may

change gene expression and provide contradicting results. MAPK3 has been demonstrated to modulate autophagy by regulating mTOR pathway and Beclin-1 expression (43). Further investigation is required to elucidate the way by which MAPK3 control autophagy in DR models and cells.

## CONCLUSIONS

To sum up, 23 potential autophagy-related genes in DR were identified by bioinformatics analysis, 10 hub genes TSC1, RAF1, RB1, ITPR1, CDKN1B, MAPK1, FOXO3, DAPK1, MAPK3, BCL2L1 were identified by constructing PPI network and identifying key modules. MAPK3 was preliminarily identified by *in vitro* experiments, which may affect the occurrence and development of DR by regulating autophagy. In the future, further experiments are needed to investigate the regulatory role of MAPK3 in DR models in order to clarify its value as potential clinical biomarkers or therapeutic targets.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

NW analyzed the data. NW and LFW drafted the first draft. DL, QYZ, LXD, and XBX edited and provided comments to improve the manuscript. SQX designed this experiment and reviewed and revised the manuscripts. All authors contributed to the article and approved the final manuscript.

## FUNDING

This study was financially supported by The National Natural Science Foundation of China (No.81974137 to SQX), The National Natural Science Foundation of China (No.82070966 to LD), The Natural Science Foundation of Hunan Province (No.2019JJ40507 to XS), and the Science and Technology Innovation Program of Hunan Province (No.2021RC3026 to LD).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.867600/full#supplementary-material

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ORIGINAL RESEARCH
published: 26 July 2022
doi: 10.3389/fendo.2022.890218

# Heat Shock Proteins 70 Regulate Cell Motility and Invadopodia-Associated Proteins Expression in Oral Squamous Cell Carcinoma

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**Specialty section:**
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology*

**Received:** *05 March 2022*
**Accepted:** *20 June 2022*
**Published:** *26 July 2022*

**Citation:**
*Ding L-X, Zhang J, Yang S-S, Wu J, Su T and Wang W-M (2022) Heat Shock Proteins 70 Regulate Cell Motility and Invadopodia-Associated Proteins Expression in Oral Squamous Cell Carcinoma. Front. Endocrinol. 13:890218. doi: 10.3389/fendo.2022.890218*

**Background:** Many studies have shown that diabetes is often closely related to oral squamous cell carcinoma (OSCC) occurrence and metastasis. Heat shock protein 70 (Hsp70) is a molecular chaperone related to diabetes complications. This study aims to investigate the role of Hsp70 in OSCC in expression of invadopodia-associated proteins.

**Methods:** The expressions and correlation of HSP70, Hif1α, MMP2, MMP14, and cortactin were examined using bioinformatics analysis and verified by OSCC tissue microarrays. Assay *in vitro* was performed to analyze cell migration capacity after treatment with or without the HSP70 inhibitor.

**Results:** The expressions of invadopodia-associated proteins were enhanced in OSCC tissues compared with paracarcinoma tissues and partially correlated with HSP70. Inhibiting HSP70 significantly decreased the cell viability, proliferation, and migration of OSCC cells.

**Conclusions:** HSP70 may be involved in invadopodia-associated proteins in OSCC cells, which provides a promising method for treatment of OSCC metastasis.

**Keywords: invadopodia-associated proteins, Hif1α, heat shock proteins 70, oral squamous cell carcinoma, bioinformatics analysis**

## INTRODUCTION

Oral squamous cell carcinoma (OSCC) is diagnosed with 350,000–400,000 new cases every year around the world (1). Although the advanced multimodal treatment strategy has improved the prognosis and survival rate of OSCC patients, the 5-year survival rate still is about 50% these years (2, 3). The cause of occurrence and metastasis of OSCC is still unclear. Previous studies have proved that the occurrence and development of oral squamous cells may be closely related to diabetes (4).

The role of heat shock proteins 70 (Hsp70) in a variety of cancers and diabetes has been paid more attention in recent years. The expression level of HSP70 was demonstrated to be elevated remarkably in various cancers (5). The high expression level of Hsp70 is associated with the tumor

resistance to chemotherapeutic agents (6, 7). HSP70 may play an important role in diabetes mellitus through a mechanism that interferes with the expression of cytokines, matrix metallopeptidase 9 (MMP9), and the antioxidant enzyme superoxide dismutase 2 (8–10). However, as far as we know, whether HSP70 is involved in the metastasis of oral cancer has not been reported.

Invadopodia, a subcellular membrane structure that can degrade the extracellular matrix (ECM), plays an essential role in the invasive migration and metastasis of cancer cells (11, 12). Proteinase like MMP2 and MMP9 are secreted at invadopodia to promote the degradation of ECM (13). The upregulation of some key invadopodia molecules in cancer cells including the matrix metalloproteinase MT1-MMP(MMP14) and the actin assembly protein (cortactin) is associated with patients poor prognosis (14). Tumor microenvironment such as growth factors, hypoxia, and PH was reported to affect the formation and function of invadopodia (15).

In this study, we attempt to explore the potential role of Hsp70 in OSCC expression of invadopodia-associated proteins. The cell viability, proliferation potential, and migratory ability of OSCC cells were examined after inhibiting Hsp70. We discovered that HSP70 may be important for the expression of invadopodia-associated protein.

# MATERIALS AND METHODS

## Ethics Statement

The present study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (Hunan, China) and was performed according to the Declaration of Helsinki guidelines on experimentation involving human subjects. Written informed consent was obtained from all participants.

## Patients

OSCC and matched paracancer tissues in the Department of Pathology, Department of Stomatology, Xiangya Hospital, Central South University were determined by two independent pathologists according to the 2006 WHO Classification System (16). In collaboration with Shanghai Biochip Co., LTD. (China), tumor tissue microarray was constructed, including 117 OSCCs and 56 matched paracancer tissues as previous described (17).

## Reagents, Antibodies, Cell Lines, and Culture

Apoptozole (APO, HSP70 inhibitor; catalog no. S8365) was purchased from Selleck China subsidiary (Shanghai, China). Antibodies HSP70 (catalog no. 4873), Hif1α (catalog no. 8690), cortactin (catalog no. 3502), and MMP2 (catalog no. 40994) were acquired from Cell Signaling Technology (Danvers, MA, USA). MT1-MMP (MMP14; catalog no. 14552-1-AP) was purchased from Proteintech (Wuhan, China). Human OSCC cell line (Cal27) was purchased from ATCC. Cal27 cells were cultured in Dulbecco's minimum essential medium (Hyclone, Logan, UT, USA), containing10% fetal bovine serum (Gibco, Carlsbad, CA, USA) in oxygen concentrations for normoxia (21% $O_2$) at 37°C in Anoxomat chambers (Mart Microbiology, Lichtenvoorde, the Netherlands).

## Immunohistochemistry

The sections of OSCC tissue arrays were deparaffinized in xylene and rehydrated in a graded series of ethanol and double-distilled water before subjected to heat-induced antigen retrieval. After incubated with primary antibodies (HSP70, 1:200; Hif1α, 1:100; MMP2, 1:200; MMP14, 1:50; cortactin, 1:100) overnight at 4°C, the secondary antibody was incubated at room temperature for 1 h. All slices were scanned by an Aperio ScanScope CS scanner (Epistem, Cambridge, MA) and quantified using Aperio Quantification software (Version 9.1, Epistem) for staining quantification. Histoscore of membrane and nuclear staining was calculated as a percentage of different positive cells using the formula (3+) ×3 + (2+) × 2 + (1+) × 1. Histoscore of pixel quantification was calculated as total intensity/total cell number. The threshold for scanning of different positive cells was set according to the standard controls provided by Aperio.

## Cell Counting Kit-8 Assay

When plated in 96-well plates at $2 \times 10^3$ cells per well, Cal27 cells were cultured with Cell Counting Kit (CCK8; BioTime, Shanghai, China) for 24 and 48 h, respectively. Absorbance at 450nm was measured with the microplate reader (FlexStation 3, Molecular Devices, USA).

## EdU (5′-Ethynl-2'-Deoxyuridine) Staining Assay

The effects of Apo on cell proliferation were assessed by the Cell-Light™ 5′-ethynl-2′-deoxyuridine (EdU) Apollo®488 *In Vitro* Imaging Kit according to the manufacturer's instructions. The number and proportion of the cells incorporated EdU were visualized and the fluorescence intensity was quantified using Image J1.42.

## Wound Healing Assay and Transwell Invasion Assay

Wound healing assay and Transwell invasion assay were performed as previously described (18) and details were described in Supplementary Material and Methods in S1 File.

## The Cancer Genome Atlas Analysis

In order to obtain a correlation between the target genes among head and neck cancer (HNSC) patients, gene expression quantification data of HNSC patients were downloaded from The Cancer Genome Atlas (TCGA) database. Then, the expression quantification data of HSPA4, HIF1A, MMP14, MMP2, and CCTN in TCGA HNSC dataset were extracted and processed by R × 64 3.5.1 with the package ggplot2, showing the inner relationship among above genes in HNSC intuitively. Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) was used to predict the interactions between proteins (19).

## Statistical Analysis

All experiments were performed at least in triplicate and each experiment was repeated for three times. GraphPad Prism Software (GraphPad Software Inc) was used for statistical analysis, and the data are presented as means ± standard error of mean (SEM). One-way analysis of variance (ANOVA) and Student's t-test were used to analyze the differences as compared with control group and among each group. The relationship among the expression of Hsp70, Hif1α, MMP14, MMP2, and cortactin was examined using two-tailed Pearson's statistics. The differences were considered statistically significant if the P-value $< 0.05$.

# RESULTS

## The Overexpression of Hsp70, Hif1α, MMP2, MMP14, and Cortactin in OSCC

We explored the expression of Hsp70, Hif1α, MMP2, MMP14, and cortactin in OSCC using tissue arrays, in which the normal adjacent tissues were used as control (denoted as normal mucosa). As shown in **Figure 1**, a significantly enhanced immunohistochemical staining was found in OSCC tissues compared with normal mucosa. The positive staining of Hsp70, MMP14, MMP2, and cortactin was mostly observed in the cytoplasmic of the carcinoma cells, whereas the nucleic positive signals were detected much more in the Hif1α immunohistochemical staining. The positive staining of MMP14 was mostly observed in the cytoplasmic and cytomembrane of the carcinoma cells. Then, we analyzed the histoscores of Hsp70 (**Figure 2A**), Hif1α (**Figure 2B**), cortactin (**Figure 2C**) MMP14 (**Figure 2D**), and MMP2 (**Figure 2E**) in both tumor and normal tissues and observed significantly higher expression levels of these proteins in OSCC tissues.

## The Correlations Among Hsp70, Hif1α, MMP2, MMP14, and Cortactin in OSCC

In order to investigate the correlations among the expressions of Hif1α, MMP2, MMP14, cortactin, and Hsp70, we calculated the histoscores of Hsp70, Hif1α, MMP14, MMP2, and cortactin, and

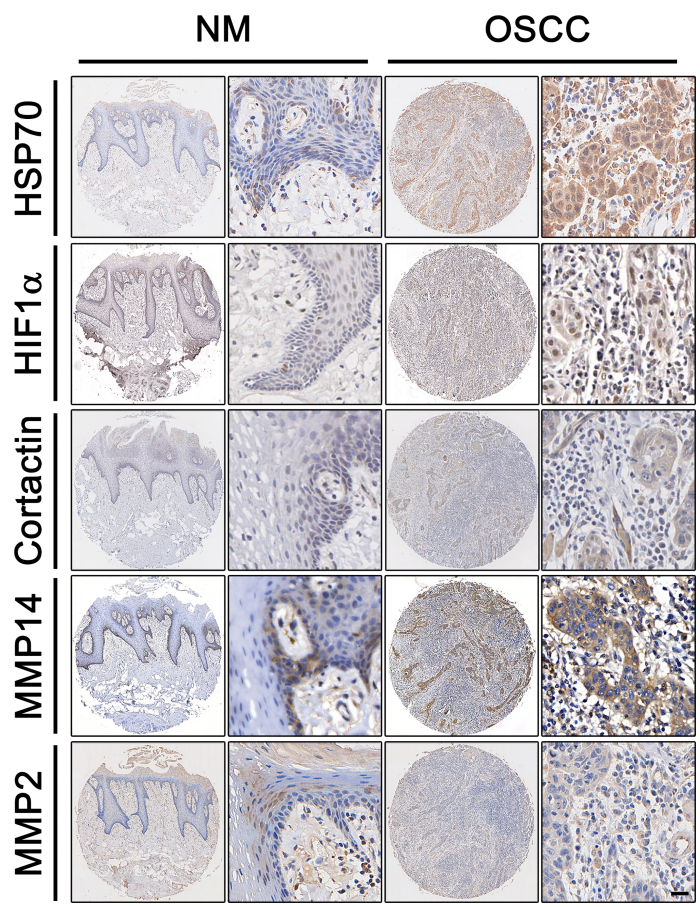


**FIGURE 1** | *Expression of HSP70, Hif1α, MMP2, MMP14, and cortactin in OSCC.* Representative immunohistochemical staining of HSP70, Hif1α, MMP2, MMP14, and cortactin expression in oral squamous cell carcinoma (OSCC) and matched paracarcinoma (NM). Scale bar, 50 μm.

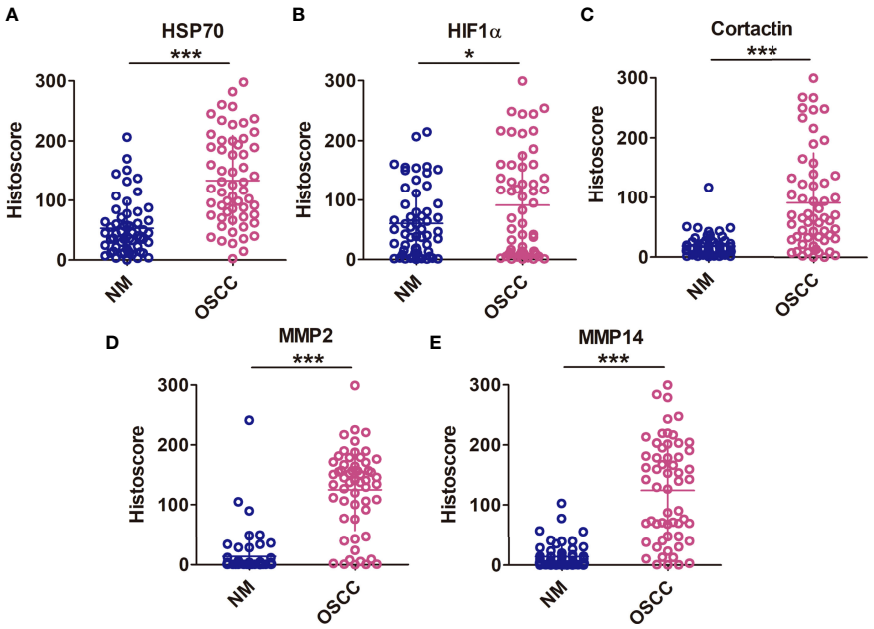


**FIGURE 2** | *High expression of HSP70, Hif1α, MMP2, MMP14, and cortactin in OSCC*. Histoscores of Hsp70 **(A)**, Hif1α **(B)**, cortactin **(C)**, MMP14 **(D)**, and MMP2 **(E)** in both tumor and normal tissues, and observed significantly higher expression levels of these proteins in OSCC tissues. Means ± SEM. *P < 0.05; ***P < 0.001 versus the NM group; Student's t-test analysis.

then picked every two proteins to analyze their correlations. Our results showed significant positive correlations between the histoscores of Hif1α, cortactin, MMP2, MMP14, and Hsp70. Moreover, the expression of cortactin in OSCC was positively correlated with the expression of Hif1α and MMP14, and the histoscores of MMP14 and MMP2 also showed a positive relationship (**Figures 3A–D**). We also extracted the above genes in OSCC samples from TCGA database and analyzed their correlations. As shown in **Figure 4**, the expression of HSPA4 and HIF1A (**Figure 4A**), HIF1A and CTTN (**Figure 4B**), CTTN and MMP4 (**Figure 4C**), as well as MMP14 and MMP2 (**Figure 4D**) also exhibited a significant positive relationship.

## Inhibiting Hsp70 Suppresses Cell Viability and Proliferation in OSCC Cells

In this study, Cal27 cells were treated with ascending concentrations of Apo for 24 h, and it was displayed in **Figure 5A** that the OSCC cell viability was reduced dose-dependently as the Apo concentration increased. The suppressive effect of inhibiting Hsp70 on OSCC cells viability was more evident after 48-h treatment (**Figure 5B**). Furthermore, we examined the possible role of inhibiting Hsp70 in cell proliferation using EdU incorporation assays. Compared with control group, Apo treatment group showed decreased EDU positive stained cells significantly (**Figure 5C**). The quantitative data suggested that, at 100 μM, Apo suppressed more than 50% proliferative potentials of OSCC cells (**Figure 5D**). In summary, inhibiting Hsp70 significantly reduced cell viability and inhibited cell proliferation in OSCC cells, exhibiting OSCC cell cytotoxicity.

## Inhibiting Hsp70 Prevents Cell Invasion of OSCC Cells

To further investigate the potential role of inhibiting Hsp70 in tumor invasion and metastasis, we employed wound healing assays and utilized Transwell chamber system to determine the effect of inhibiting Hsp70 on cell invasion. As **Figure 6A** showed, after treating 100 μM Apo for 12 h, Cal27 cells migrated more slowly in contrast to those treated without Apo. The motility of Cal27 cells was significantly inhibited after Apo treatment (**Figure 6B**). To further explore the correlation between ability of OSCC cells invasion and Apo concentration, Transwell chamber system was utilized in Cal27 cells at 0, 50, 100, and 150 μM Apo, respectively. It is revealed that the number of migrated cells significantly decreased as the Apo concentration improving in OSCC cells (**Figures 6C, D**).

# DISCUSSION

In previous studies, HSP70, overexpressed in a variety of cancers, can inhibit endogenous and exogenous apoptotic pathways, block oncogene-induced senescence, and lead to treatment resistance (20). HSP70 also mediates the occurrence of tumor-promoting immune microenvironment through TLR4, NF-κB,

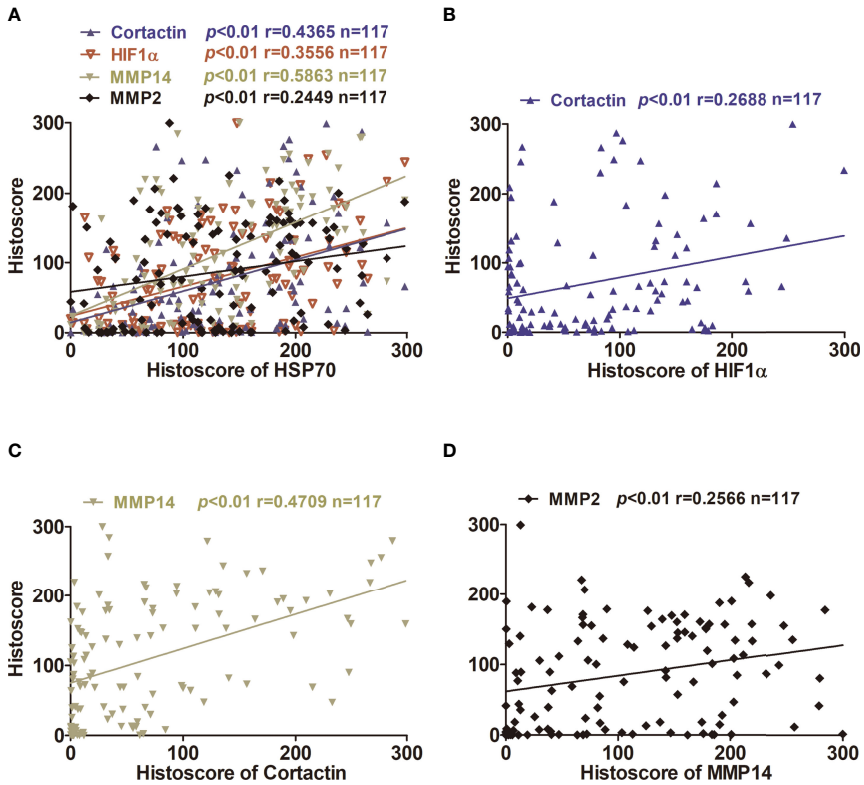


**FIGURE 3** | Correlation and regression of HSP70, Hif1α, MMP2, MMP14, and cortactin in OSCC tissues using two-tailed Pearson's test. **(A)** A correlation and regression of HSP70, Hif1α, MMP2, MMP14, and cortactin in OSCC tissues using two-tailed Pearson's test. **(B)** Correlation between Hif1α and cortactin expression levels in OSCC tissues. **(C)** Correlation between MMP14 and cortactin expression levels in OSCC tissue. **(D)** Correlation between MMP2 and MMP14 expression levels in OSCC tissues.

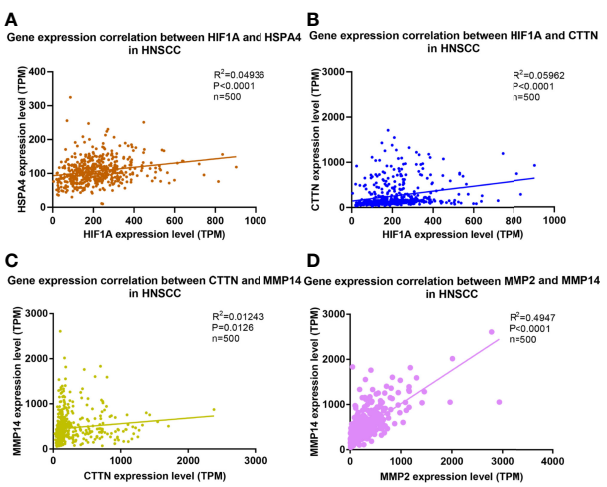


**FIGURE 4** | Correlation and regression of HSPA4, HIF-1A, MMP2, MMP14, and CTTN in TCGA database. Correlation and regression of **(A)** HSPA4, HIF-1A **(B)** HIF-1A, CTTN **(C)** CTTN, MMP14 **(D)** MMP2, and MMP14 in TCGA database.

STAT3, and other signal pathways (20, 21). The HSP70-TLR2 interaction also leads to the activation of neutrophils and the production of pro-inflammatory cytokines in diabetes vascular complications (20). Silencing of Hsp70 enhanced the metastatic properties of the HeLa, A549, and MCF7 cancer cell lines, and Hsp70 (HSP70A1A) inhibited the metastatic ability of cancer cells (22). However, heat shock protein family members such as HSC70 and HSPA2 may play inhibitory roles in cancer cell invasion and metastasis (23, 24). In OSCC, the relationship between HSP70 and tumor metastasis is unclear.

In our study, the Hsp70, Hif1α, cortactin, MMP2, and MMP14 expressions were elevated in OSCC tissue compared with normal counterparts. Cortactin is widely recognized as a marker of invadopodia. The main function of cortactin is to facilitate the interaction of Arp2/3 with F-actin to induce the formation of branched actin thereby promoting cell migration and invasion (25). Moreover, cortactin participates in matrix degradation and MMP2, MMP9, and MT1-MMP secretion in OSCC cells (26). Cortactin phosphorylation being regulated through Src-family kinases, Erk1/Erk2, and PAK is essential for invadopodia formation and ECM degradation (27). The recruitment and activation of proteases such as MMP2 and MMP9 at invadopodia sites could promote the degradation of

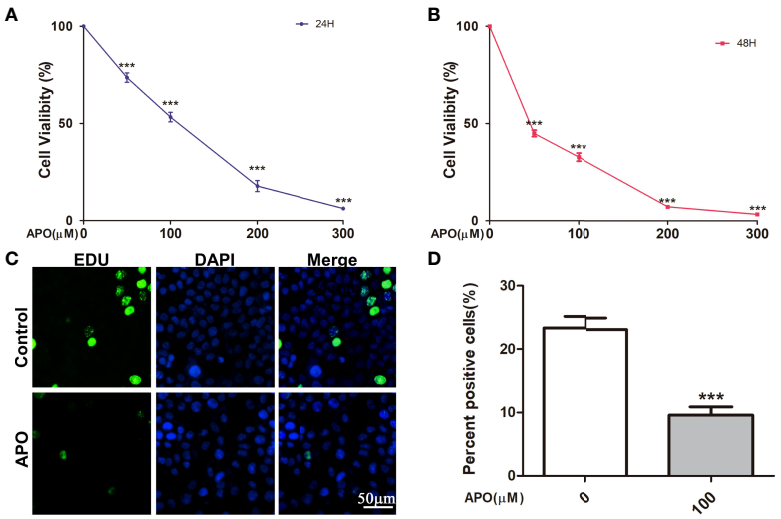


**FIGURE 5** | APO inhibits OSCC cell proliferation *in vitro*. Cell Counting Kit (CCK8) assay shows the suppressive effect of APO after 24-h **(A)** and 48-h **(B)** treatment by CCK8 assay and 5′-ethynl-2′-deoxyuridine (EdU) assay **(C)**. Means ± SEM; ***$P < 0.001$; two-way ANOVA analysis. **(D)** Quantification of EdU assay. Means ± SEM; ***$P < 0.001$; Student's t-test analysis; APO, apoptozole.

ECM so as to provide traction for cancer invasion and metastasis (28, 29). In addition, MT1-MMP also contributes to the activation of MMP2 in invadopodia and facilitate the digestion of various ECM macromolecules including collagen, fibronectin, and laminins (30).

To evaluate the relationship between the expression of invadopodia-associated proteins and Hsp70, we examined histoscore of those proteins using two-tailed Pearson's statistics. The results showed that positive relationships between Hsp70 and Hif1α, cortactin, MMP2, and MMP14 were found in OSCC tissue. Thus, we speculated that Hsp70 overexpression may be correlated with the increased invadopodia formation in OSCC. Bioinformatics on HNSC extracted from TCGA database also showed these positive relationships at the gene transcription level.

The life span of Hif1α could be prolonged owing to the formation of persistent complex between Hsp70 and Hif1α (31).

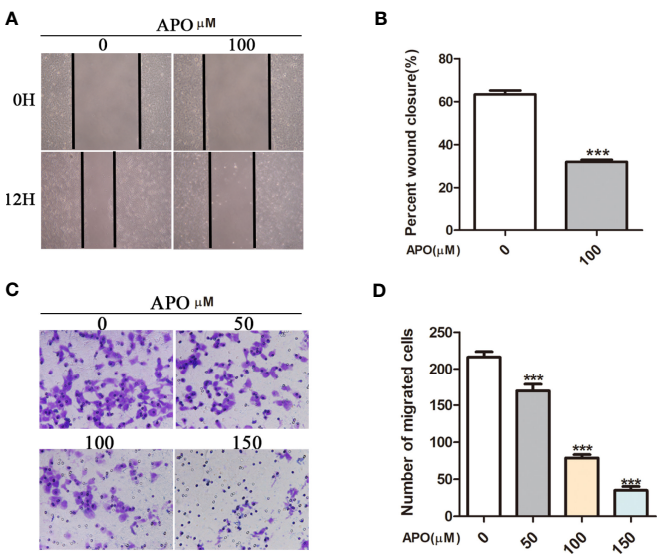


**FIGURE 6** | APO inhibits OSCC cell migration and invasion *in vitro*. Migration assessed by *in vitro* wound-healing assay **(A, B)** quantification of EdU assay. Means ± SEM; ***$P < 0.001$; Student t-test analysis. Invasion assessed and by *in vitro* Transwell assay (8-μm pore size) **(C, D)** quantification of EdU assay. Means ± SEM; ***$P < 0.001$; two-way ANOVA analysis; APO, apoptozole.

HSP70 maintains Hif1α stability dependent on the activation of phosphatidylinositol 3-kinase (PI3K)/Akt and PI3K inhibitors could downregulate HSP70 expression (32). Hif1α not only increases the levels of invadopodia-forming activity but also stimulates its increased degradative activity (15, 33). Diaz et al. found that hypoxia no longer increases invadopodia formation after Hif1α knockdown in HNSC SCC61 cell lines, and it was believed that stabilized Hif1α under hypoxia activated Notch signaling thus increasing invadopodia formation (34). Moreover, Hif1α was reported to elevate the expression of b-PIX which was identified as a fundamental driver of invadopodia formation so that augments the invasive potential in cancer cells (33). Through a literature review, we prospected that inhibiting Hsp70 may affect the formation of invadopodia through downregulating the expression of Hif1α.

In order to further explore the role of Hsp70 in OSCC cells, we cultured Cal27 with distinct concentrations of Hsp70 inhibitor, Apo. We found that Apo could not only impair the cell viability and proliferation ability of OSCC cells in a dose-dependent manner but also attenuate their migratory ability. Overall, the *in vitro* experiment suggested that inhibiting Hsp70 may cause cytotoxicity to OSCC cells and interrupt their motility. These results further confirmed that HSP70 was involved in regulating the migration ability of OSCC cells.

It is worth mentioning that this study has some limitations. First, experiments on animals actually are needed to further verify the effect of Apo on OSCC. Last, our study discussed a little about the signaling mechanism of APO inhibiting the expression of Hif1α in OSCC cells, which may be meaningful to explore in the future.

## CONCLUSION

In conclusion, our work shows that HSP70 inhibition exhibits cytotoxic effect on OSCC cells and prevents invasion and metastasis of OSCC cells through decreasing the expression of invadopodia proteins. These results may provide a new therapeutic prospect in OSCC treatment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (Hunan, China). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

L-XD: concept/design, data analysis. W-MW: concept/design. S-SY, JW, JZ: data analysis. TS, W-MW: critical revision, final approval. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was funded by the National Natural Science Foundation of China (grant no.81702708,81873717,82170973,82070966), Natural Science Foundation of Hunan (grant no. 2018JJ3862), the Science and Technology Innovation Program of Hunan Province (grant no.2021RC3026), Scientific research Project of Hunan Provincial Health Commission(grant no. 202208024658).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.890218/full#supplementary-material

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