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EDITORIAL

RSSDI's Defeat Diabetes Campaign: India takes a major leap in the direction of diabetes care capital of the world

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Before the country could recover from the first wave of COVID-19 pandemic, India witnessed a sharp rise in number of cases in the month of March 2021. The number of new cases in a single day reached its peak on 4,12,431, during the first week of May 2021. The effects have been devastating with close to half a million deaths as per the official data. Several studies have demonstrated that the risk of severe infection, respiratory distress syndrome, and mortality has been significantly higher in people with diabetes [1–4]. Even people with prediabetes and undiagnosed diabetes [5, 6] were found to be at a significantly higher risk of severe infection and mortality due to COVID-19 infection.

India has estimated 77 million people living with diabetes, and an even larger number of people with prediabetes. Various studies have also reported that 50% of people with diabetes are unaware of the condition. This unholy interaction of two ongoing pandemics—diabetes and COVID-19—has baffled medical professionals and organizations worldwide. With such a high prevalence of diabetes and its strong association with COVID-19 mortality, it is imperative that creating awareness in general public could save a lot of lives.

Defeat Diabetes Campaign

Research Society for Study of Diabetes in India (RSSDI), being the largest organization of professionals involved in the care of people with diabetes, decided to launch a massive public awareness campaign. In the second week of June 2021, a task force was created which further identified hundreds of RSSDI members who volunteered to be the ambassadors for the campaign. The goal was set to reach a hundred million people in the next 100 days with a tagline of Test, Track, and Treat.

WhatsApp messaging service was used as the channel for percolating content and information to the grassroot levels. Several WhatsApp groups were created by the task force members and ambassadors. For bulk sharing of the content received through this network, various social media platforms like Facebook, Twitter, Instagram, and YouTube were used. Various creatives that would catch the attention of general people and educate them regarding various aspects of diabetes were designed and shared through social media platforms on a daily basis.

Live video sessions for public awareness were done on a daily basis using various social media platforms. A number of community reach activities were also conducted locally by the ambassadors. These activities were being covered by local print and electronic media. Influential people from various spheres of life including social activists, politicians, bureaucrats, and Bollywood celebrities contributed to the campaign by posting about the same on their social media handles. Some of them joined the weekly live event of RSSDI—Diabetes Bytes. A separate FM campaign was launched in all major cities of India. Audio bytes of 20–40 doctors from each city were broadcasted through FM, 6 slots a day for one week.

Based on the conclusions of a systematic review by Kesavadev et al. [7], an online petition addressed to Director, WHO—"Declare Blood Sugar as Fifth Vital Sign" was published [8] and circulated among doctors.

Campaign analysis

After completion of 75 days of the 100-day campaign, interim analysis of its reach through social media was performed using online social media analytic tool "Brand

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Mentions." The analysis concluded that the campaign reach had been 123.3 million between 1 July and 23 Sept with 1747 mentions, 230.5 thousand interactions, 26.5 thousand shares, and 203.7 thousand likes. Major sources of this were Twitter and Instagram contributing to 77.68% and 21.18%, respectively. It was impossible to estimate its reach on diverse platforms like WhatsApp, FM Campaign, Print Media, and Community programs.

One Nation, One Day, One Million Tests

As part of the campaign, a massive nationwide blood sugar testing camp was planned. Rotary India, an organization with huge volunteer base, was approached, and they agreed to partner with RSSDI for this historic event. With RSSDI being a huge organization, the information related to this mega event was passed on to 21 state chapters and 700 district coordinators.

A geolocation-based webapp was developed. Doctors from all over India and clubs of Rotary India were registered on the app. A separate portal to capture a few basic parameters from history, anthropometry, and blood sugar levels was developed. This being the first attempt to check around 1 million people in one day, Asia Book of Records was approached and all their requirements for considering this as a record were fulfilled.

On the day of the event, data of over 1.1 million blood sugar tests were received, target being 1 million blood sugar tests in one day. The data was later verified by validators from Asia Book of Records and has now been published as a record for participation of maximum people in diabetes screening camps held at multiple venues in one day [9]. As published in Asia Book of Records, a total number of 10,64,989 people were screened for diabetes across 10,258 camps conducted on September 29, 2021.

Potential impact

Over a hundred million people have been educated regarding importance of screening for diabetes (test), regular monitoring of diabetes (track), and treatment to maintain glycemic control (treat). With nationwide camps, a large number of people, who were undiagnosed earlier, have been detected to be diabetic. This would surely translate into preventing complications and saving thousands of lives. The partner organizations also realized that there is a huge untapped potential that can be utilized in the future for such activities.

Campaign to movement—the way forward

This campaign witnessed an unprecedented enthusiasm from medical professionals and volunteers. During various meetings related to the campaign, it was suggested that this campaign had gradually taken the shape of a movement. The two largest partnering organizations, RSSDI and Rotary India, signed a memorandum of understanding for three years to continue with similar activities in the future. Defeat Diabetes Campaign was a first major leap in the direction of making India a diabetes care capital of the world.

Conclusion

The grand success of the Defeat Diabetes Campaign and Mammoth One Nation, One Day, One Million Blood Sugar Tests confirms that a coordinated and meticulously executed campaign by healthcare professionals and other voluntary organizations along with the use of technology can contribute to the cause of public awareness in a big way.

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CONSENSUS

RSSDI guidelines on thyroid dysfunction and diabetes

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Abstract
Diabetes and thyroid dysfunction coexist. There are several published guidances on screening detection and management of thyroid dysfunction in diabetes. The RSSDI has reviewed the evidence in the publish literature including India and provided a guidance to clinicians based on an expert review of the literature. Methods: A literature review was conducted of all published studies and the evidence was categorised by levels. The evidence was then reviewed by the expert group and a recommendation was provided with a grade. Summary: The relationship of hypothyroidism with T1DM is strong and warrants screening detection and treatment. While T2DM is frequently associated with thyroid dysfunction, the association is less strong. There appears to be a relationship between thyroid dysfunction and diabetes comorbidities. Drugs such as metformin may alter TSH levels. Thiazolidinediones may worsen dysthyroid orbitopathy. Gaps: The relationship between thyroid dysfunction and diabetes requires further study from a translational clinical and epidemiologic perspective leading to better evidence and recommendations.

Keywords Type 1 Diabetes · Type 2 Diabetes · Screening · Hypothyroidism · Thyrotoxicosis · Comorbidities · Thionamides · Insulin · Pregnancy

Abbreviations

AACE	American Association of Clinical Endocrinology
ADA	American Diabetes Association
AITD	Autoimmune thyroid disorder
AITD	Association of autoimmune thyroid disease
AMD	Italian Association of Clinical Diabetologists
AME	Association of Clinical Endocrinologists
AMPK	Adenosine monophosphate-activated protein kinase
APS3	Autoimmune polyglandular syndrome type 3 variant
ATA	American Thyroid Association
ATD	Anti-thyroid drug
BTA	British Thyroid Association
CRP	C reactive protein
CVD	Cardiovascular disease
DM	Diabetes mellitus
DKD	Diabetic kidney disease
FNAC	Fine needle aspiration cytology
FT4	Free T4
GD	Graves' disease
GLP-1	Glucagon-like peptide-1
GO	Graves' orbitopathy
HbA1c	Hemoglobin A1C
HLA	Human leukocyte antigen
HT	Hashimotos thyroiditis
LT4	Levothyroxine
NICE	National Institute for Health and Care Excellence
OHAs	Oral hypoglycemic agents

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RAI	Radioactive iodine
RCTs	Randomized clinical trials
RSSDI	Research Society for the Study of Diabetes in India
SCH	Sub clinical hypothyroidism
SU	Sulfonylurea
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TD	Thyroid disorders
TES	The Endocrine Society
TFT	Thyroid function test
TPO-Ab	Thyroxine peroxidase-antibody
TRAb	TSH receptor autoantibodies
TSH	Thyroid-stimulating hormone

Introduction

Diabetes mellitus (DM) and thyroid disorders (TD) are two common endocrine disorders, which often co-exist in clinical practice [1]. In the South Asian population, 9.83% of individuals with type 2 diabetes mellitus (T2DM) have clinical hypothyroidism, and another 5.9% have subclinical hypothyroidism [2].

In India, the prevalence of hypothyroidism in T2DM patients is approximately 26.8% and that of subclinical hypothyroidism is 22.22% [3]. In a study conducted in Eastern India, the prevalence of subclinical hypothyroidism (SCH) was found to be much higher than overt hypothyroidism [4]. SCH was seen in 23% of the population and overt hypothyroidism was found in 3% [4]. Thyroid autoantibody results were positive in 13.1% patients [4]. In the presence of other comorbidities like hypertension, the risk of hypothyroidism in T2DM patients is elevated to almost 33.5% [3]. In patients with T1DM, the prevalence of thyroid disorders is much higher than in patients with T2DM [5]. Anti-TPO and anti-thyroglobulin antibodies are positive in 51% and 25% of the patients respectively [5].

In patients with Diabetic complications such as diabetic kidney disease (DKD), the prevalence of hypothyroidism in the Indian population is 34.1%, of which, 29.3% of the cases had SCH [6]. Due to the higher prevalence of SCH in the Indian population, there is a need for regular screening and evaluation.

The correlation between TD and DM can be attributed to the overlapping functions of the thyroid and insulin hormones as well as the common autoimmune pathways between T1DM and hyperthyroidism (Fig. 1). This relationship is denominated as auto-immune polyglandular syndrome type 3 variant (APS3) where human leukocyte antigen (HLA) is the genetic determinant [7, 8].

The thyroid hormone is a regulator of the metabolic and energy expenditure processes, which is directly involved in the regulation of insulin secretion as well as the maintenance of glucose homeostasis. In patients with T2DM or insulin resistance, there is a surge in the levels of circulating insulin, which has a proliferative effect on the thyroid tissue [4, 9]. As a result, the size of the thyroid gland may increase and the risk of formation of nodules is elevated [4, 9].

The co-existence of TD and DM results in several complications, which become difficult to manage in clinical practice, especially in the absence of clear screening and treatment recommendations. Acute critical situations such as diabetes ketoacidosis, thyroid storm, and myxedema coma are reported in these cases, which necessitate emergency management following an early multi-disciplinary approach [11–13].

This guidance document will provide recommendations for the co-management, screening, and treatment of TD and DM in co-existence. It will be based on gathered clinical evidence stating levels of recommendations. Along with this, it

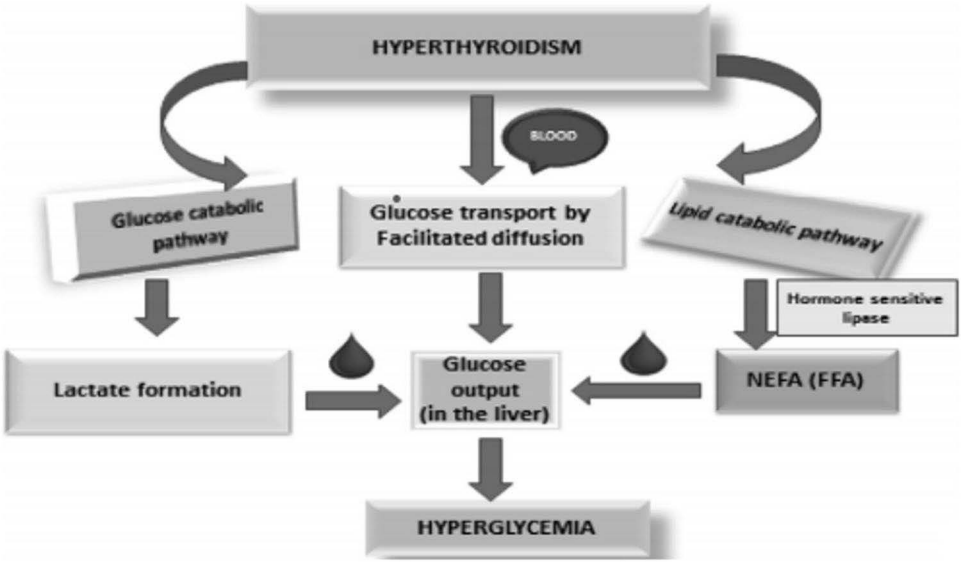

Fig. 1 The Relationship between hyperthyroidism and diabetes mellitus [10]

will combine evidence from recommendations gleaned from existing guidelines.

Methodology

RSSDI guidelines for screening and management of TD and DM were formed based on consensus-based recommendations of expert panelists including senior endocrinologists and diabetologists. These recommendations were supported by literature evidence and clinical overview obtained from existing Indian and international guidelines. Literature evidence included data and recommendations from Indian, international, and South Asian journals, which were gathered based on extensive literature research, primarily conducted in PubMed and Cochrane libraries. Published RCTs, systematic reviews, meta-analysis papers, cross-sectional studies, cohort studies, and expert opinion papers were considered for inclusion after thorough quality assessment. Standard guidelines that were referred in the paper included American Diabetes Association (ADA), American Thyroid Association (ATA), American College of Endocrinology (ACE), British Thyroid Association (BTA), and Indian guidelines such as the Indian Thyroid Society (ITS) among many others. Based on the strength of evidence, grades were awarded to each recommendation (Tables 1 and 2).

The first draft of recommendations was prepared and circulated among RSSDI panelists to gather suggestions for improvements. All authors provided written recommendations for improvements in each section following the rigorous review of the document based on their expertise in the field. Following this, the draft was revised to address the identified gaps and was sent out to the authors for further review and feedback. Since all the expert authors approved the recommendations made in the second draft, it was finalized and sent out for publication.

Discussion

Summary of evidence

Thyroid screening recommendations for patients with type 1 diabetes mellitus

- A randomized observational study of 1310 patients suggested annual thyroid functional test (TFT) and thyroid-

Table.1 Levels of recommendation based on the type of literature evidence

Level	Type of evidence
I	Systematic review (with homogeneity) of RCTs OR RCTs with a large sample size depicting significant results
II	Systematic review (with homogeneity) of cohort studies OR Small-scale RCTs with unclear results OR consistent recommendations from multiple consensus guidelines (more than 2 national / international guidelines) OR randomized observational studies
III	Individual Cohort studies or clinical studies without randomization OR "Outcomes" research OR cross-sectional studies OR evidence gathered from existing consensus guidelines
IV	Systematic review (with homogeneity) of case-control studies OR Individual Case-control studies OR guidelines with improper evidence / lack of consensus OR retrospective analysis of patient data
V	Case series OR independent case study observations OR Expert opinion without explicit critical appraisal based on standard principles or narrative reviews or literature reviews without systematic analysis

Table.2 Grades of recommendation for guiding practice implications for the physicians

Grade	Descriptor	Quantifying evidence	Implications for practice
A	Strong recommendation	Level I evidence with consistent findings from multiple studies of levels II, III, or IV	Clinicians should follow grade A recommendations unless a clear and compelling rationale for an alternative approach is defined
B	Recommendation	Levels II, III, or IV evidence with consistent findings but lack of level I evidence	Clinicians should follow grade B recommendation while remaining alert to newly published evidence and sensitive to patient preferences
C	Option	Levels II, III, or IV evidence with inconsistent findings	While considering grade C evidence for individual practice, clinicians should be flexible in their decision-making approach, patient preferences and peer opinions should have a substantial influencing role
D	Option	Level V evidence: little or no systematic empirical evidence	For a grade D evidence, the physician must consider all options in their decision making and be alert to new published evidence that clarifies the benefit versus harm of the selected approach; patient preference should have a substantial influencing role

stimulating hormone (TSH) screening of all patients with T1DM [14]. It was observed that many patients who were diagnosed to be positive for TD during the study period were asymptomatic at the time of their presentation/screening; thus, indicating the need for regular annual screening [14].

- Expert opinion and literature reviews highlight the need for screening for TD in T1DM patients for their glycemic control as well as early TD control.
- A retrospective analysis of 200 patients indicates that TSH screening must be performed more frequently in T1DM patients with SCH [15].
- Based on practice variance and survey results obtained from 374 endocrinologists, biennial screening of TSH levels was recommended instead of annual screening keeping in mind cost reduction and efficient practice management [16].
- A cohort study of 58 patients suggested that annual TSH screening must be conducted in all patients with T1DM including men and women below the age of 50 years; although women above this age group are potentially at much higher risk compared to the general population, and have formed the primary focus for screening [17].
- In a cross-sectional study conducted in Eastern India ($n = 50$), it was found that 24% of participants with T1DM had TPO antibodies, but,were asymptomatic for TD [18]. Undiagnosed TD highlighted the need for regular TSH screening as well as TPO antibody evaluation at the time of diagnosis of T1DM [18].

Grade B recommendation

- Annual TSH screening along with TPOAb testing at the time of diagnosis of T1DM is recommended in all patients regardless of their age, clinical presentation, and gender.
- In patients with SCH, more frequent TSH screening can be performed keeping in mind the economic considerations of the patient.

Thyroid screening recommendations for patients with type 2 diabetes mellitus

- A cross-sectional study of 364 patients with T2DM suggested that patients with "high-risk" condition must be screened for TD [2]. This includes patients with frequent hospital visits and poor glycemic control. Community-based TSH screening is not recommended in T2DM patients [2].
- Retrospective analysis of 339 patients with T2DM in the UK suggested targeted screening for patients with poor glycemic control [19].
- A cross-sectional study of 8258 participants identified elderly women with uncontrolled T2DM as high-risk patients and recommended annual TSH screening for them [20].
- TSH screening has also been recommended for patients with an autoimmune thyroid disorder (AITD) and Graves' orbitopathty (GO) based on expert opinions gathered from individual cases [7].
- Some retrospective analysis and meta-analytical reviews have suggested the need for routine annual screening for TD in patients with T2DM for the diagnosis of SCH and overt hypothyroidism [21, 22].

Grade B recommendation

- There is insufficient evidence to support annual TSH screening in patients with T2DM.
- Targeted screening for high-risk patients is thereby recommended including elderly women, patients with uncontrolled DM, patients with frequent hospital visits, those with existing comorbid conditions, and patients with prediagnosed AITD or GO.

Screening recommendations for thyroid cancer in patients with diabetes mellitus

- A meta-analysis of 16 cohort studies indicated that DM patients, especially women are at increased risk of thyroid cancer.
- According to the findings of a case-control prospective study of 772 patients, the risk of thyroid cancer was similar among patients with T2DM and prediabetes and suggested the need for screening for all patients with a positive family history [23].
- A strong link between DM and thyroid cancer has not yet been established; palpation of the thyroid gland in patients with is recommended with further workup that conforms to established practice.

Grade B recommendation

- Thyroid gland palpation is recommended in patients with DM with a positive family history of thyroid cancer regardless of their glycemic presentation.

Thyroid screening recommendations in children with type 1 diabetes mellitus

- A randomized controlled trial of 611 children and adolescents with T1D suggested that TPOAb and TGAb tests are best for the primary diagnosis of TD [24].
- A survey analysis of 374 respondents, suggested that TPOAb is the most suitable screening test for children and adolescents with T1DM, especially newly diagnosed cases [16].

- A cross-sectional study of 2858 subjects annual TSH screening was suggested for children and adolescents with T1DM [20].
- In a cross-sectional study with 100 children and adolescent patients with T1DM and 284 controls, both regular TSH screening and anti-TPO antibody analysis were useful for assessing thyroid dysfunction in the pediatric population [25].
- Children with frequent hypoglycemic episodes may be considered for TD evaluations [26].
- Since the thyroid disease may be asympotmatic all pediatric patients with T1DM must be screened for TD [25].

Grade A recommendation

- Children and adolescent patients with T1DM must be screened for TPOAb and TGAb at the time of diagnosis of T1DM.
- Thereafter, annual TSH evaluations must be performed.

Grade B recommendation

- Asymptomatic children and adolescents with T1DM need to be screened annually to rule out the risk of SCH.
- For those with frequent hypoglycemic episodes, more frequent TSH assessment is recommended.

Treatment recommendations

Treatment with metformin in patients with thyroid disorders

- Metformin may reduce TSH levels, thyroid volume, and nodule size in patients with TD and DM, and can be safely recommended [21].
- In a retrospective analysis treatment with metformin was associated with a decrease in TSH levels in diabetic patients. This appears through the activation of adenosine monophosphate-activated protein kinase (AMPK) [27]. This study recommended a reduction in the dose of levothyroxine in patients on Metformin [27].
- In a pilot study of 11 patients, treatment with metformin was associated with reduction in TSH levels without impacting the levels of FT4 [28]. Re-evaluation of thyroid function every 6–12 months in patients on metformin was suggested [28].

Treatment with other oral hypoglycemic agents in patients with thyroid disorders

- Sulfonylureas (SU) may reduce iodine uptake, and may elevate thyroid hormone levels [29].
- Thiazolidinediones should to be avoided in patients with clinically active Graves' orbitopathy [7]. If treatment with pioglitazone is necessitated, regular screening of TSH levels is warranted [7].
- Treatment with glucagon-like peptide-1 (GLP-1) receptor drugs must be avoided in patients with a family history of thyroid cancer. There is a possible risk of C cell hyperplasia and increase in medullary thyroid cancer [30].

Treatment with insulin in patients with thyroid disorders

- Insulin requirements of patients may be reduced or increased based on their TD; hence, their dose must be adjusted accordingly [31].

Levothyroxine dosage considerations in patients with diabetes mellitus

- In a cross sectional study in women from the treatment group as opposed to controls, it was observed that treatment with LT4 improved insulin resistance and endothelial dysfunction. It also reduced atherosclerotic risk markers.
- In a large multi-center RCT involving 611 children and adolescents, it was observed that L-T4 treatment reduced the thyroid volume in pediatric patients with AIT and T1D, but had no effect on thyroid function and serum autoantibody levels [32]. However, it helped in the prevention of goiter and had positive treatment outcomes [32].
- In a retrospective analysis of 100 patients with SCH, it was found that LT4 administration normalized the TSH levels lowering the levels of fasting and postprandial glucose levels thereby reducing fasting hyperinsulinemia, CRP and total lipids [25].
- In a retrospective, non-randomized study of 257 patients with diabetic nephropathy, it was found that Levothyroxine therapy at a starting dose of 50 µg/day for patients aged < 60 years and 25 µg/day for older patients helped in reducing the risk of CVD [33].

Treatment with anti-thyroid medications in patients with diabetes mellitus

- Treatment with anti-thyroid drugs (ATDs) or radioactive iodine (RAI) do [34]. Do not have different impacts on glycemic control and may be used based on clinical indication and patient preference.
- In patients with Graves' disease thionamid therapy will help control glycemia in addition to control of the hyperthyroid state [29].

Grade A recommendation

- Levothyroxine at a suitable starting dosage is recommended for adult and pediatric patients with hypothyroidism and DM for thyroid hormone modulation as well as improvement in insulin resistance and management of cardiovascular risk factors.
- 1.6 µg / kg per day is a suitable dosage for euthyroid children with HT.
- Metformin can be safely prescribed in patients with DM and TD; however, regular monitoring of thyroid function is recommended in patients on who are thyroxine and metformin.
- Other OHAs such as SUs, GLP-1 receptor drugs, and pioglitazone must be selected in caution because of their respective risks of hyperthyroidism, medullary thyroid cancer and complications of GO.

Grade B Recommendation

- In patients on a Metformin + Levothyroxine regimen, the dose of Levothyroxine may need to be reduced.
- Early insulin dosage is recommended for elderly patients along with necessary dose reduction.
- Clinical indication and patient preference can indicate treatment choice of ATD vs radioiodine ablation.

Summary of Indian evidence

Thyroid screening recommendations for patients with type 1 diabetes mellitus

- For Indian population, it has been recommended that annual TSH screening must be performed in all patients with T1DM at the time of their diagnosis including euthyroid subjects [16, 21].
- A comparative cross-sectional study of 30 participants with T1DM suggested that regular TSH screening must be conducted in patients with T1DM for an early diagnosis of SCH [34].
- A cross-sectional study of 66 Indian women suggested TSH levels between 3.1 and 6.2 mIU/L carried the same risk of adverse fetomaternal outcomes as TSH levels < 3 mIU/L [35]. Trimester-specific TSH range in pregnant Indian women was not found to be statistically different from non-pregnant women in a cross-sectional study of 300 women [36].

Thyroid screening recommendations for patients with type 2 diabetes mellitus

- A cross-sectional study of 234 Indian patients recommended TSH screening in T2DM patients with uncontrolled blood glucose levels [4].
- A cross-sectional observational study of 100 patients supported screening for TD in T2DM [17]. Patients with existing neuropathy and nephropathy appeared are at higher risk of TD [21].
- In a cross-sectional study of 1508 patients, conducted across multiple clinical centers in India, the authors emphasized need for regular TSH screening in patients with T2DM and hypertension [37].
- A retrospective analysis of 1152 patients in Thiruvananthapuram reflect a high prevalence of clinical and subclinical hypothyroidism in T2DM patients indicating the need for regular TSH screening [38].

Screening for diabetes mellitus in patients with thyroid disorders

- In an observational cross-sectional study of patients with TD and DM, regular screening for diabetic neuropathy and nephropathy was recommended because of their high risk in the Indian population [4].

Grade B recommendations

- Annual TSH screening must be performed in Indian patients with T1DM, especially in pregnant women in whom normal TSH cut-offs must be considered.
- For patients with T2DM, annual TSH screening is recommended in euthyroid patients with detectable TPO antibodies or TSH levels above 2.5 mU/L.
- Patients with TD and DM must regularly be screened for nephropathy and neuropathy.
- High-risk patients including patients with hypertension, neuropathy, nephropathy, and uncontrolled blood glucose levels maybe screened annually.
- Individual case presentations of the patient must be considered to decide the frequency of TSH screening since the burden of hypothyroidism is much higher in the Indian population.

Treatment recommendations

Treatment with insulin in patients with thyroid disorders

Levels of insulin resistance along with the physiological, and biochemical profile of the patient as well as the impact on treatment on the thyroid status of the patient must be considered before insulin administration [10].

Recommendation of other guidelines and societies

Thyroid screening recommendations for patients with type 1 diabetes mellitus

- International guidelines including AACE, ATA, and TES recommend screening for anti-TPO antibodies at the time of diagnosis of T1DM. If anti-TPO antibodies are present, annual TSH screening must be performed [39].

- BTA Guidelines recommend TSH screening along with antibody analysis at baseline followed by annual TSH screening, which has been supported by other guidelines and literature reviews [26].
- ADA recommends annual TSH screening in patients with T1DM who have negative TPO Abs evaluation. For those with positive TPO antibodies, more frequent screening (at 6 months) is recommended, especially in patients with goiter and/or unexplained glycemic variation. In addition to TSH and anti-TPO analysis, thyroglobulin screening and serological examination are also recommended by the ADA [7, 29].
- ADA guidelines recommend performing repeat TSH screening in patients with T1DM at 1 year after achieving glycemic control. For patients with AITD, A1C levels must be evaluated regularly [29].
- The Indian Thyroid Society describes type 1 diabetes as a risk factor for hypothyroidism in pregnancy. Untreated hypothyroidism has also been identified as a risk factor for diabetes in pregnancy and thus screening for both T1DM and TD is recommended in pregnant Indian women [29, 36].

Thyroid screening recommendations for type 2 diabetes mellitus

- The UK National Screening Committee does not recommend screening for TD in the general population since there are no established normal levels of thyroid hormones [40].
- The US Guidelines also state that there is insufficient evidence to assess the balance of benefits and harms of screening for thyroid dysfunction in non-pregnant asymptomatic adults with T1DM. While T1DM was identified to be a risk factor for TD, T2DM was not found to have a significant association [41].
- The National Institute for Health and Care Excellence (NICE) guidelines for T2DM and the American Diabetes Association (ADA) Standards of Medical Care in Diabetes do not provide any guidance for the routine monitoring of thyroid function in T2DM [42].
- The British Thyroid Association has recommended screening at the diagnosis of T2DM along with regular screening in patients with SCH [26].
- The American Thyroid Association recommends screening for thyroid disorders every 5 years in adults aged ≥ 35 years regardless of their diabetes status. This recommendation is supported by a cost-utility analysis based on a decision model for the US healthcare system [43].
- ATA and TES recommend thyroid screening every 5 years for individuals above the age of 35 years. For patients with T2DM, TSH screening must be performed at the diagnosis of DM, and 5 years henceforth [39].
- ADA recommends thyroid gland palpation in patients with T2DM at the time of diagnosis. For those with dyslipidemia and those above 50 years of age, TSH screening is also recommended [7].
- Italian Association of Clinical Endocrinologists suggests regular TSH screening in T2DM patients above the age of 65 years, especially those with macrovascular complications. Ultrasound screening is not recommended [29].

Grade B recommendation

- In patients with T1DM, anti-TPO test at the time of diagnosis along with annual TSH screening is recommended henceforth.
- For patients with T2DM, TSH screening at the time of diagnosis is recommended, followed at every 5 years in individuals above the age of 35 years.
- Annual screening can be recommended in high-risk cases including patients with macrovascular complications, SCH, positive TPOAb, and dyslipidemia.

Thyroid screening recommendations for pregnant women with type 1/type 2 diabetes mellitus

- Italian Guidelines, AME, and AMD recommend TSH and TPOAb screening in women with T1DM who are for planning pregnancy [29]. If this stage has already passed and the woman is pregnant, TSH and TPOAb screening is recommended at the earliest stage. In the case of positive TPOAbs and normal serum TSH during pregnancy, TSH levels must be evaluated at 3, 6, and 12 months postpartum [29].
- BTA Guidelines recommend TSH and TPOAb screening in patients with T1DM at the beginning of pregnancy followed by postpartum evaluations at 3, 6, and 12 months. For patients with T2DM, the frequency of this screening remains unclear [35].
- AACE recommends that TSH levels must be evaluated in all women of the childbearing age group with either T1DM or T2DM [39]. If this has not been achieved, screening during the first trimester is strongly recommended [39].
- ATA and AACE guidelines recommend TSH and anti-TPO antibody screening at the beginning of pregnancy for women with GDM/prediabetes and T2DM in addition to screening patients with T1DM [7]. Women with risk factors such as central obesity are also recommended to be screened at the beginning of pregnancy regardless of their diabetic status of control [7].
- ATA guidelines recommend that in women with T1DM, reflex anti-TPO evaluation needs to be conducted if TSH levels are between the range of 2.5 to 10 mU/L [21].

Thyroid screening recommendations for children and adolescents with type 1 diabetes mellitus

- ISPAD Guidelines recommend that screening for TD must be initiated at 10 years of age or at the beginning of puberty and must be performed at every 2 years. In children with additional risk factors such as obesity or a family history of TD, more frequent screening may be needed [7].
- Italian Guidelines, AME and AMD recommend TSH, TPOAb, and TgAb analysis at the time of diagnosis of T1DM in children. TSH and TgAB must be recommended in TPOA-negative children as well [29]. Annual TSH evaluation is recommended in TPOAb-positive children, and biennial evaluation is suited for negative cases [29].

Treatment recommendations for diabetes mellitus and thyroid disorders in co-existence

- AME and AMD indicated that treatment with metformin reduces the incidence of thyroid cancer and thus recommended its use in patients with DM and TD [29].
- European and American Thyroid Associations recommend treating both overt and subclinical hyperthyroidism including grade 1 subclinical hyperthyroidism in patients with diabetes if TSH levels are persistently low [29].

Grade B recommendation

- In women with T1DM, TPOAb screening is recommended during the planning of pregnancy. If this is missed, the test must be performed at the earliest within the first trimester.
- After parturition, TPOAb screen must be repeated at 3, 6, and 12 months, followed by regular annual TSH screening as preferred in T1DM patients.
- In women with T2DM, central obesity/a history of prediabetes as well, TPOAb is recommended at the beginning of pregnancy.
- In children and adolescents, annual TSH screening is recommended in patients with positive TPOAb at the time of diagnosis of T1DM. If TPOAb test is negative, biennial evaluation will be suitable.

Final recommendation of RSSDI

Recommendations for screening for thyroid disorders in patients with type 1 diabetes

- RSSDI recommends screening for anti-TPO antibodies as well as TSH levels at the time of diagnosis of T1DM in all patients including euthyroid patients, young patients and males. In those with positive anti-TPO, annual TSH screening is recommended. In patients with negative TPOAb test, TSH screening can be performed once in 2 years.
- In high-risk patients (uncontrolled DM, high glycemic variability, or symptoms of goiter) with positive anti-TPO evaluation, more frequent TSH screening may be necessary based on their individual risks.

Screening for thyroid disorders in patients with type 2 diabetes

- Routine screening for TD is not necessary for patients with T2DM, but it may be considered depending on their clinical profile.
- For patients above the age of 35 years without any additional risks, TSH screening is recommended every 5 years.
- Patients with TSH > 2.5 mU/L or positive anti-TPO test must be considered for annual screening.
- For patients with uncontrolled diabetes mellitus, presence of comorbid conditions like hypertension, dyslipidemia, poor glycemic control, existing complications like neuropathy, and nephropathy or macrovascular complications and those frequently visiting the hospital settings for poorly managed T2DM, annual TSH screening is recommended.
- In patients above the age of 65 years, especially women, annual TSH screening is recommended, especially in the presence of macrovascular complications/comorbidities.
- In those above the age of 50 years, annual screening may be needed if there is a history of dyslipidemia.

RSSDI recommendations for screening pregnant women and children with diabetes mellitus

- Pregnant women must be screened for TPOAb before pregnancy or at least during the first trimester. TSH screening is also recommended along with TPOAb at the time of planning of pregnancy in women with T1DM.
- Women with positive TPOAb must be screened at 3 and 6 months at (12) months postpartum.
- In patients with T2DM, prediabetes, GDM, and risk factors such as obesity may also be screened for TD during the first trimester of pregnancy regardless of their levels of diabetes control or a euthyroid profile.
- TPOAb evaluation as well as TSH screening is recommended in children and adolescents at the time of diagnosis of T1DM.
- In children with positive TPO antibodies, annual TSH screening is recommended whereas in children with negative TPOAb, TSH evaluation can be performed every 2 years.

RSSDI recommendations for screening thyroid cancer

- Palpation of the thyroid gland must be performed at the time of diagnosis of diabetes mellitus in patients with a family history of cancer.

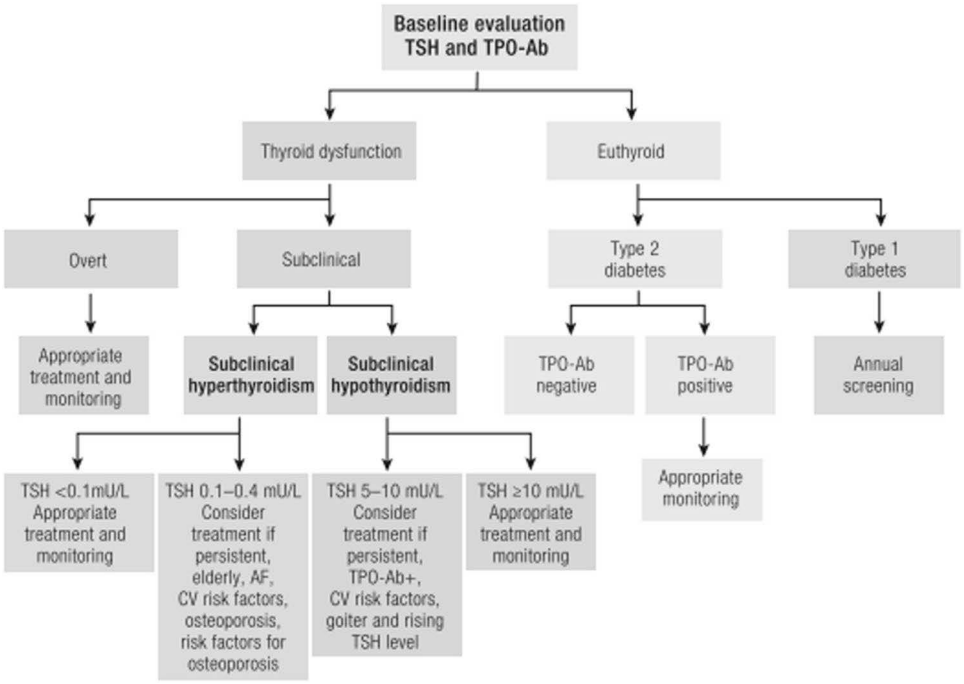

Fig. 2 Summary of screening, diagnosis and treatment recommendations for co-existing TD and DM [7]

RSSDI treatment recommendations for patients with thyroid disorder

- Metformin can be safely prescribed in patients with DM and TD for regulating blood glucose levels when prescribed at standard dosages.
- The daily dose of insulin must be calculated based on individual profile of the patient Insulin dose reduction may be needed in some patients (Fig. 2).

Gaps and directions for future research

A major gap identified during the preparation of the guidance document was the lack of RCTs with clinically significant results that provide clear recommendations for screening of thyroid disorders in Indian patients with diabetes mellitus, especially T2DM. Although evidence from clinical trials have been included in this paper, it was observed that no large-scale multi-centre RCTs have so far been conducted. Further, the negative impact of lack of screening have not been clearly identified. Thus, future research must be planned in the form of multi-centre RCT with a large sample size where the symptom profile of patients who were screened annually for TSH levels is compared with patients who were not scheduled for screening to state the strength of this recommendation. This research must involve patients with both T1DM and T2DM so that clear recommendations can be formed for the latter population, which is missing in most consensus guidelines.

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REVIEW ARTICLE

In-hospital fasting hyperglycemia and increased risk of mortality after acute coronary syndrome: a systematic overview and meta-analysis

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Abstract

Background Several studies noted that increased fasting blood glucose in hospital is associated with increasing mortality of acute coronary syndrome (ACS). We conducted a meta-analysis to assess the risk of mortality after ACS in patients who had fasting hyperglycemia (FH) in hospital.

Methods We conducted searches on PubMed, Cochrane Library, Web of Science, and Embase for relevant studies published before August 31, 2019. We pooled odds ratios (OR) from individual studies using a random effects model. Data abstraction was conducted by two independent reviewers, and the meta-analysis was performed using Review Manager version 5.3.

Results Eight studies were included into our research. Patients with FH showed a 3.09-fold (OR 3.09, CI 2.64–3.61; $p < 0.00001$) increased mortality of patients during admission compared with those who had normal fasting blood glucose (FBG). A statistically significant difference in post hospitalization mortality between patients with and without in-hospital FH was also detected (OR 3.40, CI 2.80–4.14; $p < 0.00001$).

Conclusions The available evidence suggests that in-hospital FH could increase the risk of in-hospital and out-of-hospital (30-day and long-term) mortalities after ACS.

Keywords Diabetes · Fasting hyperglycemia · Acute coronary syndrome

Introduction

Cardiovascular disease is one of the most important complications of patients with diabetes. Elevated blood glucose is also a well-recognized risk factor associated with increasing mortality of acute coronary syndrome (ACS) [1]. But the specific association between fasting blood glucose and outcome of ACS is not clear. Several studies had noted that elevated fasting blood glucose on admission is associated with increasing mortality of ACS. It appears to be an important prognostic marker for all causes of death in patients with ACS, whether or not they had previously been diagnosed as diabetics [2–4].

Recently, more and more studies highlighted the positive correlation between the in-hospital fasting hyperglycemia (FH) and the occurrence of major adverse cardiovascular events [5–12]. These studies indicated that FH might be an important and potential modifiable risk factor for poor outcome of adverse cardiovascular events. However, several areas of uncertainty remain, particularly relating to the impact of FH on in-hospital and out-of-hospital mortalities in patients after ACS. Moreover, the large multicenter clinical study

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including different ethnicities is rare. A meta-analysis is a good way to summarize the available data to provide more robust results than the individual study.

We therefore searched for, reviewed, and evaluated published evidence on the association between fasting hyperglycemia and mortality on admission in patients with or without diabetes.

Methods

Identification of studies and data extraction

According to the Preferred Reporting Items for Systematic Reviews And Meta-Analysis guidelines [13], all the studies that we captured were published before August 31, 2019 in PubMed, Embase, Web of science, and the Cochrane Library based on the following search terms: (1)"hyperglycemia," "hyperglycemias," "fasting hyperglycemia," "fasting hyperglycemias," "high fasting glucose," "fasting blood glucose," and "fasting plasma glucose"; (2)"acute coronary syndrome," "myocardial infarction," and "ischemic heart disease"; (3) "death," "mortality," and "prognosis." Ongoing and unpublished studies were identified by searching the web site of the Chinese State Food and Drug Administration, clinicaltrialsregister.eu, and clinicaltrials.gov. No restriction was imposed on search language. To be eligible, studies had to meet the following criteria: (1) they were prospective study, retrospective study, or cross-sectional study, and (2) they reported the extractable data on values of fasting blood glucose and mortality after ACS. Case-only studies, case reports, and reviews were all excluded. Identification of relevant abstracts and the selection of studies were performed by two independent investigators. The conflicts were solved through discussion and consensus with a third investigator. The review protocol has been registered at prospero.

In our analysis, we define the FH in acute coronary syndrome as the FBG ≥ 126 mg/dL (7 mmol/L) if the authors did not define the FH among their own studies [14]. The patient we defined as ACS should be assigned to one of the following categories: ST elevation myocardial infarction (STEMI), non-ST elevated myocardial infarction (NSTEMI), or unstable angina [15].

The following information was sought from each trial: authors, ethnicity, study design, sample sizes, duration of follow-up, and with or without diabetes. Relevant information is shown in detail in Table 1.

Statistical analysis

The meta-analysis was done using Review Manager version 5.3 (the Nordic Cochrane Centre, Copenhagen, Denmark) from the Cochrane Collaboration. The heterogeneity was assessed using the Cochran Q statistic (significance level at $p < 0.10$) and the I^2 statistic which was also quantitative analysis of heterogeneity ($I^2 = 0$–25%: low heterogeneity; $I^2 = 25$–50%: moderate heterogeneity; $I^2 = 50$–75%: substantial heterogeneity; $I^2 = 75$–100%: extreme heterogeneity) [16].

We summarized the risk of bias across studies for selection bias, performance bias, attrition bias, and reporting bias. We tested for publication bias by using a funnel plot. The plot resembles a symmetrical inverted funnel in the absence of bias and is skewed if there is a bias [17]. In addition, we sequentially excluded one study each time to see whether the findings were robust.

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

Study	Year of publication	Ethnicity	Design	Definition of fasting hyperglycemia	Duration of follow-up	First fasting glucose
Mahmoud Suleiman	2005	Israel	Prospective study	126 mg/dL	30 days	Overnight fast of 8 h within 24 h of admission
Guojing Luo	2014	Asia	Cross-sectional study	7 mmol/L	In-hospital	The second day after admission
Louis Kolman	2009	Caucasia	Cross-sectional study	126 mg/dL	6 months	First fasting glucose of the admission
Avital Porter	2007	Israel	Cross-sectional study	126 mg/dL	6 months	2–4 days after admission
F.Schiele	2006	Caucasia	Prospective study	7.7 mmol/L	1 year	First fasting glucose of the admission
Bruno Verges	2007	Caucasia	Prospective study	126 mg/dL	30 days	4–5 days after admission
Shi-Wei Yang	2013	Asia	Cross-sectional study	7 mmol/L	3 years	The morning after admission
Peter R. Sinnaeve	2009	Caucasia	Cross-sectional study	126 mg/dL	6 months	First fasting glucose of the admission

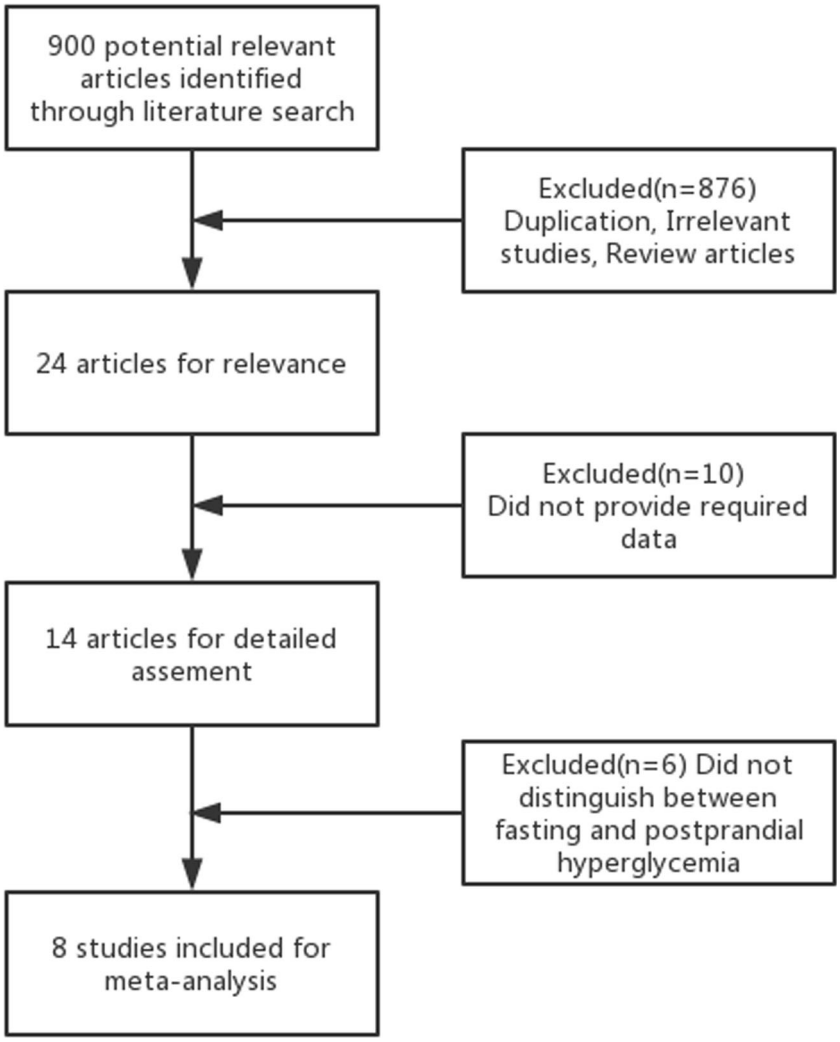

Fig. 1 Flow chart of study selection in the systematic review

Results

Study selection

Overall, we screened 990 articles from PubMed, the Cochrane Library, Web of Science, and Embase. The majority of articles were excluded because of duplication. From the titles and abstracts, 24 citations were thought to be retrieved for detailed assessment. Sixteen articles were excluded: 10 studies did not provide the data of fasting blood glucose that we required. The rest 6 studies did not separate the fasting hyperglycemia from prandial hyperglycemia. Finally, 8 studies were retained for the following meta-analysis [5–12]. Three of them were prospective studies, and the rest were cross-sectional studies. We show the flow chart of study selection in Fig. 1.

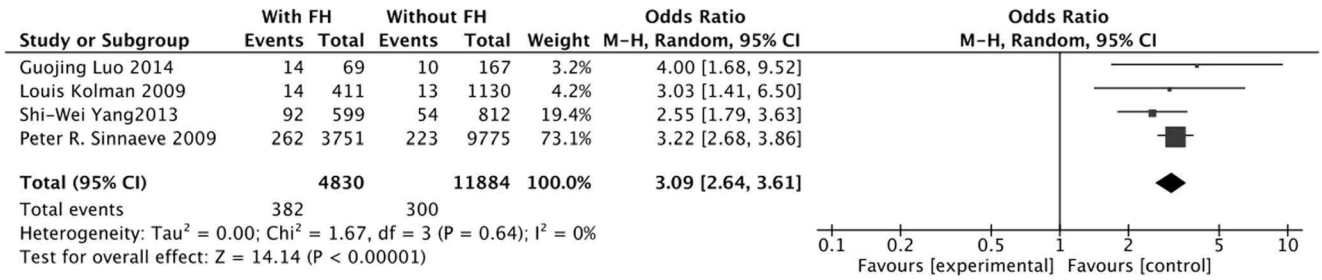

Fig. 2 Odds ratio of in-hospital mortality in patients with and without fasting hyperglycemia

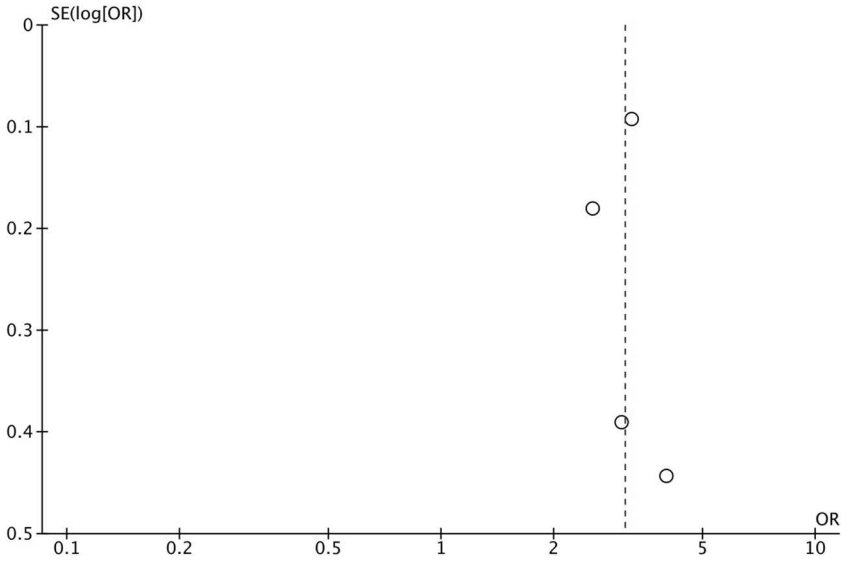

Fig. 3 The funnel of studies evaluating in-hospital mortality

Study characteristics

The features of the eight included studies are presented in Table 1. Three prospective studies were conducted in Caucasia and Israel. The other five cross-sectional studies were conducted in Asia, Caucasia, and Israel. The sample size of enrolled studies ranged from 155 to 13,526. The duration of the follow-up ranged from 30 days to 3 years.

In-hospital mortality

Four studies [8, 9, 11, 12] evaluated the impact of fasting hyperglycemia on in-hospital mortality among 3107 patients. Patients with FH showed a 3.09-fold (OR 3.09, CI 2.64–3.61; $p < 0.00001$) increased mortality of patients on admission compared with those who had normal fasting blood glucose (Fig. 2). The heterogeneity of in-hospital mortality is low ($I^2 = 0\%$, $p = 0.64$). In a sensitivity analysis, we did not find substantial modification of the estimates. However, considering true heterogeneity caused by clinical and methodological heterogeneity, a random effects meta-analysis model was used. Funnel plot for the included studies did not show any significant publication bias (Fig. 3).

Out-of-hospital mortality

We investigated the association between in-hospital FH and out-of-hospital mortality by including 6 studies. A statistically significant difference in out-of-hospital mortality between patients with and without in-hospital FH was detected (OR 3.40, CI 2.80–4.14; $p < 0.00001$) (Fig. 4). The I^2 value of 16% indicates low heterogeneity among the included studies. There was no significant publication bias according to the funnel plot for the 6 studies (Fig. 5).

For further analysis, we divided the patients who included the analysis of out-of-hospital mortality into 30-day and long-term group (up to 6 months). As shown in Fig. 6, the results indicated that in-hospital FH could increase the risk of mortality during a follow-up of 30 days (OR 3.73, CI 2.21–6.30; $p < 0.00001$). The results of the long-term mortality are shown in Fig. 7; the risk of mortality in patients with in-hospital mortality showed a 3.09-fold compared with controls (OR 3.09, CI 2.46–3.87; $p < 0.00001$).

As ethnicity may affect the mortality of ACS, we performed subgroup analysis. In the subgroup analysis of ethnicities, the results of out-of-hospital mortality were as follows: in Caucasia OR = 3.65, 95% CI (2.80, 4.77), $p < 0.00001$; in Israel OR = 2.38, 95% CI (0.62, 9.20), $p = 0.21$; and in Asia OR = 3.12,

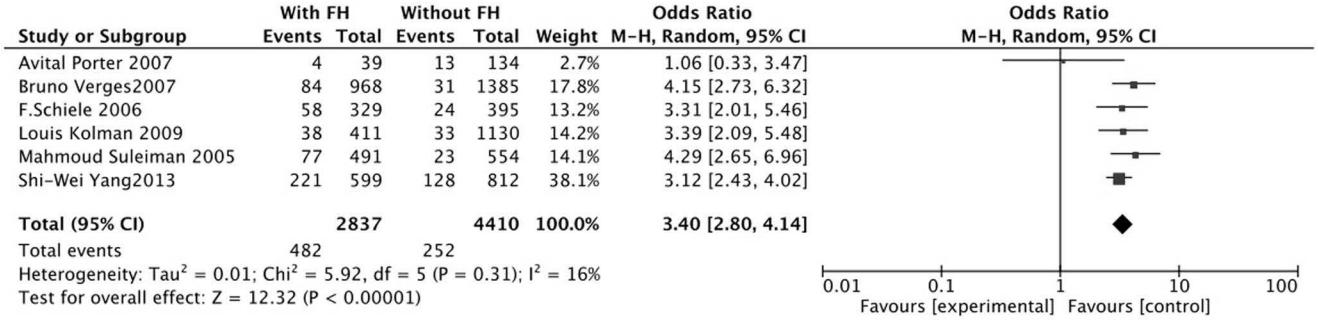

Fig. 4 Odds ratio of out-of-hospital mortality in patients with and without fasting hyperglycemia

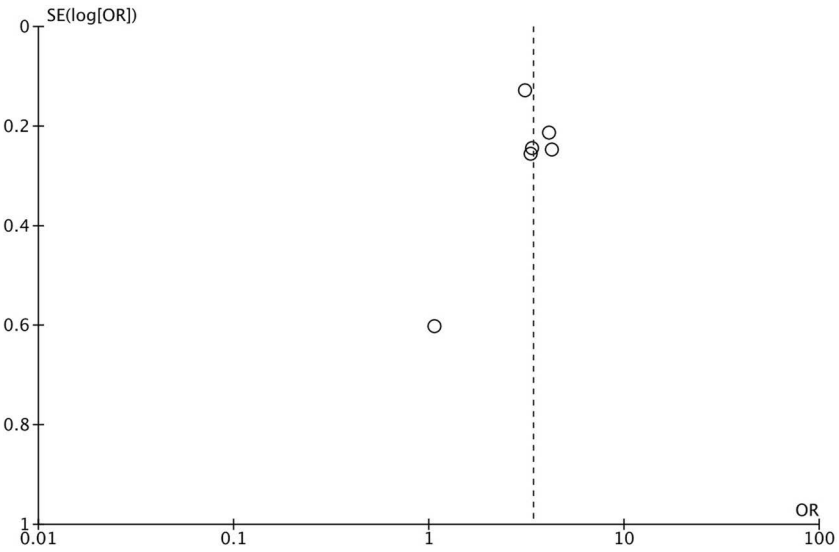

Fig. 5 The funnel of studies evaluating out-of-hospital mortality

95% CI (2.43, 4.02), $p < 0.00001$. The odds ratio difference among Caucasia, Israel, and Asia was further compared by chi-square test. The p value of subgroup difference was 0.62, which indicated that there was no difference among them (Fig. 8).

We conducted the sensitivity analysis by excluding individual study one by one. The results of out-of-hospital mortality revealed that Peter's study had extreme heterogeneity; thus, we excluded it in our analysis. In the analysis of in-hospital mortality, we did not find substantial modification of the estimate.

Discussion

Several previous studies have demonstrated that elevated fasting plasma glucose on admission increased risk of mortality after ACS [5–12]. However, there is rare comprehensive analysis between in-hospital FH and mortality of ACS with or without diabetes in patients. In this systematic review, we conducted a complete search, integration, and analysis of data to evaluate the association between the mortality and fasting hyperglycemia. Eight studies focusing on the in-hospital fasting hyperglycemia were included into the meta-analysis. The main finding of the present study is that the in-hospital FH not only increase the risk of in-hospital mortality but also run the risk of out-of-hospital mortality.

There are several potential underlying mechanisms associated with the higher risk of mortality in patients with FH during ACS. Firstly, the lack of insulin related to FH could decrease the glycolytic substrate and increase the free fatty acids which may reduce myocardial contractility at increased oxygen cost [18, 19]. FH could also increase platelet aggregation, impairing coronary endothelial function and increasing the release of inflammatory and vasoconstrictive factors which may lead to poor outcome after ACS [20–22]. In addition, ACS may lead to a greater rise in stress hormones (promoting glycogenolysis and hyperglycemia) and may also increase the risk of congestive heart failure and mortality [23]. Finally, hyperglycemia may precipitate an osmotic diuresis which may increase the risk of failing left ventricle [24, 25]. Although, the definite mechanism is still unknown.

After evaluating the out-of-hospital mortality, we found that both short- and long-term mortalities increased. It indicated that in-hospital FH might have long-term effect on prognosis of ACS. Due to the important relationship between mortality and in-hospital fasting hyperglycemia, each patient's fasting blood glucose on admission should be monitored more frequently, because patients with normal random or 2-h

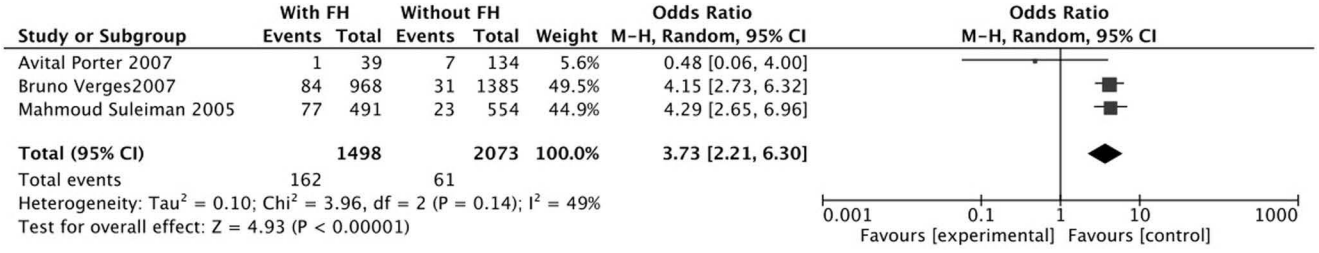

Fig. 6 Odds ratio of 30-day mortality in patients with and without fasting hyperglycemia

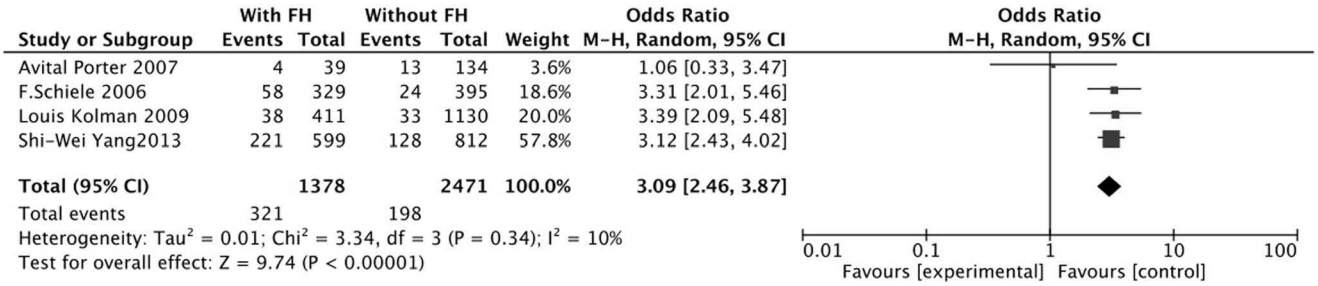

Fig. 7 Odds ratio of long-term mortality in patients with and without fasting hyperglycemia

postprandial blood glucose could also accompany with fasting hyperglycemia. In addition, we should control patients' fasting blood glucose on admission more strictly. The appropriate glucose management strategies may potentially improve outcomes in patients with FH. Several studies were conducted to evaluate the effect of glycemic control in ACS patients [26, 27]. However, the clinical benefits of an aggressive treatment with insulin are yet unproven, because hypoglycemia along with aggressive treatment might also increase the mortality.

Our meta-analysis has several limitations. The definition of hyperglycemia and concomitant treatment differed in several including studies. Comparatively, few prospective studies are included in this study which could hamper our detection abilities. When we analyzed the out-of-hospital mortality, we excluded Peter's study because of the extreme heterogeneity. The total number of outcome events in the pooled studies was small. In addition, only published studies were included.

Conclusion

The available evidence suggests that in-hospital FH could increase the risk of in-hospital and out-of-hospital (30-day and long-term) mortalities after ACS. Further research is required to find whether reversal of in-hospital FH could improve the clinical outcome for the patients after ACS.

Authors' contributions Hui Li and Chen Yiting conceived and designed the study. Xuehua Jiao, Heming Guo, Guodong Zhang, and Xueyan Yin collected and analyzed the data. Xuehua Jiao and Heming Guo wrote the manuscript. All authors read and approved the manuscript.

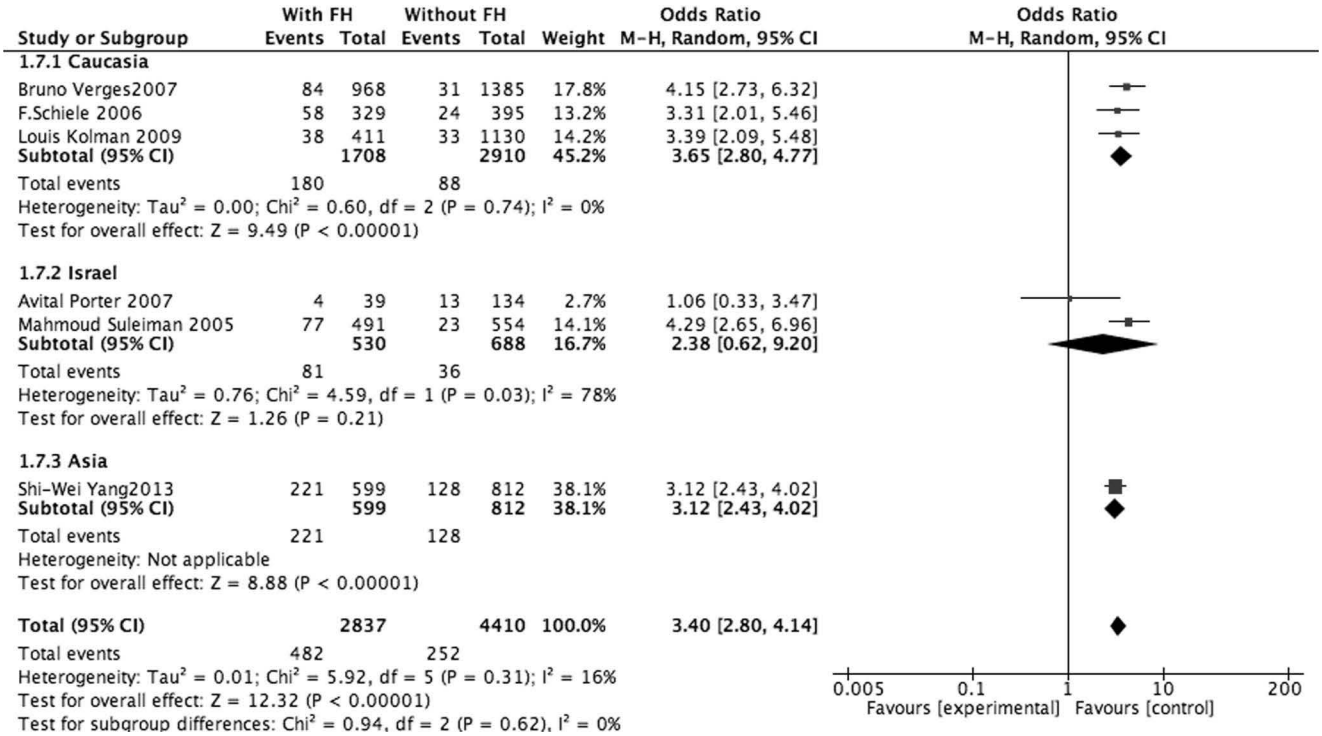

Fig. 8 Subgroup analyses for mortality according to ethnicity

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Compliance with ethical standards

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

Ethical approval Not applicable.

Informed consent Not applicable.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Abbreviations ACS, acute coronary syndrome; OR, odds ratios; FH, fasting hyperglycemia; FBG, fasting blood glucose; STEMI, ST elevation myocardial infarction; NSTEMI, non-ST elevated myocardial infarction

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REVIEW ARTICLE

Elevated levels of fasting serum GIP may be protective factors for diabetic retinopathy in type 2 diabetes mellitus

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Abstract

Objective Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone which has been ascribed a positive role in cardiovascular function. However, little is known about the association between GIP and microvascular complications including retina and kidney. In the present study, we conducted a cross-sectional study to investigate the relationship between fasting serum GIP and microvascular complications in type 2 diabetes mellitus (T2DM).

Methods A cross-sectional study was performed in 295 T2DM patients in our endocrine ward in order to investigate the relationship between fasting serum GIP and microvascular complications.

Results Among the 295 T2DM patients, the levels of median fasting serum GIP of all were 431.36pg/ml, interquartile range of which were 333.26~531.96pg/ml and the prevalence of diabetic retinopathy (DR) and diabetic nephropathy (DN) were 37.63% and 38.64% respectively. Our study observed that the prevalence of DR was significantly higher in low-levels GIP group compared with those in high-levels GIP group (p=0.007) (46.26% versus 31.08%) and the levels of fasting serum GIP were also higher in T2DM patients without DR than those with DR (p=0.019) (440.99pg/ml versus 405.90pg/ml). Spearman's correlation and multiple stepwise regression analysis showed that prevalence of DR was independently and negatively correlated with fasting serum GIP in T2DM (r=−0.134, p=0.021) (DR,β= −0.279; 95% CI, −0.512~−0.047, p = 0.019). However, there were no differences in fasting serum GIP between non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) (p=0.951). Similarly, we did not find any association between fasting serum GIP and prevalence of DN by using various statistical analyses.

Conclusions Prevalence of DR was independently and negatively correlated with fasting serum GIP in T2DM, indicating that elevated levels of fasting serum GIP may act as protective factors for DR.

Keywords Glucose-dependent insulinotropic polypeptide (GIP) · Diabetic retinopathy (DR) · Type 2 diabetes mellitus (T2DM) · Microvascular complications

Introduction

Epidemiology indicates that the number of people with diabetes has quadrupled in the past three decades globally, this estimate is projected to rise to 642 million by 2040 [1]. Over 90% of diabetes mellitus cases are type 2 diabetes mellitus (T2DM), most of the patients with T2DM have at least one complication. The epidemic of T2DM and its complications pose a major global health threat and has contributed tremendously to the burden of mortality and disability worldwide.

Microvascular complications of T2DM are very common, about half of T2DM patients presenting with microvascular complications [1]. The microvascular complications of T2DM have generally been referred to diabetic retinopathy (DR) and diabetic nephropathy (DN). DR is a common and serious microvascular complication that remains a leading cause of permanent visual loss in adults worldwide currently. With the development of T2DM, retinal changes are associated with severe microvascular complications including capillary leakage, edematous changes, capillary dilatation, loss of pericytes,

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and thickening of the capillary basement membrane [2]. The most popular treatment for DR includes laser surgery and pharmacotherapy, but these therapies could result in retinal neuron destruction and irreversible retinal damage [3]. As another serious microvascular complication of diabetes, DN is characterized by albuminuria and progressive loss of kidney function. Approximately, 10% of deaths in people with T2DM are attributable to renal failure [1]. Therefore, searching forecast index of the microvascular complications is contributed to the diagnosis and treatment of them.

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone with extrapancreatic effects beyond glycemic control. In addition to stimulating secretion of insulin, glucagon-like peptide-1, and glucagon, GIP has been ascribed a positive role in cardiovascular function [4, 5]. Clinical studies have found that patients with cardiovascular disease such as peripheral systemic atherosclerosis, myocardial infarction, and stroke have elevated circulating GIP levels [6, 7]. Further experimental studies identified that GIP was an endogenous counterregulatory vaso-protective peptide, which attenuated atherosclerotic plaque inflammation in vivo and abrogated inflammatory macrophage activation in vitro [7]. Previous studies of dogs showed that GIP infusion increased blood flow into the superior mesenteric artery and portal vein, while decreasing it in the pancreatic and hepatic arteries [8, 9]. Recent research of human indicated that GIP infusion had a dual effect on blood flow, with a prominent increase in jejunal blood flow paralleled by a decrease in pancreatic blood flow [10].

As previously mentioned, increasing evidence suggested that GIP played an important role in cardiovascular function. However, the association between GIP and microvascular complications including retina and kidney is largely unknown in humans even though GIP mRNA expression has been confirmed in the rat retina and their levels were upregulated in diabetic rats in an early report [2]. In view of vascular effects of GIP, it is reasonable to speculate that there is a correlation between GIP and microvascular complications in T2DM. In the present study, a cross-sectional study was performed to explore the relationship between GIP and microvascular complications in T2DM. On the other hand, because of the high prevalence of complications and limit treatment of microvascular complications in T2DM, searching forecast index could provide a new thinking for the diagnosis and treatment of them.

Methods

Study design and participants

The study consisted of 451 T2DM patients who were hospitalized in our endocrine ward at the Second Affiliated Hospital of Fujian Medical University, during the period from July 2019 to December 2019. Because of the loss to follow-up, drug using and several reasons, analysis was limited to 306 T2DM patients who received fundus examination, albuminuria creatinine ratio and fasting serum GIP measurements. Furthermore, 11 subjects were excluded from the study due to a lack of levels of glycosylated hemoglobinA1c (HbA1c) (n=7), fasting plasma glucose (FPG), total cholesterol (TC), triglyceride (TG), and uric acid (UA) (n=4). Eventually, 295 T2DM patients were enrolled. There was no significant difference in clinical parameters between the excluded and included subjects. People eligible for the study were informed about the research, its purposes and requested to provide written consent of participation.

Diagnosis of the T2DM

The T2DM patients were diagnosed using the criteria recommended by American Diabetes Association (ADA). This was the case if people had two or more of the following criteria: fasting plasma glucose (FPG)>126.00mg/dL (7.00mmol/L), 2-h postprandial blood glucose (PG) >200.00mg/dL (11.10 mmol/L) during OGTT, HbA1C>6.50% (48.00mmol/mol), a patient with classic symptoms of hyperglycemia or hypoglycemic crisis, a random plasma glucose > 200.00mg/dL (11.10mmol/L).

Determination of fasting serum GIP levels

A 10 ~ 15 ml peripheral venous blood sample was obtained from each participant after an 8-h fasting period. Withdrawn samples were centrifuged at 4 °C for 10 min at 3000 rpm, which were stored at −80 °C until analysis. The levels of serum total GIP were measured using human GIP enzyme-linked immunosorbent assays (ELISA) (MEIMIAN, Jiangsu Meimian Industrial Co., Ltd.) methods according to the kit manufacturers' instructions (range of detection from 0 to 4000 pg/ml).

Screening of microvascular complications

For diabetic retinopathy (DR), non-mydriatic fundus photographs were examined using digital retinal camera (Canon, Kawasaki Kanagawa Japan, CR-2 Plus AF) by an ophthalmologist and then patients were divided into without DR and with DR. To further assess the severity of DR, the fundus of patients with DR was acquired as per the International Clinical Diabetic Retinopathy Disease Severity Scale (2002).

For diabetic nephropathy (DN), the patients were asked to collect their 24-h urine in whole day. Subsequently, we examined the levels of microalbuminuria and urinary creatinine and computed albuminuria creatinine ratio (ACR). Finally, patients were divided into two groups, namely, without

microalbuminuria (ACR<30mg/dl) and with microalbuminuria (ACR≥30mg/dl).

Data collection and biochemical analyses

A questionnaire survey of the medical records of all subjects was performed, including age, gender, and duration of T2DM. We analyzed such variables as HbA1c, FPG, TC, TG, UA, and other diverse clinical characteristics. Serum levels of HbA1c were measured using high performance liquid chromatography (Bio Rad, DuoFlow-40). Serum levels of FPG, TC, TG, and UA were measured using electrochemiluminescence analysis (Hoffmann-La Roche Ltd., COBAS-8000).

Statistical analysis

All analyses were carried out using the Statistical Package for Social Sciences software version 23 (SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used for variables with skewed distributions. We used Blom's formula to transform the skewed distribution to normal. Continuous variables were normally distributed and shown as mean ± standard deviation ($\overline{x} \pm s$) and irregularly distributed data were expressed as medium (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%). Differences between numeric variables in groups were tested with Student's *t* test or post hoc analysis of one-way analysis of variance (ANOVA). Pearson's correlation coefficient or Spearman's correlation coefficients were calculated to evaluate the relationships between GIP and clinical parameters. To assess independent relationships between GIP and clinical parameters, a multiple linear regression analysis was performed. All reported *p* values were two-tailed and a value of $p < 0.05$ was considered statistically significant.

Results

Characteristics of T2DM patients

The baseline characteristics and clinical parameters of the cross-sectional study were summarized in Table 1.The mean age of the subjects in this study was 55.10 years, and 60.68% were male. The subjects were with a median disease duration of 7.00 years and their median glucose status was 8.62mmol/L fasting glucose and 9.00% HbA1c. The levels of median fasting serum GIP of all were 431.36pg/ml, interquartile range of which were 333.26~531.96 pg/ml. Among the 295 T2DM patients, the prevalence of DR and DN were 37.63% and 38.64% respectively.

Table 1 Demographic and clinical characteristics of T2DM patients (*n*=295)

Parameters	
Age (year)	55.10±12.45
Gender (male%)	60.68
Duration (year)	7.00 (2.00~10.00)
GIP (pg/ml)	431.36 (333.26~531.96)
HbA1c (%)	9.00 (7.20~11.10)
FPG (mmol/L)	8.62 (6.83~12.28)
TC (mmol/L)	4.45 (3.66~5.47)
TG (mmol/L)	1.54 (1.09~2.41)
UA (μmol/L)	346.00 (274.00~393.00)
DR (%)	37.63
DN (%)	38.64

Data are presented as mean±standard error ($\overline{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

GIP glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid, *DR* diabetic retinopathy, *DN* diabetic nephropathy

The prevalence of DR were significantly higher in low-levels GIP group compared with those in high-levels GIP group

To determine the relationship between fasting serum GIP and clinical parameters, the T2DM patients were divided into two groups based on the median of fasting serum GIP levels in our study, as follows: GIP < 431.36pg/ml and GIP≥431.36pg/ml. The medians of low-levels GIP group and high-levels GIP group were 333.26pg/ml and 531.90pg/ml, interquartile ranges of which were respectively 252.71~393.96pg/ml and 466.80~685.13pg/ml. In contrast to the high-levels GIP group, the prevalence of DR was significantly higher in the low-levels GIP group (p=0.007) (46.26% versus 31.08%) (Table 2), whereas there was no significant difference in the prevalence of DN between these two groups (41.50% vs 33.78%) (P =0.173). Also, no differences were found between groups for age, gender ratio, duration, HbA1c, FPG, TC, TG, and UA (Table 2).

The levels of fasting serum GIP were higher in T2DM patients without DR than those with DR

To compare the clinical parameters between T2DM without DR and those with DR, our study divided T2DM patients into two groups based on whether they had DR, as follows: T2DM without DR and T2DM with DR. The result showed that the levels of fasting serum GIP were different in DR in these

Table 2 Clinical characteristics of the T2DM patients in different levels of fasting serum GIP

Parameters	GIP<431.36pg/ml (*n*=147)	GIP≥431.36pg/ml (*n*=148)	*p*
Age (year)	54.95±12.52	55.24±12.41	0.843
Gender (male%)	60.54	60.81	0.963
Duration (year)	7.00 (2.00~10.00)	6.00 (1.00~10.00)	0.191
GIP (pg/ml)	333.26 (252.71~393.96)	531.90 (466.80~685.13)	<0.001**
HbA1c (%)	8.80 (7.20~10.90)	9.15 (7.43~11.48)	0.395
FPG (mmol/L)	8.56 (6.83~12.30)	8.70 (6.81~12.12)	0.956
TC (mmol/L)	4.35 (3.58~5.39)	4.56 (3.72~5.50)	0.558
TG (mmol/L)	1.59 (1.15~2.26)	1.53 (1.03~2.61)	0.830
UA (μmol/L)	350.00 (280.00~393.00)	335.00 (272.00~398.75)	0.546
DR (%)	46.26	31.08	0.007**
DN (%)	41.50	33.78	0.173

Data are presented as mean±standard error ($\bar{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

**$p < 0.01$

GIP glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid, *DR* diabetic retinopathy, *DN* diabetic nephropathy

microvascular complications (p=0.019) (Table 3). The levels of fasting serum GIP were significantly higher in T2DM patients without DR than those with DR (440.99pg/ml versus 405.90pg/ml) (Fig. 1). In addition, age and duration of T2DM were higher in T2DM patients with DR. Between the groups, there were no significant differences in HbA1c, FPG, TC, TG, and UA.

Meanwhile, same subjects were divided into two groups according to whether they had DN, as follows: T2DM without DN and T2DM with DN. However, no significant differences were observed on the levels of fasting serum GIP between T2DM without DN and those with DN (p=0.273) (Table 4). Moreover, compared with the T2DM patients without DN, those with DN had longer duration of T2DM, higher FBG, TG, and UA.

The levels of fasting serum GIP of T2DM patients without both DR and DN were higher in contrast to those with diabetic microvascular complications and those with DR only

To determine the relationship between fasting serum GIP and diabetic microvascular complications (DR and DN), the T2DM patients were divided into four groups, as follows: T2DM without DR and DN, T2DM with DR only, T2DM with DN only and T2DM with DR and DN. Our study indicated that the levels of fasting serum GIP of T2DM patients

Table 3 Clinical characteristics were compared with or without DR in T2DM

Parameters	Without DR (*n*=181)	DR (*n*=114)	*p*
Age (year)	53.16±13.28	58.18±10.33	0.001**
Gender (Male%)	63.54	56.14	0.207
Duration (year)	5.00 (1.00~10.00)	9.00 (4.00~11.25)	0.000**
GIP (pg/ml)	440.99 (354.98~559.93)	405.90 (321.98~506.60)	0.019*
HbA1c (%)	8.60 (7.00~11.45)	9.10 (7.45~10.50)	0.742
FPG (mmol/L)	8.53 (6.76~12.42)	8.81 (6.88~11.89)	0.687
TC (mmol/L)	4.56 (3.62~5.50)	4.29 (3.74~5.35)	0.558
TG (mmol/L)	1.57 (1.06~2.30)	1.51 (1.12~2.72)	0.633
UA (μmol/L)	344.00 (274.00~385.50)	349.00 (277.75~403.25)	0.411

Data are presented as mean±standard error ($\bar{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

*$p < 0.05$

**$p < 0.01$

DR diabetic retinopathy, *GIP* glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid

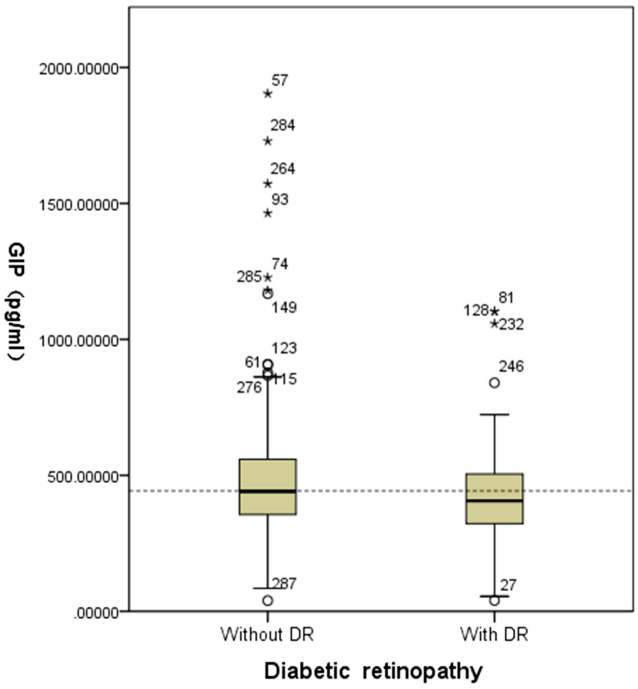

Fig. 1 Comparison of fasting serum GIP levels between T2DM patients without DR and with DR

without DR and DN were higher compared with those with diabetic microvascular complications and with DR only (Table 5). Although there were no significant differences in fasting serum GIP among the groups by using one-way ANOVA, post hoc shown that in contrast to the T2DM without DR and DN, T2DM with DR only (p=0.052) (441.54pg/ml versus 417.57pg/ml) and T2DM with DR and DN (p=0.046) (441.54pg/ml versus 403.85pg/ml) had lower levels of fasting serum GIP. Additionally, there were significant differences in age and duration of T2DM among the groups. It is worth mentioning that TG differed among the four groups, which tended to be higher in T2DM patients with both DR and DN.

Prevalence of DR was independently and negatively correlated with fasting serum GIP in T2DM

We next evaluated the correlation between fasting serum GIP and clinical parameters. In all the patients, the Spearman's correlation analysis showed that prevalence of DR was negatively correlated with fasting serum GIP in T2DM (r=−0.134, p=0.021) (Table 6). However, no significant differences were observed in fasting serum GIP with age, gender ratio, duration, HbA1c, FPG, TC, TG, UA, and prevalence of DN.

To further determine which clinical parameters were independently associated with fasting serum GIP, multiple stepwise regression analysis was performed (Table 7). We selected the parameters such as age, gender, duration, HbA1c, FPG, TC, TG, UA, prevalence of DR, and prevalence of DN as independent, fasting serum GIP as dependent. As a result, prevalence of DR was independently associated with fasting serum GIP even when adjusted for age, gender, duration, HbA1c, FPG, TC, TG, UA, and prevalence of DN (DR: β = −0.279, 95% CI: −0.512 ~ −0.047, p = 0.019).

No differences were observed in fasting serum GIP betweens NPDR and PDR

The fundus of T2DM patients with DR was acquired as per the International Clinical Diabetic Retinopathy Disease Severity Scale (2002) to further assess the severity of DR. In our further study, the population of T2DM patients with DR, those with DR in only one eye (n=4), those with different binocular stage of DR (n=3), and those who had cataract

Table 4 Clinical characteristics were compared with or without DR DN in T2DM

Parameters	Without DN (n=184)	DN (n=111)	p
Age (year)	54.08±12.08	56.78±12.91	0.060
Gender (male%)	60.87	60.36	0.931
Duration (year)	5.00 (1.00~10.00)	10.00 (4.00~11.00)	0.001**
GIP (pg/ml)	436.56 (352.12~538.26)	418.90 (324.78~512.12)	0.273
HbA1c (%)	8.65 (7.10~10.85)	9.40 (7.80~11.60)	0.061
FPG (mmol/L)	8.12 (6.64~11.47)	9.50 (7.41~12.49)	0.024*
TC (mmol/L)	4.43 (3.61~5.46)	4.57 (3.72~5.47)	0.416
TG (mmol/L)	1.43 (1.02~2.19)	1.69 (1.21~2.95)	0.002**
UA (μmol/L)	337.50 (270.50~381.50)	360.00 (285.00~404.00)	0.048*

Data are presented as mean±standard error ($\bar{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

*$p < 0.05$

**$p < 0.01$

DN diabetic nephropathy, *GIP* glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid

Table 5 Clinical characteristics were compared with or without diabetic microvascular complications in T2DM

Parameters	Without DR and DN (*n*=132)	DR only (*n*=52)	DN only (*n*=49)	DR and DN (*n*=62)	*p*	Post hoc
Age (year)	53.26±12.60	56.17±10.45	52.88±15.08	59.87±9.99	0.005**	0.001[c] 0.006[f]
Gender (male%)	62.12	57.69	67.35	54.84	0.554	NS
Duration (year)	5.00 (0.87~10.00)	7.50 (2.25~10.00)	7.00 (2.50~10.00)	10.00 (5.00~14.00)	0.000**	0.009[a] 0.000[c] 0.011 [f]
GIP (pg/ml)	441.54 (362.55~570.08)	417.57 (283.00~509.79)	440.99 (305.45~526.51)	403.85 (331.83~500.00)	0.112	0.052[a] 0.046[c]
HbA1c (%)	8.50 (6.90~10.98)	8.80 (7.30~10.50)	9.30 (7.80~12.45)	9.45 (7.65~10.55)	0.258	NS
FPG (mmol/L)	8.28 (6.79~11.77)	7.85 (6.42~10.34)	9.19 (6.73~13.65)	9.68 (7.48~12.42)	0.080	0.029[d] 0.028[e]
TC (mmol/L)	4.56 (3.57~5.50)	4.13 (3.73~5.24)	4.57 (3.69~5.52)	4.56 (3.85~5.44)	0.619	NS
TG (mmol/L)	1.52 (1.01~2.30)	1.27 (1.05~1.87)	1.68 (1.22~2.47)	1.83 (1.21~3.60)	0.004**	0.009[c] 0.036[d] 0.000[e]
UA (μmol/L)	340.50 (274.00~382.00)	320.50 (258.50~379.25)	354.00 (278.00~388.50)	366.50 (286.50~420.25)	0.120	0.044[c] 0.026[e]

Data are presented as mean±standard error ($\bar{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

**$p < 0.01$

DR diabetic retinopathy, *DN* diabetic nephropathy, *GIP* glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid, *NS* not significant.

[a] Without DR and DN versus DR only

[b] Without DR and DN versus DN only

[c] Without DR and DN versus DR and DN

[d] DR only versus DN only

[e] DR only versus DR and DN

[f] DN only versus DR and DN

Table 6 Evaluated the correlation between fasting serum GIP and clinical parameters

Parameters	Correlation coefficient (*r*)	*p* value
Age	0.031	0.592
Gender	0.009[b]	0.874
Duration	−0.021	0.715
HbA1c	0.019	0.743
FPG	−0.026	0.656
TC	0.045	0.446
TG	−0.072	0.215
UA	−0.020	0.732
DR	−0.134[b]	0.021*
DN	−0.065[b]	0.265
DR and DN	−0.135[b]	0.020*

[b] Spearman's correlation coefficients

*$p < 0.05$

HbA1c glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid, *DR* diabetic retinopathy, *DN* diabetic nephropathy

meanwhile which caused their fundus were unclear (*n*=5) were excluded. Finally, 102 T2DM patients with DR were included. Subsequently, a total of 102 T2DM patients with DR were divided into two groups based on the presence of neovascularization, as follows: T2DM patients with non-proliferative diabetic retinopathy (NPDR) and T2DM patients with proliferative diabetic retinopathy (PDR). As shown in Table 8, there were no differences in fasting serum GIP between NPDR and PDR (*p*=0.971).

Table 7 Multiple stepwise regression analysis to determine independently associated between fasting serum GIP and clinical parameters

Parameters	β	95% CI for β	*t*	*p*
Constant	0.108	−0.036~0.253	1.471	0.142
DR	−0.279	−0.512~−0.047	−2.366	0.019*

DR diabetic retinopathy

*$p < 0.05$

Table 8 Clinical characteristics were compared in different stage of DR

Parameters	NPDR (*n*=92)	PDR (*n*=10)	*p*
Age (year)	57.94±10.43	56.60±6.28	0.614
Gender (male%)	57.61	50.00	0.648
Duration (year)	9.00 (4.00~10.00)	5.50 (0.85~14.25)	0.613
GIP (pg/ml)	413.09 (325.34~516.92)	413.91 (359.09~470.26)	0.971
HbA1c(%)	9.10 (7.53~10.50)	7.60 (6.43~10.95)	0.255
FPG (mmol/L)	8.50 (6.84~11.29)	9.19 (5.82~11.24)	0.853
TC (mmol/L)	4.45 (3.76~5.32)	3.95 (3.41~6.49)	0.932
TG (mmol/L)	1.48 (1.12~2.42)	1.34 (1.10~2.22)	0.754
UA (μmol/L)	350.00 (269.50~404.00)	313.00 (269.50~371.50)	0.486

Data are presented as mean±standard error ($\bar{x} \pm s$) or median (interquartile range). Categorical outcomes were shown as absolute and relative prevalence of complications (%)

NPDR non-proliferative diabetic retinopathy, *PDR* proliferative diabetic retinopathy, *GIP* glucose-dependent insulinotropic polypeptide, *HbA1c* glycosylated hemoglobinA1c, *FPG* fasting plasma glucose, *TC* total cholesterol, *TG* triglyceride, *UA* uric acid

Discussion

In the present clinical study, we observed that the prevalence of diabetic retinopathy (DR) was significantly higher in low-levels glucose-dependent insulinotropic polypeptide (GIP) group and the levels of fasting serum GIP were also higher in type 2 diabetes mellitus (T2DM) patients without DR. Furthermore, prevalence of DR was independently and negatively correlated with fasting serum GIP in T2DM patients. To our knowledge, this is the first clinical study revealing the association between fasting serum GIP and prevalence of DR. The conclusion of our research applies to T2DM who meet the characteristic such as the age was about 55.10 years, the disease duration was about 7.00 years and the HbA1c was about 9.00%.

DR is a common and specific microvascular complication of diabetes, which remains the leading cause of decreased vision and eventual blindness. The multi-hospital-based epidemiological investigations in large Chinese sample had shown that approximately one-third of patients with diabetes mellitus had DR [11]. In addition, finding from epidemiological studies suggested that DR was a risk marker for systemic vascular complications including stroke, coronary heart disease, and heart failure [12]. The pathophysiology of DR is multifactorial and complex, which is induced by multiple factors involving retinal inflammation, elevated retinal vascular permeability, and breakdown of blood-retinal barrier [12–14]. Chronic exposure to hyperglycemia is believed to initiate a cascade of biochemical and physiological changes that ultimately lead to microvascular damage and retinal dysfunction [15]. Meanwhile, it is indisputable that vascular endothelial growth factor (VEGF) plays an important role in the pathogenesis of DR [12, 16]. The upregulation of VEGF is a major cause of vascular leakage and retinal neovascularization because VEGF could enhance vascular permeability dramatically by weakening inter-endothelial junctions and inducing fenestrations [13]. Proinflammatory molecules also play prominent parts in the pathogenesis of DR [17]. In response to hyperglycemia, an array of inflammatory mediators is upregulated that might cause abnormal leucocyte-endothelial interactions and ultimately retinal microvascular damage [12, 13]. Moreover, oxidative stress is a vital contributor to vasculopathy in DR, which might exacerbate breakdown of the blood-retinal barrier by increased vascular leakage and VEGF expression [18].

The control group of non-diabetes population was not designed in our study, because many researches have focused on the comparison between T2DM and non-diabetic people in GIP levels so far [19, 20]. It has been confirmed that the levels of GIP in T2DM were significantly higher than non-diabetic people in both fasting and postprandial. As been reported, the mean levels of fasting serum GIP were 319±18pg/ml in non-diabetic population [21] and it was 272pg/ml in another research [22]. In present study, the levels of median fasting serum GIP of all were 431.36pg/ml which was obviously higher than the levels of fasting serum GIP in non-diabetes population as previously reported [21]. These differences were similar to the previous studies. Therefore, we believe that the levels of fasting serum GIP in this study are highly reliable.

In the present study, we measured the GIP levels of the subjects after an 8 h fasting period, so we considered that the effect of diet on the levels of fasting serum GIP was very little. After selecting all T2DM, we eliminated the patients who were treated with GLP-1 analog and DPP-4 inhibitor. The included subjects were grouped according to the type of drug used and there were no significant differences in the levels of fasting serum GIP among groups.

Although impaired GIP effect has been reported in patients with T2DM, increasing evidence suggests that fasting GIP

plays an important role in the pathophysiology of T2DM and basal GIP concentration plays a significant role in glucose homeostasis [23, 24]. In addition, recent study shows a significant association between fasting GIP and cardiovascular disease [25]. Meanwhile, increased fasting GIP concentration is also associated with risk of cardiovascular mortality. Hence, our study mainly focused on the fasting serum GIP.

Limited number of studies of animal experiments indicated that the expression of GIP and GIP receptor (GIPR) were not only in the rat retina but their levels were marked increment in the diabetic rat as compared to controls [2]. Similarly, a nested case-control study found that fasting total GIP levels were higher in the incident diabetes group than in the control group and in them were associated with an increased risk of diabetes independent of other risk factors which provided clue that GIP may be a risk factor for the development of T2DM [26]. Considering their localization and increased expression, the GIP and GIPR might play a role in retinal physiology, which were related to the retinal vascular dysfunction under DR. These conclusions have not been confirmed in clinical trials so far. Here, we demonstrate the amazed correlation between fasting serum GIP and prevalence of DR. Although the exact mechanism by which fasting serum GIP are associated with prevalence of DR remains unclear, possible explanations for the association deserve consideration. As an incretin hormone, GIP has been ascribed a positive role in cardiovascular function. Various studies have shown that GIP exerts direct vasoprotective and anti-atherogenic effects [4, 5, 7]. Activation of GIPR has been indicated to be protective against atherosclerosis as well [6]. Summary of numbers of researches find that there is a substantial difference in the hemodynamic responses to GIP among splanchnic organs and vessels, for example, the increased blood flow of superior mesenteric artery [8], femoral artery [27], portal vein [9], and jejunum [10] and the decreased blood flow of hepatic artery [9] and pancreas [10] are observed after GIP infusion. These would suggest that GIP could modulate blood flow in different ways. Consistent with the pathophysiology of DR, GIP exerts anti-inflammatory and antioxidant effects in vasculature [28]. The experiments performed in vitro conclude that GIP could suppress the formation of macrophage foam cell, abrogate the activation of inflammatory macrophage, and block the proinflammatory pathways in macrophages and then plays a positive part in anti-atherogenic effects [5, 7]. Additionally, GIP has been confirmed stimulating endothelial NO production by a GIPR/PLC/CaMKKb/AMPK/eNOS pathway [29]. Nitric oxide (NO), as a potent and short-lived vasodilator, is a clear mediator of the protective effects of GIP against peripheral arterial remodeling [29, 30]. Given that critical effect in the anti-atherogenic process of GIP, we speculate that GIP might play a role in retinal vascular dysfunction under DR and the mechanism of the correlation between fasting serum GIP and prevalence of DR might be related to the anti-inflammatory and antioxidant effects of GIP in endothelial cells of retinal vasculature. However, the causal interpretation remains unclear, which needs further validation.

Based on the presence of neovascularization, DR can be classified as either non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR). In NPDR, the manifestations include vascular permeability, microaneurysms, hemorrhages, and venous abnormalities. In PDR, the characteristics involve neovascularization in the retina and posterior surface of the vitreous, which is accompanied by unavoidable vision loss generally [31]. Unexpectedly, our study did not observe any differences on fasting serum GIP between NPDR and PDR. This may be due to the count of T2DM patients with PDR included in our study is small because they usually visit the ophthalmology department for vision loss. Therefore, this result is worthy of discussion, which needs more cases to determine in the future.

One of the vital goals of T2DM management is to reduce microvascular complications including DR. Early detection and medical treatment are essential to preserve eyesight because it might result in visual loss or even blindness if patients are undiagnosed and untreated before the manifestation of symptoms [32]. Visual loss resulting from DR exerts a significant negative effect on the life quality. Recently, although great advances have been made in therapy of DR, the most popular treatment for DR such as laser surgery could lead to irreversible retinal damage as well [33]. Hence, it is still a need to identify early-stage DR biomarkers and search new diagnoses and treatment avenues for DR. The results obtained in the present study could allude that elevated levels of fasting serum GIP were protective factors for DR and low levels of fasting serum GIP were associated with a significantly increased risk of DR in T2DM, which might open new diagnoses and treatment avenues for T2DM patients with DR. Obviously, the decreased levels of fasting serum GIP could predict the occurrence of DR in T2DM, while it could not predict the development of DR. Currently, numerous drug studies which were based on the structure and function of GIP have been carried out to improve the efficiency of it in humans [34, 35]. With our conclusion as a clue, the development of these drugs may be a benefit to control the blood glucose and prevent the occurrence of DR.

It is worth mentioning that we did not find any association between fasting serum GIP and prevalence of DN. As previously mentioned, there is a substantial difference in the hemodynamic responses to GIP among splanchnic organs and vessels, which might depend on the vascular bed affected [36]. Therefore, we infer that the differences in the correlation between fasting serum GIP and microvascular complications between different splanchnic organs might be related to the different hemodynamic responses.

We acknowledge the limitations of our study. First, the current study, as a hospital-based study, was conducted in a

small number of single-center cohort patients, which led to the possibility of selection bias. The patients recruited into our study may not be representative of the overall population with T2DM. Secondly, in addition to age, other clinical parameters such as duration, GIP, HbA1c, FPG, TC, TG, and UA were not normally distributed. These skewed distributions might be mainly related to the sample size because the sample population in our study was small exactly. We will expand the sample size in future experiments to further verify the conclusions of this study. Third, the cross-sectional design is known to have many sources of bias, we minimized confounders by adjusting for relevant risk factors using multiple stepwise regression analyses. Even though cross-sectional design could only provide clues to the correlation, but could not support causal interpretation, which needs prospective studies to confirm. Finally, the count of T2DM patients with PDR included in our study is small because they usually visit the ophthalmology department for vision loss. Therefore, the result that no differences were observed on fasting serum GIP between NPDR and PDR is worthy of discussion, which needs more cases enrolled to determine in the future.

Taken all together, prevalence of DR was independently and negatively correlated with fasting serum GIP in T2DM, indicating that elevated levels of fasting serum GIP were protective factors for DR and low levels of fasting serum GIP were associated with a significantly increased risk of DR in T2DM, and the mechanism needs to be further discussed.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Declarations

Competing interests The authors have declared that no competing interest exists.

Ethical considerations The research protocol was approved by the local Bioethics Committee.

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ORIGINAL ARTICLE

Association Between Homocysteine and Type 2 Diabetes Mellitus: a Systematic Review and Meta-analysis

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Abstract

Background Several studies have been performed to assess the relationship between hyperhomocysteinemia and type 2 diabetes mellitus (T2DM). However, inconsistent results have been obtained. Therefore, we performed this meta-analysis to address this knowledge gap.

Methods We searched PubMed, Cochrane library, and EMBASE database for studies that evaluated the relationship between blood homocysteine (HCY) level and T2DM from inception to Jun 2019. The quality of all included studies was assessed by the Newcastle Ottawa Scale (NOS) and the Agency for Healthcare Research Quality (AHRQ). RevMan5.3 and Stata12.0 were used for data analyses.

Results Twenty-five studies (including 1881 cases and 2868 controls) on blood HCY level in T2DM were pooled in our meta-analysis. The blood HCY level in the T2DM patients was significantly higher than in the healthy individuals (SMD = 0.63, 95% CI = 0.43–0.84, and $p < 0.001$, $I^2 = 89\%$, $p < 0.001$), ignores the effects of age, sex, cardiovascular and cerebrovascular disease conditions, and other comorbidity. Additionally, in T2DM patients with nephropathy or retinopathy, blood HCY level was also significantly higher than in those with only T2DM (SMD = 1.17, 95% CI = 0.76–1.58, $p < 0.001$; $I^2 = 90\%$, $p < 0.001$ and SMD = 0.91, 95% CI = 0.39–1.44, $p < 0.001$; $I^2 = 82\%$, $p < 0.001$, respectively).

Conclusion Our meta-analysis revealed the HCY level in the blood of T2DM patients was significantly higher than those of the health subjects, especially in patients with diabetic nephropathy (DN) and diabetic retinopathy (DR).

Keywords Homocysteine · Type 2 diabetes mellitus · Systematic review · Meta-analysis

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Introduction

With the expanding globalization, human lifestyle and behavior have significantly changed, leading to a substantial increase in diabetes mellitus prevalence. Currently, diabetes mellitus is one of the most common chronic diseases worldwide. Estimates showed that 382 million people all over the world had diabetes mellitus in 2013, and this number is expected to increase to 592 million by 2035 [1].

Two main forms of diabetes exist, type 1 diabetes mellitus and type 2 diabetes mellitus (T2DM). In this study, we focused mainly on T2DM. T2DM is characterized by insulin resistance and/or abnormal insulin secretion, whereby blood glucose cannot be normally metabolized by insulin, which leads to hyperglycemia [2]. However, chronic hyperglycemia in T2DM patients was evidenced to be related to long-term damage or dysfunction in many organs and systems, including the eyes, kidneys, nerve system, heart, and the blood vessels [3]. The most common long-term complications of T2DM are macrovascular and microvascular diseases. Diabetic

retinopathy (DR) and diabetic nephropathy (DN) are among the most significant microvascular complications of T2DM that lead to vision loss and renal failure [3], correspondingly. These complications seriously affect the quality of life and individuals' survival. Therefore, establishing the potential risk factors is necessary to effectively prevent and treat T2DM.

Homocysteine (HCY), a sulfur-containing non-protein amino acid, is an intermediary in the metabolism of methionine. The metabolism of HCY in the blood is realized by two pathways: (1) remethylation to methionine, which requires the presence of folic acid and vitamin B12; (2) transsulfuration to cystathionine, which necessitates the availability of pyridoxal-5′-phosphate, the active coenzyme form of vitamin B6 [4]. Therefore, any deficiency of elements in the two pathways may lead to an elevation in blood HCY level, and, eventually, to the development of hyperhomocysteinemia. Furthermore, blood HCY level is also affected by renal dysfunction [5], abnormal liver function [6], insulin resistance [7], *etc*. Hyperhomocysteinemia is an emerging risk factor for T2DM, diabetics complications, cardiovascular disease, and cerebrovascular disease [8–12].

In recent years, the relationship between hyperhomocysteinemia and T2DM has been extensively investigated [11–35], but the obtained results are controversial or inconclusive. The mechanism of elevated blood HCY in patients with T2DM is still not fully understood. Therefore, we conducted a systemic review and meta-analysis to evaluate the relationship between blood HCY level and T2DM.

Materials and methods

Search strategy

According to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines [36], a systematic search of studies was carried out among papers written in English that investigated the association of plasma HCY level and T2DM patients, regardless of the publication status (published, in press, or in process). PubMed, EMBASE, and Cochrane Library databases were independently searched by two investigators using expanded Medical Subject Headings (MeSH) terms and corresponding terms as keywords or text words to retrieve related original studies published from inception to Jun 2019. The following terms were used for the search: (MeSH exp "Diabetes Mellitus, Type 2" and keywords/text words "type 2 diabetes mellitus" or "T2DM" or "niddm" or "mody*" or "DMT2" or "stable daibet*"), and (MeSH exp "Homocysteine" and keywords/text words "Homocystine" or "Hcy" or "homocysteinemia" or "hyperhomocysteine" or "HHcy"). In addition, reference lists of all closely related literature, meta-analysis and review articles were also reviewed to avoid missing important literature.

Study selection

Studies that met the following inclusion criteria were included in this meta-analysis: (1) case-control, cross-sectional, cohort, and other prospectively designed studies that assessed the association between blood HCY level and T2DM within human studies; (2) the diagnosis of T2DM patients was clearly defined according to recognized diagnostic standards; (3) the literature clearly indicated the number of cases and controls and provided the value of mean with 95% confidence intervals (95%CIs) and standard deviation (SD) or sufficient data for calculation; (4) participants were not less than 18 years old, regardless of nationality, profession, sex, ethnicity, etc.

Data extraction

Each identified study was carefully reviewed by two independent investigators to determine if it met the inclusion criteria of the present meta-analysis. When there was a conflicting evaluation, a discussion was carried out until an agreement was reached. If a consensus could not be reached, a third experienced investigator was consulted. Data were also extracted independently by two investigators to ensure that the precise targeted data were collected. The following data were extracted from each of the included studies: name of the first author, year of publication, journal, study location, number of cases and controls, study design, mean BMI and age, duration of diabetes mellitus, diabetic complications, and mean values with standard deviation (SD) of fasting blood HCY level in both the experimental cases and the control group.

Quality score assessment

We used the Newcastle Ottawa Scale (NOS) [37] to assess the quality of the included case-control and prospective studies. The Agency for Healthcare Research Quality (AHRQ) guidelines were adopted for evaluating the inclusion of cross-sectional studies. The highest score for NOS was 10 points. Studies with NOS score from 5 to 7 and greater than 7 were considered "medium-" and "high-quality" studies, respectively. On the other hand, studies with NOS score lower than 5 points were considered "low-quality" studies. The quality scores of included case-control and prospective studies ranged from 8 to 9. The maximum AHRQ score was 11. The quality scores of included cross-control studies scoped from 6 to 10 (low quality: 1–3, moderate quality: 4–7, high quality: 8–11).

Statistical analysis

This meta-analysis was carried out using RevMan5.3 software and Stata12.0 software. We used mean ± standard (SD) to extract HCY values from each included study. Due to the different measurement methods and units of the blood HCY

level among studies, we used standardized mean difference (SMD) and the corresponding 95% confidence intervals (CIs) as a measure of the effect size to evaluate the differences in the level of HCY in the blood between healthy individuals and T2DM patients.

I-square (I^2) statistic was used to assess the statistical heterogeneity among studies. The heterogeneity was considered significant when the I^2 was greater than 50% [38]. A random effects model was adopted in cases of significant heterogeneity, whereas a fixed effect model was employed in the absence of heterogeneity.

Sensitivity analysis was performed by sequentially omitting one single study every time to assess the influence of each individual study on the pooled measures and then the summarized SMD for the remainders was calculated. Potential publication bias was assessed using Begg's funnel plots and further confirmed by Egger's test [39, 40].

Results

Study selection

Our literature selected 1122 studies after 253 duplicate studies were removed, and 872 studies were excluded because they did not meet the purpose of this meta-analysis. One hundred and thirty-four investigations were potentially eligible for inclusion. After reviewing the full texts, 25 studies were considered eligible and were eventually included in this meta-analysis. The exclusion criteria are presented in Fig. 1.

Characteristics of the included studies

With respect to blood HCY level in T2DM patients, the characteristics of all the included studies were shown in Table 1. Meanwhile, eight studies further investigated the relationship between blood HCY level and T2DM with nephropathy [11, 15, 17, 18, 28, 30, 33, 35]. Furthermore, the relationships between blood HCY level and T2DM with retinopathy were also investigated in three studies [12, 21, 27]. The participants recruited were from 23 to 689, with an average age from 48 to 73 years. The mean BMI of the participants ranged from 21 to 33. Thirteen of the 25 included studies were conducted in Asia, six in Africa, three in Europe, and three in South America. Seventeen were cross-sectional [11–16, 18, 20, 21, 23, 25, 26, 28, 29, 33–35], six case-control [19, 22, 24, 27, 31, 32], and two were prospective studies [17, 30]. The quality assessment parameters and data of each of the included study are listed in Table 1. Two of these studies were evaluated and established to be of medium quality, whereas the other 23 were of high quality according to AHRQ and NOS criteria and other basic characteristics of the T2DM patients, such as the criteria for T2DM diagnosis, the HCY detection method, the sample size, BMI, age, *etc*.

Meta-analysis

All 25 studies [11–35] with 1881 cases and 2868 controls reported blood HCY level. Meta-analysis of the 25 studies showed that the blood HCY level in healthy controls was significantly lower than those in the T2DM patients (SMD = 0.63, 95% CI = 0.43–0.84, and $p < 0.001$: Fig. 2) and ignores the effects of age, sex, cardiovascular and cerebrovascular disease conditions, and other comorbidity. Significantly statistical heterogeneity was observed among studies ($I^2 = 89\%$, $p < 0.001$). However, sensitivity analysis showed that there was no obvious change in the pooled estimates. Based on the types of the studies, subgroup analysis showed significantly lower blood HCY level in the healthy controls than in the T2DM patients (SMD = 0.61, 95% CI = 0.42–0.79, and $p < 0.001$) in 17 cross-sectional studies. Lower blood HCY level was also found in healthy controls as compared with those in T2DM patients (SMD = 0.79, 95% CI = 0.11–1.48, and $p = 0.02$) in six case-control studies. The two prospective studies were pooled in the subgroup analysis; no significant difference in the blood HCY level was detected in the healthy controls and T2DM patients (SMD = 0.58, 95% CI = −1.12–2.29, and $p = 0.50$. Fig. 2), and no statistically significant heterogeneity was observed among the subgroups ($I^2 = 0\%$, $p = 0.88$).

To further investigate the differences in the blood HCY level between the T2DM patients without complications and those with DN or DR, subgroup analyses were performed. All eight studies [11, 15, 17, 18, 28, 30, 33, 35] including 512 DN patients and 773 T2DM patients without DN or DR were pooled in the meta-analysis. The blood HCY level of the T2DM patients was significantly lower (SMD = 1.17, 95% CI = 0.76–1.58, and $p < 0.001$: Figs. 3 and 4) than that of the DN patients, with statistically significant heterogeneity among the eight studies ($I^2 = 90\%$, $p < 0.001$). A total number of three studies [12, 21, 27], including 252 DR patients and 213 T2DM patients without DN or DR, were also pooled in our meta-analysis. The level of HCY in blood was significantly increased in DR patients than T2DM patients (SMD = 0.91, 95% CI = 0.39–1.44, and $p < 0.001$: Fig. 3). A significantly statistical heterogeneity was observed among the three studies as well ($I^2 = 82\%$, $p < 0.001$). Moreover, for each of the two subgroup analyses above, no significant publication bias was observed ($P_{Egger's} = 0.57$), and omission of any single study did not significantly change the overall SMD.

Subgroup analyses were performed based on BMI and ethnicity to evaluate the relationship between the studied clinic parameters of the T2DM patients and HCY level. If data were not reported in a certain study, it would be classified as a non-reporting group (NR). Only eight studies had no information about mean BMI. T2DM patients were

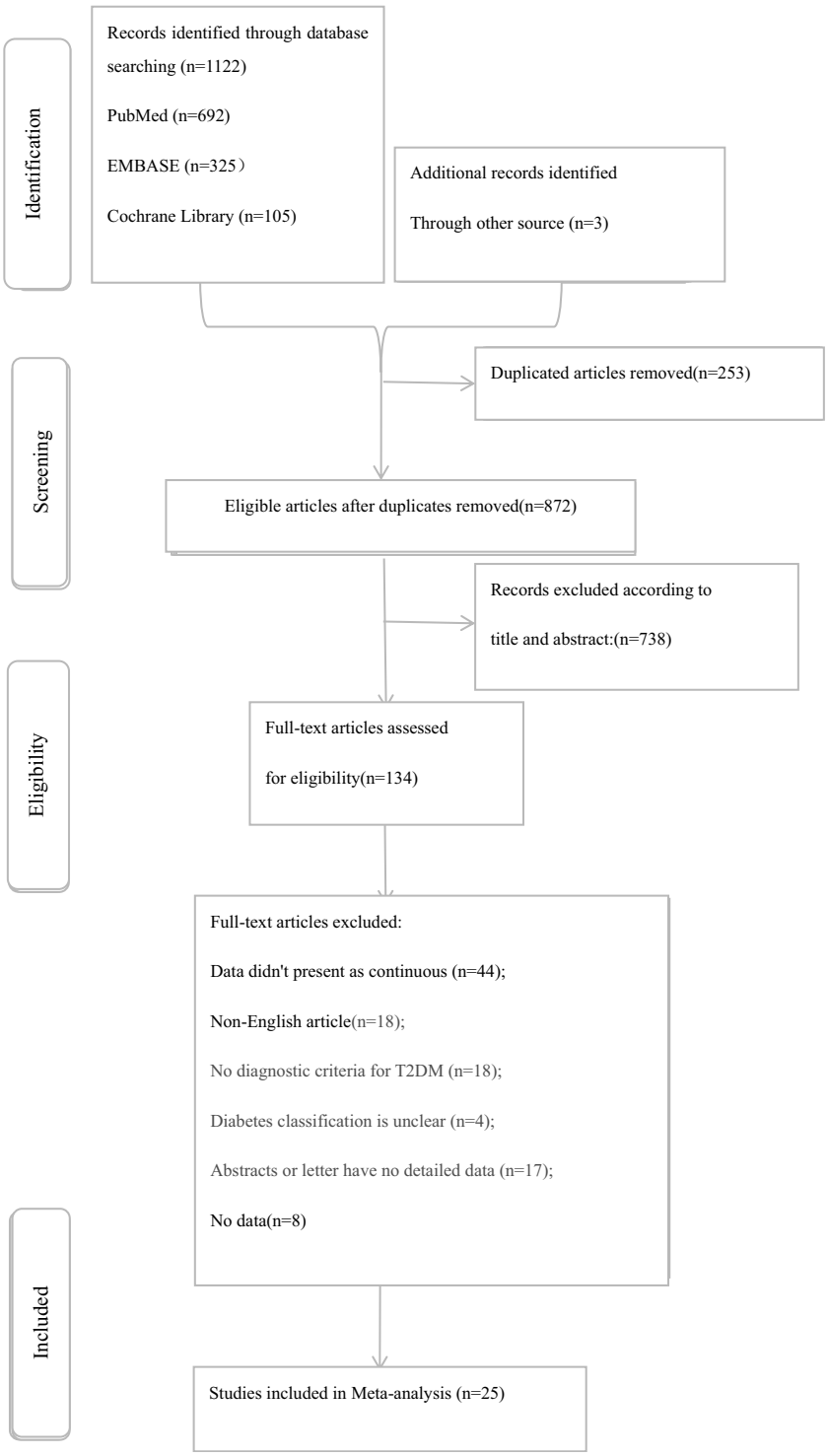

Fig. 1 PRISMA Flow diagram of selection process for this meta-analysis

stratified taking 25 as the tipping point for BMI, 17 studies were classified as mean BMI ≥ 25 (overweight and obese T2DM patients) and mean BMI < 25 (normal weight patients), respectively. Eight studies that did not report mean BMI were classified as NR (Fig. A1). Based on ethnic group, the 25 included studies were divided into four major groups: Asian, African, European, and South American (Fig. A2). As can be seen in Table 2, the blood HCY level in healthy controls was significantly lower than in the T2DM patients in all predefined classifications for BMI level and ethnic except in the South American group. A significant heterogeneity was still observed in all subgroup analyses. However, sensitivity analysis showed that there was no obvious change in the pooled estimates as well.

Table 1 Main characteristics of studies included in this meta-analysis

Study, year	Country	Study design	Control/Case1/Case2/Case 3					Quality score
			N	Mean age (years)	Mean BMI	Diagnosis criteria (T2DM)	Detecting method (HCY)	
Emoto. M, 2001a/b,c,d [11]	Japan	CS	54/39/17,9,10 /NA	53.8/54.6/53.9,57,64/NA	22/22.2/22.8,22.4,22.8/NA	ADA	EIA	8[b]
Aso, Y, 2003 [16]	Japan	CS	45/103/NA/NA	55.1/59.2/NA/NA	23.8/27.7/NA/NA	WHO	HPLC	9[b]
Ozdemir, G. 2005a/b [18]	Turkey	CS	20/31/17/NA	50.4/50/56.9/NA	27.4/29.3/31.9/NA	ADA	IFA	6[b]
Helfenstein, T. 2005 [19]	Brazil	CC	56/50/NA/NA	58/59/NA/NA	27.9/30.1/NA/NA	ADA or WHO	HPLC	8[a]
Sandhu, J. S. 2004a/b [17]	North India	PP	25/25/50/NA	NA/NA/NA/NA	21.5/26.2/24.3/NA	WHO	ELISA	8[a]
Soares, A. L. 2008 [22]	Brazil	CC	16/7/NA/NA	52.3/52.1/NA/NA	25.1/28.1/NA/NA	WHO	AXSYM	8[a]
Masuda, Y. 2008a [23]	Japan	CS	391/196/NA/NA	56.14/60.76/NA/NA	23.51/23.81/NA	JDS	HPLC	10[b]
Masuda, Y. 2008 b [23]	Japan	CS	605/84/NA/NA	56.38/64.73/NA/NA	21.72/23.42/NA/NA	JDS	HPLC	10[b]
Alis, R. 2014 [29]	Spain	CS	69/75/NA/NA	48.1/63/NA/NA	33/30.6/NA/NA	ADA	IFA	8[b]
Al-Maskari, M.Y. 2012 [24]	Sultan	CC	50/50/NA/NA	NA/NA/NA/NA	28/27/NA/NA	ADA	Competitive Immunoassay	8[a]
Okada, E. 1999 [13]	Japan	CS	99/46/NA/NA	63.9/64.5/NA/NA	23.6/22.2/NA/NA	ADA	NR	8[b]
Das, S. 1999 [14]	India	CS	10/20/NA/NA	48.7/54.6/NA/NA	23.87/21.75/NA/NA	the National Diabetes Data Group	HPLC	9[b]
Wang, H. 2015a/b,c [30]	China	PP	49/72/85,51/NA	62.16/60.47/61.79,62.07/NA	23.33/23.68/23.18,22.57/NA	WHO	FPIA	8[a]
Ebesunun M. O.2012 [25]	Nigeria	CS	30/70/NA/NA	49/54/NA/NA	24/30/NA/NA	WHO	ELISA	8[b]
Wang, T. 2013 [28]	China	CS	56/51/60,57/NA	49.2/48.2/51.3,54/NA	NA/NA/NA/NA	WHO	ECLIA	9[b]
Huang, T. 2012 [26]	China	CS	150/104/NA/NA	48.4/57.3/NA/NA	24.3/24.6/NA/NA	WHO	FPIA	10[b]
Dong, N. 2017 [32]	China	CC	126/126/NA/NA	63.6/63.6/NA/NA	24.3/24.6/NA/NA	WHO	HPLC	8[a]
Srivastav, K. 2016a/b,c [12]	India	CS	20/20/NA/20,20	53.26/56/NA/54.3,51.02	NA/NA/NA/NA	ADA	ELISA	8[b]
Ozmen, B.2002a/b [15]	Spain	CS	40/25/40/NA	54.6/55/56.13/NA	NA/NA/NA/NA	ADA	HPLC	7[b]
Dominguez, R. O. 2005 [20]	Brazil	CS	19/18/NA/NA	73.89/66.74/NA/NA	NA/NA/NA/NA	WHO	FPIA	8[b]
Amrane, M.2012a/b [27]	Sétif	CC	60/57/NA/121	48.6/57.3/NA/55.5	NA/NA/NA/NA	WHO	NR	9[a]
Mtiraoui N, 2007a/b [33]	Tunisia	CS	400/267/93/NA	57.6/57.3/59.3/NA	24.5/27.6/28.3/NA	WHO	NR	9[b]
Agullo-OrtunoMT, 2002 [34]	Spain	CS	54/32/NA/NA	33.5/58/NA/NA	NA/NA/NA/NA	the National Diabetes Data Group	FPIA	9[b]
Sharaf SM 2012a/b [35]	Egypt	CS	20/27/23/NA	54.6/52.4/58.4/NA	NA/NA/NA/NA	ADA	Chemiluminescence	8[b]
Huang.E.J.2006a/b [21]	China	CS	204/116/NA/91	NA/NA/NA/NA	NA/NA/NA/NA	WHO	HPLC	9[b]
Fekih-Mrissa.N.2017 [31]	Tunis	CC	300/160/NA/NA	50.5/49.37/NA/NA	25.41/28.47/NA/NA	WHO	Competitive immunoassay	9[a]

CS cross-sectional study, *CC* case-control study, *PP* prospective study, *Case1* type 2 diabetes mellitus, *Case2* type 2 diabetic with nephropathy, *Case3* type 2 diabetic with retinopathy, *NA* not available, *ADA* American Diabetes Association, *WHO* World Health Organization, *JDS* Japan Diabetes Society, *EIA* enzyme immunoassay, *HPLC* high-pressure liquid chromatography, *IFA* immunofluorescence assay, *ELISA* enzyme-linked immunosorbent assay, *AXSYM* Axsym system, *FPIA* fluorescence polarization immunoassay, *ECLIA* electrochemiluminescence immunoassay, *NR* non-reporting

[a] The Newcastle Ottawa Scale (NOS) was used for quality scoring in the included studies

[b] The Agency for Healthcare Research and Quality (AHRQ) was used for quality scoring in the included studies.

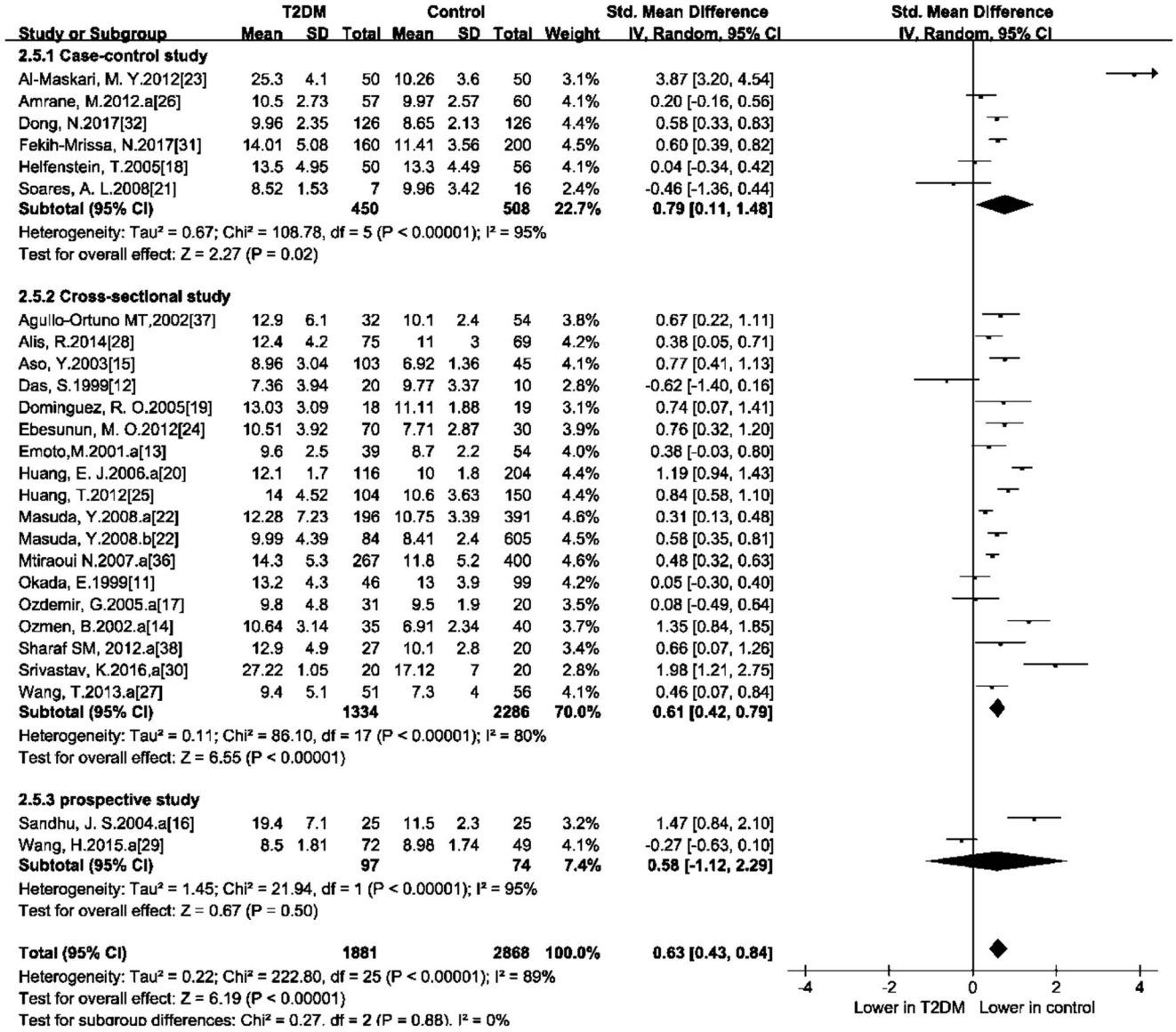

Fig. 2 Meta-analysis of homocysteine levels in T2DM vs healthy controls

Sensitivity analysis and publication bias

During the sensitivity analysis, we found that the pooled SMD and their 95%CI were not obviously altered when sequentially omitting a single study at a time. Hence, the results of the sensitivity analysis suggested that the pooled SMD values for T2DM remained stable.

No significant publication bias was observed in this meta-analysis based on the results from the Begg's funnel plot (Fig. A3). The shapes of the funnel plot for the 25 included studies

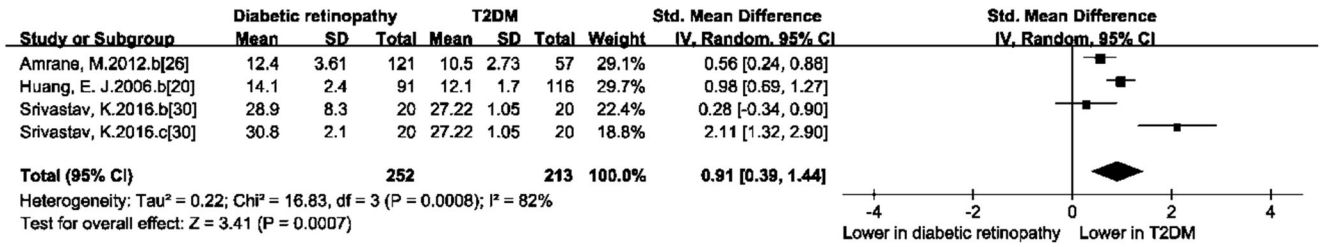

Fig. 3 Meta-analysis of homocysteine levels in T2DM with retinopathy vs T2DM controls

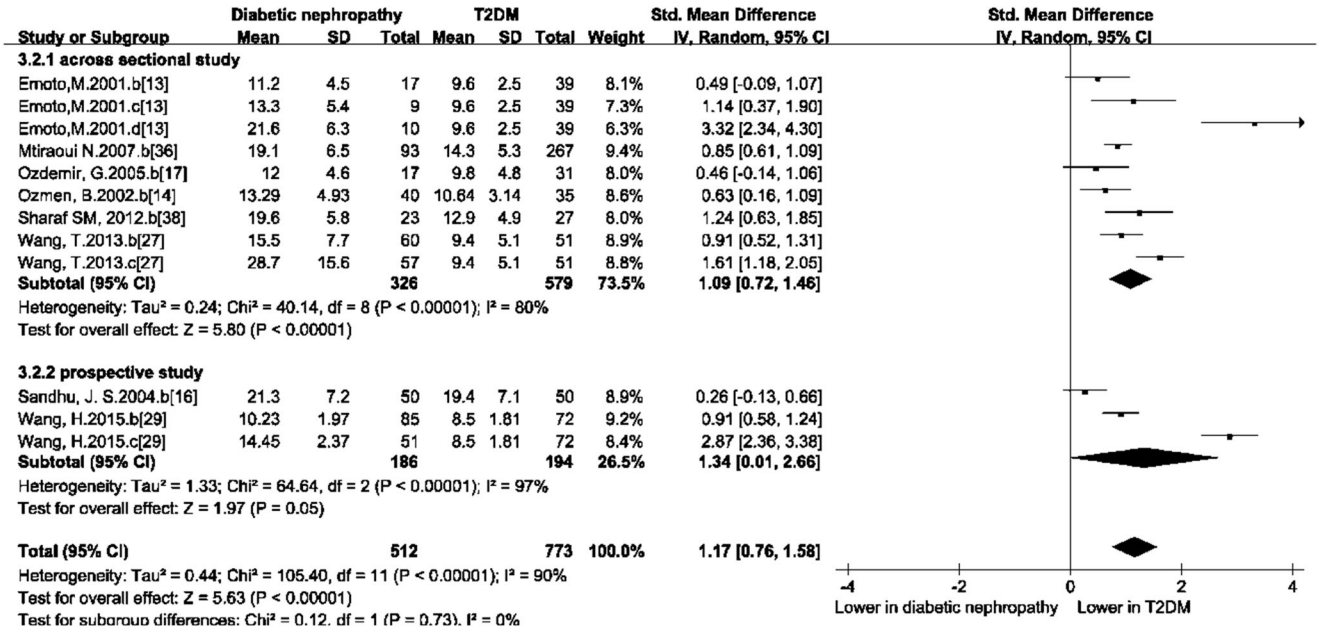

Fig. 4 Meta-analysis of homocysteine levels in T2DM with nephropathy vs T2DM controls

on the HCY level had no obvious asymmetry; the Egger's test confirmed these results (t = 1.71, $P_{Egger's}$ = 0.10, 95% CI = −0.53–5.67).

Discussion

In recent years, the relationship between blood HCY level and T2DM has drawn research attention. In this meta-analysis, we investigated the relationship between blood HCY level and T2DM patients. Moreover, we further studied the relationship between blood HCY level and DN or DR. Overall, the blood HCY level in the T2DM patients was significantly higher than in the healthy control group regardless of the effects of confounders, such as the body mass index, age, gender, ethnicity, diabetes treatment regimen, duration of diabetes, and fasting glucose levels. Importantly, the blood HCY level was significantly higher in the DN and DR patients. However, statistically significant heterogeneity was observed among the studies. Hence, the random-effects model was adopted in our meta-analysis. Moreover, the sensitivity analysis showed no obvious change in the pooled estimates, and no evidence of publication bias was noted as well. These results implicate the presence of an association between blood HCY level and T2DM, especially in patients with DN and DR. Our results are consistent with those of previous meta-analysis conducted by Xu *et al*. [41], although many differences exist. Based on the results of our meta-analysis and those of previous studies, we speculated that

Table 2 Subgroup meta-analysis of the included studies

Subgroup	No. of studies	No. of T2DM	No. of control	SMD(95%CI)	p value	Heterogeneity	
						I^2%	p value
Overall	25	1881	2868	0.63 (0.43–0.84)	<0.001	89	<0.001
BMI							
BMI<25	8	790	1529	0.36 (0.12–0.59)	0.003	82	<0.001
BMI≥25	9	735	866	0.78 (0.32–1.24)	<0.001	93	<0.001
NR	8	356	473	0.87 (0.50–1.23)	<0.001	81	<0.001
Ethnicity							
Asians	10	968	1799	0.47 (0.24–0.71)	<0.001	86	<0.001
African	9	696	815	1.01 (0.51–1.51)	<0.001	94	<0.001
Europeans	3	142	163	0.77 (0.23–1.32)	0.005	80	0.007
South America	3	75	91	0.14 (−0.43, 0.72)	0.62	60	0.08

BMI body mass index, *SMD* standardized mean difference, *CI* confidence interval, *NR* non-reporting

hyperhomocysteinemia may be a useful biomarker for increased risk of T2DM. Moreover, HCY may also increase the risk of diabetic complications, such as DN and DR. Hence, reducing HCY level has guiding significance for the prevention or clinical therapy of the T2DM and diabetic complications.

Moreover, the relationship between elevated blood HCY level and BMI and ethnic in T2DM patients was further investigated. The subgroup analysis of BMI showed that the blood HCY level was significantly higher in both the normal weight and the overweight patients as well as in the obese patients with T2DM. Meanwhile, the blood HCY level was more markedly elevated in the overweight and obese patients with T2DM. In the subgroup analysis of ethnicity, the blood HCY level was significantly more elevated in T2DM patients than in healthy controls of Asian, African, and European populations. However, in the South American group, the level of HCY had no significant difference between T2DM patients and healthy controls. The difference in HCY level in different ethnic might be related to genetic background, type of nutrition, and level of obesity. These factors probably cause the significant heterogeneity of our results, which need to be further investigated. These results implicate an association between blood HCY level and BMI or ethnicity.

The blood HCY level might be affected by multiple factors, such as age, sex, genetic background, duration of diabetic, fasting glucose level, BMI, dietary habit, therapeutic schedule, impaired renal function, and HCY metabolism pathways. However, the main mechanism for affecting blood HCY level in T2DM patients could be listed as follows: First, the insulin resistance (IR) plays a vital role in the pathogenesis of T2DM. Previous studies revealed that blood HCY level was elevated in patients with IR [42–44]. These studies also showed that IR was an independent determinant of blood HCY level in T2DM patients [11, 45]. Moreover, hyperhomocysteinemia was found to contribute to the development of insulin resistance [46, 47]. In addition, HCY is considered to be a neurotoxin. As pancreatic beta cells share many phenotypic similarities with neuronal cells, HCY could cause dysfunctional insulin secretion from pancreatic beta cells [48]. Therefore, we could speculate that hyperhomocysteinemia not only deteriorates IR but also affects insulin secretion by damaging pancreatic beta cells. IR is a well-known characteristic of T2DM, which is accompanied by insufficient insulin secretion. Hence, we speculate that blood HCY level is affected by IR in T2DM patients. Hyperhomocysteinemia in turn deteriorates IR, causing a vicious cycle that leads to higher HCY level in blood. However, it is not clear whether blood HCY is involved in the pathogenesis of T2DM. Second, hyperhomocysteinemia can promote the vascular endothelial injury and microvascular damage, resulting in impaired microcirculation and microthrombosis in the kidneys and the liver [49–51]. It should be noted that HCY is mainly metabolized and cleared through the kidneys [52, 53]. Blood HCY is ultrafiltrated through the glomeruli, almost entirely reabsorbed in the tubuli, and degraded in kidney tissue by transsulfuration and transmethylation [53]. Thus, chronic kidney disease can lead to an increase in blood HCY level. Meanwhile, increased HCY level can produce toxic effects on kidney, which accelerates the deterioration of renal function [54]. Previous studies have demonstrated that the blood HCY level was significantly elevated in T2DM patients with diabetic retinopathy [55, 56]. In addition, the level of HCY in blood, vitreous body, and aqueous humor are also increased in T2DM patients with proliferative retinopathy [57]. Higher HCY level is associated with retinal vein occlusion [58]. Taken together, the blood HCY level is considered as a potential risk factor for accelerating the deterioration of DN and DR. However, the mechanisms for elevating blood HCY level in DN and DR patients are still unclear and need to be investigated further.

Although this meta-analysis was very rigorous, certain limitations are to be acknowledged. First of all, statistical significant heterogeneity was present among the included studies. Although subgroup analysis was employed to evaluate heterogeneity, we failed to detect the main source of heterogeneity. Second, due to the use of the diagnostic criteria of T2DM, the detection methods of blood HCY level, fasting blood glucose level, and the duration of diabetes and diabetes treatment varied among the included studies, which could have affected the precision of the HCY level determined. Third, only studies published in English were included, which lowered the comprehensiveness of our investigation. Fourth, only two prospective studies were included in this meta-analysis due to the lack of sufficient prospective studies to evaluate the association between T2DM patients and the blood HCY level. Hence, further prospective studies are necessary to reveal the causal role of HCY in the development of T2DM. Finally, due to limited data available, we did not investigate the stratified effect of hypoglycemic medications on the blood HCY level in T2DM patients and also the lack of sufficient data to assess the relationship among the HCY and DM and coronary artery disease (CAD). Thus, the effect of antidiabetic medications and CAD on the HCY level in patients with T2DM will be explored in the future.

Conclusions

In the present meta-analysis, we found higher blood HCY level in T2DM patients, especially in those with DN and DR. However, the role of HCY in the pathogenesis of T2DM and its complications remain unclear. More studies need to be performed in the future to determine the causal role of HCY in the development of T2DM. Furthermore, considering the limitations of this meta-analysis, a larger number of

prospective and large-sample studies should be conducted to confirm our current findings.

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Author contribution Jin-Xiang Wang and Rui Liao designed the study, conducted the literature search and screening, as well the data extraction and analyses. Jin-Xiang Wang wrote the first draft of the manuscript. Ding-Yun You analyzed the statistical data. All the authors revised the subsequent drafts for important intellectual content, read, and approved the final version of the manuscript.

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Declarations

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

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ORIGINAL ARTICLE

Genetic characterization of suspected MODY patients in Pakistan by next generation sequencing—a pilot study

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Abstract

Background Maturity onset diabetes of the young (MODY) is the genetic form of diabetes inherited in autosomal dominant pattern. The diagnosis of MODY is challenging and confirmed by genetic testing. The objective of this study was to investigate MODY in Pakistani patients using next-generation sequencing.

Methods The next-generation sequencing was performed to know the cause of disease in 07 patients phenotypically suspected for MODY from Pakistan. These patients were selected based on the high probability score through MODY probability calculator. Only those genetic variants were retained which were having low frequency as per available data bases and their potential effects on protein as assessed by bioinformatics prediction tools.

Results Four heterozygous variants were found among patients. This includes mutation in *HNF1a* gene (c.526+1G>A) that is already reported for causative for MODY type 3 which is most prevalent among all MODY sub-types. Other variants were identified in *GCK* (c.-45G>A), *KLF11* (c.*96dupA), and *CEL* (c.1235C>T).

Conclusion This study was the first in Pakistan to investigate the MODY using next-generation sequencing for clinical applications. There is huge public health burden of diabetes in the country. The study identified mutation in one known MODY gene. More extensive studies involving large sample size are required in future.

Keywords Diabetes · Genetics · Monogenic

Introduction

Maturity onset diabetes of the young is the type of monogenic diabetes inherited in autosomal dominant pattern. The clinical presentation is heterogenous and often characterized by the defects in insulin secretion and beta cell dysfunction [1]. The classical symptoms are onset of diabetes below 25 years of age, diabetes in one of the parents, and absence of auto antibodies [2]. The proportion of MODY comprises 1–5% of all the diabetic patients [3]. Its probability score can be calculated by the MODY probability calculator followed by molecular confirmation [4]. Fourteen different genes have been described to be involved in pathogenesis of the disease including *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, and *APPL1.* The common among them are *HNF4α*, *GCK*, *HNF1α*, *PDX1*, *HNF1β*, and *NEUROD1* [1]. The prevalence of these MODY subtypes varies across the globe; for example, MODY type 2 (*GCK*) is the common subtype in United Kingdom, Italy, Spain, Canada, and Brazil while MODY type

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3 (*HNF1α*) is the prevalent subtype in China, Japan, France, Germany, and Norway [5]. There is scarcity of data on MODY from Pakistan [6]; however, the prevalence of diabetes is suspected to be higher in developing countries. A National Diabetes Survey from Pakistan in 2016–2017 reported 26.3% prevalence of diabetes mellitus among adults indicating a huge public health burden over the healthcare system of the country [7].

The diagnosis of MODY is often challenging owing to the similar clinical pictures of MODY, type 1 and type 2 diabetes. Furthermore, the MODY subtype distinction is not possible clinically which is necessary because treatment choices vary as per the MODY subtype, and misdiagnosis could lead to inappropriate therapy [8]; for example, *HNF1a* MODY subtype is treated with sulphonylureas as first-line oral antidiabetic medications while *GCK* MODY subtype does not require any medications as it causes only mild hyperglycemia with no associated complications. Hence, the necessity is to molecularly characterize the MODY that could help the physician to decide treatment plan.

Conventionally, testing of MODY was limited to Sanger sequencing of genes involved but the method is laborious and time consuming [9]. Nowadays, the advancements in molecular genetics and new technologies greatly facilitated the diagnosis of MODY and have replaced the Sanger sequencing [10]. This study was carried out to identify the MODY and its subtypes via next-generation sequencing in suspected patients among diagnosed diabetics.

Materials and methods

The study was conducted as per Helsinki Declaration. The ethical clearance was provided by the Institutional Bioethics Committee of PHRC (4-87-1/16/IBC/RDC/3231). The written informed consent was taken from all participants prior to enrolment.

The patients were enrolled from Diabetic Clinic of Baqai Institute of Diabetology and Endocrinology, Karachi, Pakistan, on the following criteria:

- Diagnosed as diabetics, onset of diabetes below 25 years of age
- Having direct family history (one of the parents was having diabetes)

Among them, 07 patients were selected for next-generation sequencing based on their high probability score calculated by the MODY probability calculator and presenting clinical pictures. The MODY probability calculator is based on the information related to current age, age at diagnosis, BMI, HbA1c, family history, and treatment details (currently treated with insulin and time to insulin treatment). The demographic information including current age, onset age, gender, family history, and medication received was recorded on structured proforma. The height was recorded in centimeters and weight in kilograms for BMI. The biochemical information, i.e., HbA1c was also collected from hospital patient records. The serum was separated from blood, and glutamic acid decarboxylase (GAD) autoantibody test was done by GAD autoantibody ELISA Kit (Kronus, USA). The ZnT8 (Zinc transporter antibodies) test was also performed by ELISA. These antibody tests helped in excluding type 1 diabetes patients.

Next-generation sequencing

The blood samples were taken from patients and serum separated. The genomic DNA was extracted from patients' blood using DNA purification kit (Promega Corporation USA) as per user manual instructions. The DNA quality was assessed by gel electrophoresis and quantity by ultraviolet spectrophotometer. The whole exome sequencing was performed for which sure select V6 post Kit was used for enrichment while the library sequences were subjected to Illumina Sequencer for sequencing.

Bioinformatic analysis

The Fastq files were aligned for map referencing using Burrows-Wheeler Alignment (BWA) Tool. The duplications were marked using PICARD tool. The variant calling and filtering was done by using Genome analysis toolkit (GATK). Finally, the variant annotation was conducted via SnpEff.

The variants obtained were filtered according to the following criteria; variants in known 14 genes of MODY, heterozygous, non-synonymous coding variants, splice site affecting variants, frame shift indels, and frequency of variants in different databases. The variants were interpreted using Ensembl, dbSNP, ExAC, genomAD, HGMD, ClinVar, 1000Genome database, SIFT, MutationTaster, PROVEAN, and polyphen.

Sanger sequencing

The Sanger sequencing was conducted to confirm the pathogenic variants. The genetic variants were amplified by polymerase chain reaction from DNA samples using forward primers (GGGCTCCATAACTGCTTTCA) and reverse primers (CCTTTCCATCTACCTGTCTGTG) with annealing temperature of 60°C The oligo-nucleotide primers were designed by Primer 3 software. The Sanger sequencing was performed and analyzed by ChromasPro software. For sequence obtained through Sanger sequencing, variants were

labeled as per HGVS nomenclature and classified as per ACMG guidelines

Results

The demographic and clinical characteristics of the seven selected patients are presented in Table 1 while the family history and mode of inheritance are shown in Fig. 1. Among them, three were male patients while four were female patients. The mean age of the patients was 15.7 years (range from 12 to 21 years), mean BMI was 24.3 (range from 18.3 to 29.3), and mean HbA1c was 76.2 mmol/mol (range from 48 to 125).

Out of seven, four patients (M46, M64, M13, and M6) were negative for both GAD-65 and ZnT8 antibodies while two were positive for GAD-65 (M15 and M24) and one (M54) was positive for ZnT8 antibodies.

After filtering and prioritization, variants were identified in four patients. Three patients could not be identified with any variant in known 14 genes. The identified variants include one splice donor, one 5′ UTR, one 3′ UTR variant, and one missense. All variants have previously been reported elsewhere and are presented in Table 2. Two variants (c.526+1G>A in *HNF1a* and c.1235C>T in *CEL*) were identified in patients (M6 and M64) who were negative for both antibodies. .

The splice donor variant was identified in *HNF1a* gene in patient M6. The heterozygous mutation was c.526+1G>A. The patient develops the disease at the age of 17 years. The BMI was 24.6 kg/m^2. The pancreatic auto-antibodies (GAD-65 and ZnT8) were negative. The Sanger sequencing confirmed the presence of this mutation (Fig. 2).

In patient M15, we found five prime UTR variants in *GCK* gene: c.-45G>A having a dbSNP ID as rs150560724. It has a frequency of 0.002 in South Asian database. The patient was female diagnosed at 15 years and having positive auto-antibodies therefore excluded from the final analysis

The patient M54 was diagnosed at the age of 15 years. The GAD-65 auto-antibodies were negative while ZnT8 antibodies were positive. The next-generation sequencing revealed a three prime UTR variant c.*96dupA in *KLF11* gene. It is reported as uncertain significance, but interpreted condition is MODY.

Discussion and conclusion

In this study, we performed the next-generation sequencing in suspected MODY patients to detect genetic variants causing the disease. To the best of our knowledge, this is the first study from Pakistan to investigate the types of MODY using next-generation sequencing. The next-generation sequencing resulted in huge amount of genetic variants and assessment of their causative role is difficult [9]. This problem was overcome by the prioritization and filtration strategy based on allele frequency and predictions of impact of mutations on protein as assessed by bioinformatics prediction tools [11].

The seven patients were selected for next-generation sequencing based on high MODY probability score; however, clinical tests showed auto antibodies for GAD 65 and ZnT8 in three patients and four were negative for both antibodies. The MODY probability score calculator was developed for Caucasian population; therefore, there is a need to validate this probability calculator in different population settings other than where it was developed. Same was reported by other studies as well [12, 13]

We identified three likely pathogenic variants in three patients. This includes one of the variant in the gene *HNF1a* (MODY type 3) which is most common among all MODY types. The two MODY types (*GCK* and *HNF1a*) together constitute about 70% of the MODY cases [5, 14]. In a study from UK, it was reported that 52% of the monogenic cases were due to variants in HNF1a gene [15]. In this type, most of the mutation carriers develop this disease before 25 years of age [16]. This type of MODY was reported in different parts of the world including Ireland [17], Croatia [18], and India [19].

The hepatocyte nuclear factor 1 alpha encodes for transcription factor expresses in pancreas, kidney, liver, and

Table 1 Presentation of demographic and clinical characteristics of patients

S.No	Patient ID No.	Gender	Age at diagnosis (years)	BMI (kg/m^2)	HbA1c (mmol/mol)	GAD65 (IU/ml)	ZnT8	MODY probability score	Family history (mother/father affected)
1.	M46	Male	17	24.2	48	Negative	Negative	>75	Yes
2.	M54	Female	15	24.87	125	Negative	Positive	>75	Yes
3.	M64	Female	21	22.3	108	Negative	Negative	>75	Yes
4.	M13	Female	13	29.3	65	Negative	Negative	>75	Yes
5.	M6	Male	17	24.6	66	Negative	Negative	>75	Yes
6.	M15	Female	15	18.3	57	Positive	NA	>75	Yes
7.	M24	Male	12	26.9	65	Positive	NA	>75	Yes

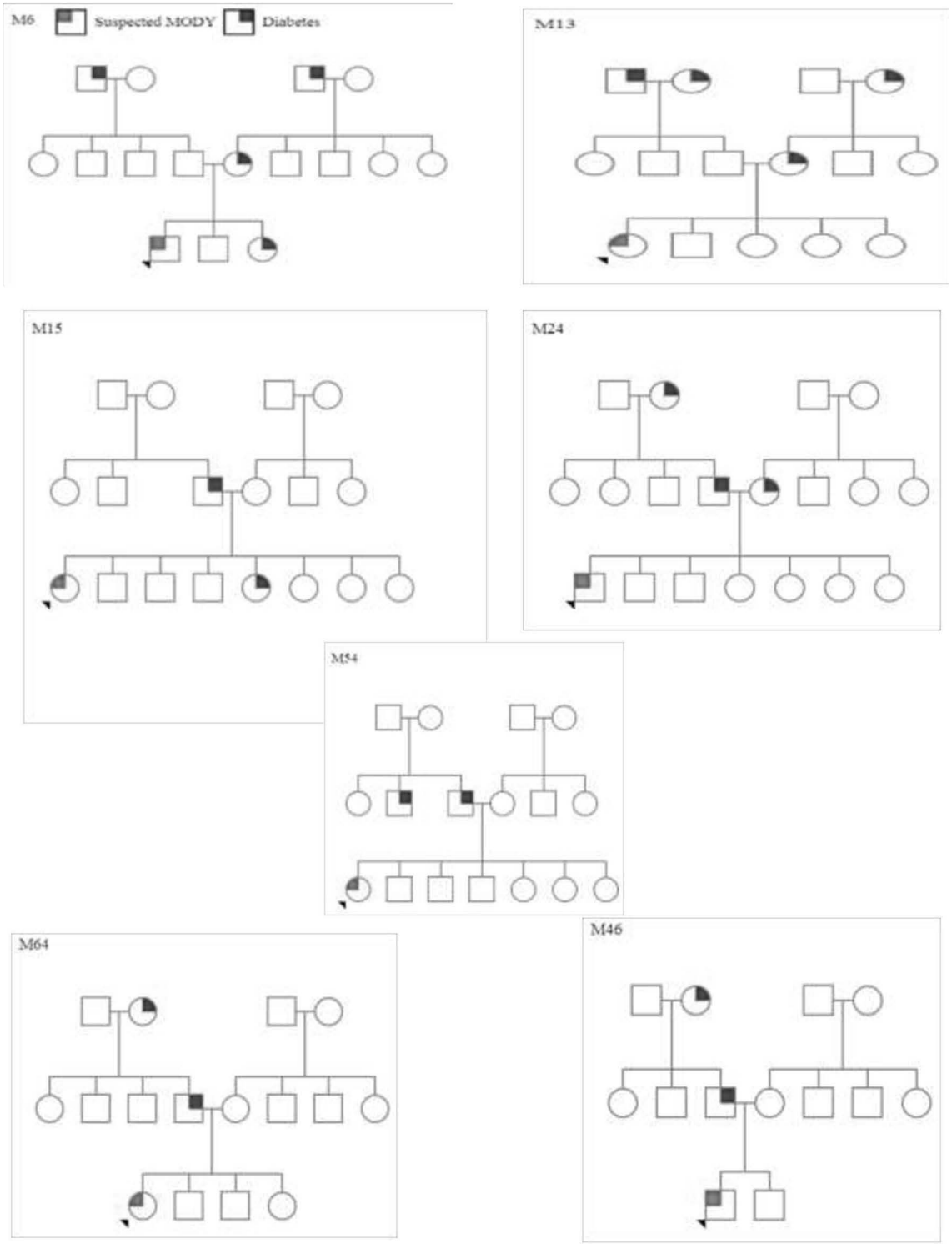

Fig. 1 Overview of pedigree structure of 07 patient families enrolled as suspected MODY. Red-filled circles showed the suspected MODY cases. Blue shows the diabetes family members and unaffected are shown by open symbols

intestine. Its deficiency leads to defects in insulin secretion causing hyperglycemia. The *HNF1a* protein binds to the promoter regions of the gene and activates the transcription to mRNA, and by this, it controls gene functions which are involved in insulin synthesis, pancreatic development, glucose transport, and metabolism in the cell [20]. There is lot of phenotypic variability involved with this type; in some cases, it develops during childhood and in some cases in late adulthood [21, 22]. The mutation found in this study was c.526+1G>A that was a splice donor variant and is previously

Table 2 Variants identified in patients with gene name, nucleotide change, and bioinformatics predictions

S.No	Patient ID	Gene	OMIM number	Mutation type	Nucleotide change	Amino acid change	Prediction of SIFT score	Prediction of Polyphen 2 score	Frequency in South Asia
1.	M6	*HNF1a*	* 142410	splice_donor_variant&intron_variant	c.526+1G>A	-	-	-	-
2.	M15	*GCK*	* 138079	5_prime_UTR_variant	c.-45G>A	-	-	-	0.002
3.	M54	*KLF11*	* 603301	3_prime_UTR_variant	c.*96dupA	-	-	-	-
4.	M64	*CEL*	* 114840	Missense	c.1235C>T	p.Thr412Ile	Damaging (0.008)	Damaging (1.0)	-

reported in a study from Saudi Arabia [23], China [24], and Norway [25]. It results in mild hyperglycemia in early adulthood as evident from the clinical characteristics of the patient. This sequence change affects the donor splice site in intron 2 of the *HNF1a* gene. The prediction algorithm suggests that this variant results in disrupting the consensus splice site resulting in disrupting of the RNA splicing which might result in disrupted or absent protein. The donor and acceptor splice sites generally lead to loss of function, and loss of function in *HNF1a* gene is known to be pathogenic [26–29]. The patient was counseled through the treating physician and treatment was prescribed as per standard guidelines.

The other retained variant was from three prime UTR variant from c.*96dupA located on KLF11 gene which is involved in MODY type 7. This gene Kruppel like factor 11 encodes zinc finger transcription factor that binds to promoters, inhibits growth, and causes apoptosis [30]. This variant was reported as of uncertain significance in clinvar [31].

This study contributed to the better understanding of the pathophysiology of the diabetes using genetic characterization. The identification of *HNF1a* mutation in M6 patient confirms the change in treatment pattern. Genetic cause cannot be identified in other patients. This might be due to overlap of clinical characteristics of MODY and other form of diabetes. One other explanation to this fact can be that there may be some other genetic factors in young onset of diabetes. The MODY types were not prevalent in suspected patients as reported from Tunis [32], Jordan [33, 34], Muscat [35], and Iran

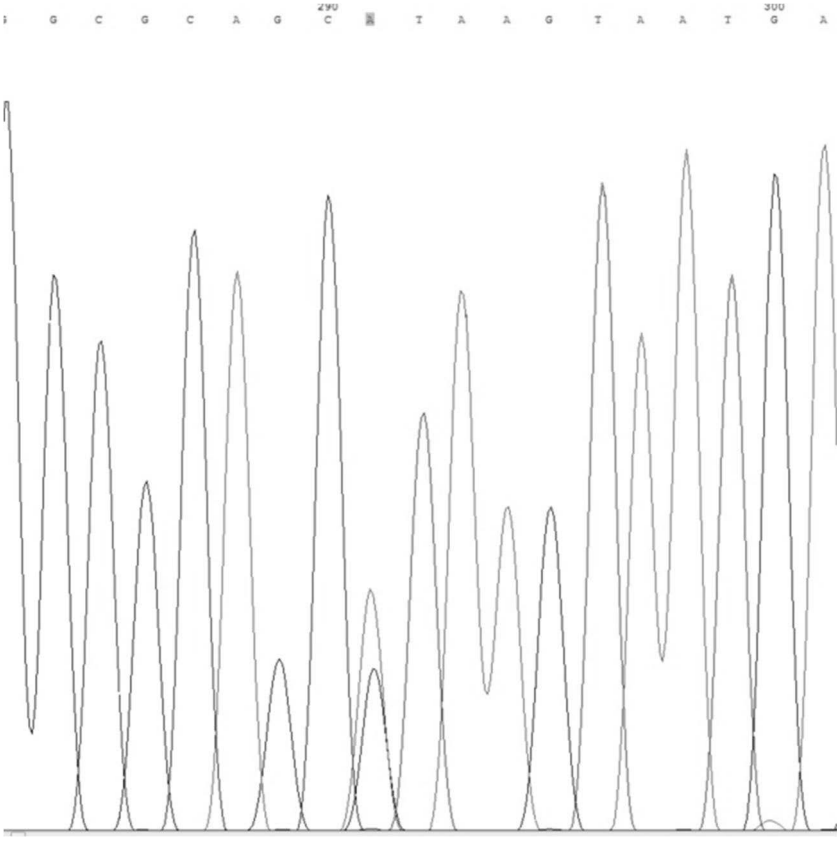

Fig. 2 Confirmation of heterozygous mutation c.526+1G>A in *HNF1a* gene in patient M6. Overlapping peaks at 291 position confirming the mutation

[36]. The population in these countries is socio-culturally different from other developed countries as these have consanguineous marriages. In Pakistan, two studies from different provinces reported consanguinity more than 50% [37, 38].

Next-generation sequencing is recently used in different studies for identification of mutations in MODY genes. We used the same technique with similar inclusion criteria. The identification of MODY carries immense importance as it alters the treatment pattern for the patient. Having huge burden of diabetes in our country, it is of utmost importance to identify and detect MODY among diabetic patients and create awareness among physicians regarding its diagnosis. There is a need to conduct large-scale studies on adult diabetic patients to screen for MODY.

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Availability of data and material (data transparency) The data will be available.

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Declarations

Ethics approval and consent to participate The ethical approval has been taken from Institutional Bioethics Committee of Pakistan Health Research Council. The written informed consent was taken from participants prior to the enrolment in the study.

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

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ORIGINAL ARTICLE

Circulating microRNAs associated with prediabetes and geographic location in Latinos

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Abstract

Background Globally, type 2 diabetes is highly prevalent in individuals of Latino ancestry. The reasons underlying this high prevalence are not well understood, but both genetic and lifestyle factors are contributors. Circulating microRNAs are readily detectable in blood and are promising biomarkers to characterize biological responses (i.e., changes in gene expression) to lifestyle factors. Prior studies identified relationships between circulating microRNAs and risk for type 2 diabetes, but Latinos have largely been under-represented in these study samples.

Aims/hypothesis The aim of this study was to assess for differences in expression levels of three candidate microRNAs (miR-126, miR-146, miR-15) between individuals who had prediabetes compared to normal glycemic status and between individuals who self-identified with Latino ancestry in the United States (US) and native Mexicans living in or near Leon, Mexico.

Methods This was a cross-sectional study that included 45 Mexicans and 21 Latino participants from the US. Prediabetes was defined as fasting glucose 100–125 mg/dL or 2-h post-glucose challenge between 140 and 199 mg/dL. Expression levels of microRNAs from plasma were measured by qPCR. Linear and logistic regression models were used to assess relationships between individual microRNAs and glycemic status or geographic site.

Results None of the three microRNAs was associated with risk for type 2 diabetes. MiR-146a and miR-15 were significantly lower in the study sample from Mexico compared to the US. There was a significant interaction between miR-146a and BMI associated with fasting blood glucose.

Conclusions/interpretation This study did not replicate in Latinos prior observations from other racial groups of associations between miR-126, miR-146a, and miR-15 and risk for type 2 diabetes. Future studies should consider other microRNAs related to different biological pathways as possible biomarkers for type 2 diabetes in Latinos.

Keywords microRNA · Diabetes · Fasting blood glucose · Biomarker

Introduction

Type 2 diabetes is highly prevalent in individuals of Latino ancestry in both native countries of origin and in immigrants to other countries. The prevalence of type 2 diabetes in Latinos living in the United States (US) is 12.7% [1] and the prevalence of type 2 diabetes in Mexico is 14.8% [2]. Progression to type 2 diabetes occurs on a continuum, and even in the prediabetes

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state, harmful complications begin to occur [3]. Genetic risk factors for type 2 diabetes are common in some individuals of Latino ancestry [4, 5]. However, Latinos are characterized by highly heterogeneous genetic admixture [6], and genetic risk for type 2 diabetes between individuals who identify as Latino may vary considerably by geographic ancestry. Furthermore, environmental, social, and lifestyle factors are also important contributors to risk for type 2 diabetes [7]. The complex etiology of type 2 diabetes makes it hard to accurately identify which individuals are at greatest risk and the specific mechanisms underlying risk for a given individual or population.

MicroRNAs are short (i.e., 18–26 nucleotide) regulatory elements of translation of messenger RNAs to amino acids. Circulating microRNAs found in serum and plasma are easily measured in blood and are potential biomarkers for risk for development of type 2 diabetes, characterizing changes in expression levels prior to the onset of prediabetes or type 2 diabetes [8, 9]. Because microRNAs capture both underlying genetic risk as well as responses to environmental, social, and lifestyle factors [10, 11], they may be useful as biomarkers in two ways. The first is improved identification of which individuals are at greatest risk for type 2 diabetes. The second is information about specific patterns of gene expression in individuals at risk for type 2 diabetes, which is of particular interest for this complex condition because gene expression is driven by interactions between underlying genetic predisposition and environmental and lifestyle factors.

Prior studies on microRNAs associated with risk for type 2 diabetes have primarily been focused on non-Hispanic white and Asian populations [12]. The purpose of this study was to assess relationships between circulating microRNAs and prediabetes in individuals of Latino ancestry. We selected three microRNAs (i.e., miR-126, miR-146a, miR-15) previously shown to be associated with risk for type 2 diabetes in other racial groups to assess in this study [13, 14]. We evaluated differences in microRNA expression levels between individuals who self-identified with Latino ancestry in the United States (US) and native Mexicans living in or near Leon, Mexico, and individuals who were prediabetic compared to normal glycemic status.

Research design and methods

Recruitment

This was a multi-center observational cross-sectional study carried out at two different research institutions in Mexico and the US.

Mexico

Participants of Mexican ancestry were recruited from primary care health centers that serve the general population from the city of Leon in Guanajuato, Mexico. Participants included were between 35 and 65 years old without a previous diagnosis of prediabetes. Participants presenting with fasting glucose of 100–125 mg/dL or 2-h glucose between 140 and 199 mg/dL after an oral glucose tolerance test (OGTT) were categorized as having prediabetes (n = 36). Participants with fasting glucose < 100 mg/dL and 2-h OGTT < 140 mg/dL were categorized as having normal glucose tolerance (n = 30). Exclusion criteria included history of diabetes, hypertension, thyroid, hepatic, immune, neoplastic, or endocrine disorder; statin, glucocorticoid, or anticonvulsant use; current smoking; and > 2 drinks of alcohol/day.

United States

The US study sample included participants from the previously completed Practicing Restorative Yoga Metabolic Syndrome (PRYSMS) study (clinicaltrials.gov identifier NCT01024816), which tested the effects of restorative yoga versus active stretching on blood glucose in adults at risk for type 2 diabetes. Participants in the PRYSMS study were recruited from the San Francisco and San Diego areas and met the International Diabetes Federation criteria for metabolic syndrome [15]. The subset of participants who self-identified as Latino were included in this study (n = 21). Exclusion criteria included fasting glucose ≥ 126 mg/dL, HbA1c ≥ 7.0%, fasting triglycerides ≥ 300 mg/dL, weight ≥ 400 lbs, chronic disease, and neurological conditions that limited mobility, hospitalization for coronary heart disease within the past 6 months, current pregnancy or lactation, history of bariatric surgery, substance abuse, and use of medications affecting metabolic factors. The PRYSMS trial and the study described in this paper were approved by the Institutional Review Board at the University of California, San Francisco. All participants provided informed consent to participate in the study.

Data Collection

Mexico

The enrollment visit at the Mexico site included a brief medical history and family history for diabetes using the American Diabetes Association criteria [16]. Physical activity was assessed using the Spanish version of the self-administered short International Physical Activity Questionnaire (IPAQ) forms [17]. Participant height and weight were measured with a standardized stadiometer (Seca) and scale (Tanita BC-536). Hip and waist girth were measured with an anthropometric tape (Lufkin), and body composition was assessed using a bioimpedance device (InBody). Blood glucose and lipid levels were measured from serum samples by colorimetric enzymatic assays (Spinreact). Insulin was measured from serum using an ELISA kit (ALPCO) as per the manufacturer's instructions.

Hemoglobin A1c (HbA1c) was analyzed via chromatography with Labona Check equipment and reagents in plasma. Plasma was stored at − 80 °C at both locations until used.

United States

The full clinical data collection protocol for the PRYSMS trial has been reported previously [18]. Participant weight was measured on a standard balance beam scale and height using a stadiometer. Waist circumference was measured using a Gullick II tape spring-tension measure at the site of maximum circumference midway between the lower ribs and the anterior superior iliac spine. The mean of two waist circumference measurements was calculated. Blood glucose was measured using an automated analyzer with an immobilized enzyme biosensor (YSI 2300 STAT Plus, YSI Life Sciences, Yellow Sprints, OH). Total cholesterol, triglycerides, and HDL-cholesterol were measured by enzymatic colorimetric methods (Quest Diagnostics, San Jose, CA), and LDL-cholesterol was calculated using the Friedewald equation [19]. Blood used for banking of plasma was collected by venipuncture. Blood was collected into vacutainers containing the preservative EDTA, centrifuged at 4 °C to separate plasma from cellular blood components, and stored at − 80 °C.

MicroRNA quantitation

Study personnel from the Mexico site were trained in the isolation and quantitation of microRNAs at the US site. Both sites employed the same study protocol for all assays. RNA was extracted from 200 μL of plasma using the miRNeasy serum/plasma kit (Qiagen). Purified RNA was converted to cDNA using the miScript II RT Kit (Qiagen) in 20 μL reaction volumes using the miScript HiSpec buffer. Real-time quantitative polymerase chain reaction (qPCR) was used to assess relative expression of candidate microRNAs using the miScript kit (Qiagen). Experiments were carried out using a 384-well (US) or 96-well (Mexico) plate format on a Bio-Rad CFX real-time PCR machine using the manufacturer's recommended cycling conditions. A standard curve was constructed for each microRNA target using a series of five serial dilutions. Both sites obtained at least three replicates measures per sample for each microRNA target. MicroRNA expression levels were normalized using cel-miR-39 and the global geometric mean signal of all reliably detected microRNAs [20, 21], and relative expression levels were calculated using the ΔΔCt method [22].

Statistical analysis

Descriptive statistics and Student's *t* test were used to evaluate demographic and clinical characteristics of participants between study sites and glycemic status (Stata version 13, College Station, TX). Pearson's correlation coefficients were used to determine relationships between fasting blood glucose and covariates that are continuous variables. Logistic regression models were used to determine whether individual microRNAs were associated with prediabetes compared to normal glucose tolerance. Logistic regression models were also used to determine whether individual microRNAs were associated with the study site with the US as the reference site. Linear regression models were used to determine whether individual microRNAs were associated with fasting blood glucose. For all regression models, unadjusted models were first created. Next, variables that were significantly associated with prediabetes or study site were included as covariates in adjusted models. Finally, we included interaction terms for covariates that were significantly associated with individual microRNAs.

Results

A total of 45 participants were enrolled in Leon, Mexico, and 21 participants from the US-based PRYSMS trial who self-identified as Latino were included in the study. Participants from Mexico were younger (46 ± 8 years versus 51 ± 7 years, $p < 0.05$) and had lower BMI (29.8 ± 3.8 kg/m^2 versus 35.9 ± 8.1 kg/m^2, $p < 0.05$) and weight (81.8 ± 11.4 kg versus 92.3 ± 18.2 kg, $p < 0.05$) (Table 1). While there were no differences in fasting blood glucose, hemoglobin A1c was higher in Latinos from the US compared to Mexicans from Mexico (6.0 ± 0.3% versus 4.4 ± 0.5%, $p < 0.001$) (Table 1). In a multivariate-adjusted logistic regression model, BMI but not age or sex was significantly lower in individuals from Mexico compared to the US (OR 0.82 (95% CI 0.71, 0.94)).

In the full study sample, 60% ($n = 36$) of participants had prediabetes compared to normal glucose tolerance (Table 2). There was no difference in the proportion with prediabetes by study site. A higher proportion of individuals with prediabetes were female (83% ($n = 30$) versus 57% ($n = 17$), $p < 0.05$) and had higher BMI (34.4 ± 6.0 kg/m^2 versus 29.7 ± 5.8 kg/m^2, $p < 0.05$). Fasting blood glucose was higher in individuals with prediabetes (109 ± 8 mg/dL versus 89 ± 8 mg/dL, $p < 0.05$) but there were no differences in hemoglobin A1c. In a multivariate-adjusted logistic regression model, BMI, but not age or sex, was significantly associated with risk for prediabetes (OR 1.12 (95% CI 1.00, 1.26)).

All three microRNAs were strongly significantly correlated with each other (Table 3). MiR-146a was significantly associated with BMI ($r^2 = 0.28$, $p < 0.05$). There were no other significant correlations between individual microRNAs and age, sex, or BMI.

The distribution of normalized (i.e., ΔCt) expression for each microRNA by study site is shown in Fig. 1. In unadjusted logistic regression models, both miR-146a (OR 0.83 (95% CI 0.67, 0.99)) and miR-15 (OR 0.79 (0.65, 0.97)) were

Table 1 Demographic and clinical characteristics by study site

% (*n*) or average ± standard deviation	US Latinos (*n* = 21)	Mexican (*n* = 45)	*p* value
Age	51 ± 7	46 ± 8	< 0.05
Sex (male)	24 (5)	31 (14)	0.54
Body mass index (kg/m^2)	35.9 ± 8.1	29.8 ± 3.8	< 0.05
Weight (kg)	92.3 ± 18.2	81.8 ± 11.4	< 0.05
Fasting blood glucose (mg/dL)	101 ± 11	99 ± 14	0.44
Hemoglobin A1c (%)	6.0 ± 0.3	4.4 ± 0.5	< 0.001
Prediabetes (%)	57 (12)	67 (24)	0.77
Total cholesterol (mg/dL)	216 ± 44	194 ± 34	0.06
LDL-c (mg/dL)	134 ± 37	120 ± 30	0.13
HDL-c (mg/dL)	47 ± 10	39 ± 11	< 0.05
Triglycerides (mg/dL)	176 ± 68	165 ± 6	0.58

significantly decreased in individuals from the Mexico study site compared to the US site (Table 4). In a model adjusted for age and BMI, miR-15 remained significantly lower in individuals from Mexico compared to the US (OR 0.76, (95% CI 0.60, 0.97)). When we further added hemoglobin A1c to the model, which was higher in participants from the US compared to Mexico, miR-15 was no longer significant. There was no interaction between miR-15 and hemoglobin A1c. There was a significant interaction between miR-146a and BMI in both unadjusted and age- and BMI-adjusted linear regression models for fasting blood glucose.

The distribution of normalized (i.e., ΔCt) expression for each microRNA by glycemic status is shown in Fig. 2. In unadjusted and sex- and BMI-adjusted logistic regression models, no microRNAs were significantly associated with higher odds for prediabetes. In unadjusted and sex- and BMI-adjusted linear regression models, no microRNAs were significantly associated with fasting blood glucose. However, there was a significant interaction between miR-146a and BMI in a linear regression model for fasting blood glucose ($\beta = -0.16$, 95% CI (-0.32, -0.01)). The test for interaction was not significant for miR-126 and miR-15.

Discussion

Compared to prior studies focused on relationships between circulating microRNAs and risk for type 2 diabetes, we did not find significant associations between miR-126, miR-146a, or miR-15 and prediabetes. Prior studies were primarily conducted in European or Asian populations [12, 23, 24], whereas we studied Latinos. We did identify a significant association between miR-146a and BMI, which is a relationship that has previously been observed in several studies of Europeans [25]. We also identified differences in expression levels of miR-146a and miR-15 between individuals living in or near Leon, Mexico, compared with individuals living in the US who self-identified as Latino.

MiR-146a has previously been associated with risk for type 2 diabetes in numerous studies, including a meta-analysis [24, 26]. Inflammation is one of the potential mechanisms by which miR-146a is hypothesized to have an effect on risk for type 2 diabetes and related conditions [27, 28]. There is strong experimental evidence that biological pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) are targeted by miR-146, including ones related to inflammation

Table 2 Demographic and clinical characteristics by glycemic status

	Normal Glucose Tolerance (*n* = 30)	Prediabetes (*n* = 36)	*p* value
Study site (Mexico)	70 (21)	67 (24)	0.77
Age (years)	47 ± 9	48 ± 8	0.40
Sex (male)	43 (13)	17 (6)	< 0.05
Body mass index (kg/m^2)	29.7 ± 5.8	34.4 ± 6.0	< 0.05
Weight (kg)	81.2 ± 13.6	88.6 ± 14.5	0.05
Fasting blood glucose (mg/dL)	89 ± 8	109 ± 8	< 0.001
Hemoglobin A1c (%)	4.8 ± 0.7	5.1 ± 0.9	0.18
Total cholesterol (mg/dL)	201 ± 35	201 ± 41	0.96
LDL-c (mg/dL)	125 ± 27	123 ± 37	0.85
HDL-c (mg/dL)	41 ± 9	42 ± 13	0.67
Triglycerides (mg/dL)	170 ± 82	167 ± 77	0.86

Table 3 Correlation coefficients between individual microRNAs and covariates

	MiR-126	MiR-146a	MiR-15	Age	Sex	BMI	FBG
MiR-126	1.0000						
MiR-146a	*0.9538*	1.0000					
	< 0.001						
MiR-15	*0.7406*	*0.6792*	1.0000				
	< 0.001	*< 0.001*					
Age	− 0.1470	− 0.0997	− 0.0646	1.0000			
	0.2388	0.4258	0.6063				
Sex	− 0.2197	− 0.1761	− 0.1401	0.3649	1.0000		
	0.0763	0.1573	0.2620	0.0026			
BMI	0.1687	*0.2815*	0.0362	*0.3245*	0.2314	1.0000	
	0.1757	*0.0220*	0.7730	*0.0078*	0.0616		
FBG	0.0971	0.1235	0.1928	0.0917	0.1446	*0.3144*	1.0000
	0.4379	0.3232	0.1208	0.4639	0.2466	*0.0101*	

Italicized font indicates values that were statistically significant

BMI, body mass index; *FBG*, fasting blood glucose

(e.g., nuclear factor-κβ (NF-κβ) signaling pathway, Toll-like receptor signaling pathway, tumor necrosis factor-α signaling pathway) [29]. Findings from prior studies have been inconsistent in terms of the direction of the association (i.e., increased versus decreased expression of miR-146a and increased risk for type 2 diabetes). These discrepancies may partly result from differences in the tissue source from which microRNAs were obtained (e.g., plasma versus peripheral blood mononuclear cells (PBMCs)) and cross-sectional study design, which does not allow for characterization of where an individual lies on the glycemic spectrum. Our study did not find any association between miR-146a and prediabetes, which may be attributed in part due to differences in study design, including the examples listed above [26]. Another possible explanation for our null findings is that prior studies were primarily focused on European and Asian populations [12]. One prior study of microRNAs and risk for type 2 diabetes included Mexicans [30]. MiR-146a was significantly decreased in individuals with type 2 diabetes compared to healthy controls and was significantly correlated with BMI [30]; however, microRNAs were obtained from PBMCs, whereas our study focused on circulating microRNAs from plasma. Another study identified decreased expression of miR-146a associated with type 2 diabetes in Ecuadoreans and correlations between miR-146a and inflammatory markers [31]. Both of these prior studies were cross-sectional and included individuals with a diagnosis of type 2 diabetes, whereas our study focused on prediabetes. There are many physiological changes across the glycemic spectrum from normal glucose tolerance to impaired fasting glucose (i.e., prediabetes) to type 2 diabetes that are not captured in cross-sectional study designs that use only fasting blood glucose to assess glycemic status.

Table 4 Odds ratios for prediabetes and study site

	miR-126, OR (95% CI)	miR-146a, OR (95% CI)	miR-15, OR (95% CI)
Prediabetes			
Unadjusted	1.03 (0.88, 1.22)	1.02 (0.89, 1.19)	1.05 (0.92, 1.21)
Adjusted*	1.05 (0.87, 1.27)	1.02 (0.85, 1.22)	1.09 (0.93, 1.27)
Study site#			
Unadjusted	0.86 (0.70, 1.05)	*0.83 (0.67, 0.99)*	*0.79 (0.65, 0.97)*
Adjusted$	0.99 (0.71, 1.14)	0.87 (0.69, 1.10)	*0.76 (0.60, 0.97)*

Italicized font indicates values that were statistically significant

* Models are adjusted for sex and BMI

United States is the reference site

$ Models are adjusted for age and BMI

BMI, body mass index; *CI* confidence interval; *OR*, odds ratio

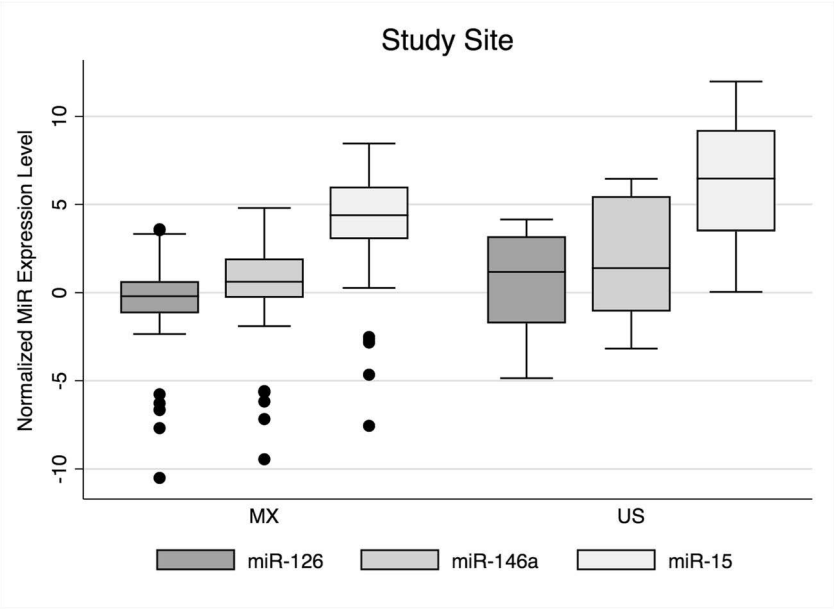

Fig. 1 Distribution of normalized microRNA expression levels by study site. Box and whisker plots show maximum (upper horizontal line), 75th percentile (upper border of box), 50th percentile/ median (mid-line of box), 25th percentile (lower border of box), and minimum (lower horizontal line). Black dots represent outliers. MiR, microRNA; MX, Mexico; US, United States

Overweight and obesity are major risk factors for type 2 diabetes, with approximately 40–70% of individuals at high risk for type 2 diabetes being overweight or obese [32]. We observed a significant correlation between miR-146a and BMI and a significant interaction between these two variables in determining odds for prediabetes versus normal glucose tolerance. MiR-146a has previously been associated with obesity in animal models [33, 34] and obesity-related inflammation in human adipocytes [35]. In human studies, miR-146a was increased in obese Chinese children and Chinese adults with type 2 diabetes and in functional in vitro and animal model studies increased miR-146a impaired β-cell function and insulin secretion [23]. Another KEGG pathway targeted by miR-146a is the adipocytokine signaling pathway. Overweight and obesity cause inflammation in part through the activity of adipocytokines, which are inflammatory molecules generated in adipose tissue [36–38]. The significant interaction that we observed between miR-146 and BMI suggests that the effect of BMI on prediabetes depends on the expression level of miR-146a, or vice versa. The relationship between miR-146a and risk for type 2 diabetes may be linked to its effect on body composition and/or obesity-related inflammation. Future studies that include a longitudinal design, gold-standard assessments of body composition and glycemic status, and functional analysis of the impact of miR-146a on genes related to inflammation and obesity may further shed light on these relationships.

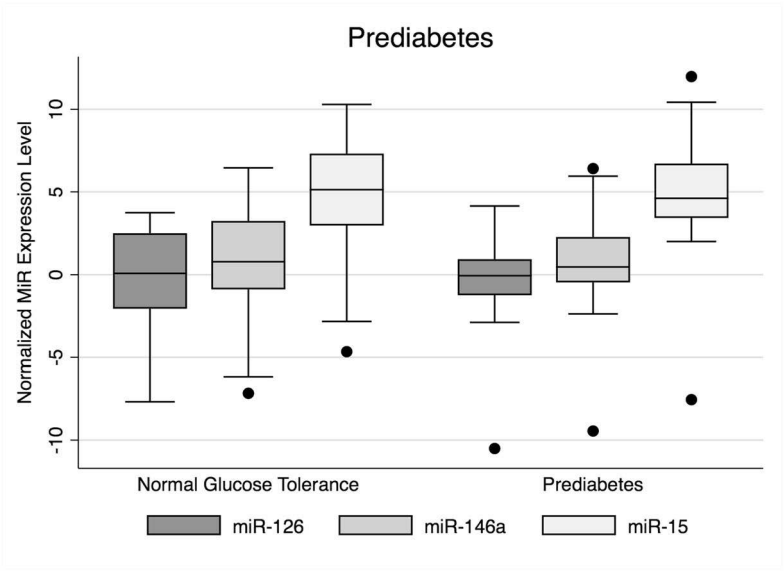

Fig. 2 Distribution of normalized microRNA expression levels by glycemic status. Box and whisker plots show 95th percentile (upper horizontal line), 75th percentile (upper border of box), 50th percentile/median (mid-line of box), 25th percentile (lower border of box), and minimum (lower horizontal line). Black dots represent outliers. MiR, microRNA; MX, Mexico; US, United States

Prior studies identified associations between miR-15 and risk for type 2 diabetes [39, 40]. Baseline levels of miR-15 were lower in Spanish individuals who developed type 2 diabetes after 5 years, though miR-15 was not significantly associated with other measures of risk for type 2 diabetes (i.e., fasting blood glucose, hemoglobin A1c, measures of insulin sensitivity) [40]. A study of African-Americans identified a U-shaped curve in the relationship between miR-15 and the glycemic trajectory, with lower expression of miR-15 in individuals with prediabetes compared to individuals with normal fasting glucose or type 2 diabetes [39]. In the group with type 2 diabetes, miR-15 was associated with body weight and body mass index, but not hemoglobin A1c, and none of these associations was observed in the group with prediabetes [39]. In addition, miR-15 was able to discriminate between type 2 diabetes and prediabetes and between prediabetes and normal blood glucose, although these models were not compared to any other predictive or discriminatory models [39]. Our study showed that hemoglobin A1c, which differed between study sites (i.e., US versus Mexico) attenuated the relationship between miR-15 and study site. The relationship between miR-15 and hemoglobin A1c remains relatively unstudied. Mechanistic studies of miR-15 identified regulation of NF-κβ [41, 42] with corresponding increases in the inflammatory interleukin-8 and interferon-γ markers [42], suggesting that miR-15 may also contribute to regulation of inflammation observed in individuals at risk for type 2 diabetes.

MiR-126 was previously associated with risk for type 2 diabetes in numerous studies [14, 43]. Insights from mechanistic studies of miR-126 showed that this microRNA is associated with endothelial cell function [44–46], and therefore, differential expression may be associated with consequences from type 2 diabetes and elevated blood glucose levels [47]. Prior studies that identified associations between miR-126 and risk for type 2 diabetes focused on primarily European and Asian racial groups [43, 48–51]. Very little has been reported about microRNAs associated with risk for type 2 diabetes in Latino populations. Further studies are needed to validate our finding that miR-126 may not be associated with prediabetes in Latinos.

The Latino racial group category includes individuals from vast geographic regions with highly admixed genetic characteristics [6]. Characterization of individuals by this broad criterion may lack specificity about the degree of genetic similarity. For example, Latinos living in California have different genetic characteristics compared with Latinos living on the East Coast of the US or in Mexico and Central and South America [52]. Prior studies that included individuals of Latino origin (e.g., Ecuadorean) may have been genetically dissimilar to our study sample. In order to accurately assess genetic similarity between individuals from the Latino racial group, genetic admixture analysis is needed. Furthermore, behavioral and lifestyle factors that impact risk for type 2 diabetes vary considerably between individuals who are broadly categorized as Latino. Inconsistencies in the associations between individual microRNAs and risk factors for type 2 diabetes may be the result of not only differences between racial groups in terms of genetic admixture/genetic risk and behavioral/lifestyle factors but also differences within racial groups (i.e., Latinos) that are unaccounted for by this very general categorization.

A limitation of this study and the majority of studies to date is the cross-sectional design. Development of type 2 diabetes occurs on a continuum, and cross-sectional studies fail to identify where on this continuum an individual may fall. Even within the clinically assigned categories of normal glucose tolerance, prediabetes, and type 2 diabetes, there may be differences in the underlying pathophysiology that impact expression levels of circulating microRNAs. Clinical and molecular data collection for this study was carried out at separate study sites. However, the laboratory protocols were identical at both sites, and study personnel were trained on the molecular data collection protocols at the US site. All data analysis was performed at a single site (US). There were some differences in demographic and clinical characteristics between the study sites, which were included as covariates in models. The genetic ancestry of all participants is not known, and the degree of genetic similarity between individuals is not known. Environmental and lifestyle factors were not assessed and conclusions about the impact of these potential risk factors on the associations between individual microRNAs and prediabetes cannot be made.

Circulating microRNAs are emerging as promising biomarkers for risk for type 2 diabetes. However, the majority of studies to date have primarily included individuals who identify as non-Hispanic white/European or Asian. Latinos have a particularly high prevalence of type 2 diabetes, and the reasons for this are not well understood. Given that circulating microRNAs capture the combined impact of genetic predisposition with responses to environmental and lifestyle factors, they may provide new insights about the reasons for increased risk for type 2 diabetes in some individuals and populations.

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Compliance with ethical standards

Our research was approved by the University of California, San Francisco, and Institutional Review Boards and all procedures followed were in accordance with the ethical standards of the IRBs and the Helsinki Declaration of 1975, as revised in 2000.

Conflict of interest The authors do not have any relevant disclosures.

Ethical considerations The study was approved by the Institutional Committee of Bioethics of the University of Guanajuato (CIBIUG-P05-2016) and by the Research Committee of the VII Health Jurisdiction of the Health Secretary of Guanajuato (JS7-02-280217). All procedures were performed according to the Mexican General Health Laws and the Declaration of Helsinki. All participants provided informed consent to participate in the study.

Informed consent was obtained from all participants included in the study.

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ORIGINAL ARTICLE

MiRNA expression analysis emphasized the role of miR-424 in diabetic cardiovascular complications

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Abstract

Background The discovery of miRNAs as promising new biomarkers in the field of cardiovascular disease has caused great expectations. Stability in the bloodstream, specific regulation, and high sensitivity and specificity suggest that the feasibility of miRNAs as cardiovascular biomarkers can even displace protein-based biomarkers. The objective of our study was to determine the plasma expression profile of miRNAs, which are reported to have important correlations with cardiovascular diseases, in patients with type 2 diabetes mellitus in relation to cardiovascular complications.

Methods We isolated plasma miRNAs from 6 patients with type 2 diabetes mellitus without cardiovascular disease (control group) and 9 patients with type 2 diabetes mellitus and cardiovascular disease (target group). Following reverse transcription and subsequent real-time PCR analysis of the same amount of starting miRNAs, the following miRNAs were successfully analyzed: miR-16-5p, miR-155-3p, miR-155-5p, miR-210, miR-221-3p, and miR-424-5p.

Results The relative expression analysis showed a statistically significant increase in the expression of all these miRNAs in the target group. The highest level of increase was established for miR-424-5p with an average relative quantification value of 10.5, followed by miR-155-5p with an average value of 7.5.

Conclusion Cardiovascular risk assessment, supported by emerging circulating biomarkers, such as miRNAs, is important for stratifying high-risk individuals, optimizing treatment strategies, and enhancing our understanding of basic biology. Our study showed the highest increase in expression levels of miR-424-5p in target group and emphasized its role as a biomarker for cardiovascular damage in patients with type 2 diabetes mellitus.

Keywords miRNA · Type 2 diabetes mellitus · Cardiovascular disease · Biomarkers

Introduction

Diabetes mellitus is a very common disease, and its frequency continues to rise worldwide every year. It was estimated that in 2017 the people with diabetes were 451 million (age 18–99), and their number is expected to rise to 693 million in 2045. Cardiovascular complications in diabetes are among the main causes of death—diabetic patients are two to three times more likely to develop cardiovascular disease (CVD) in comparison with people without diabetes [1]. People with diabetes without myocardial infarction (MI) have the same risk for developing coronary artery disease (CAD) like people with previous MI [2].

Epigenetics studies the heritable changes in the phenotype that do not involve alterations in DNA. These changes may be a result of external or environmental factors, or be a part of the normal development [3]. Inherited or sporadic epimutations or dysregulation of the epigenome could lead to disease. The mechanisms that are known to affect the epigenome are DNA methylation, histone modification, and noncoding RNAs.

Noncoding RNAs comprise several classes of RNAs that are classified in different groups according to several characteristics—length, localization, and function. MicroRNAs (miRNAs) are small noncoding RNA molecules that consist

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of 21 to 23 nucleotides and act as posttranslational regulators of gene expression. They bind to target mRNAs and modify them to suppress protein translation [4]. It is found that a single miRNA can bind to several mRNAs, and several miRNAs can regulate a single mRNA. MiRNAs modulate different physiological and pathological processes in diseases such as diabetes and CVD [5]. They are known to regulate a wide range of pathways in cardiovascular disease such as myocardial remodeling and fibrosis, vascular inflammation [6]. Altered microRNA expression affects the progression of diabetic complications.

In 2008, extracellular miRNA was detected in body fluids [7]. It was found that miRNAs circulate in the blood and are resistant to degradation unlike most RNAs [8]. Evidence suggests that they are protected from degradation due to packaging in extracellular vesicles or association with RNA-binding protein complexes [9]. On the other hand, studies show the potential role of extracellular miRNAs as mediators of communication between cells. MiRNAs encapsulated in vesicles can be taken up by cells and can lead to changes in gene expression and function in those particular cells [10]. It was found that circulating miRNA may be abundant in different diseases and may result in alterations in any cells that internalize them. MiRNA expression is different in healthy people and in those with CVD [8].

The authors identified 158 dysregulated miRNAs in seven different major sample types. The functional role of miRNAs in diabetes is expressed by one of the following mechanisms: (i) negative regulation of b-cell survival [12, 13], (ii) proliferation control, in particular inhibition of b-cell proliferation [14, 15], (iii) determinant in modulating insulin sensitivity by regulation of molecules in the insulin-signaling pathway in peripheral tissues [16], and (iv) essential for cell differentiation [17, 18].

A systematic study of dysregulated miRNA in T2DM reviewed 59 independent studies, selected only from human patient samples to investigate the functional involvement of miRNAs in human T2DM pathology [11]. In skeletal muscles, there were 29 miRNAs with decreased expression and 31 with increased expression compared with healthy controls [19, 20]. The affected miRNAs in adipose tissues interact with multiple transcription factors, such as PPARs (peroxisome proliferator-activated receptors), including PPARG, also known as PPARg, and adipocyte-enriched genes (GLUT4 [also known as SLC2A4], SOCS1, SOCS3, GRB2, INSR, and PPARG), to regulate many aspects of the lipid and glucose metabolisms [21]. The dysregulated miRNAs and their interacting mRNA targets may provide new insights into the T2DM pathology and provide new disease monitoring and management tools.

Several studies show that different miRNAs are altered in diabetes and CVD. Good glycemic control attenuates some of the peripheral diabetic symptoms, but it is found that diabetic cardiomyopathy progresses even after normalization of blood glucose. A study showed that even after glycemic control in diabetic mice, miRNA dysregulation of many myocardial damage pathways (dysregulated miRNA-221, miRNA-146a, miRNA-34a, miRNA-210, miRNA-19b, miRNA-27a, miRNA-155) persisted [22].

The objective of the present of study was to determine the plasma expression profile of miRNAs, which are suggested to have important correlations with cardiovascular diseases, in patients with type 2 diabetes mellitus with and without CVD in order to evaluate their potential role as markers for these complications.

Materials and methods

Materials

Blood samples were collected from patients with type 2 diabetes mellitus. All patients signed an informed consent to participate in the study. The personal/laboratory characteristics of patients are given in Table 1. According to cardiovascular complications, patients with and without CVD complications were selected, following the next exclusion criteria:

- Patients on hormone replacement therapy
- Patients with respiratory failure and chronic pulmonary disease
- Patients with chronic kidney disease
- Patients with neoplastic diseases
- Patients with autoimmune diseases or immunosuppressive therapy

Methods

Isolation of total RNA from tissue material and reverse transcription

Total RNA was isolated from plasma samples using a kit for RNA extraction miRNeasy Serum/Plasma Kit (Qiagen,

Table 1 Personal/laboratory characteristic of patients in the study

Parameters	T2DM without CVD	T2DM with CVD
Number	6	9
Gender (male/female)	3/3	5/4
Age (years)	52.8±4.2	55.7±7.7
BMI (kg/m^2)	34.6±3.4	34.0±2.7
HbA1c (%)	8.8±1.8	8.7±2.1

Hilden, Germany). The quality and quantity of the total RNA samples were assessed by NanoDrop 2000 (Thermo Fisher Scientific, Walmington, DE, USA).

Fifty nanograms of total RNA of each sample was used to prepare cDNA by using miScript II RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. After RT, samples were processed further or stored at −20° C until use.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The expression of mature miRNAs was assayed using miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) on a 7900HT Fast Real Time PCR System (Applied Biosystems, CA, USA). The miScript Primer assays (Qiagen, Hilden, Germany) were used for six mature miRNAs: miR-16, miR-155-3p, miR-155-5p, miR-210-3p, miR-221-3p, and miR-424-5p. Each reaction was performed in triplicate in a total volume of 10 μL, according to the manufacturer's protocol. Expression levels of miRNAs were normalized to the internal control RNU6-2 (Qiagen, Hilden, Germany). The relative quantification (RQ) of miRNAs plasma samples of the target group was analyzed by the 2-ΔΔCt method, as previously published. A RQ≥2 was defined as overexpression, RQ<0.5 as underexpression, and RQ between 1.99 and 0.5 as no change in expression. The analyzed miRNAs and their function are shown in Table 2.

Statistical analysis

Data analysis was performed with the SPSS software version 23.0 for Windows (IBM SPSS, NY, USA). A value of $p<0.05$ was considered statistically significant.

Results

In our study, we isolated plasma miRNAs from diabetic patients with and without CVD. The quality and quantity of the miRNAs obtained were evaluated on a spectrophotometer. Subsequent analysis included 6 patients with type 2 diabetes mellitus without CVD (control group) with the mean age of 52.8 years and 9 patients with type 2 diabetes mellitus and CVD (target group) with the mean age of 55.7 years. Following reverse transcription and subsequent real-time PCR analysis of the same amount of starting miRNAs, the following miRNAs were successfully analyzed: miR-16-5p, miR-155-3p, miR-155-5p, miR-210, miR-221-3p, and miR-424-5p. The relative expression analysis showed a statistically significant increase in the expression of all these miRNAs in the target group compared with the control group (Fig. 1; Table 3). The average relative expression levels of the analyzed miRNAs are presented in Table 4.

The highest level of increase was established for miR-424-5p with average RQ of 10.5, followed by miR-155-5p with average RQ of 7.5. One of target mRNAs for miR-424-5p is *HIF1A*—the master transcriptional regulator of the adaptive response to hypoxia, and one target for miR-155-5p is *ICAM-1*—with a role in inflammation and in the regulation of vascular permeability, affected in atherosclerosis (Fig. 2).

Table 2 List of the analyzed miRNAs and the processes they mediate

miRNA	Processes mediated by miRNA
miR-16	Proliferation, apoptosis, and cell cycle progression
miR-155-3p, 5p	Inflammation and immune regulation
miR-210-3p	Hypoxia, angiogenesis, cardiomyocyte apoptosis
miR-221-3p	Vascular smooth muscle cell proliferation and neointimal hyperplasia
miR-424-5p	Hypoxia and regulation of endothelial differentiation

Table 3 Number of patients from target group with different degree of expression (in comparison to control group) for each of miRNAs

	Increased expression (no. of patients)	No change (no. of patients)	Decreased expression (no. of patients)
miR-16	6	1	1
miR-155-3p	5	3	1
miR-155-5p	8	1	0
miR-210-3p	8	1	0
miR-221-3p	8	1	0
miR-424-5p	9	0	0

Discussion

Data from previous studies showed important correlations of tested miRNAs with cardiovascular diseases. All of the analyzed miRNAs showed elevated levels in plasma of diabetic patients with CVD compared with patients without CVD. The lowest increase was detected for miR-155-3p and miR-16-5p

Table 4 Average relative expression of the analyzed miRNAs

miRNA	Average relative expression (RQ)
miR-155-3p	2.2
miR-16-5p	2.5
miR-210-3p	5.1
miR-221-3p	6.2
miR-155-5p	7.5
miR-424-5p	10.5

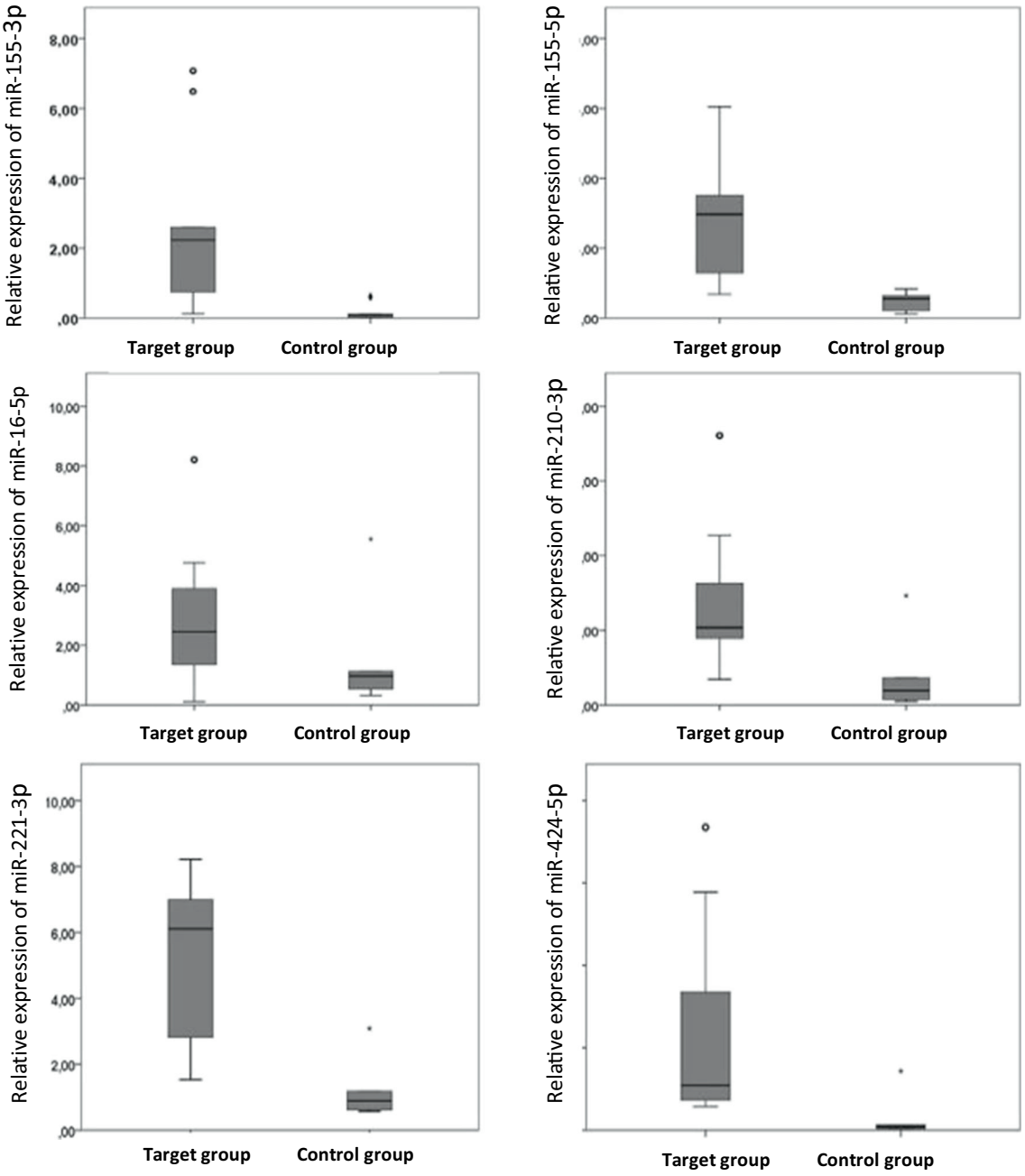

Fig. 1 Relative expression levels (axis) of the analyzed miRNAs in target group compared with control group

(2.2 and 2.5 times), followed by high increase in the levels of miR-210-3p (5.1 times), miR-221-3p (6.2 times), miR-155-5p (7.5 times), and miR-424-5p (10.5 times).

Nielsen et al. [23] found that immediately after exercise, several miRNAs, including miR-16, significantly decreased in circulation. Emerging data suggest that this is important for the heart's response to physiological stress during exercise. Given the well-known cardiovascular benefits of exercise, elucidating the contribution of such miRNAs to this response has the potential not only to uncover new aspects of cardiovascular biology but also to identify new targets for therapeutic intervention. Our study does not show remarkable change

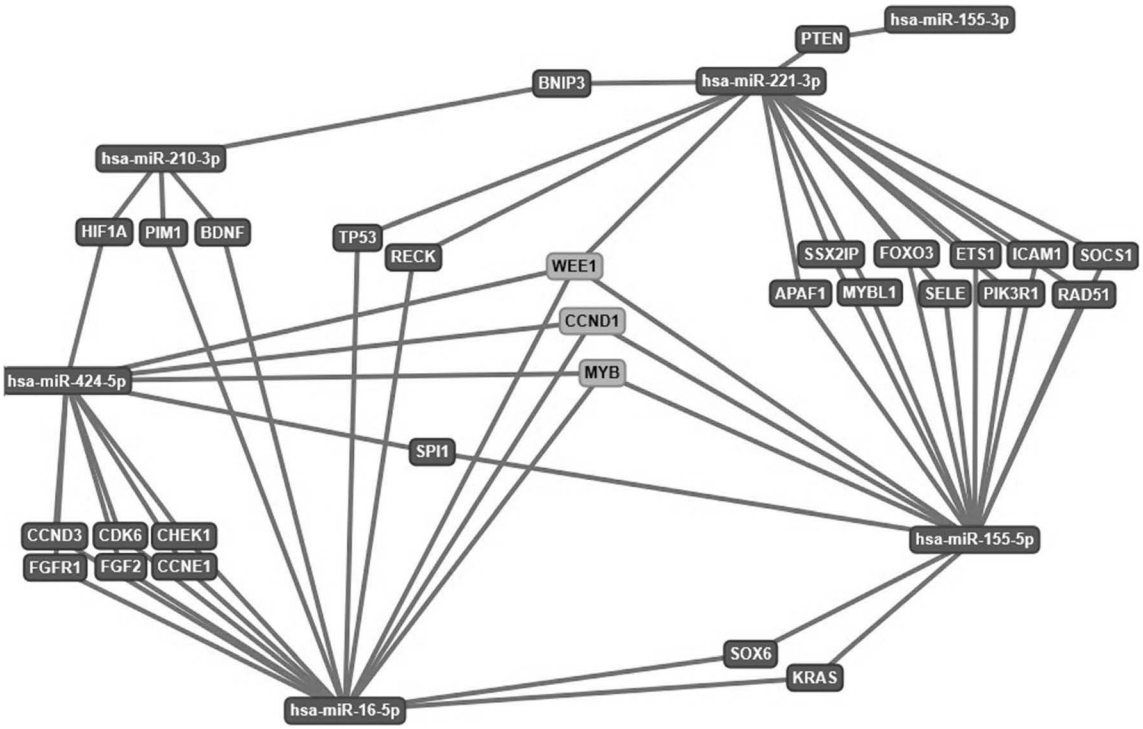

Fig. 2 Graphical representation of target genes for all analyzed miRNAs

in the expression of miR-16, and further investigations are needed for correlating it to the physical activity of the patients.

Cakmak et al. [24] have shown the relationship between miRNA expression and electrocardiogram parameters related to the left ventricular mass index (LVMI) in patients with chronic heart failure (CHF) [25]. The results of this study indicate that 29 miRNAs are altered in patients with CHF. Among these 29 miRNAs, miR-155 showed a direct correlation with LVMI. The expression of miRNA-155 has been found to be elevated in human plaques suggesting their role in atherosclerosis [26]. Our study found that expression levels of miR-155-5p were among the most elevated in patients in the target group.

Another highly elevated miRNA from our study was miR-221. Studies using human umbilical vein endothelial cells (HUVECs) showed that miR-221 regulates angiogenesis in response to stem cell factor (SCF), possibly by directly suppressing cKit levels and attenuating cell survival, migration, and vascular formation. Although it is not clear whether miR-221 expression is altered during the physiological capillary formation process in response to SCF, it is clear from overexpression studies that modulation of miR-221 represents a potential pathway for therapeutic modulation of angiogenesis. Patients with chronic arterial disease show elevated levels of miR-221 in endothelial progenitor cells, and this results in their mobilization [27, 28]. The association of miR-221 with the cardiovascular system is known. On this basis, expression levels were evaluated in serum samples from stroke patients—the results of this study indicate that miR-221 is a new biomarker for stroke [29]. It is known that circulating miRNA-221 is elevated in patients after acute myocardial infarction (MI) [30]. On the other hand, their expression is reduced in patients with heart failure and severe cardiac fibrosis. That might suggest that miRNA-221 plays an important role in regulating cell survival in patients with CVD [31].

Expression of miR-210 was also increased in the patients with CVD from our study. It is known to be increased during hypoxic conditions and has been shown to have pro-angiogenic properties in vitro [32, 33]. Overexpression of miR-210 in endothelial cells results in accelerated vessel formation under normal conditions and increased VEGF-dependent migration. The results of these studies indicate that miR-210 should be further investigated for a potential cardioprotective role after ischemic injury. Studies confirm that miRNA-210 takes a role in the regulation of hypoxia-induced cell response. They are upregulated in ischemic conditions such as myocardial infarction [34] and heart failure in patients with diabetes [35]. Although no clinical study has reported the precise association between miR-210 and cardiovascular protection, alterations in miR-210 expression are often observed in peripheral blood or damaged tissues from patients with CVD, including atherosclerosis, acute coronary syndrome, valvular heart disease, pulmonary arterial hypertension, and heart failure [36]. In clinical studies, elevated miR-210 has been detected in both the plasma and urine of adolescent type I diabetes mellitus patients [37]. Amr et al. [38] evaluated miR-210 expression in type 2 diabetes with or without CAD and reported that it is upregulated in both groups of diabetic patients. However, between these two groups,

patients with CAD had a more significant miRNA change than those with diabetes alone.

The highest elevation was detected for the levels of miR-424 in patients with CVD from our study. A prospective case-control study was conducted with a 10-year follow-up, and many circulating miRNAs (among them miR-424-5p) were identified predicting future fatal acute myocardial infarction in healthy participants in the Nord-Trøndelag Health Study (HUNT). Sayed et al. suggest that circulating miRNA-149, miRNA-424, and miRNA-765 might be novel indicators for the diagnosis of CAD [36]. On the other hand, miRNA-424 is known to promote angiogenesis during hypoxia [39]. The expression of miR-424 is reported to be elevated during compensated cardiac growth, and such overexpression is thought to be limited by the progression of heart failure at the chronic phase. Therefore, miR-424 may be useful to monitor heart failure progression but seems unsuitable for prediction of sudden cardiac death [40].

CVD risk assessment, supported by emerging circulating biomarkers, such as miRNAs, is important for stratifying high-risk individuals, optimizing treatment strategies, and enhancing our understanding of basic biology. Our study showed the highest increase in expression levels of miR-424-5p in the target group and emphasized its role as a biomarker for cardiovascular damage in patients with type 2 diabetes.

Author contribution All authors contributed to the study conception and design. Methodology: Ivelina Mihaleva, Silva Kyurkchiyan, Rumyana Dodova, Ivanka Dimova. Formal analysis and investigation: Ivelina Mihaleva, Pavlina Gateva, Rumen Nikolov, Tsvetanka Markova, Ivanka Dimova. Writing—original draft preparation: Ivelina Mihaleva, Ivanka Dimova. Writing—review and editing: Pavlina Gateva, Rumen Nikolov, Tsvetanka Markova. Funding acquisition: Ivanka Dimova. Resources: Ivanka Dimova. Supervision: Ivanka Dimova

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Availability of data and material All data and material are available at the Molecular Medicine Centre, Medical University Sofia.

Declarations

Ethics approval The collection of patients' samples was approved by the institutional ethical committee (Medical University Sofia) with the approval no. 1209/2018. The research does not include animals.

Consent to participate All participants in the study signed the informed consent before collection of the samples.

Consent for publication It is included in the text of the informed consent signed by the patient.

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

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ORIGINAL ARTICLE

Arbutin alleviates diabetic symptoms by attenuating oxidative stress in a mouse model of type 1 diabetes

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Abstract

Background Arbutin is a well-known tyrosinase inhibitor that prevents the formation of melanin through the inhibition of tyrosinase. Therefore, it has been widely used as a cosmetic skin-lightening agent. Arbutin is able to scavenge free radicals within cells and previous studies have found that it also exhibited useful activities for the treatment of diuresis, bacterial infections, and cancer, as well as anti-inflammatory and anti-tussive activities. This study analyzed the effects of arbutin on streptozotocin (STZ)-induced diabetes mellitus in a murine model.

Methods Healthy male adult C57BL/6 mice (7 weeks old) were randomly allocated into one of the following three groups of six animals: Normal control with no STZ administration, STZ-induced diabetes, and STZ-induced diabetes treated with 0.3 g/kg/day of arbutin. After 12 days, the levels of insulin, C-peptide, and HbA1c were measured in serum, and the expression and enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were analyzed in pancreatic tissues by western blotting.

Results Arbutin was found to significantly inhibit the increase in blood glucose and the loss of body weight in diabetic mice. Arbutin increased plasma insulin levels in mice with STZ-induced diabetes, whereas there was no detection of insulin in untreated diabetic mice. In addition, there was an increased expression and activity of SOD, CAT, and GPx in diabetic mice treated with arbutin.

Conclusions This investigation demonstrated that arbutin possesses antioxidant activities and can alleviate symptoms of type-1 diabetes mellitus (T1DM) in mice.

Keywords Arbutin · Type-1 diabetes · Oxidative stress · Glucose · Streptozotocin

Abbreviations

STZ	streptozotocin
CAT	catalase
DM	diabetes mellitus
T1DM	type-1 diabetes mellitus
SOD	superoxide dismutase
GPx	glutathione peroxidase
ROS	reactive oxygen species

Introduction

Diabetes mellitus (DM), commonly referred to as diabetes, is a chronic metabolic disorder characterized by persistent hyperglycemia. Diabetes poses major health challenges and is recognized as one of the most urgent medical problems worldwide [1]. DM is characterized by absolute or relative

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deficiencies in insulin secretion, which is due to either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced. Symptoms of diabetes include frequent urination, increased thirst, increased hunger, and weight loss.

Chronic hyperglycemia is associated with abnormalities in carbohydrate, lipid, and protein metabolism [1]. Hyperglycemia is one of the major factors responsible for intense oxidative stress in diabetes. It is also an important source of reactive oxygen species (ROS) and is likely to be responsible for glucose autoxidation, which causes oxidative damage, particularly to the heart, kidneys, eyes, nerves, liver, small and large blood vessels, and the gastrointestinal system [2, 3].

Plant polyphenols are naturally occurring compounds that are widely distributed in fruits and vegetables. In previous studies, plant polyphenols have been reported to exhibit many beneficial effects including antioxidant, anti-inflammatory, antibacterial, antitumor, and other biological activities [4, 5]. Arbutin (C12H16O7), a naturally occurring glucoside of hydroquinone, is widely distributed in animals, plants, and microbes [6]. Arbutin is widely used as an ingredient in skincare products and has been traditionally used in Japan to treat pigmentary disorders [7]. Its depigmenting mechanism in humans is thought to involve the inhibition of melanosome tyrosinase activity [8, 9]. Arbutin is safe for humans and may have the potential to be an antitumor agent at high doses [10, 11]. It has been reported that arbutin can inhibit TCCSUP human bladder cancer cell proliferation via the upregulation of p21 [12]. Arbutin has exhibited many biological activities, including antioxidant, diuretic, antibacterial, anti-inflammatory, and antitumor [13]. To our knowledge, there are no reports about the effect of arbutin on type-1 diabetes. In this study, we have investigated the effects of arbutin on type-1 diabetes.

Materials and methods

Chemicals and biological materials

Streptozotocin (STZ) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rat/mouse insulin ELISA kits were purchased from EMD Millipore. Arbutin was purchased from Wuhan Fude Chemical Co. (Wuhan, China) and dissolved in ultrapure water to a concentration of 50 mM.

Experimental animals

Healthy male adult C57BL/6 mice (7 weeks old) were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine, China. All of the experimental procedures were approved by the ethics committee of the Nanjing University of Chinese Medicine. The animals were maintained at a constant temperature (22 ± 2 °C) and relative humidity (60–70%) with a 12 h light-dark cycle. All mice were housed at six mice per cage, fed with a standard pellet diet, and allowed free access to water.

Induction of experimental diabetes

STZ was dissolved in freshly prepared 0.1 M sodium citrate buffer (pH 4.5). Diabetes was induced by intraperitoneal injection (i.p.) of a single 130 mg/kg body weight dose of STZ. On day 3 after the STZ injection, all mice were fasted for 4 h, and their blood glucose levels were monitored from the tip of the tail vein. The mice were considered to have type-1 like DM when their fasting blood glucose levels were above 250 mg/dL.

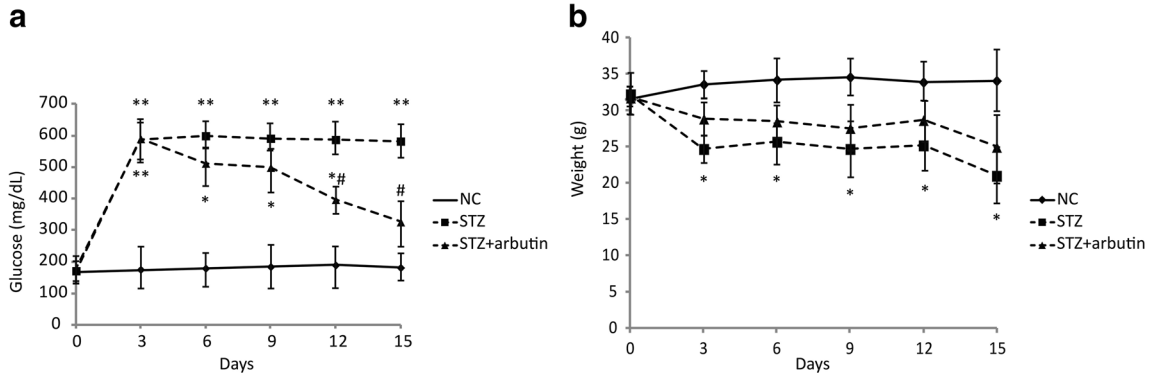

Fig. 1 Effect of arbutin on blood glucose levels and weight. (**a**) Glucose levels and (**b**) body weights of the three groups of mice during the 15-day treatment. The values represent the means of $n = 6$. Significant differences were determined with multiple -tests. Groups: NC, normal control; STZ, streptozotocin-induced diabetic mice; STZ + arbutin, arbutin-treated STZ-induced diabetic mice. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ with respect to the healthy group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$ with respect to the group of mice with STZ-induced diabetes

Experimental groups and treatment

The mice were randomly allocated into three groups of six animals each and received the following treatments: Group I, normal control (NC), having no STZ administration; Group II, diabetes (STZ), STZ-induced diabetes; and Group III, diabetic mice treated with 0.3 g/kg/day of arbutin (STZ + arbutin). Freshly prepared extracts of arbutin were orally administered daily for 12 days. Body weights and blood glucose levels were measured at 3-day intervals after the mice were fasted for 4 h.

Measurements of serum insulin, C-peptide, and HbA1c

Blood was collected at the end of the treatment and centrifuged at 1,200 x *g* for 5 min. A mouse insulin enzyme-linked immunosorbent (ELISA) kit (EMD Millipore, Kankakee, Illinois, USA) was used to determine the fasting serum insulin levels according to the manufacturer's instructions. C-peptide enzyme immune assay (EIA, Sigma-Aldrich) and HbA1c ELISA (Cusa-Bio Biotech Co., Ltd., Wuhan, China) kits were used to quantify these parameters according to the manufacturers' protocol.

Effects of arbutin on parameters of oxidative stress

At the end of the treatment, the animals were sacrificed, and each pancreas was quickly removed from each of the animals in the study groups. The pancreatic tissues were homogenized (around 200 mg) in 5–10 mL of cold 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram tissue) using a Bullet Blender (Next Advance, New York, New York State, USA). Then centrifuged at 1,500 x *g* for 5 min at 4 °C and the supernatant was collected and stored at −80 °C. The enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) was analyzed by assay kits (Cayman Chemical Company, Ann Arbor, Michigan, USA).

Antibodies and western blot analysis

The antibodies purchased were as follows: β-actin mouse mAb (Cell Signaling Technology, Beverly, MA, USA); rabbit anti-human SOD polyclonal antibody (Abcam, Cambridge,

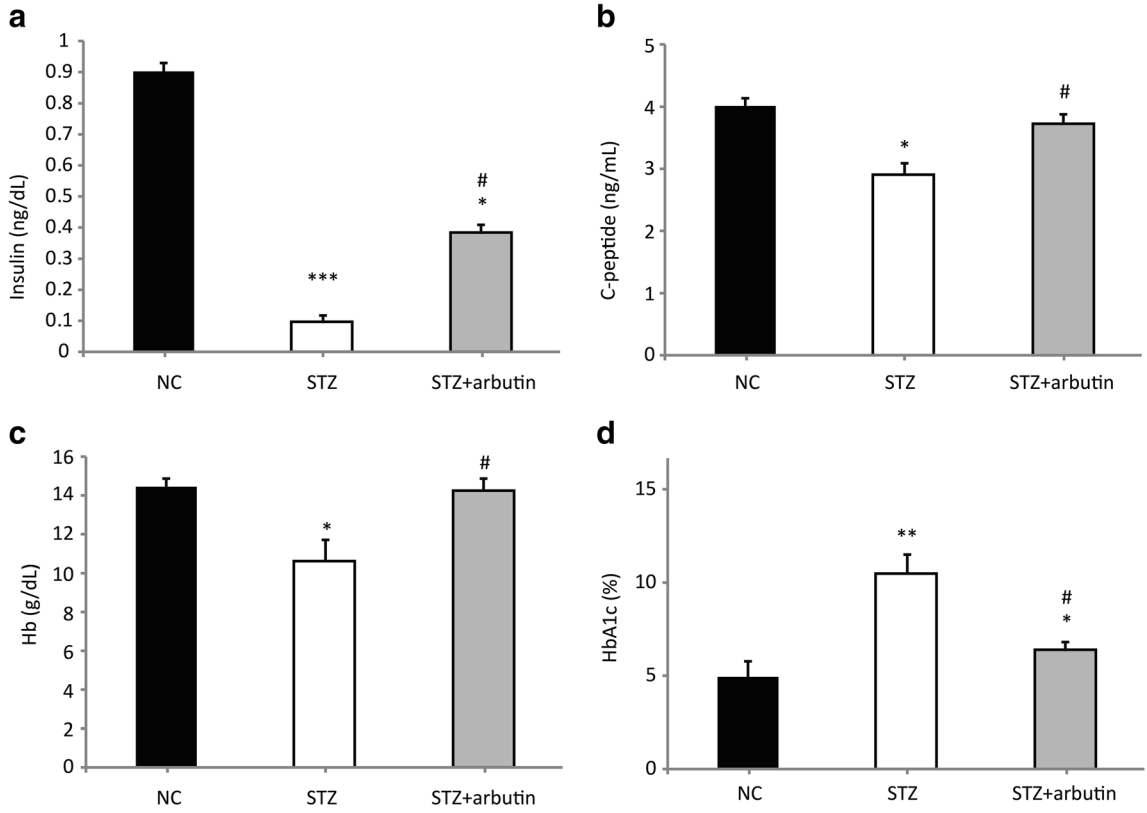

Fig. 2 Effects of arbutin on serum levels of insulin, C-peptide, glycated hemoglobin (HbA1c), hemoglobin (Hb). Serum insulin (**a**), C-peptide (**b**), glycated hemoglobin (**c**), and hemoglobin (**d**) were analyzed with arbutin treatment. The values represent the means of $n = 6$. Significant differences were determined with multiple *t* tests. Groups: NC, normal control; STZ, streptozotocin-induced diabetic mice; STZ + arbutin, arbutin-treated STZ-induced diabetic mice. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ with respect to the healthy group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$ with respect to the diabetic group. HbA1c, glycated hemoglobin; Hb, hemoglobin

UK); rabbit anti-human CAT polyclonal antibody (Abcam); and goat anti-human GPx IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For western blot analysis, the pancreatic tissues were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, Texas, USA) with PMSF (Santa Cruz Biotechnology) for 30 min on ice. Supernatants were collected via centrifugation at 12,000 x *g* for 10 min at 4 °C. The total protein concentration in the supernatants was determined using a BCA protein assay kit (Santa Cruz Biotechnology). The sample protein was separated by electrophoresis in 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, California, USA). Primary antibodies were used to detect the immunoblots at 4 °C overnight. After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Membranes were subsequently developed using Western Blotting Luminal Reagent (Santa Cruz Biotechnology) for immunological detection.

Statistical analysis

All data were presented as the means ± standard error. Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA) using GraphPad Prism (version 6.0) statistical analysis software. $p < 0.05$ was considered to indicate statistical significance.

Results

Effects of arbutin on the blood glucose level

In NC mice, constant glucose levels were maintained below 200 mg/dL during the whole treatment period. The glucose levels of the two groups of mice that received the STZ treatment increased to more than 500 mg/dL by day 3. The mice with STZ-induced diabetes (STZ) showed constant hyperglycemia from day 3 until day 15, whereas the glucose levels of the arbutin-treated group (STZ + arbutin) decreased steadily until the end of the treatment period. On day 15, the glucose levels of the mice with STZ-induced diabetes treated with arbutin were lower than untreated mice and not significantly different from that of the NC group (Fig. 1a).

Effects of arbutin on body weight

The diabetic mice (STZ) had a significant loss of body weight compared with that of the NC group from days 3 to 15. In contrast, the administration of arbutin had a considerable

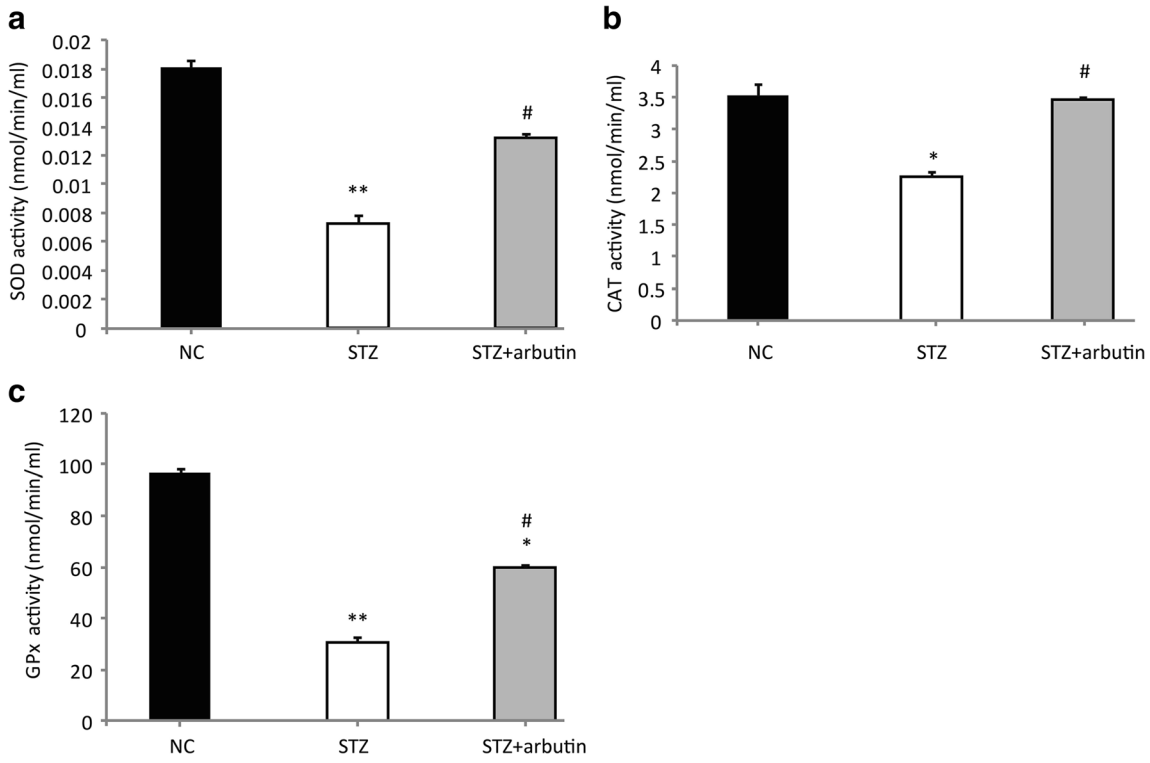

Fig. 3 Effects of arbutin on the enzymatic activities of SOD, CAT, and GPx. Enzymatic activities of SOD (**a**), CAT (**b**), and GPx (**c**) in healthy mice, diabetic mice, and diabetic mice treated with arbutin. The values represent the means of $n = 6$. Significant differences were determined with multiple tests. Groups: NC, normal control; STZ, streptozotocin-induced diabetic mice; STZ + arbutin, arbutin-treated STZ-induced diabetic mice. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ with respect to the healthy group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$ with respect to the group of mice with STZ-induced diabetes

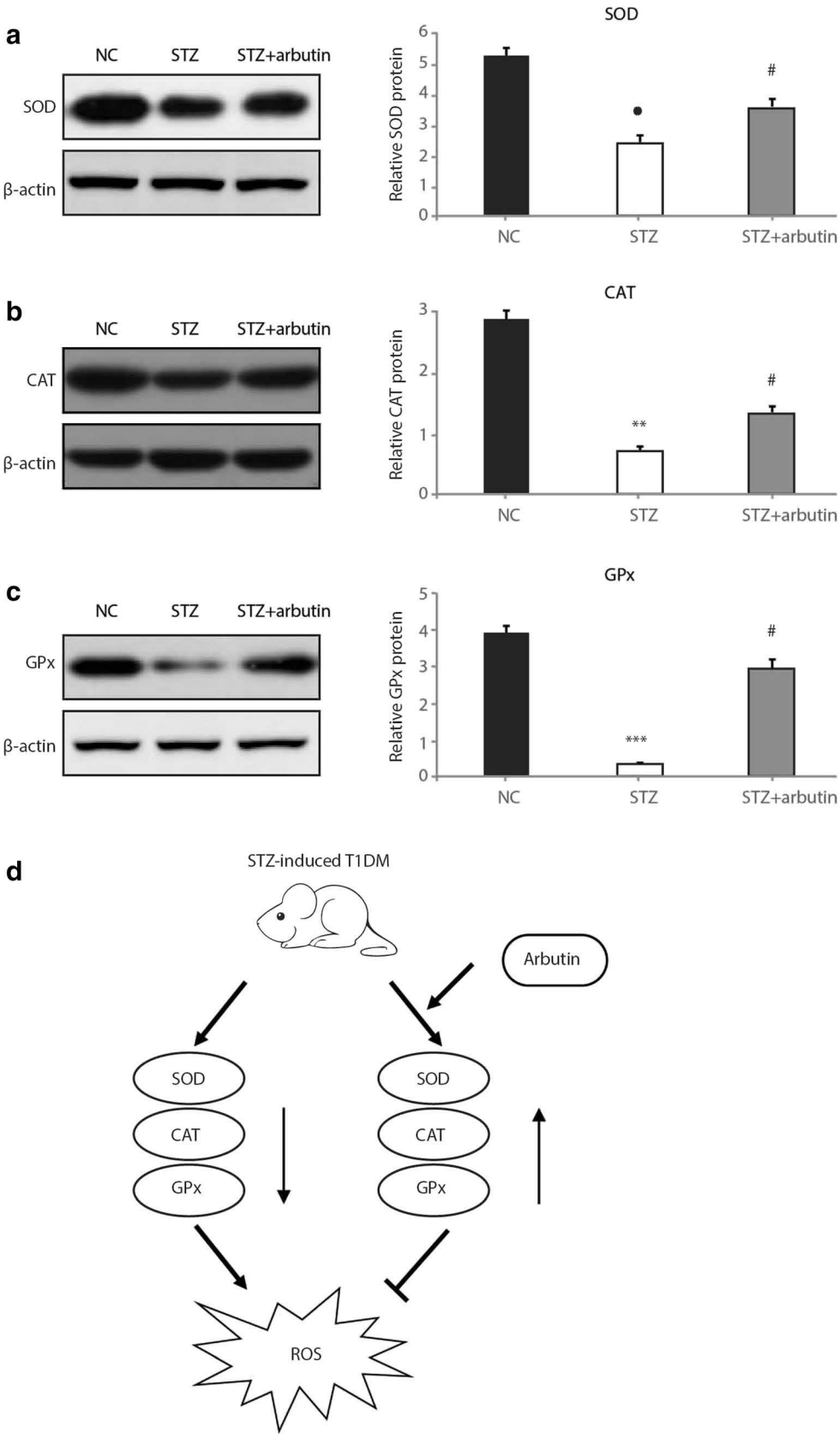

Fig. 4 Effects of arbutin on the protein expression level of SOD, CAT, and GPx. (**a**) western blot assay and densitometer analysis of SOD protein levels in healthy mice, diabetic mice, and diabetic mice treated with arbutin. (**b**) Western blot assay and densitometer analysis of CAT protein levels in healthy mice, diabetic mice and diabetic mice treated with arbutin. (**c**) Western blot assay and densitometer analysis of GPx protein levels in healthy mice, diabetic mice, and diabetic mice treated with arbutin. (**d**) A diagram of arbutin attenuates oxidative stress in STZ-induced type-1 diabetes mellitus mice. Groups: NC, normal control; STZ, streptozotocin-induced diabetic mice; STZ + arbutin, arbutin-treated STZ-induced diabetic mice

effect on the weight loss of diabetic mice. Compared with the diabetic mice, the diabetic mice treated with arbutin had significantly greater body weights of above 25 g; the NC mice maintained the highest weight (over 30 g). At the end of treatment, the weights of the mice in the arbutin-treated group (STZ + arbutin) were significantly different to those of the diabetic mice (Fig. 1b).

Effects of arbutin on serum insulin levels

Insulin levels were determined in the experimental groups at the end of treatment. The insulin levels were 0.9 ng/mL in NC mice and 0.1 ng/mL in diabetic mice, whereas insulin levels of 0.3 ng/mL were recorded in diabetic mice treated with arbutin, which was significantly different to those found in the diabetic mice (Fig. 2a).

Effects of arbutin on serum C-peptide, HbA1c, and Hb levels

Compared with the mice in the NC group, the serum C-peptide and blood Hb levels of the diabetic group were significantly decreased. In contrast, arbutin treatment restored these hematological parameters in diabetic mice (Figs. 2b, c). In addition, levels of HbA1c were significantly elevated in the STZ-induced diabetic mice; however, levels were near to normal in the arbutin-treated group (Fig. 2d).

Effects of arbutin on the activity of antioxidative enzymes

A constant high level of glucose in diabetes causes oxidative stress by increasing the levels of ROS and decreasing the antioxidant defenses of the organism. The enzymatic activities of three enzymes involved in the antioxidant system (SOD, CAT, and GPx) were determined at the end of the experiment using colorimetric methods. The enzymatic activities of these three enzymes were decreased in the diabetic mice. Whereas, the enzymatic activities of SOD, CAT, and GPx increased in the diabetic mice treated with arbutin (Fig. 3). This result indicated that arbutin could alleviate oxidative stress in diabetes.

Effects of arbutin on the expression of antioxidative enzymes

The expression levels of SOD, CAT, and GPx in pancreatic tissues were determined by western blot analysis (Figs. 4a–c). In comparison with the healthy mice, the protein expression levels of SOD, CAT, and GPx decreased in the diabetic mice, whereas the protein levels of SOD, CAT, and GPx increased with arbutin treatment (Figs. 4a–c).

Discussion

The toxic conditions in hyperglycemia disrupt pancreatic β-cells, leading to a reduction of insulin secretion in type-1 diabetes [14, 15]. Subsequently, hyperglycemia causes disturbances in all the biochemical mechanisms of the body, including abnormalities in carbohydrate, lipid, and protein metabolism [16].

More and more natural plant compounds have been found to have a therapeutic effect against diabetes with fewer side effects [17]. However, the effect of arbutin has not been evaluated in diabetic mice. Thus, in the present study, we have investigated the antidiabetic properties and underlying mechanisms of arbutin using mice with STZ-induced diabetes. First, STZ-induced high glucose levels were gradually decreased by arbutin, and the body weight loss of diabetic mice was also alleviated after 15 days of arbutin treatment. Second, decreased levels of serum insulin in diabetic mice caused by the destruction of pancreatic β-cells were restored by arbutin treatment (Fig. 2a).

The levels of hematological parameters, such as HbA1c, have been found to be proportional to blood glucose levels [18]. In addition, C-peptide is a protein connecting the α- and β-chains of insulin in the proinsulin molecule [19]. Therefore, C-peptide is thought to play a crucial role in controlling glucose concentration [20]. As expected, arbutin treatment increased serum HbA1c and C-peptide to a relatively normal level in diabetic mice.

In this study, we have reported that the administration of arbutin in diabetic mice restored the activity and expression of the antioxidant enzymes SOD, CAT, and GPx (Figs. 3 and 4). The mechanism by which arbutin may act is through its potent antioxidant capacity. The three antioxidant enzymes could protect the cells in the islet of Langerhans from damage and promote the secretion of insulin.

This investigation demonstrated that arbutin possessed hypoglycemic and antioxidant activities and provided a novel insight into the prevention of type-1 diabetes (Fig. 4d). However, more studies are needed to increase the knowledge of all the biological activities of arbutin, its potential molecular mechanisms, and possible adverse effects. Research is underway to further explore the long-term administration and application of arbutin to type-2 diabetes. It would also be very interesting to access the effect of arbutin combined with insulin on both type-1 and type-2 diabetes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethnical approval All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine, China. All of the experimental procedures were approved by the ethics committee of the Nanjing University of Chinese Medicine.

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ORIGINAL ARTICLE

Effects of anti-CD20 monoclonal antibody and IL-10 on pancreatic β cell regeneration in nonobese diabetic mice

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Abstract

Objective To investigate the effects of anti-CD20 monoclonal antibody (mAb) combined with IL-10 mediated by adenovirus on the regeneration of pancreatic β cells in nonobese diabetic (NOD) mice.

Methods Serum IL-10, TGF-β, TNF-α, IFN-γ, C-peptide, and insulin were determined using commercial ELISA. Western blot assay and real-time PCR were performed to determine the expression of mRNA and protein expression, respectively. Besides, immunohistochemistry and immunofluorescence were performed.

Results Immunohistochemisty assay indicated Ngn3 expression showed decrease in the anti-CD20+IL-10 group compared with the other groups ($p < 0.05$). Immunofluorescence assay showed Pdx-1 and insulin coexpressing cells showed increase in the anti-CD20+IL-10 group. Real-time PCR indicated that the expression of Pdx-1, Pax4, Nkx6.1, and Mafa mRNA showed significant increase in the anti-CD20+IL-10 group compared with the other groups ($p < 0.05$). The expression of Pdx-1, Pax4, Nkx6.1, and Mafa protein was significantly upregulated in the anti-CD20+IL-10 group compared with that of the other groups ($p < 0.05$).

Conclusions Anti-CD20 combined with IL-10 promoted insulin secretion in NOD mice and the controlling of blood glucose. Besides, the combination induced the decrease of TNF-α and IFN-γ, and the elevation of TGF-β. Besides, it may activate the Pdx-1-Ngn3-Pax4-Nkx2.2-Nkx6.1 signaling pathway, and trigger the regeneration of pancreatic β cells.

Keywords Adenovirus · Diabetes · Pancreatic β cells · IL-20

Introduction

Type 1 diabetes mellitus (T1DM) is a type of autoimmune disease featured by progressive injury of pancreatic β cells and insufficient insulin secretion. Currently, the treatment of T1DM is mainly relied on insulin; however, it cannot inhibit the apoptosis of pancreatic β cells. In patients with T1DM, there are remnant pancreatic β cells with the capacity of self-replication and self-regeneration [1]. Therefore, extensive studies have been focusing on the remnant pancreatic β cells, for the management of T1DM patients with acute or chronic inflammation as well as brittle diabetes.

To date, several mechanisms have been reported to be associated with the pathogenesis of T1DM, among which the Th1/Th2 subset imbalance and excessive activation of B lymphocytes are considered to be crucial for the immune response in these patients [2–4]. As an important cytokine of Th2, IL-10 was reported to correct the Th subset imbalance [5]. Besides, CD20 considered as a crucial molecule on the B cell surface is associated with the apoptosis of B cells [6]. In this study, NOD mice with T1DM were treated using IL-10 and

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anti-CD20 monoclonal antibody (mAb), in order to determine their roles in modulating the apoptosis and/or proliferation of pancreatic β cells.

Materials and methods

Animals

Female NOD/Lt mice (specified pathogen free, 14–16 weeks old) with a body weight of 20–25 g were purchased from the Chinese Academy of Medical Sciences Institute of Experimental Animals. The animals were acclimated for 3 weeks under a temperature of 23 ± 2 °C with a humidity of 58 ± 2 °C in a cycle of 12-h light/12-h dark. All animals had free access to animal diet and water. The animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies.

T1DM induction

T1DM induction was carried out according to previous description. Briefly, the animals were fed using a complete formula granulated feed (Beyotime, Shanghai, China). Mice with urine glucose were selected for the blood glucose. T1DM was defined as blood glucose of ≥ 13.9 mmol/L randomly determined at least twice [7].

Experimental design

Those newly diagnosed with T1D were randomly divided into four groups, including (i) anti-CD20 mAb (anti-CD20) group ($n = 6$), subject to anti-CD20 (500 μg; d1) via caudal vein injection, followed by caudal vein injection of anti-CD20 (250 μg; d3, d6, and d9); (ii) anti-CD20 mAb plus Ad-mIL-10 (anti-CD20+IL-10) group ($n = 6$), subject to anti-CD20 (500 μg; d1) and Ad-mIL-10 (1×10^{11}PFU/ml, 0.1 ml) via caudal vein injection, followed by caudal vein injection of anti-CD20 (250 μg) and Ad-mIL-10 (1×10^{11}PFU/ml, 0.1 ml) on d3, d6, and d9, respectively; (iii) Ad-mIL-10 group ($n = 6$), subject to caudal vein injection of Ad-mIL-10 (1×10^{11}PFU/ml, 0.1 ml) on d1, d3, d6, and d9, respectively; and (iv) normal saline (NS) group, subject to equal volume (0.1 ml) of NS via caudal vein injection on d1, d3, d6, and d9, respectively. Mice were depleted of B cells using anti-murine CD20 IgG2a antibody (clone 18B12; Biogen Idec, Weston, MA, USA) at indicated schedules and dosages [8].

Preparation of anti-CD20 monoclonal antibody

Mice were depleted of B cells using anti-murine CD20 IgG2a antibody (clone 18B12; Biogen Idec, Weston, MA, USA) at indicated schedules and dosages [8].

ELISA

Blood samples were collected from the eyeballs of each mouse. Serum was collected from the mice. Serum IL-10, TGF-β, TNF-α, IFN-γ, C-peptide, and insulin were determined using commercial ELISA kits purchased from R&D Inc. (CA, USA), according to the manufacturer's instructions. All tests were performed at least in triplicate.

Immunohistochemical evaluation

The pancreatic tissues were fixed and embedded, followed by antibody recovery. Then, Fox01, Ngn3, and Pdx-1 primary antibodies were added and incubated at 4 °C overnight. After washing with MPBS, the biotinylated IgG was added and incubated at 37 °C for 20 min. Subsequently, DAB was used for staining, followed by hematoxylin counter staining. Five pancreatic tissues were randomly selected under a magnification of × 400. Cells with cytoplasmic granules stained in brown were considered to be positive cells. The IHS is calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score) as follows: no staining is scored as 0, 1–10% of cells stained scored as 1, 11–50% as 2, 51–80% as 3, and 81–100% as 4. Staining intensity is rated on a scale of 0 to 3, with 0 as negative, 1 as weak, 2 as moderate, and 3 as strong.

Immunofluorescence

The pancreatic tissues were fixed and embedded according to the conventional procedures. Then, the sections were incubated with the primary antibodies, followed by incubating at 4 °C overnight. Subsequently, the secondary antibodies were added. Finally, the images were observed under a light microscope.

Real-time PCR

Total RNA was extracted from pancreatic tissues using RNAiso Plus Kit (Takara, Tokyo, Japan) according to manufacturer's instructions. The cDNA synthesis was carried out with approximately 2 μg RNA using ExTaq (Takara, Tokyo, Japan). Real-Time PCR was conducted using SYBR (Takara, Tokyo, Japan) on an ABI system with the primers listed in Table 1. The mRNA level was normalized by β-actin. PCR reactions were performed in a total volume of 20 μl containing

Table 1 Comparison of body weight in each group

Group	0 week	3 weeks	6 weeks	9 weeks
Anti-CD20 ($n = 6$)	25.132 ± 0.864	25.658 ± 1.046	26.462 ± 0.884	26.360 ± 0.679
Anti-CD20+IL-10 ($n = 6$)	25.163 ± 1.232	25.868 ± 1.022	26.875 ± 0.663	27.017 ± 0.665
IL-10 ($n = 6$)	25.612 ± 0.855	25.955 ± 1.204	26.275 ± 1.142	25.710 ± 0.749
NS ($n = 6$)	24.988 ± 1.036	25.503 ± 1.326	25.938 ± 0.985	25.302 ± 0.784

10 μl 2 × SYBR premix, 0.5 μl each specific primer to a final concentration of 10 μM, and 2 μl cDNA template. The PCR conditions consisted of denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 34 s. The amplification result for the real-time PCR was calculated as $2^{(-\Delta\Delta Ct)}$.

Western blot analysis

The pancreatic tissues were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Proteins were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a Hybond-P PVDF membrane. The membrane was blocked in 5% nonfat milk and incubated with primary antibodies (Pdx-1, 1: 1000, Abcam; Pax4, 1:500, Santa Cruz; Mafa, 1:500, Santa Cruz; Nkx6, 1:500, Santa Cruz) overnight at 4 °C. Then the mixture was incubated with the HRP-conjugated secondary antibodies for 1 h at room temperature. After washing with PBS, the bound primary antibody was visualized with the enhanced chemiluminescence system from Amersham (Piscataway, NJ, USA) and exposed to film. The same membrane was probed for β-actin for loading control. The relative density of PAR to β-actin was analyzed with the ImageJ software.

Statistical analysis

SPSS16.0 software was used for the statistical analysis. All data were presented as mean ± standard deviation. Measurement data that were normally distributed are subject to homogeneity of variance. Log/Ln/Sin or Sqrt transition was performed to the data that were not normally distributed. Data normally distributed with homogenous variance were compared with the one-factor analysis of variance and LSD test among the groups. The Games-Howell test and Kruskal-Wallis rank test were used for the data not normally distributed or with heterogenous variance even after Log/Ln/Sin or Sqrt transition. Ranked data were analyzed using the Kruskal-Wallis rank test. $p < 0.05$ was considered to be statistically significant.

Results

Comparison of body weight and blood glucose in each group

No statistical difference was noticed in the body weight at the baseline level among the 4 groups ($p > 0.05$; Table 1). Besides, no statistical differences were noticed in the body weight at weeks 6 and 9 compared with that of week 3 in each group ($p > 0.05$).

For the glucose, no statistical difference was observed at the baseline level ($p > 0.05$; Table 2). At week 3, the glucose level in the anti-CD20 group and anti-CD20+IL-10 group was significantly lower than that of the IL-10 group and NS group, respectively ($p < 0.05$). At week 6, the glucose level in the anti-CD20+IL-10 group was significantly lower than that of the anti-CD20 group, IL-10 group, and NS group, respectively ($p < 0.05$).

Comparison of IL-10, insulin and C-peptide level, TNF-α, IFN-γ, and TGF-β

The serum IL-10 showed significant elevation in the IL-10 group compared with that of the other groups ($p < 0.05$; Table 3). Besides, the serum IL-10 in the anti-CD20+IL-10 group was significantly higher than that of the anti-CD20

Table 2 Comparison of blood glucose in each group

	0 week	3 weeks	6 weeks	9 weeks
Anti-CD20 ($n = 6$)	17.633 ± 2.609	17.583 ± 4.788a	23.650 ± 4.563c	25.200 ± 4.660c
Anti-CD20+IL-10 ($n = 6$)	17.283 ± 1.514	15.883 ± 4.958a	16.283 ± 5.758ab	16.567 ± 4.689ab
IL-10 ($n = 6$)	18.617 ± 1.988	21.067 ± 6.868	22.233 ± 5.207a	24.500 ± 6.743
NS ($n = 6$)	19.367 ± 1.429	24.833 ± 4.313	28.467 ± 3.565	30.150 ± 3.844

[a] $p < 0.05$ vs NS group; [b] $p < 0.05$ vs L-10 group; [c] $p < 0.05$ vs anti-CD20+IL-10 group

Table 3 Comparison of serum IL-10, insulin, and C-peptide

	IL-10 (pg/ml)	Insulin (pmol/g)	C-peptide (ng/l)
Anti-CD20	12.911 ± 4.682bc	94.299 ± 20.539c	9.245 ± 0.307 ac
Anti-CD20+IL-10	37.490 ± 4.769ab	144.230 ± 24.551ab	10.513 ± 0.610ab
IL-10	54.813 ± 7.457a	96.218 ± 27.345	9.129 ± 0.322a
NS	12.953 ± 4.870	96.886 ± 28.768	5.756 ± 0.545

[a] $p < 0.05$ vs NS group; [b] $p < 0.05$ vs L-10 group; [c] $p < 0.05$ vs anti-CD20+IL-10 group

group and NS group, respectively ($p < 0.05$). The serum C-peptide in the anti-CD20+IL-10 group was significantly higher than that of the anti-CD20 group, IL-10 group, and NS group, respectively ($p < 0.05$). For the insulin level, the concentration in the anti-CD20+IL-10 group was significantly higher than that of the other groups ($p < 0.05$). The TNF-α and IFN-γ in the anti-CD20+IL-10 group was significantly lower than those of the anti-CD20 group, IL-10 group, and NS group, respectively ($p < 0.05$). The TGF-β in the anti-CD20+IL-10 group was significantly higher than that of the anti-CD20 group, IL-10 group, and NS group, respectively ($p < 0.05$; Table 4).

Comparison of immunohistochemical evaluation and immunofluorescence assay

Immunohistochemical evaluation of the CD20 revealed that there was no statistical difference among the 4 groups ($p > 0.05$; Fig. 1). The expression of Ngn3 in the anti-CD20+IL-10 group was significantly higher than that of the other groups ($p < 0.05$; Fig. 1). For the immunofluorescence assay, coexpression of Pdx-1 and insulin in the anti-CD20+ IL-10 group was significantly higher than that of the other groups ($p < 0.05$; Fig. 2).

Comparison of Pdx-1, Pax4, TGF-β, Mafa, and Nkx6 mRNA

The expression of Pdx-1, Pax4, TGF-β, Mafa, and Nkx6 mRNA in the anti-CD20+IL-10 group was significantly higher than that of the other groups ($p < 0.05$; Table 5).

Comparison of Pdx-1, Pax4, Mafa, and Nkx6 protein

In this section, we compared the expression of Pdx-1, Pax4, Mafa, and Nkx6 protein in the pancreatic β cells. The expression of Pdx-1 protein in the anti-CD20+IL-10 group was significantly upregulated compared with that of the other groups ($p < 0.05$; Fig. 3). Similarly, the expression of Pax4, Nkx6, and Mafa protein in the anti-CD20+IL-10 group was significantly upregulated compared with that of the other groups ($p < 0.05$).

Discussion

T1DM, with a high prevalence worldwide, is a public threat to the health conditions. Increasing evidence indicates that immune injury plays a crucial role in the pathogenesis of T1DM; however, the exact mechanism is still not well defined [2, 9, 10]. In this study, NOD mice with T1DM were treated using IL-10 and anti-CD20 monoclonal antibody, in order to determine their roles in modulating the apoptosis and/or proliferation of pancreatic β cells.

As a specific molecule on the surface of β cells, CD20 is expressed on the surface of pro-B cells and mature B cells [2, 9, 10]. The anti-CD20 mAb could modulate the immune failure of these cells rather than inducing damage to the stem cells [11]. Therefore, anti-CD20 mAb therapy could induce gradual elevation of B cells after a sharp decrease [12]. In this study, the pancreatic CD20 expression showed no statistical difference among the four groups as revealed by the immunohistochemical technique, which was consistent with previous findings.

Table 4 Comparison of TNF-α, IFN-γ, and TGF-β

	TNF-α (ng/l)	IFN-γ (pg/ml)	TGF-β (ng/l)
Anti-CD20	48.684 ± 1.220c	49.755 ± 0.925c	62.932 ± 2.004c
Anti-CD20+IL-10	46.438 ± 1.460ab	43.795 ± 0.587ab	93.648 ± 3.077ab
IL-10	50.087 ± 0.492a	49.808 ± 0.960a	63.398 ± 2.383a
NS	90.736 ± 2.806	67.280 ± 0.834	53.776 ± 2.100

[a] $p < 0.05$ vs NS group; [b] $p < 0.05$ vs L-10 group; [c] $p < 0.05$ vs anti-CD20+IL-10 group

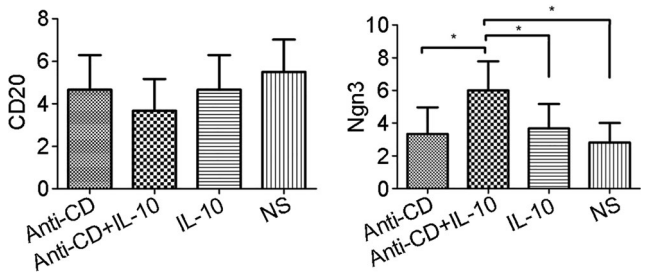

Fig. 1 Immunohistochemical results. $*p < 0.05$

For the effects of the anti-CD20+IL-10 on glucose, the glucose level in the anti-CD20+IL-10 group was significantly lower than that of the IL-10 group and anti-CD20 group at weeks 3, 6, and 9, respectively. However, no statistical differences were noticed in the body weight after various interferences. The serum C-peptide in the anti-CD20+IL-10 group was higher than that of the IL-10 group, anti-CD20 group, and NS group, respectively. Similarly, the expression of insulin in the anti-CD20+IL-10 group was significantly higher than that of the other groups. Therefore, the anti-CD20+IL-10 contributed to the decrease of glucose and secretion of insulin in NOD mice.

In this study, TNF-α and IFN-γ expression was determined using ELISA assay, which revealed the TNF-α and IFN-γ expression in the anti-CD20+IL-10 group was significantly lower than that of the IL-10 group, anti-CD20 group, and NS group. This indicated that anti-CD20 mAb combined with IL-20 could inhibit the inflammatory reactions in NOD mice with T1DM. TGF-β is an immunologic suppressor factor secreted by CD4+CD25+Treg cells, and its function is mainly through the following mechanisms: (i) It is involved in the immunologic tolerance through modulating the cell number of CD4+CD25+Treg. (ii) It may contribute to the generation and differentiation of iTreg cells that secret IL-10, which then regulates the proportion of Th cell subsets and maintenance of the homeostasis [13–15]. Using real-

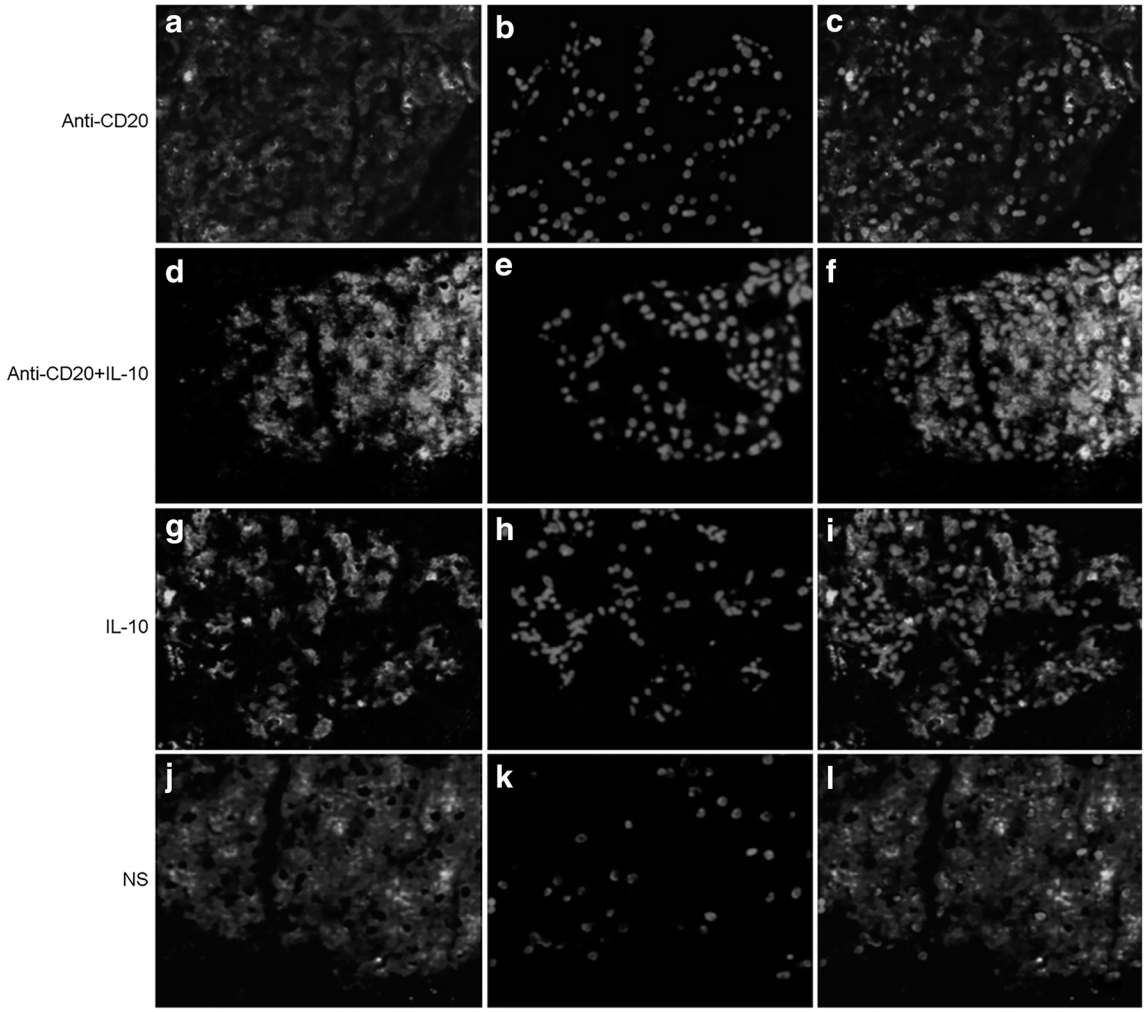

Fig. 2 Immunofluorescence of Pdx-1 and insulin. **a**–**c** Single staining for insulin and Pdx-1, as well as the costaining for Pdx-1 and insulin in the anti-CD20 group. **d**–**f** Single staining for insulin and Pdx-1, as well as the costaining for Pdx-1 and insulin in the anti-CD20+IL-10 group. **g**–**i** Single staining for insulin and Pdx-1, as well as the costaining for Pdx-1 and insulin in the IL-10 group. **j**–**l** Single staining for insulin and Pdx-1, as well as the costaining for Pdx-1 and insulin in the NS group

Table 5 Expression of Pdx-1, Pax4, TGF-β, Mafa, and Nkx6 mRNA

Group	Pdx-1	Pax4	TGF-β	Mafa	Nkx6
Anti-CD20	1.097 ± 0.351c	1.412 ± 0.053abc	1.322 ± 0.044 ac	1.277 ± 0.036abc	1.301 ± 0.056 ac
Anti-CD20+IL-10	2.823 ± 0.991ab	1.781 ± 0.031ab	1.715 ± 0.006ab	1.734 ± 0.027ab	1.917 ± 0.031ab
IL-10	1.334 ± 0.458	1.297 ± 0.055a	1.319 ± 0.030a	1.356 ± 0.035a	1.244 ± 0.039a
NS	0.471 ± 0.158	0.755 ± 0.029	1.240 ± 0.020	0.734 ± 0.038	0.731 ± 0.029

[a] $p < 0.05$ vs NS group; [b] $p < 0.05$ vs L-10 group; [c] $p < 0.05$ vs anti-CD20+IL-10 group

time PCR, we determined the expression of TGF-β mRNA in pancreatic tissues, which indicated the expression of TGF-β mRNA in the anti-CD20+IL-10 group was significantly higher than that of the other groups. For the serum TGF-β protein, the expression in the anti-CD20+IL-10 group was obviously higher than that of the other groups. This implied that the combination of anti-CD20 and IL-10 contributed to the expression of TGF-β, which then inhibited the immune response.

Pancreatic β cells play an important role in the immune injury in T1DM patients. In recent years, regeneration of pancreatic β cells is presented in T1DM patients [16]. Pdx gene is a major regulatory factor for the pancreatic development, as well as the differentiation of pancreatic progenitor cells to the islet cells, and is expressed in the development of the whole pancreas [17, 18]. Ngn3 is a protein marker of islet cells that can activate the NeuroD and the subsequent expression of isl-1, Pax6, Pax4, Nkx2.2, and Nkx6.1 [19]. Pax4, as a transcriptional factor of paired-box family, is expressed in the development of pancreas, which is crucial for the growth of B cells [20]. Besides, it is also considered as an important element for the Nkx6.1 expression. Moreover, Pax4 is regulated by the Arx that serves as an Aristaless family member. To our best knowledge, Atx was mainly reported to be associated with the generation of pancreatic α cells, while the Pax4 was mainly associated with the formation of β cells. Nkx2.2 activation is crucial for the maturity of β cells, and its expression is regulated by Nkx6, both of which are transcriptional factors of homeo-box family. In the presence of Nkx2.2 mutation, massive immature β cells were observed [21], which showed a lack of GLUT2 and glucokinase that were specific for the advanced β cells. Therefore, Nkx2.2 is crucial for the regulation of differentiation of β cells [22]. Meanwhile, the expression of Nkx2.2 and Pax4 was of prime importance for the survival of β cells, and deletion of pancreatic β cells may present in cases of any mutation in these two genes [23]. Nkx6.1 is a downstream gene of Nkx2.2, and is highly expressed in the proliferated pancreatic progenitor cells and the differentiated β cells. Therefore, it is considered as an important marker for the regeneration of beta cells [22]. On this basis, we speculated that the activation of the Pdx-1-Ngn3-Pax4-Nkx2.2-Nkx6.1 signaling pathway was closely related to the regeneration and maturity of the beta cells.

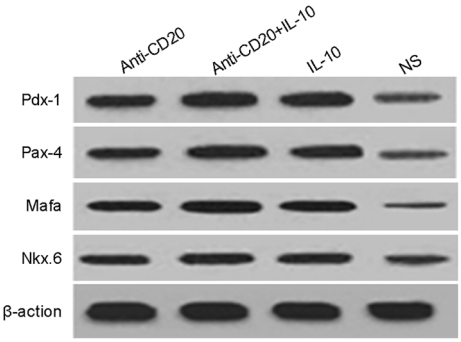

Fig. 3 Protein expression of pancreatic β cells in each group

The expression of mRNA and protein of Pdx-1, Pax4, and Pax6.1 in the anti-CD20+IL-10 group was significantly higher than that of the other groups. Using immunohistochemical technique, the expression of Ngn3 in the anti-CD20+IL-10 group was significantly higher compared to that of the other groups. Besides, immunofluorescence technique indicated that the number of Pdx-1 and insulin coexpressing cells in the pancreatic tissues in the anti-CD20+IL-10 group was obviously larger than that of the other groups. These indicated that anti-CD20+IL-10 treatment could decrease the blood glucose and contribute to the secretion of insulin through activating the Pdx-1-Ngn3-Pax4-Nkx2.2-Nkx6.1 signaling pathway.

Mafa is a transcriptional factor closely related to the function of β cells. It is a member of leucine zipper family, and is merely expressed in the β cells. It could synergize with Pdx-1 and NeuroD genes, which then promotes the transcription of genes encoding the insulin. The gene transcription of Mafa was regulated by the circulating glucose [24, 25]. In this study, the Mafa gene expression in the anti-CD20+IL-10 group was obviously higher than that of the other groups. Also, the Mafa protein showed a similar pattern as revealed by Western blot analysis. The anti-CD20+IL-10 could contribute to the expression of Mafa, which then regulates the transcription of insulin genes.

In conclusion, the combination of anti-CD20 mAb plus Ad-mIL-10 contributed to the secretion of insulin in NOD mice as well as controlling blood glucose. The combination

could attenuate the expression of TNF-α and IFN-γ, and increase the TGF-β synthesis and maintenance of immune homeostasis. Meanwhile, the combination of anti-CD20 mAb plus Ad-mIL-10 could activate the Pdx-1-Ngn3-Pax4-Nkx2.2-Nkx6.1 signaling pathway, and contribute to the regeneration of β cells though upregulating the expression of Mafa.

Authors' contributions TF drafted the article or revised it critically for important intellectual content; LT finally approved the version to be published; LC, ZY, JJ, CZH, and ZLJ contributed to the conception and design or acquisition of data or analysis and interpretation of data.

Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Ethical considerations The study protocols were approved by the EC of Qingdao Women and Children Hospital.

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ORIGINAL ARTICLE

Influence of a 12-week physical activity program on leptin resistance in metabolic syndrome: a preliminary study

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Abstract

Purpose Metabolic syndrome is a condition with clustering of risk factors like insulin resistance, obesity, dyslipidemia, and hypertension. Leptin is a protein of obese gene produced by white adipose tissue. Leptin resistance is the insensitivity of leptin in hypothalamus despite high amounts in blood causing obesity and metabolic syndrome. The study focused on the influence of a 12-week physical activity promotion program on leptin resistance in people with metabolic syndrome

Methods After approval from institutional ethics committee (IEC 343-2018), 18 participants (males *N*= 4, females *N*=14) of age group 45.0±7.6 years with metabolic syndrome according to (NCEP ATP-III) criteria were included in the study. The participants underwent a 12-week physical activity program consisting of 150 min of moderate to vigorous activity per week as per GPAQ domains—work, transport, and recreation. The outcomes were measured at baseline and after 12 weeks.

Results Out of 18 participants, 10 participants who completed the study were analysed. Twelve-week physical activity showed significant changes in waist circumference (p=0.047), post prandial blood glucose (p=0.0396), triglycerides (p=0.0323), body mass index (p=0.0056), subcutaneous fat (p=0.0354), and basal metabolic rate (p=0.0035). Fasting blood glucose (p=0.254), lipid profiles (total cholesterol (p=0.062)), high-density lipoprotein (p=0.367), low-density lipoprotein (p=0.641), and leptin showed insignificant change (p=0.328). Global physical activity questionnaire showed significant change (p=0.0254) suggesting changes in physical activity behaviors.

Conclusion From present study, it is concluded that a 12-week physical activity promotion program brought marginal changes in leptin levels and has potential to modify metabolic syndrome parameters and improve physical activity.

Keywords Metabolic syndrome · Leptin resistance · Physical activity · Body composition

Introduction

Metabolic syndrome (MetS) is a condition characterized by clustering of several known risk factors like insulin resistance (IR), obesity, atherogenic dyslipidemia, and hypertension (HTN) [1]. It was named as syndrome X by Gerald Raven in 1988 with aggregation of several important cardiovascular risk factors. Visceral fat is considered as a major risk factor for coronary heart disease and type 2 diabetes mellitus [2]. In addition to this, elevated serum levels of fibrinogen and tissue plasminogen activator inhibitor (PAI) are related to metabolic factors of CHD. Low-density lipoprotein (LDL) is associated with insulin resistance, obesity, and type 2 diabetes. Dyslipidemia is also considered as one of the risk factors of MetS with increased serum triglycerides, decreased high-density lipoprotein (HDL), and increased LDL. Other contributing conditions of MetS include endothelial dysfunction [3], inflammation, and oxidative stress [4].

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Leptin is a 167-amino acid protein, a product of obese gene produced by white adipose tissue. It functions as endocrine organ with numerous functions apart from fat storage [5]. It works on hypothalamus and acts to decrease food intake and increase metabolism. It helps in decreasing appetite, increasing energy expenditure, and regulating glucose homeostasis [6]. Congenital leptin deficiency has shown to cause severe obesity, glucose intolerance, and insulin resistance in humans. Increased serum leptin can be due to increased FFA, insulin stimulation, oestrogen, TNF α, and impaired renal clearance. Increased leptin levels are associated with metabolic syndrome [7]. High levels of leptin are seen in obese individuals due to defect in leptin receptor, inhibition in leptin signalling, and alterations in leptin transport in blood brain barrier [8]. Leptin resistance occurs in β cells which may lead to hyperinsulinemia which in turn causes less energy expenditure and leads to metabolic syndrome. There are no specific criteria to diagnose leptin resistance. Studies showed that hyperleptinemia is an important marker for leptin resistance [9, 10]. Leptin resistance could be due to reduction in expression of short and long isoforms of leptin receptors (OBRa & OBRb) in hypothalamus, hepatocytes, adipose tissue, and muscles [11, 12].

Strong evidence exists that both children and adults benefit from being physically active. Benefits of physical activity include lower risk of chronic diseases such as obesity, heart disease, diabetes, cancer, and depression [13–19]. It is widely accepted that physical activity is a low-cost alternative to disease treatment and prevention [20]. Phillip Tuso discussed the five strategies to be physically active which are to (1) measure physical activity as a vital sign; (2) encourage patients to be physically active at least 150 min per week; (3) create healthy environments by making it easier for patients to be physically active where they live, learn, work, play, and pray; (4) monitor disease incidence of patients who are physically active vs those who are not physically active; and (5) spread best practices [20]. MetS affects people whose excess body weight, and sedentary lifestyle determine the phenotype expression of genetic trait [21]. Maintaining normal weight and practising physical activity remain the most effective prevention strategy of MetS [22]. Management of MetS should be based on effective weight loss program and exercise program which needs specific strategies for the maintenance of the program. Weight loss is associated with significant improvements in clinical components of MetS, and there is association between moderate weight loss and improvements in metabolic profile [23]. Physical activity (PA) has a major role in the treatment of MetS. Cardiopulmonary fitness has shown to modify cardiovascular events, thus providing a strong and protective effect on cardiovascular health. Several studies have shown that increased levels of physical activity are associated with less prevalence and incidence of MetS [24].

However, the effect of PA promotion on leptin resistance among people with metabolic syndrome is not known. The purpose of this study is to determine the influence of a 12-week PA promotion program on leptin resistance in people with metabolic syndrome (Fig. 1).

Methods

After approval from institutional ethics committee (IEC 343-2018), 18 participants (males *N*= 4, females *N*=14) with age group 45.0±7.6 with 3 out of 5 criteria of metabolic syndrome according to the National Cholesterol Education Program Adult Treatment Protocol III (NCEP ATP-III) criteria were included in the study (Fig. 2). In the present study, the screened participants have been advised to continue the medication as per the physician advice. Standard diet prescription was given by the hospital dietician. Participants were excluded if they have a history of smoking past 6 months, cardiovascular disease, stroke, transient ischemic attack (TIA), any cognitive impairment, any systemic illness, and if they are having any other contraindications for PA as mentioned in PARQ+ physical activity readiness questionnaire.

Study design

The study is preliminary pre-post non-randomized interventional with a single group of participants with metabolic syndrome. The sample size for this study was estimated as general rule of thumb as suggested by Julious [25]. The participants were selected using convenience sampling from the Kasturba Hospital after a thorough medical evaluation and clearance for physical activity from the physician.

Procedure

The participants were enrolled into the study after physician clearance and low risk as per AHA/ACSM pre-participation questionnaire and PARQ+ physical activity readiness questionnaire. If the participants were found to be under moderate or severe risk category, they were advised to consult a physician before the start of any physical activity. Participants who were included in the study were assessed for fasting blood sugar (FBS), waist circumference (WC), blood pressure (BP), body composition, physical activity, leptin, lipid profile, and basal metabolic rate (BMR). BP is taken with digital sphygmomanometer *Omron* HEM-7113 (Omron Health Care Inc, Illinois) (Accuracy: Pressure: ±3 mmHg Pulse: ±5% of display reading) after making the participant rest for 3 min in a quiet room.

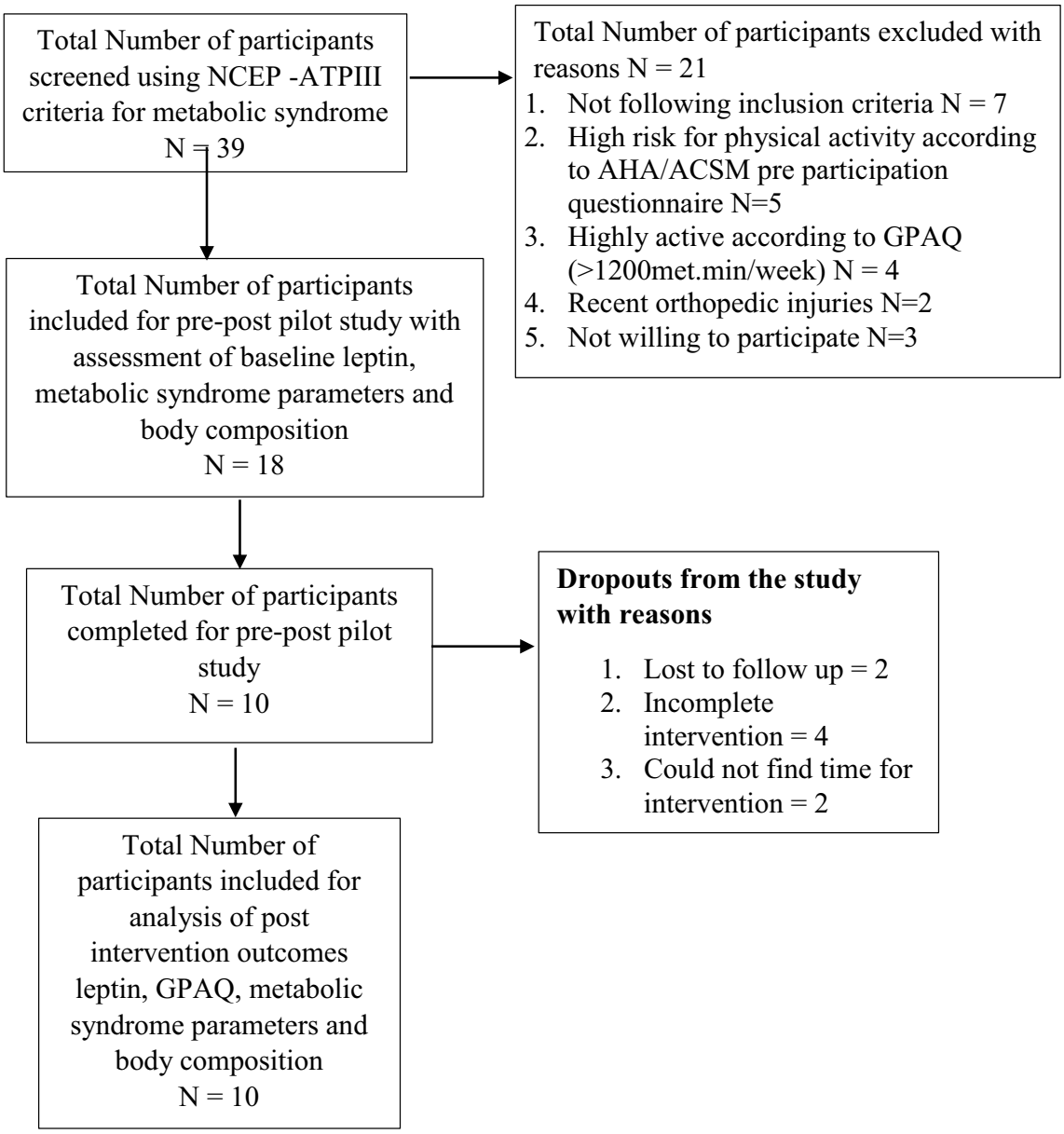

Fig. 1 Flow chart of the participant recruitment and analysis

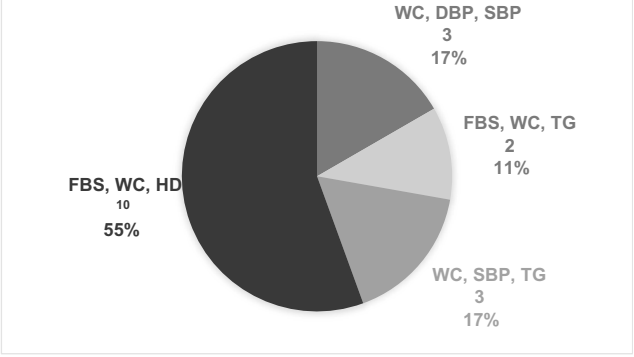

Fig. 2 Diagnostic criteria of metabolic syndrome according to NCEP-ATP III

Body composition and anthropometry

Body composition (BC) was analysed using Omron Karada Scan HBF 362 bio impedance analyser (BIA) (Omron Health Care, Inc, Illinois). Body composition included total body fat (TBF), subcutaneous fat (SCF), visceral fat (VF), musculoskeletal mass (MSM), and basal metabolic rate (BMR). For assessing BC using BIA, the participant was instructed to wear light clothing, 8-h fasting, avoiding of caffeine intake, alcohol intake and exercise 2 h prior to test, and no fluid intake 2 h prior to the test. The participant age, gender, and height are entered into the analyser, and the participant is made to stand on the BIA platform. TBF, SCF, VF, MSM, and BMR readings are recorded. WC is taken in centimetres with a non-

stretching tape at the level above iliac crest. Basal metabolic rate was calculated using Mifflin-St Jeor equation

$$\text{BMR (kcal/day)} = 10\text{*weight (kg)} + 6.25\text{*height (cm)} - 5\text{*age (y)} + \text{s (kcal/day)},$$

where s is +5 for males and −161 for females.

Biochemical measurements

Blood sample was taken after 8 h of fasting state to measure FBS, total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL), low-density lipoprotein (LDL), and leptin. FBS was analysed using hexokinase method, TC was analysed using CE-CHOD-POD method (Atlas Medicals, Cambridge), triglycerides were analysed using GPO trinder method (BQ kits Inc, San Francisco, CA), HDL was assessed using direct-homogenous assay, and LDL was calculated using the Friedewald formula. Leptin was analysed by enzyme-linked immunosorbent assay (ELISA) sandwich technique using commercially available kits DBC ELISA (Diagnostic Biochem Canada Inc) with specificity of 0.50 ng/ml, intra assay CV 3.7%, and interassay CV of 5.9 %.

Physical activity measurement

PA was measured using the World Health Organization global physical activity questionnaire (WHO-GPAQ). It contains 4 domains: work, transport, recreational, and sedentary time. GPAQ demonstrated good-to-very good test-retest reliability with time intervals that ranged from 3 days to 2 weeks [21]. GPAQ is used to assess the PA for a week and is expressed in met.min/week. The individual is said to be sedentary if the GPAQ score is less than 600 met.min/week.

PA promotion program

After assessing the PA using GPAQ, the participants were given a 12-week PA program which consisted of 150 min/week moderate to vigorous PA. Before giving the PA, the participants were asked about the daily routine and explained how they can modify their work routine into PA by monitoring themselves using commercially available fitness trackers which measure the step count. The participants using these fitness trackers were instructed to maintain 10,000 steps/day. People who did not have access to fitness trackers were given PA program according to the WHO compendium of physical activities which contain list of activities with specific MET values. To maintain adherence, the participants were advised to do PA in their convenient time without disturbing their daily routine but were instructed to maintain 150 min moderate to vigorous PA per week. The PA included various activities like brisk walking, gardening, house chores, workplace physical activity, and leisure time physical activities like group activities in a park which included yoga, general drills, and light jogging. To monitor PA, the participants were called by telephone every week to enquire about the activities they are doing. The progression of PA was measured every third week using GPAQ. The target was to achieve 150 min of physical activity per week. All the participants had less than 60 min/week of physical activity at the start of the study. The target was to increase the physical activity in a steady phase until they reach the required 150 min/week target. After 12 weeks, the participants are re-assessed for the outcomes like leptin, GPAQ, BP, TC, TG, HDL, LDL, BMR, SCF, VF, MSM, and WC.

Statistical analysis

Descriptive statistics were presented as mean and standard deviation. Shapiro-Wilk test was performed for normal distribution. Paired *t* test was performed to analyse the difference pre- and post-intervention. *p* value was set at $p<0.05$ at 95% CI.

Results

Participants (N=18) aged 30–50 years of either gender who were diagnosed with metabolic syndrome under NCEP ATP

Table 1 Baseline characteristics of the participants

Baseline characteristics of the participants $N = 18$	
Variable	Mean ± SD
Age (years)	47.2±4.86
GPAQ (met.min/wk)	231.7±138.18
LEPTIN (ng/ml)	25.9±10.69
Metabolic syndrome parameters	Mean ± SD
WC (cm)	101.8±8.5
SBP (mmhg)	134.2±3.96
DBP (mmhg)	88.2±4.62
FBS (mg/dl)	152.5±42.44
HDL (mg/dl)	43.3±10.14
LDL (mg/dl)	129.1±32.7
TG (mg/dl)	177.1±34.81
BMI (kg/m^2)	28.3±4.57
PPBS (mg/dl)	188.8±44.39
Body composition	Mean ± SD
TC (mg/dl)	214.1±29.24
SCF (%)	28.9±3.7
VF(%)	11.2±1.6
MSM(%)	23.4±5.8
BMR (Kcal)	2025.54±138.6
TF (%)	31.3±8.5

III criteria were recruited for the study. Ten participants who completed the study were included in the analysis. The main parameters of the MetS are presented in Table 1 which included all 18 participants. All the participants had increased waist circumference, dyslipidemia, hyperglycemia, and elevated blood pressure according to NCEP ATP III for metabolic syndrome (Fig. 3) . Table 2 describes the changes in all outcomes with a 12-week physical activity of the 10 participants who completed the study. Two participants who were enrolled into the study were not available for the post-intervention analysis. One participant was available but followed intervention less than 6 weeks so could not be analysed; 3 participants had adherence issues due to their work-related stress and stopped intervention abruptly. Two participants who did not find time for the intervention described of unable to balance time due to change of their job. The results showed that the mean physical activity levels of the participants were 330.4±85.6 met.min/week which is less than the recommended levels of >600met.min/week according to GPAQ. This clearly had shown the elevation of MetS parameters showing the importance of physical activity. All the participants were diagnosed as leptin resistant with elevated leptin levels (25.5±9.3 ng/ml) which correlated with the elevation of BMI (27.5±2.36 kg/m^2). We also observed that all the participants had elevated BMR levels (1954.35±142.5 kcal) suggesting decreased lean mass and increased total fat mass (30.7±6.8). The 12-week physical activity program brought changes in some parameters of MetS. The participants were motivated and explained the benefits of physical activity which brought a significant change in physical activity levels (p = 0.0254). As the physical activity levels increased, WC changed significantly (p=0.047). PPBS has shown significant change (p=0.0396), whereas FBS did not show significant change (p=0.254). No significant changes were seen in lipid profiles (TC (p=0.062), HDL (p=0.367), LDL (p=0.641)). But triglycerides have shown significant change in 12 weeks (p=0.0323). Body composition assessment has shown significant changes in BMI (p=0.0056), SCF (p=0.0354), and BMR (p=0.0035). Leptin levels did not show significant change (p=0.328) despite changes in BMI and SF. Out of 10 participants who were analysed, 1participant had improvement in three criteria of metabolic syndrome (WC, FBS, and TG), 2 participants had improvements in 2 criteria of Mets (FBS and WC), 1 participant showed improvement in parameters (WC, TG), 2 participants showed improvement in SBP and DBP, and 4 participants showed changes in BMI (Fig. 2).

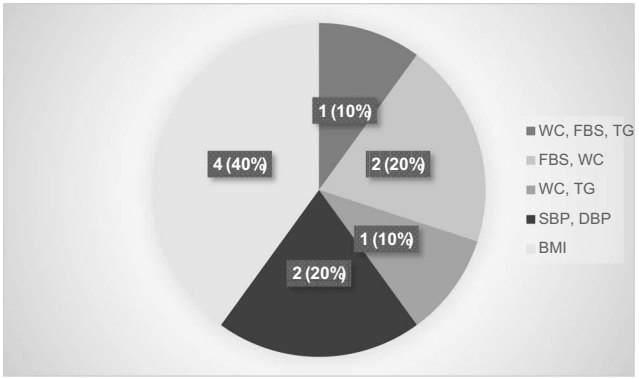

Fig. 3 Changes in individual parameters of metabolic syndrome according to NCEP-ATP III

Table 2 Mean change in leptin, body composition, and metabolic syndrome parameters of the participants who completed the study

Variable	Baseline (N=10)	12 weeks (N=10)	p value
Leptin (ng/ml)	25.5±9.3	23.7±11.5	0.328
GPAQ (met.min/wk)	330.4±85.6	420.5±122.4	0.0254*
Metabolic syndrome parameters			
WC (cm)	98.6±5.4	96.4±2.8	0.047*
SBP (mmhg)	132.5±4.25	131.5±3.68	0.549
DBP (mmhg)	86.2±5.83	86.8±2.95	0.269
FBS (mg/dl)	148.5±39.65	147.7±38.63	0.254
PPBS (mg/dl)	185±41.36	183.54±36.52	0.0396*
Lipid profile			
TC (mg/dl)	213.4±29.24	212.2±25.63	0.062
HDL (mg/dl)	45.3±9.6	43.9±3.8	0.367
LDL (mg/dl)	132.4±27.5	130.7±26.8	0.641
TG (mg/dl)	180.6±26.3	176.3±26.7	0.0323*
Body composition			
BMI (kg/m^2)	27.5±2.36	25.3±2.89	0.0056*
SCF (%)	29.3±5.6	26.5±2.92	0.0354*
VF (%)	10.8±0.8	9.7±1.6	0.296
MSM (%)	23.5±4.6	22.8±4.9	0.074
BMR (Kcal)	1954.35±142.5	2026.4±142.9	0.0035*
TF (%)	30.7±6.8	28.9±5.6	0.086

*p value < 0.05

Discussion

The present study aimed to find the effect of a 12-week physical activity promotion on leptin resistance in people with metabolic syndrome. This preliminary study results showed changes in some parameters of metabolic syndrome, body composition, and changes in PA behavior. Out of 18 participants, 10 participants completed the study (Fig. 1). The reason could be that unlike exercise, PA is more generalized and does not follow a structured protocol, and we observed that participants were not aware of the difference between an exercise protocol and physical activity. Moreover, awareness and self-motivation play an important role in maintaining the adherence to program. Previous studies were in line with our findings about the adherence to physical activity [26]. In our study, the dropped-out participants reported that they did not have time for exercise mostly due to their work schedule and type of work. Participants who enrolled into the study were

clearly explained the importance of physical activity to maintain optimum health and bring changes in metabolic syndrome parameters and lead a healthy lifestyle. Participants were encouraged to use technology like smart wearables and smart phone apps which monitor and provide motivation to increase physical activity and continue the new active lifestyle. There is a strong evidence on benefits of technology in improving physical activity [27–31]. Overall, the 12-week PA promotion program has brought changes in most metabolic syndrome parameters. Leptin levels did not change significantly despite significant decrease in BMI levels. Previous studies done by Xenachis C et al. [32] found that a 7% decrease in body weight was associated with a 22% reduction in serum leptin; Bastard et al. [33] found that decrease in leptin with a decrease of 2.1 kg/m^2 in BMI was followed by a 45% reduction in leptin levels. The discrepancy in leptin levels in our study may be due to sample size being too low to determine the effect of this intervention in decreasing leptin resistance. Leptin levels remained stable despite decrease in SCF. The participants were mostly females, and they have comparatively higher fat content than male population. This could be the reason of unchanged leptin levels. The changes in BMR can be attributed to change in SF and non-significant increase in MSM. Because BMR is the marker of lean body mass, increase in MSM and decrease in SF could decrease in BMR suggesting healthy weight loss. We have observed a statistically insignificant change in VF. The reason could be the unique characteristics of VF like increased blood flow and more response to norepinephrine. Studies have shown that increase in VF could lead to increased BMR [34]. In our study, MSM and VF have shown insignificant changes supporting the statement of higher BMR values. Further assessment is needed to assess the difference in BMR per unit decrease in VF and per unit increase in MSM. SBP and DBP did not show a significant change suggesting that the intervention could not bring out much change in sympathetic system which regulates the blood pressure. The results for SBP and DBP were inconsistent with other studies [35–37] probably due to small sample size. Although there was insignificant change in TC, TG had shown a significant change post-intervention suggesting that a moderate amount of physical activity for longer duration could help in fat metabolism especially TG which is source of energy for long duration steady-state activity. E.G. Oh et al. [38] found a decrease in TG with a 3-month total lifestyle modification which supported our results concerning decrease in TG. In the present study, based on the WHO-GPAQ questionnaire, all the participants were sedentary with the MET value <600met.min/week. Our main purpose of the study was to sensitize people about the physical activity and to include physical activity in their day-to-day life. Based on our results, we observed that all participants' physical activity has been improved. However, as a part of ongoing research, we had followed them telephonically regarding the continuation physical activity promotion, but the data was not reported in the present study.

The strengths of the study were that the 12-week physical activity promotion program could bring changes in metabolic syndrome parameters, decrease sedentary time, and improve physical activity. Waist circumference showed a significant change with the intervention which suggests that healthy lifestyle with physical activity as a daily routine could bring out weight loss and help in improving optimum health.

The limitations of the study were small sample size and disparity between gender which could have interfered with the results. As this is a longitudinal study and no control group was involved, there was no comparison for the results. Another limitation of the study was high attrition rate showing poor adherence to the program which needs to be addressed in the future studies. Future studies should investigate a randomized controlled trial with appropriate control group and address the above issues for more clear results and the effect of intervention.

Future studies should also investigate association between leptin resistance and BMR, total fat, and effect of the PA intervention on these variables. However, our study was able to show a considerable change in the metabolic syndrome parameters, some components of body composition, and anthropometry.

Conclusion

From the present study, we have concluded that a 12-week physical activity promotion program brought marginal changes in the leptin levels and has a potential to modify the metabolic syndrome parameters and changes in body composition and improve physical activity behavior.

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Centre for Diabetic Foot Care and Research.

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Declarations

Conflict of interest The authors declare no competing interests.

Informed consent Informed consent has been obtained from all the participants prior to the inclusion into the study.

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ORIGINAL ARTICLE

Predicted HbA1c and fructosaminated HbA1c: evaluating their role as an indicator of glycemic status in diabetes mellitus: a hospital based cross-sectional study

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Abstract

Background Glycated hemoglobin has been a popular investigation to access the glycemic status. It is a marker for intracellular glycation, and hence, an investigation which can comply with both intracellular and extracellular glycation statuses is preferred.
Methodology In this regard, fructosaminated HbA1c ($FHbA_{1c}$), predicted HbA1c ($PHbA_{1c}$) and glycation gap (GG) were evaluated in 57 cases of diabetes mellitus (DM) without complication and having a normal serum protein and albumin levels. Fifty controls were also evaluated. $FHbA_{1c}$, $PHbA_{1c}$ and post-prandial predicted HbA1c ($PPHbA_{1c}$) were plotted using population linear regression equation and calculated individually against each subject. The statistical calculation was evaluated using SPSS version 21.
Results $FHbA_{1c}$, $PHbA_{1c}$, $PPHbA_{1c}$ and GG were found to be significantly elevated in cases (p value < 0.001). $FHbA_{1c}$ and GG show significant correlation with the glycemic indices in cases compared to controls. The correlation with FBS and PPBS increases as we move from controls to cases. Area under curve (AUC) in case of $FHbA_{1c}$ is 96.8%, and cut-off level of 5.85% can result in sensitivity of 88.2% and specificity of 90% for the diagnosis of DM.
Conclusion $FHbA_{1c}$ can be used as an additional investigation in cases of DM. Along with GG, it is increased in DM and correlates with the glycemic indices. It has a decent AUC on plotting ROC. Thus, $FHbA_{1c}$ and GG can be used as a part of glyco-metabolic profile and can provide insight into the individualised glycation tendency of the cell.

Keywords Diabetes mellitus · Fructosamine · HbA_{1c} · Fructosaminated HbA_{1c} · Glycation gap

Introduction

Diabetes mellitus (DM) continues to grapple India and the world. The global prevalence of diabetes amongst adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014 [1]. The fatality of this disease lies in its microvascular and macrovascular complications which can effect up to 46% of diabetics.

Key messages Fructosaminated HbA_{1c} and glycation gap can be used as a part of glyco-metabolic profile in diabetes mellitus.

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Although glycated hemoglobin (HbA1c) is the most popular indicator of glycemia, but it has its own discrepancies and variations. Around 1/3rd of HbA1c variance is not explained by mean blood glucose and the terms such as "high glycators" and "low glycators" tell the story of incompleteness of HbA1c as an indicator of glycemia[2, 3]. HbA1c represents Hemoglobin glycation capacity only and hence an indicator of intracellular protein glycation. The rate of glycation of extracellular protein, albumin, the most dominant extracellular protein, is said to be around 9–10 times higher than hemoglobin and hence a dominant factor of glycemic status [4]. The glycation of proteins is responsible for the formation of advanced glycation end products (AGEs) and, in turn, paves the way for the development of various complications in DM [5]. In addition, due to the long lifespan of RBCs (2–3 months), the response to changes in treatment is not visible early.

The two parameters proposed to counter the aforementioned queries to a satisfactory extent are predicted HbA1c (PHbA1c) and fructosaminated HbA1c (FHbA1c). PHbA1c is the value of

HbA1c calculated from its regression with fasting blood glucose. FHbA1c is predicted from fructosamine (an index of extracellular protein glycation) based on the HbA1c–fructosamine regression equation [2, 6, 7]. It is the value of HbA1c standardised with reference to extracellular protein glycation. Glycation gap (GG) can be calculated from HbA1c. In this study, an attempt will be made to evaluate PHbA1c and FHbA1c as a marker of glycemic status.

Aims and objectives

(1) To evaluate PHbA1c and FHbA1c in the cases and controls.
(2) To correlate the values of PHbA1c and FHbA1c with the average fasting blood glucose(AFBG) and average post-prandial blood glucose (APPBG) levels and assess whether the parameters can be used as a reliable indicator of glycemic status
(3) To calculate GG and see whether it is correlated with the glycemic indices
(4) To propose a diagnostic cut-off level of FHbA1c for DM

Material and methods

Study design

The present cross-sectional study was carried out in the Department of Biochemistry, All India Institute of Medical Sciences (AIIMS), Mangalagiri, Andhra Pradesh, from 30 August 2019 to 23 October 2019 as a part of the Indian Council of Medical Research (ICMR) Project. Fifty cases of DM, diagnosed as per guidelines laid down by the American Diabetic Association (ADA), were selected such as that their blood glucose levels do not show more than 20% variation assessed 30 days apart. Only the average of the 2 fasting blood glucose (FBG) levels was taken. Similar criteria were followed for average 2-h post-prandial blood glucose (APPBG) in our study. Serum fructosamine was evaluated with the same blood sample used for estimation of HbA1c. To avoid any influence on fructosamine measurement, only cases with total protein and serum albumin levels in the standard reference range only were included [8]. A detailed history and physical examination were done in all the participants enrolled. Fifty age and sex matched controls were also taken for the study. The samples were collected after proper informed consent.

Exclusion criteria

Individuals with liver diseases, proteinuria, hemoglobinopathy or erythrocyte disorder were excluded from the study. None should have suffered from an acute disease or had blood transfusion recently or have recently modified lifestyle or treatment.

Ethical approval

The present study was approved by the Institutional Ethics Committee, AIIMS Mangalagiri, with reference no. AIIMS/MG/IEC/2019-20/03 dated 28 August 2019. Before being enrolled for the study, informed consent was obtained from the patients and controls to use their clinical data for research. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Biochemical analysis

The sample was collected in the sample collection room, OPD building, AIIMS, Mangalagiri, under aseptic conditions after obtaining informed consent from the cases and controls. Vacutainers were obtained from BD Bioscience. Biochemical parameters were measured using Biosystems A25 automated clinical chemistry analyser, Barcelona, Spain, using assay kits from Biolabo, Germany, and Biosystems, Spain. Haematological parameters were evaluated on LH-750 Beckman Coulter haematology analyser (Beckman Coulter, USA). Plasma glucose was analysed by glucose oxidase-peroxidase method [9]. Fructosamine was measured by the nitroblue tetrazolium (NBT) photometric procedure based on the reducing ability of fructosamine in alkaline solution resulting in the synthesis of formazan [10]. HbA1c was determined in HLC 723 GX (automated glycohemoglobin analyser, Tosoh) by high performance liquid chromatography (HPLC) which is certified by National Glyco-Hemoglobin Standardisation Programme (NGSP). Serum protein and albumin were estimated by biuret method [11, 12] and bromocresol green method [13], respectively. Serum creatinine was evaluated by Jaffe's test using Biolabo kits [14]. Westgard's rules were followed for monitoring the internal quality control on the analyser. Proteinuria is detected by urine dipstick test by using Siemen's Uristix protein and glucose. During the course of the study, there was no change in the equipment, reagents, calibration standards and controls.

Statistical analysis

Statistical analysis of the data was performed using SPSS statistical software version 21. Continuous variables were expressed as means ± SD values and compared by Student's *t* test for the parametric data. A regression equation of HbA1c, termed fructosaminated HbA1c (FHbA1c), was plotted on fructosamine derived from the data of study group involved in the study. FHbA1c was then calculated for each by using the regression equation: FHbA1c = 0.01 ×

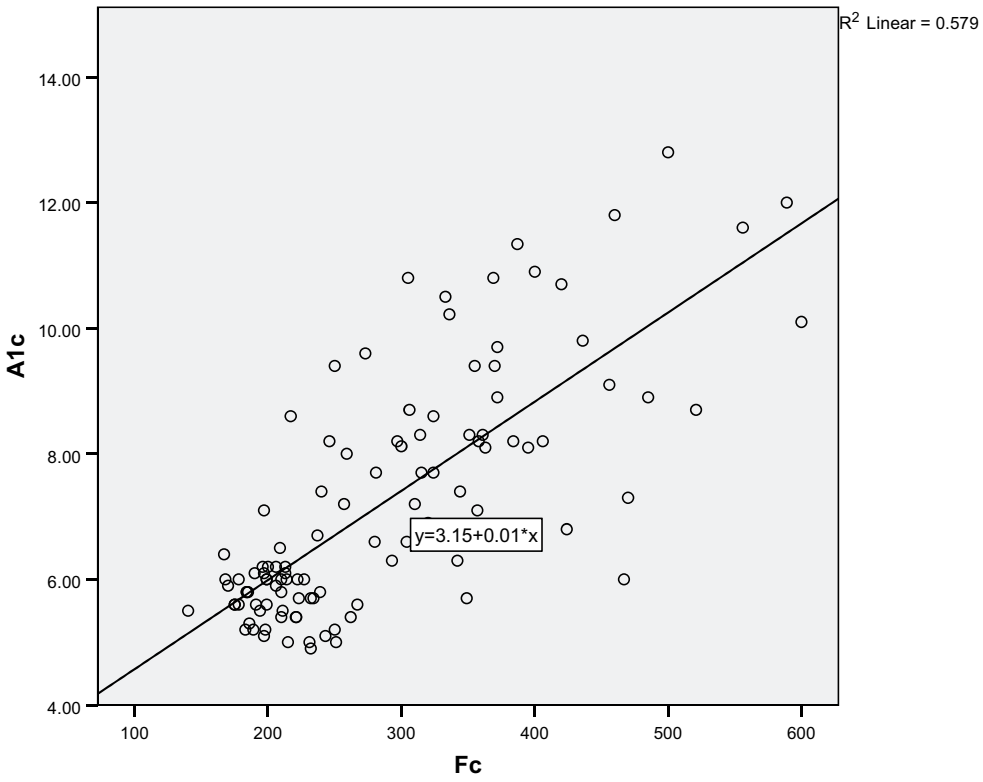

Fig. 1 Relationship between HbA1c (A1c) in percent glycation of hemoglobin and fructosamine (Fc) in μmol/l

fructosamine + 3.15. The glycation gap (GG) for each subject was calculated by subtracting FHbA1c from measured HbA1c [15]. Predicted fasting HbA1c (PHbA1c) and predicted post-prandial HbA1c (PPHbA1c) were also calculated similarly as FHbA1c by using the corresponding fasting blood glucose and post-prandial blood glucose values, respectively. The association of FHbA1c, GG and PHbA1c with the glycemic indices was analysed between

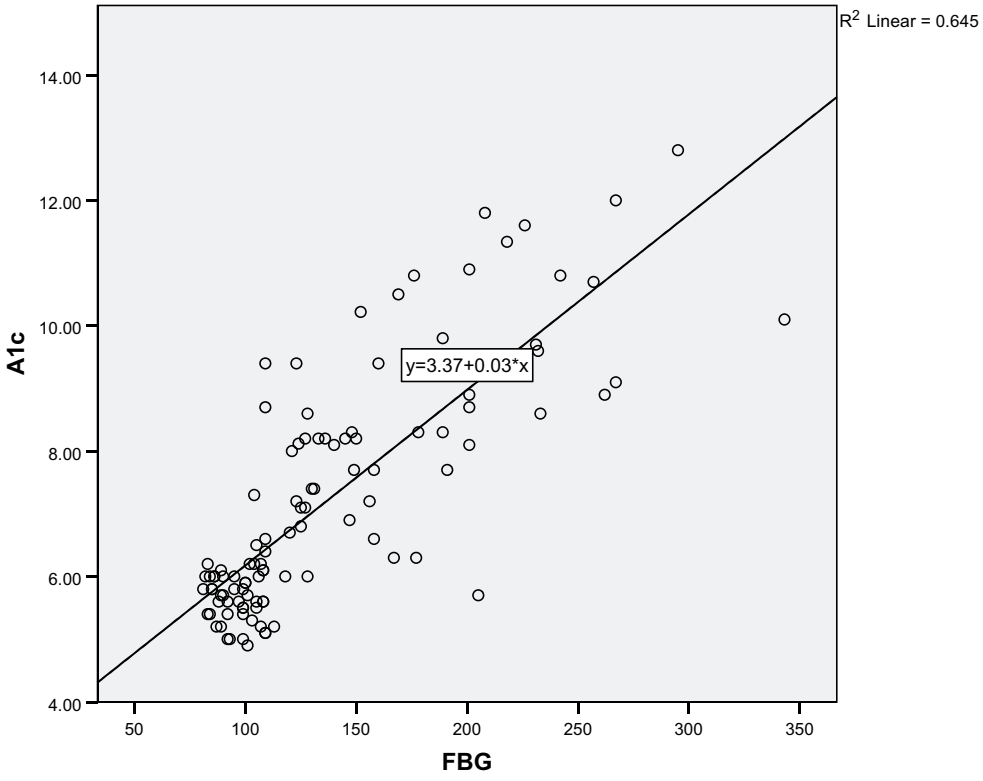

Fig. 2 Relationship between HbA_{1c} (A_{1c}) in percent glycation of hemoglobin and fasting blood glucose (FBG) in mg/dl

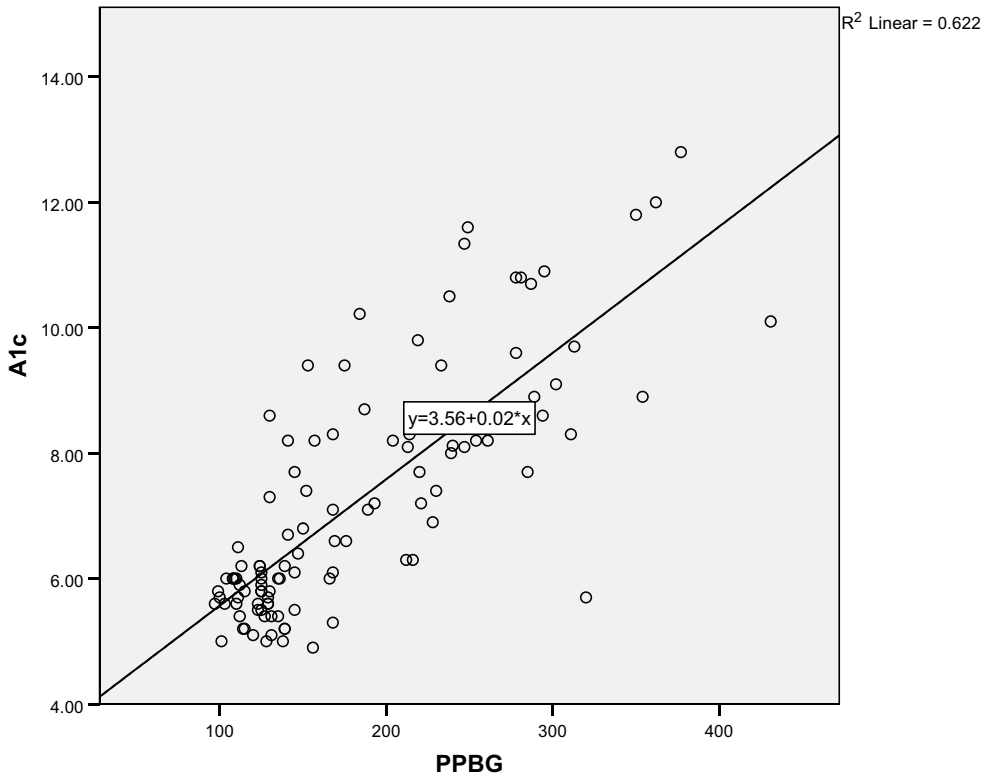

Fig. 3 Relationship between HbA_{1c} (A_{1c}) in percent glycation of hemoglobin and post-prandial blood glucose (PPBG) in mg/dl

the study groups by using Pearson's correlation analysis. A p value < 0.05 was considered statistically significant. Receiver operating curve (ROC) of the parameters was plotted to assess the diagnostic significance in terms of area under curve (AUC) and to present a cut-off level with maximum sensitivity and specificity.

Observations and results

The study consists of 100 subjects (including cases and controls); 39.25% were males, and 60.74% were females. The mean age in the control group was found to be 41.70 ± 8.45 years whereas the same for the case group was 45.25 ± 10.55 years.

Table 1 Glycemic indices and GG in controls and cases

Parameter	Controls	Cases	p value
FBG (in mg/dl)	96.86 ± 9.60	172.39 ± 53.86	< 0.001#
PPBG (in mg/dl)	124.58 ± 16.33	231.81 ± 70.11	< 0.001#
HbA_{1c} (in % of total Hb)	5.67 ± 0.39	8.58 ± 1.67	< 0.001#
Fructosamine (in μmol/l)	205.70 ± 26.21	356.95 ± 91.77	< 0.001#
$FHbA_{1c}$ (in % of total Hb)	5.21 ± 0.26	6.72 ± 0.92	< 0.001#
$PHbA_{1c}$ (in % of total Hb)	6.28 ± 0.29	8.54 ± 1.62	< 0.001#
$PPHbA_{1c}$ (in % of total Hb)	6.05 ± 0.33	8.20 ± 1.40	< 0.001#
GG	0.46 ± 0.53	1.86 ± 1.45	< 0.001#

As depicted in Figs. 1, 2 and 3, the scatter plot of HbA_{1c} (in % of total hemoglobin) with fructosamine (in μmol/l), FBG (in mg/dl) and PPBG (in mg/dl) is plotted. The corresponding HbA_{1c} as predicted is given by the following equation as derived:

- HbA_{1c} as predicted from Fructosamine ($FHbA_{1c}$) = 0.01 × fructosamine + 3.15
- HbA_{1c} as predicted from FBG ($PHbA_{1c}$) = 0.03 × FBG + 3.37
- HbA_{1c} as predicted from PPBG ($PPHbA_{1c}$) = 0.02 × PPBG + 3.56

#The mean difference is significant at the level of 0.001 amongst controls and cases

Table 2 Correlation analysis of $FHbA_{1c}$ and GG with the glycemic indices in the study groups

		HbA1c	FBG	PPBG
$FHbA_{1c}$	Cases	0.496**	0.529**	0.551**
	Controls	−0.301*	−0.258	−0.050
GG	Cases	0.835**	0.372**	0.300*
	Controls	0.881**	0.096	−0.035
PHbA1c	Cases	0.615**	1.000**	0.857**
	Controls	−0.043	1.000**	0.534**

*Correlation is significant at 0.05 level

**Correlation is significant at 0.01 level

Table 3 Area under the curve

Variable(s)	Area	Std. error	Significance level	95% confidence interval	
				Lower bound	Upper bound
$FHbA_{1c}$	0.968	0.016	0.000	0.937	0.998
A_{1c}	0.986	0.010	0.000	0.967	1.000
$PHbA_{1c}$	0.987	0.008	0.000	0.971	1.000
$PPHbA_{1c}$	0.964	0.017	0.000	0.931	0.998

$FHbA_{1c}$ as highlighted in Table 1 was found to be significantly elevated (p value < 0.001) in cases (6.72 ± 0.92) in comparison to controls (5.21 ± 0.26). Similarly, $PHbA_{1c}$ in cases (8.54 ± 1.62) and $PPHbA_{1c}$ in cases (8.20 ± 1.40) were found to be significantly elevated when compared to their controls. GG was found to be significantly elevated (p value < 0.001) in cases (1.86 ± 1.45).

As shown in Table 2, FHbA1c is found to be significantly correlated with the commonly used glycemic indices (HbA_{1c}, FBG, PPBG) in cases but not so in controls. The correlation with PPBG (Pearson's correlation coefficient, r = 0.773) is more as compared to FBG (r = 0.751). Also, GG shows a significant correlation with the glycemic indices. It is more significantly correlated with FBG (r = 0.584) as compared to the rest. The correlation of $PHbA_{1c}$ and $PPHbA_{1c}$ is the same as FBG and PPBG.

As evident from Table 3 and Fig. 4, $FHbA_{1c}$ corresponds to an area of 96.8% under the curve (AUC).

Discussion

Owing to the differences in the extent of glycemia as evaluated by HbA_{1c} and other glycemic indices, the hunt for other glycemic indicators stands unabated. HbA_{1c} is controlled by a lot of factors including heredity [16]. $FHbA_{1c}$ takes into account both the intracellular and extracellular glycation components and hence has been evaluated as a marker of glycemia in this study. GG as predicted can signify the difference in the susceptibility of intracellular vs extracellular glycation and hence can perhaps answer the individualised susceptibility to various complications in diabetes mellitus [17]. This is also

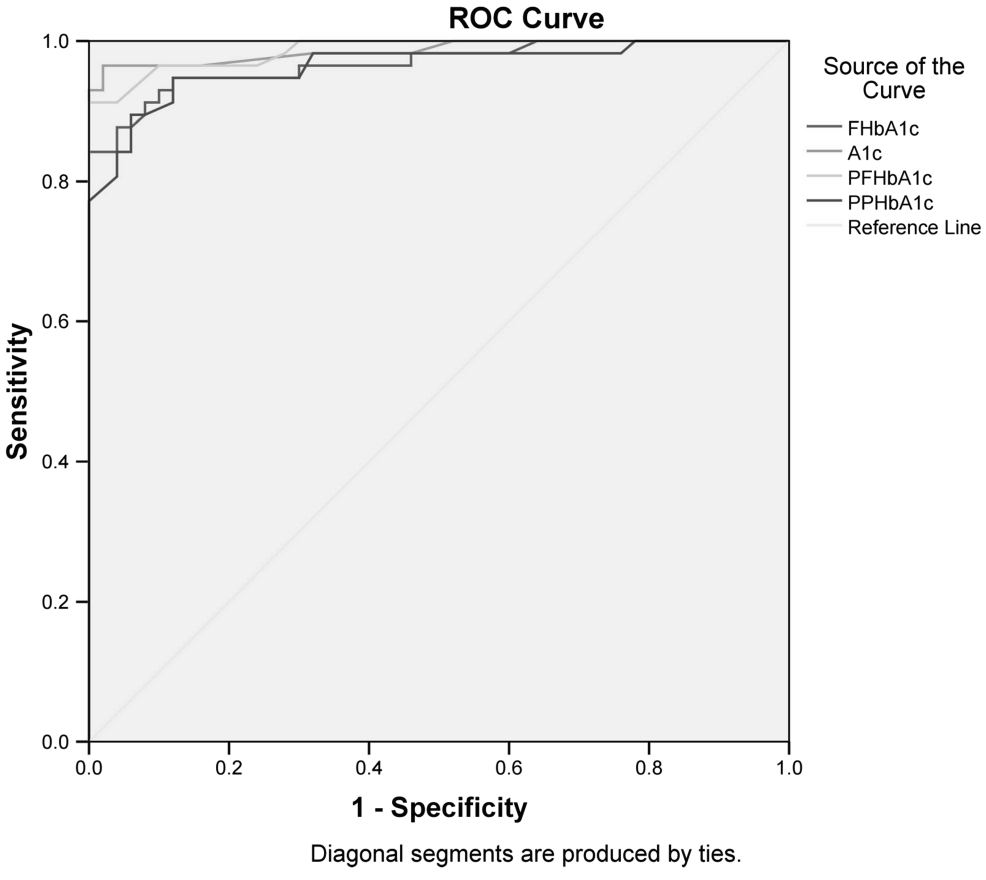

Fig. 4 ROC curve of the glycemic indices

perhaps one of the few studies to propose a cut-off level for $FHbA_{1c}$ in India to ensure maximum sensitivity and specificity. In this study, an attempt has been made to calculate PHbA1c and $PPHbA_{1c}$ on the basis of local population data by using population linear regression and see if they can provide additional information.

Although many studies have been conducted to evaluate the effectiveness of fructosamine as an indicator of glycemia, not much studies exist where HbA_{1c} derived from population regression equation of fructosamine has been evaluated as an indicator of glycemic status. Malmstorm et al. in their study on 10987 healthy cohorts found a strong correlation of fructosamine with HbA_{1c} ($r = 0.67–0.75$) when measured on 3 separate occasions within a span of 290 days. A fructosamine level of 2.5 mmol/l exhibited an area under curve of 0.91–0.95 [18]. Neelofar et al. in their study highlighted that patients of diabetes with complications exhibited a higher glycation gap [19]. But the calculation of $FHbA_{1c}$ was based on a standard equation [20], and serum protein or albumin was not taken into account during the study which might be a source of error in the interpretation. They found that fructosamine correlated with HbA_{1c} significantly only in diabetics, and the correlation increases with increase in complications in DM. Also $PHbA_{1c}$ was calculated on the basis of mean blood glucose (MBG). GG was calculated in the study, and it showed a significant association with the diabetic nephropathy.

As shown in Table 1, the mean age of the controls in the study was 41.70 ± 8.45 years, and the same in the case group was found to be 45.25 ± 10.55 years. The age group conforms to the present trend of patients attending the Outpatient Department (OPD) in AIIMS, Mangalagiri. Cosson et al. observed a mean age of 57.8 ± 9.3 years in their study [5]. This was because the patients were enrolled with complications, and hence, longer period of disease exposure was favoured. Increased enrolment of females in this study might be because of the reason that most of them are housewives and can attend the OPD in office hours.

The population regression curve of fructosamine over HbA_{1c} is plotted by using the local population data. In our study, R_2 (coefficient of determination) was found to be 0.579. It indicates what amount of variation in the value of HbA1c can be attributed to fructosamine. Cosson et al. [5] found the corresponding value to be of 0.534. Similarly, the $PHbA_{1c}$ and $PPHbA_{1c}$ values have been evaluated and the mean is presented in Table 3. A mean fructosamine value of 356.95 ± 91.77 μmol/l is calculated in diabetics as per our study. Zafon et al. in their study on 508 diabetic patients obtained a value of mean fructosamine value of 283 μmol/l [21]. The inclusion of metformin treated cases might account for the difference of fructosamine as noted between the two studies as metformin is said to increase the transport of glucose inside the cells and hence decrease in extracellular concentration of glucose.

The mean value of $FHbA_{1c}$ in diabetics without complications as evaluated in our study (6.72 ± 0.92) is in sync with the observations made by Neelofar et al. [19] (6.7 ± 0.63) in their study. But their study did not take into account the serum proteins and albumin level which might account for some bias in the study. To avoid such bias, the cases and controls included in our study had serum protein and albumin levels in the standard reference range only. As evident in Table 2, $FHbA_{1c}$ in diabetics shows significant correlation with other glycemic indices mainly HbA_{1c}, FBG and PPBG. In controls, $FHbA_{1c}$ shows a significant negative correlation with HbA_{1c}. Similar finding was observed by Dziedzic et al. [15].

HbA_{1c} represents the non-enzymatic glycation of hemoglobin within the RBCs, that is, it is a marker of intracellular glycation of proteins, whereas fructosamine is a measure of extracellular glycation of proteins. Therefore, in controls, as the glycation of proteins of intracellular compartment increases, it indicates a shift of glucose to the intracellular compartment due to highly efficient transporters, and hence, the extracellular glycation decreases. But as the concentration of sugars increase, both the compartments show a significant gain of glucose, and both the parameters increase linearly.

GG represents a non-glycemic determinant of HbA_{1c}. It can be considered a marker of intracellular susceptibility for glycation [22] and represents the extent of deviation of HbA_{1c} from its expected value. As highlighted in Table 1, GG in cases is significantly higher than the controls (p value < 0.001). As highlighted in Table 2, GG is seen to be significantly correlated with all the major glycemic indices. Since it is the difference between measured HbA_{1c} and $FHbA_{1c}$, it is negatively correlated with $FHbA_{1c}$. But the value as well as correlation seems to move towards positive side as glycemia increases. Similar findings were observed by Nayak et al. [23]. The analysis of ROC curve for FHbA1c reveals that a significant AUC of 96.8% is present which makes it a good diagnostic tool as depicted in Table 3 and Fig. 4. A specific cut-off level can be selected to suit the requirement. At a cut-off value of 5.85%, $FHbA_{1c}$ has a sensitivity of 88.2% and 90% specificity.

Conclusion

$FHbA_{1c}$ can be used as an additional investigation in cases of DM. $FHbA_{1c}$ and GG are significantly increased in DM. It is highly correlated with the glycemic indices and has a decent AUC on plotting ROC. Thus, FHbA1c and GG can be used as a part of glyco-metabolic profile and can provide insight into the individualised glycation tendency of the cell.

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Declarations

Ethics approval The present study was approved by the Institutional Ethics Committee, AIIMS Mangalagiri, Andhra Pradesh, India, with reference no. AIIMS/MG/IEC/2019-20/03 dated 28-08-2019.

Consent to participate Taken

Consent for publication Taken

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

High prevalence of prolonged QTc interval among individuals in ambulatory diabetic care in southwestern Uganda

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Abstract

Background Heart rate-corrected QT (QTc) interval is associated with increased risk for cardiovascular events and mortality among individuals with diabetes mellitus (DM). Little is known about the epidemiology of prolonged QTc among people with DM in resource-limited settings.

Methods We conducted a cross-sectional study among adults with diabetes in ambulatory care at the Mbarara Regional Referral Hospital, from November 2018 to April 2019. Twelve-lead ECG recordings were performed on all participants. We collected clinical and laboratory data related to diabetes disease status and treatment control. We estimated QTc using Bazett's formula and categorized it according to standardized sex-adjusted thresholds. Linear regression analysis was performed to identify correlates of QTc.

Results We recruited 299 participants with a mean age of 50.1 years (SD±9.8) and mean HbA1c of 9.7 % (SD±2.6), and 69.6% were female. We detected prolonged and borderline QTc in 6.4% (19/299, 95% CI: 3.9–9.7%) and 23.4% (70/299, 95% CI: 18.7–28.6%) of participants, respectively. In multivariate models, factors associated with increasing QTc interval were mean arterial pressure (β=0.34; 95% CI: 0.07–0.63, p=0.019) and female sex (β=15.26; 95% CI: 7.58–22.94, p<0.001).

Conclusions The prevalence of abnormal QTc among individuals in routine diabetes care in southwestern Uganda was high. Female sex and mean arterial pressure were correlated with QTc interval. Given these findings, future studies should explore the clinical impact of abnormal QTc in this patient population.

Keywords QT interval · Prevalence · Diabetes · QTc dispersion · QTc prolongation · Uganda

Introduction

Morbidity and mortality of diabetes are rising rapidly in low-income countries [1]. In Africa particularly, approximately five percent of deaths are now attributable to diabetes among adults aged 20–60 years [1]. Cardiovascular disease (CVD) in diabetic individuals is often unrecognized, yet it is by far the leading cause of morbidity and mortality in these individuals [2].

A major risk factor for CVD events and mortality in people with diabetes are abnormalities of the cardiac rhythm, including abnormal QT interval. In diabetic individuals, prolonged heart rate-corrected QT (QTc) interval is associated with increased risk

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of stroke, ischemic heart disease, and all-cause mortality and cardiovascular mortality, including sudden death [3, 4]. Thus, QTc interval on the ECG can potentially be utilized to identify diabetic individuals at high risk for cardiovascular events [5–7].

Whereas the prevalence of long QTc interval among individuals with diabetes varies widely, with higher estimates reported in type 2 diabetes (15 to 67%) [8–10] compared to type 1 diabetes (8%) [11], there are scanty data on its prevalence and correlates in sub-Saharan Africa, where an estimated 25 million people with diabetes reside [1]. We sought to determine the prevalence of QTc interval abnormalities and associated factors among patients with diabetes attending ambulatory care at the Mbarara Regional Referral Hospital (MRRH) in southwestern Uganda, to fill this knowledge gap.

Methods

Study population and setting

Methods for the parent cohort study have already been published previously[12]. Briefly, we conducted a cross-sectional study among patients with diabetes attending the outpatient diabetes and endocrinology clinic of the Mbarara Regional Referral Hospital (MRRH) from November 2018 to April 2019. We included diabetic patients aged 18 to 65 years using consecutive sampling of eligible participants. We defined diabetes in participants with fasting capillary glucose of ≥7.0 mmol/L or those who were already on treatment for diabetes mellitus. We excluded individuals with acute febrile illnesses in the previous 48 h, those with electrolyte abnormalities, and those with other endocrine disorders (e.g., thyroid hormone disorders). We also excluded those who were actively taking drugs known to affect the cardiac rhythm or QTc interval including antiarrhythmic drugs (class I and class III), antihypertensive medications, antidepressants, or digitalis in the prior 24 h. Lastly, we excluded individuals with self-reported or documented history of cardiac (heart failure, ischemic heart disease), renal, or hepatic disease.

Study definitions and procedures

We collected socio-demographic and clinical characteristics, including data on alcohol use, smoking, level of physical activity, and diabetes treatment history of the study participants through a structured questionnaire. We measured weight and height to the nearest 0.1kg and 0.1 cm, respectively, with participants putting on light clothes and removing shoes. We calculated body mass index (BMI) as body weight in kilograms divided by the square of the body height in meters. We measured waist circumference at the level of umbilicus with inelastic tapeline (to the nearest 0.1 cm), at the end of normal expiration.

Blood was collected for glycosylated hemoglobin (HbA1c, Cobas Integra 400, Roche diagnostics, Basel, Switzerland). Fasting blood sugar measurements were obtained using a Freestyle Glucometer (Abbott Diabetes Care Inc., Maidenhead, UK) after at least 8 h of fasting. Blood pressure was recorded in a sitting position using an automatic sphygmomanometer in the upper arm (Omron HEM 705 LP, Omron Healthcare, Inc., Bannockburn, IL, USA). We calculated the pulse pressure as the difference between systolic and diastolic blood pressures in mmHg and computed the mean arterial pressure as the sum of diastolic blood pressure and one-third of pulse pressure. After pupillary dilation by an ophthalmologist, the presence of diabetic retinopathy was diagnosed with direct ophthalmoscopy and categorized as non-proliferative or proliferative diabetic retinopathy.

Measurement and classification of QTc interval

We performed 12 lead ECG recordings on all study participants using a portable ECG machine (Edan Instruments, Inc., Hessen, Germany). Participants were instructed to rest for 5 min in supine position before all the ECG recordings. We measured the QT interval from the beginning of the earliest onset of the QRS complex to the end of the T wave where it crosses the isoelectric line. In case a U wave was present, we measured the QT interval up to the bottom of the angle between the T and U waves. We considered QT as the mean of QT from five consecutive cycles in lead V5. We calculated the QTc interval according to Bazett's formula ($QTc=QT/RR^{1/2}$) for heart rates between 60 and 100 beats/minute and Fredericia's formula ($QTc=QT/RR^{1/3)}$ for heart rates less than 60 or greater than 100 beats/minute because Bazett's formula tends to over-correct at high heart rates and under-correct at low heart rates [13]. We further classified the QTc interval as normal (<430ms in males, <450ms in females), borderline (430–450ms in males, 450–470ms in females), or prolonged (>450ms in males, >470ms in females) to further adjust for sex, according to a criteria previously described [13]. Finally, we estimated QTc dispersion (QTd) as the difference between the maximum and minimum QTc intervals in V5. QTd of >80ms was considered an abnormally prolonged [9]. To ensure quality control, two independent observers (D.C.A and G.K), who were blinded to participants' data, measured the QT and RR intervals and an average measure taken. We estimated Pearson's correlation coefficient to estimate inter-reader agreement between the two observers.

Sample size and statistical analyses

For the parent study, we calculated a sample size of 296 participants to enable a 5% precision with a 95% confidence interval around an estimate of our primary outcome, cardiovascular autonomic neuropathy (CAN), with prevalence of 20%, after consideration of a 10% non-response rate [12].

Statistical analyses were performed using Stata version 13 (StataCorp, College Station, TX, USA).

For this analysis, our outcome of interest was QTc interval. We first categorized the QTc interval into three categories (normal, borderline, and prolonged), according to the following cut-offs: normal (<430ms in males, <450ms in females), borderline (430–450ms in males, 450–470ms in females), and prolonged (>450ms in males, >470ms in females), as per recognized criteria [13]. We determined the prevalence of QTc interval abnormalities as a proportion of participants meeting the definitions of the respective categories. We considered participants to have abnormally long QTc interval if they fell into the category of borderline or prolonged QTc interval. We then described the socio-demographic and clinical characteristics of study participants by the subgrouping of QTc interval and compared differences in socio-demographic and clinical characteristics of those with QTc interval>440ms and those with QTc interval≤440ms, using chi-square test for categorical variables, Student *t*-tests for continuous normally distributed variables (expressed as means with standard deviations), and Wilcoxon rank-sum test for non-normally distributed continuous variables (expressed as medians with inter-quartile ranges). We performed univariable and multivariable linear regression analyses to determine the factors associated with QTc interval, reporting beta regression coefficients with their 95% confidence intervals as our measures of association. Variables that were considered to have an association ($p<0.1$) in univariable analyses were entered in the multivariable models through stepwise backward method. The entry and removal probabilities for stepwise were 0.05 and 0.1, respectively. Variables in the multivariable model with *p*-values <0.05 were considered statistically significant.

Results

We analyzed the data of 299 participants from a total of 512 individuals screened for inclusion into the study. Of the 512 screened individuals, 213 met the exclusion criteria including five with thyroid disorders, 94 who had taken cardiotropic medications (calcium channel blockers, beta blockers) in the prior 24-h period, and 17 with other underlying medical conditions (cardiac, renal, or hepatic disease).

Table 1 Demographic and clinical characteristics of study participants by QTc interval category

	QTc interval >440ms	QTc interval ≤440ms	
Characteristic	*n* = 104	*n* = 195	*p* value
Age in years, mean (±SD)	51.3 (±9.4)	49.5 (±10.0)	0.136
Female sex, *n* (%)	87 (83.7)	121 (62.1)	<0.001
Height in meters, mean (±SD)	1.62 (0.07)	1.62 (0.09)	0.798
Weight in kg, mean (±SD)			
BMI in kg/m^2, mean (±SD)	27.9 (±6.1)	27.2 (±5.1)	0.300
Waist circumference in cm, mean (±SD)	99.0 (±14.0)	97.8 (±13.4)	0.460
Ever smoked, *n* (%)	26 (25.0)	43 (22.1)	0.564
Duration of diabetes in year, median (IQR)	4 (2.9)	4 (1.8)	0.237
Vigorous physical activity (≥600 METS/week) (%)	66 (63.5)	107 (54.9)	0.152
Fasting blood sugar in mmol/L, mean (±SD)	11.3 (±5.1)	11.1 (±4.7)	0.669
HbA1c (%), mean (±SD)	9.6 (±2.6)	9.8 (±2.6)	0.505
History of hypertension, *n* (%)	82 (78.9)	125 (64.1)	0.009
Resting systolic blood pressure in mmHg, mean (±SD)	146 (±23)	140 (±22)	0.030
Resting diastolic blood pressure in mmHg, mean (±SD)	89 (±10)	86 (±11)	0.023
Mean arterial pressure in mmHg, mean (±SD)	108 (±13)	104(±13)	0.014
Pulse pressure in mmHg, mean (±SD)	57 (±18)	54 (±18)	0.165
Presence of diabetic retinopathy, *n* (%)			0.790
None	81 (77.9)	150 (76.9)	
Non-proliferative	19 (18.3)	34 (17.4)	
Proliferative	4 (3.9)	11 (5.6)	
Symptoms of neuropathy in past 6 months *n* (%)			
Palpitations	58 (55.8)	91 (46.7)	0.134
Fainting	41 (39.4)	73 (37.4)	0.736
Numbness in feet	67 (64.4)	116 (59.5)	0.404
Use of antihypertensive drugs *n* (%)			0.246
None	3 (5.5)	14 (17.5)	
Diuretic	6 (10.9)	7 (8.8)	
Beta blocker	3 (5.5)	6 (7.5)	
Calcium channel blocker	44 (32.6)	21 (26.3)	
ACEI/ARB	17 (30.9)	25 (31.3)	

SD standard deviation, *IQR* inter-quartile range, *METS* metabolic equivalents, *ACEI* angiotensin-converting enzyme inhibitors, *ARB* angiotensin receptor blockers

Socio-demographic and clinical characteristics

Participants' characteristics are summarized in Table 1. Of the 299 participants analyzed, 69.6% were female; mean age was 50.1 years (SD±9.8), mean HbA1c was 9.7% (SD±6.7), and mean duration of diabetes was 5.8 (SD±5.9) years. We found a higher crude prevalence of QTc prolongation among women, those with higher resting systolic and diastolic blood pressures and those with higher pulse pressure and higher mean arterial pressure. Other characteristics were similar between the two groups.

Prevalence of QTc interval abnormalities

Pearson's correlation coefficient for the agreement between the two observers for the QTc intervals was 0.92. We detected abnormally long QTc interval in 89/299 participants (29.8%, 95% CI: 24.6–35.3%), with a prevalence of prolonged QTc of 6.4% (19/299, 95% CI: 3.9–9.7%) and a prevalence of borderline QTc of 23.4% (70/299, 95% CI: 18.7–28.6%). There was no significant difference in the distribution of different categories of QTc interval in males and females, as seen in Fig. 1. No participants had abnormally prolonged QTc dispersion (QTd).

Factors associated with QTc interval

Results of univariable and multivariable linear regression models for factors associated with QTc interval are presented in Table 2. In unadjusted analyses, female sex (p<0.001), resting systolic blood pressure (p=0.020), resting diastolic blood pressure (p=0.010), history of hypertension (p=0.007), mean arterial pressure (p<0.001), and history of taking calcium channel blockers (p=0.006) were significantly associated with increasing QTc interval.

In the final multivariate model (Table 2), after controlling for the duration of diabetes, age, and antihypertensive medications, significant factors associated with QTc interval were mean arterial pressure (β=0.34; 95% CI: 0.07–0.63, p=0.019) and female sex (β=15.26; 95% CI: 7.58–22.94, p<0.001). The predicted QTc interval for females was higher across all age groups (Fig. 2).

Discussion

We found a high prevalence of abnormally long QTc interval among individuals with diabetes in ambulatory care in southwestern Uganda and identified female sex and mean arterial pressure as key correlates of increased QTc interval. The prevalence of abnormally long QTc interval of 30% (95% CI: 24.6–35.3%) reported in our study is consistent with studies in Italy and China that have reported prevalence estimates ranging from 24 to 35% among diabetic individuals [9, 10]. In contrast, our study demonstrates much higher prevalence of abnormally long QTc than others from Nigeria and Iraq, which reported much lower estimates of 12% and 9%, respectively, among diabetic individuals [14, 15]. Our study also revealed a significantly higher prevalence (p<0.001) than the 11.7% prevalence recently reported in the general population in southwestern Uganda [16].

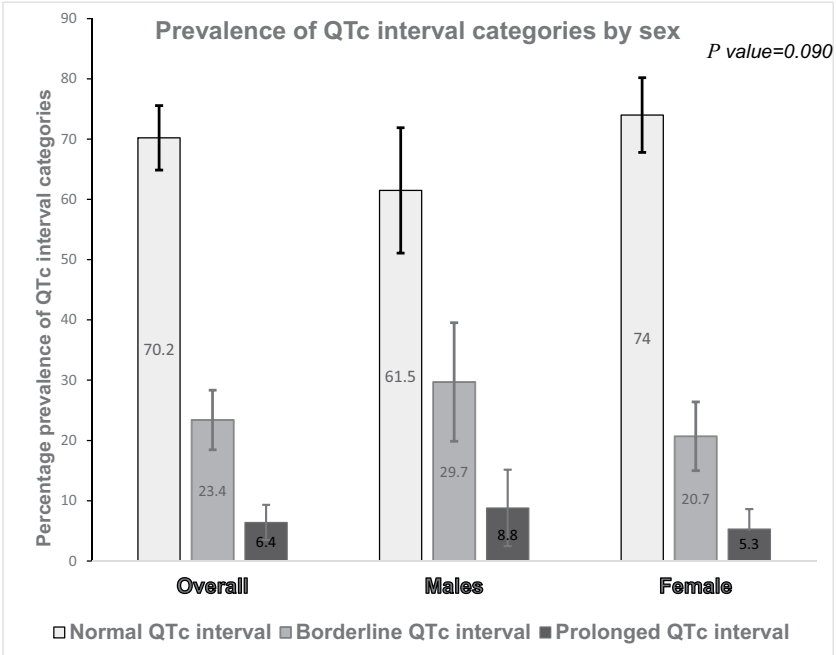

Fig. 1 Distribution of QTc interval abnormalities by sex among study participants

Table 2 Univariate and multivariate linear regression analyses for factors associated with QTc interval

Variable	Unadjusted linear regression analysis		Adjusted linear regression analysis	
	β coefficient (95% CI)	p value	β coefficient (95% CI)	p value
Age in years	0.25 (−0.001 to 0.51)	0.050	0.09 (−0.17 to 0.35)	0.491
Female sex	15.52 (10.29–20.61)	<0.001	15.26 (7.58–22.94)	<0.001
BMI in kg/m^2	0.31 (−0.14 to 0.76)	0.182		
Waist circumference in cm	0.09 (−0.09 to 027)	0.334		
Duration of diabetes in years	0.38 (−0.04 to 0.80)	0.074	0.18 (−0.75 to 0.39)	0.532
Fasting blood sugar in mmol/L	−0.06 (−0.57 to 0.46)	0.832		
HbA1c (%)	−0.07 (−1.04 to 0.89)	0.887		
History of hypertension	7.39 (2.02–12.76)	0.007		
Resting systolic blood pressure in mmHg	0.18 (0.07–0.29)	0.002		
Resting diastolic blood pressure in mmHg	0.41 (0.18–0.64)	0.001		
Mean arterial pressure in mmHg	0.34 (0.16–0.53)	<0.001	0.34 (0.07–0.63)	0.019
Pulse pressure in mmHg	0.14 (−0.01 to 0.28)	0.057		
Antihypertensive drugs				
None	Ref		Ref	
Diuretic	11.72 (−4.25 to 27.69)	0.149	10.07 (−4.97 to 25.11)	0.187
ACEI/ARB	11.77 (−0.69 to 24.23)	0.064	10.70 (−1.19 to 22.58)	0.077
Beta blocker	8.63 (−9.23 to 26.50)	0.341	4.55 (−13.09 to 22.19)	0.611
Calcium channel blocker	17.34 (4.97–29.72)	0.006	14.65 (−0.67 to 26.57)	0.054

CI confidence interval, *BMI* body mass index, *HbA1c* glycosylated hemoglobin, *ARB* angiotensin receptor blocker, *ACEI* angiotensin-converting enzyme inhibitor, *Ref* reference category

These results reinforce previous findings that suggest that individuals with diabetes have increased QTc intervals compared to non-diabetic individuals [9]. This phenomenon is hypothesized to be due to persistent hyperglycemia, which increases intracellular levels of calcium, causes production of free radicals, alters cardiac sympathovagal tone balance, and contributes to diminished nitric oxide production [17]. Low levels of nitric oxide in turn may cause impaired functioning of primary active transport mechanisms within cardiomyocytes, resulting in prolongation of myocardial repolarization [18]. Furthermore, hyperglycemia-mediated free reactive species have been shown to impair functioning of the P-glycoprotein, a protein that is responsible for intracellular extrusion of multiple pro-arrhythmic agents [19] and I_{Kr} channels, the main channels that contribute to potassium efflux during repolarization [20]. This combination of factors is believed to alter cardiomyocyte repolarization, ultimately affecting the QTc interval [20].

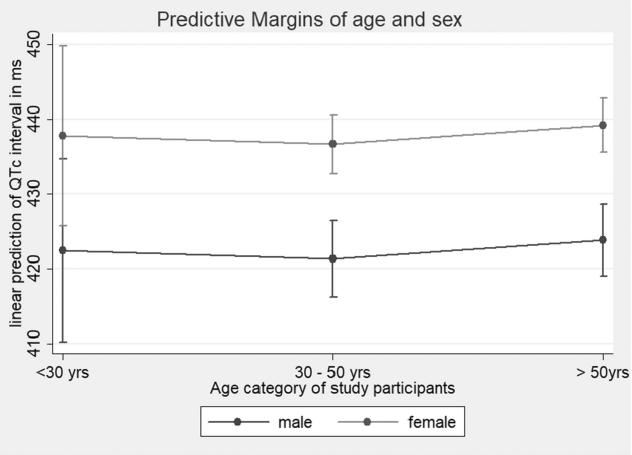

Fig. 2 Linear prediction of QTc interval by age and sex with 95% confidence intervals

Our results demonstrated a correlation between mean arterial pressure and QTc interval, in agreement with previous studies [21–24]. Of note, the association between blood pressure and QTc interval has also previously been demonstrated in hypertensive mice [25]. Although the mechanisms by which arterial blood pressure influences QTc interval are not well understood, it is postulated to be due to chronic alterations in the ultrastructure of cardiomyocytes, as a result of chronically increased afterload [26]. Additionally, the predominance of sympathetic nervous system activation, often seen in hypertensive patients, may result into increased QTc interval [27]. The influence of arterial blood pressure on QTc interval demonstrated in our study has clinical implications, as it further confirms the need to better control blood pressure in diabetic patients, so as to reduce the risk of adverse cardiovascular events. Future longitudinal studies will be required to ascertain whether control of blood pressure could potentially result into improvement in QTc interval and cardiovascular events in the study population.

Finally, we corroborated a well-established correlation between QTc interval and sex, with females having a higher QTc interval compared to males, in agreement with previous findings [15, 28]. After puberty, females tend to have a longer baseline QTc interval due to sex-hormone-related differences in modulation of ionic currents within the myocardium [29].

In individuals with diabetes, these sex-related differences in QTc interval duration may be amplified. This finding is in keeping with a possible increased risk for QTc-related cardiovascular events observed in females [30].

Limitations

We cannot demonstrate a causal relationship between measured correlates and QTc interval duration because of the cross-sectional nature of our study design. Our findings are also prone to residual and unmeasured confounding from factors that are known to affect QTc interval, such as serum electrolytes, levels of thyroid hormones, or unreported medications including different antidiabetic medications that may prolong QTc interval. Finally, we conducted a single-center, cross-sectional study without longitudinal outcome data, so we are unable to describe the clinical consequence of traditionally defined QTc prolongation in our study population.

Conclusion

The prevalence of abnormally long QTc interval was high among ambulatory individuals with diabetes in rural Southwestern Uganda, detected in about one-third of the study participants. Female sex and mean arterial pressure were correlated with longer QTc intervals. Our findings give support to consider QTc screening before initiation of QT prolonging agents among diabetics in the region and should lead to additional study of the clinical consequences of prolonged QTc interval in this patient population.

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Availability of data and materials The datasets generated and analyzed during the study are available from the corresponding author on request.

Author's contribution R.M., A.M., D.C.A, S.L.M, and M.S conceived the study, contributed to discussion, and reviewed, edited, and wrote the manuscript. G.K. and D.C.A. reviewed the ECG recordings. R.M and M.S. analyzed the data. R.M. is the guarantor of this research work and, as such, had full access to all the data for the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate The study got an approval from the institutional ethics review board of the Mbarara University of Science and Technology (MUST-REC). We also received approval for the study from the Uganda National Council of Science and Technology (UNCST) and from the Research Secretariat in the Office of the President of Uganda, in accordance with the national guidelines. All study participants provided written informed consent before recruitment and participation. Participants who could not write gave consent with a thumbprint.

Conflict of interests The authors declare no competing interests.

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ORIGINAL ARTICLE

Albuminuria increased the risk of left ventricular hypertrophy in type 2 diabetes patients with early renal insufficiency

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Abstract

Aim Albuminuria and left ventricular hypertrophy are important predictors of cardiovascular disease. We speculated that albuminuria increases the risk of left ventricular hypertrophy in patients with type 2 diabetes and early renal impairment.

Methods A total of 330 patients with type 2 diabetes and early renal insufficiency were recruited and classified according to albuminuria level (normal albuminuria, microalbuminuria, and macroalbuminuria). Plasma glucose, glycated hemoglobin (HbA1c), creatinine, uric acid, fasting insulin, albuminuria/urine creatinine ratio, renal function, insulin sensitivity, visceral fat index, body mass index, blood pressure, and left ventricular mass were assessed. The relationship between albuminuria and left ventricular hypertrophy was examined using logistic regression analysis and multiple linear regression.

Results The risk of left ventricular hypertrophy was higher in the microalbuminuria group (odds ratio [OR] 14.602, 95% confidence interval [CI] 7.050–30.243) and macroalbuminuria group (OR 21.455, 95% CI 8.613–53.443) than in the normal albuminuria group.

Conclusions Albuminuria increases the risk of left ventricular hypertrophy in patients with type 2 diabetes and early renal insufficiency.

Keywords Albuminuria · Ventricular hypertrophy · Cardiovascular disease · Type 2 diabetes · Ventricular mass index · Early renal insufficiency

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in patients with type 2 diabetes mellitus (T2DM), accounting for 50.54% of cases [1]. Hyperglycemia is a risk factor for atherosclerosis and contributes to heart failure (HF) [2]. Some studies have shown that left ventricular hypertrophy (LVH) is present in up to 71% of patients with T2DM and is an early independent predictor of CVD [3, 4]. Left ventricular mass index (LVMI) is an important predictor of the severity of LVH. Albuminuria has also been shown to predict CVD in patients with diabetes [5, 6]. Although some studies have found that albuminuria can increase the risk of LVH in T2DM patients with renal insufficiency [7, 8], another demonstrated that albuminuria was not significantly associated with the risk of LVH [9]. Currently, there is no consensus on whether albuminuria is related to LVH in diabetic patients, especially when renal function is not significantly impaired. Therefore, we hypothesized that LVH in T2DM patients is associated with albuminuria. And we conducted a cross-sectional study to confirm our hypothesis that albuminuria is associated with an increased risk of LVH and high LVMI in T2DM patients with early renal insufficiency.

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Materials and methods

Materials

This cross-sectional population study included T2DM patients with normal albuminuria (n=67), microalbuminuria (n=179), and macroalbuminuria (n=84). None of the patients had a

history of HF (chest pain, dyspnea, malignant arrhythmia), cerebrovascular disease, or renal failure. T2DM was diagnosed based on the World Health Organization Diabetes Diagnostic Criteria, 1999. Patients were recruited from the National Metabolic Management Center (MMC) and Department of Endocrinology, Nanhai People's Hospital, the Second School of Clinical Medicine, Southern Medical University, Foshan City, China, between July 2018 and March 2019. The study adhered to the principles of the Declaration of Helsinki. Ethical approval for this study was obtained from the Ethics Committee of the People's Hospital of Nanhai District, Foshan (NO.: 20180712).

Methods

Blood samples were collected from all patients after 8–12 h of fasting to evaluate plasma glucose, glycated hemoglobin (HbA1c), creatinine, and uric acid levels. Fasting insulin levels were measured using standard chemiluminescence. The albuminuria/urine creatinine ratio (ACR) was calculated by collecting the first morning urine sample. ACR was used to divide patients into the normal albuminuria group (ACR <3 mg/mmol), microalbuminuria group (ACR 3–30 mg/mmol), and macroalbuminuria group (ACR ≥30 mg/mmol). Renal function was assessed using the Modification of Diet in Renal Disease Study equation [10] to calculate the glomerular filtration rate (eGFR). Renal insufficiency was defined as eGFR >75 ml/min/1.73 m^2. Insulin sensitivity was assessed by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) [11], using the formula

$$\text{HOMA}-\text{IR} = \text{insulin (mIU/L)} \times \text{glycemia (mmol/L)}/22.5.$$

The visceral fat index (VFI) was measured by a dedicated operator using an OMRON visceral fat detection device (HDS-2000 DUALSCAN). Body mass index (BMI) was calculated using the formula weight (kg) / height $(m)^2$. Blood pressure (BP) was measured in the supine position with a cuff after at least 5 min of rest. Left ventricular mass (LVM) was detected using the American PHILIPS EPIQ5 Color Doppler Ultrasound System with a probe frequency of 2.5 MHz and was operated by an experienced deputy chief physician of the Department of Ultrasound Medicine. Left ventricular end-diastolic diameter (LVEDD), interventricular septal thickness (IVST), and left ventricular posterior wall thickness (LVPWT) were continuously measured for 3 cardiac cycles and averaged. LVMI was calculated using the Devereux formula and normalized by body surface area (BSA), LVM(g) = 0.8 × 1.04 × [(LVEDD + IVST + LVPWT) ^{3} − $LVEDD^3$] + 0.6; BSA (m^2) = 0.0057 × height (cm) + 0.0121 × body weight (kg) + 0.0882 (male); BSA (m^2) = 0.0073 × height (cm) + 0.073 × body weight (kg) − 0.2106 (female). LVH was defined as LVMI ≥ 115 g/m^2 (male) or LVMI ≥ 95 g/m^2 (female) [12].

Statistical analysis

All data were stratified by the level of albuminuria (normal albuminuria, microalbuminuria, and macroalbuminuria). Descriptive data that fit the normal distribution are expressed as mean ± standard deviation or 95% confidence interval and compared by the *t*-test. Non-normally distributed data are expressed as median (25–75th percentile). Categorical variables are expressed as percentages and compared using the chi-square test. Continuous variables were compared using one-way ANOVA or the Kruskal-Wallis H test. Linear regression models and Pearson's correlation were used to assess the correlation between LVMI and ACR. Logistic regression models were used to assess the correlation between LVH and albuminuria level. All analyses were performed using SPSS Windows version 26.0, and statistical significance was set at $p < 0.05$.

Results

Table 1 presents the clinical characteristics and biochemical indicators of the 330 T2DM patients stratified by the level of albuminuria (normal albuminuria, microalbuminuria, and macroalbuminuria). Compared to patients with microalbuminuria, patients with macroalbuminuria had significantly higher rates of hypertension (53.6%, $p = 0.000$), a family history of T2DM (50%, $p = 0.035$), and a family history of CVD (11.9%, $p = 0.014$). In addition, they had higher fasting blood glucose, total cholesterol, and low-density lipoprotein levels and were more likely to be treated with insulin, glucagon-like peptide (GLP)-1 receptor agonists, and calcium antagonists.

Color Doppler revealed that none of the patients had HF, but patients with macroalbuminuria had higher IVST than those with microalbuminuria (10.22 ± 1.78 vs 9.77 ± 1.53; $p = 0.035$). LVEDD and LVPWT did not reach statistical significance ($p = 0.202$ and $p = 0.308$, respectively). LVM and LVMI were higher in patients with macroalbuminuria than in those with normal albuminuria and microalbuminuria ($p = 0.000$ for both). However, there was no statistical difference between the LVM and LVMI in the normal albuminuria and microalbuminuria groups.

In the forward stepwise logistic regression analysis, the odds ratio (OR) was 14.602 (95% confidence interval (CI) 7.050–30.243) for LVH in the microalbuminuria subgroup, and in the macroalbuminuria subgroup, the OR was 21.455 (95% CI 8.613–53.443), after adjusting for age, sex, HbA1c, fasting plasma glucose, diastolic and systolic BP, hypertension, BMI, VFI, uric acid, eGFR, smoking, HOMA-IR, triglycerides, total cholesterol, low-density lipoprotein, high-

Table 1 The main clinical characteristics and biochemical indicators of patients stratified by level of albuminuria

Characteristic	Normal albuminuria[#] $N = 67$	Microalbuminuria[$] $N = 179$	Macroalbuminuria[&] $N = 84$	Between groups (p-value)	Post hoc multiple comparisons (p-value)
Age (years)	53.5 ± 12.19	54.1±11.5	56.2±10.2	0.254	
Sex male *n* (%)	35 (52.2%)	103 (57.5%)	44 (52.4%)	0.636	
Smoking *n* (%)	26 (38.8%)	60 (33.5%)	26 (31.0%)	0.590	
Hypertension *n* (%)	23 (34.3%)	54 (30.2%)	45 (53.6%)	0.001	# vs & 0.018; $ vs & 0.000
Family history *n* (%)					
T2DM	27 (40.3%)	65 (36.9%)	42 (50%)	0.108	$ vs & 0.035
CVD	6 (8.9%)	7 (3.9%)	10 (11.9%)	0.046	$ vs & 0.014
BMI (kg/m^2)	26.09 ± 3.28	24.84 ± 3.74	25.11 ± 3.24	0.048	# vs $ 0.014
BSA (m^2)	1.84 ± 0.19	1.79 ± 0.18	1.78 ± 0.18	0.089	# vs $ 0.045; # vs & 0.048; $ vs & 0.783
Blood pressure (mmHg)					
Systolic pressure	125.48 ± 13.28	128.04 ± 14.39	131.60 ± 13.21	0.024	# vs & 0.007
Diastolic pressure	77.24 ± 8.96	79.89 ± 12.60	79.87 ± 8.89	0.220	
VFI (cm^2)	86.79 ± 34.31	90.29 ± 43.47	89.86 ± 40.14	0.831	
Medications n (%)					
Insulin	25 (37.3%)	51 (28.5%)	38 (45.2%)	0.025	
Metformin	58 (86.6%)	135 (75.4%)	60 (71.4%)	0.077	# vs & 0.025
GLP-1R agonist	13 (19.4%)	79 (44.1%)	44 (52.4%)	0.000	# vs $ 0.000; # vs & 0.000
ACE inhibitor	8 (11.9%)	14 (7.8%)	3 (3.6%)	0.000	# vs $ 0.000; # vs & 0.000
Angiotensin II receptor blocker	16 (23.9%)	36 (20.1%)	24 (28.6%)	0.310	
Calcium antagonist	8 (11.9%)	23 (12.8%)	18 (21.4%)	0.143	
Beta blocker	7 (10.4%)	13(7.3%)	6 (7.1%)	0.682	
Diuretic	9 (13.4%)	10 (5.6%)	6 (7.1%)	0.116	
Blood samples					
Fasting blood glucose (mmol/l)	8.89 ± 3.41	10.27 ± 4.72	11.59 ± 5.77	0.003	# vs $ 0.046; # vs & 0.001; $ vs & 0.037
HbAlc (%)	8.93 ± 2.57	8.39 ± 2.97	8.33 ± 2.59	0.351	
HOMA-IR	3.79 ± 2.37	3.07 ± 3.06	3.71 ± 2.37	0.089	
Triglyceride (mmol/l)	2.87 ± 2.73	2.16 ± 2.26	2.35 ± 2.42	0.122	# vs $ 0.040
Total cholesterol (mmol/l)	4.95 ± 1.23	5.08 ± 1.24	5.27 ± 1.51	0.309	
Low-density lipoprotein (mmol/l)	2.50 ± 1.16	2.93 ± 1.04	3.01 ± 1.27	0.011	# vs $ 0.007; # vs & 0.006
High-density lipoprotein (mmol/l)	1.41 ± 0.51	1.42 ± 0.96	1.19 ± 0.36	0.063	$ vs & 0.023
Uric acid (mmol/l)	401.66 ± 113.31	395.93 ± 114.69	406.64 ± 127.18	0.781	
Creatinine (umol/l)	64.09 ± 14.94	64.97 ± 15.48	65.62 ± 16.99	0.894	
eGFR (ml/min/1.73m^2)	110.50 ± 24.46	112.05 ± 32.16	110.03 ± 34.03	0.869	
Color Doppler measurement					
LVEDD (mm)	45.64 ± 4.44	45.35 ± 3.98	46.31 ± 3.92	0.202	
IVST (mm)	9.87 ± 1.67	9.77 ± 1.53	10.22 ± 1.78	0.104	$ vs & 0.035
LVPWT (mm)	9.50 ± 1.63	9.61 ± 1.38	9.85 (9.52–10.17)	0.308	
LVM (g)	167.09 ± 49.60	229.14 (221.79–236.49)	247.96 ± 62.36	0.000	# vs $ 0.000; # vs & 0.000; $ vs & 0.008
LVMI	122.04[%] (103.10–141.15)	125.35[%] (107.84–144.44)	132.74[%] (120.48–149.71)	0.000[*]	# vs $ 0.000; # vs & 0.000

LVEDD left ventricular end-diastolic diameter, *IVST* interventricular septal thickness, *LVPWT* left ventricular posterior wall thickness, *LVM* left ventricular mass, *LVMI* left ventricular mass index, *ACR* albuminuria/urine creatinine ratio

#: ACR < 3mg/mmol; $: ACR ≥3mg/mmol and ACR < 30 mg/mmol; &: ACR ≥ 30 mg/mmol; %: median (25–75th percentile); *: Kruskal-Wallis H test

Table 2 Forward stepwise logistic regression analysis of level of albuminuria and LVH

	Normal albuminuria[#]	Microalbuminuria[$] OR[^] (95% CI)	Macroalbuminuria[%] OR[^] (95% CI)	p-value
Model 1*	1.000	15.123 (7.504, 30.479)	25.009 (10.270, 60.896)	# vs $ 0.000; # vs % 0.000
Model 2**	1.000	14.602 (7.050, 30.243)	21.455 (8.613, 53.443)	# vs $ 0.000; # vs % 0.000

*Adjusted for age, sex, glycated hemoglobin, fasting blood glucose, diastolic blood pressure, systolic blood pressure, hypertension, body mass index, vascularization flow index, uric acid, epidermal growth factor receptor, smoke, homeostasis model assessment of insulin resistance, triglyceride, total cholesterol, low-density lipoprotein cholesterol-L, high-density lipoprotein-L

**Adjusted for further medications: glucagon-like peptide-1 receptor, angiotensin-converting enzyme inhibitor, angiotensin II receptor, metformin, calcium, beta, diuretic, and insulin

^*OR* odds ratio, *95%CI* 95% confidence interval

density lipoprotein, and medications (Table 2). In the selected variable multicollinearity test, variance inflation factor (VIF) of all variables was less than 10, confirming that there was no significant multicollinearity between the various variables.

In stepwise multiple linear regression models and Pearson's correlation analysis, LVMI and ACR were linearly correlated after square root conversion ($p = 0.000$), and LVMI was higher with increasing ACR. In addition, treatment with a GLP-1R agonist showed a positive linear correlation with the prevalence of LVH ($p = 0.006$) (Table 3).

Discussion

In our study, T2DM patients with macroalbuminuria had significantly higher LVMI than those with normal albuminuria and microalbuminuria. Further, logistic regression analysis showed that a higher level of albuminuria was associated with a higher risk of LVH. In addition, LVMI was positively associated with the ACR.

Previous studies have reported an association between LVH and albuminuria [7–9]. Nabbaale et al. reported a positive correlation between microalbuminuria and LVH ($r = 0.185$, $p = 0.003$) [13]. Wu et al. found that the risk of LVH in patients with microalbuminuria (OR 2.473, 95% CI 1.370–4.464) and macroalbuminuria (OR 3.940 [95% CI 1.553–9.993]) was significantly higher compared to non-diabetic patients without kidney disease [14]. Nguyen et al. reported that microalbuminuria (OR 2.2, 95% CI 1.4–3.2, $p<0.0001$) was predictive of LVH [15]. Liu et al. found a higher prevalence of LVH (49%) in T2DM patients with macroalbuminuria compared to those with microalbuminuria [7]. Guerra et al. reported that patients with microalbuminuria had a 2-fold higher risk of LVMI [16]. Unlike the majority of previous studies which focused on causative factors for CVD, such as obesity, hypertension, and late chronic kidney disease (CKD stage 3-5), our study selected patients with preserved eGFR (eGFR>75 ml/min/1.73 m^2). For example, most of the patients included in the study by Wu et al. had stage 2 CKD (eGFR>60 ml/min/1.73 m^2). These findings suggest that albuminuria is an early marker of CVD risk. Our study demonstrated an association between albuminuria and the risk of LVH in T2DM patients with early renal insufficiency.

Advanced glycation end products (AGEs) are formed by the Maillard reaction which involves non-enzymatic reactions between reducing sugars and amino groups of large biomolecules, including proteins, nucleic acids, and lipids [17]. Smokers and patients with a history of diabetes and CKD have increased AGEs [18]. Accumulation of AGEs in the extracellular matrix interacts with the receptor of AGEs (RAGEs) to activate TGF-β1, p38 mitogen-activated protein kinase (MAPK), and nuclear factor κB (NF-κB) through protein kinase C (PKC), elevating reactive oxygen species (ROS) to cause vascular calcification and fibrosis [19]. These changes are inextricably related to the process of diabetic kidney disease and cardiomyocyte hypertrophy. In addition, the AGE/RAGE signaling pathway enhances intracellular oxidative stress to upregulate the activity of the ryanodine receptor

Table 3 [&]The stepwise multiple linear regression models and Pearson's correlation of ACR[ε] and LVMI[ε]

Variable	Standardized coefficients	Standard error	*t*	*p*-value
ACR	0.084	0.011	7.768	0.000
GLP-1R agonist	0.399	0.145	2.743	0.006

[&] Adjusted for systolic blood pressure, hypertension, body mass index, vascularization flow index, low-density lipoprotein cholesterol-C, angiotensin-converting enzyme inhibitor, angiotensin II receptor, and beta

[ε] Both of albuminuria/urine creatinine ratio and left ventricular mass index were converted by square root

(RyR) in the sarcoplasmic reticulum (SR), leading to leakage of Ga^{2+} in the SR. These changes disrupt Ga^{2+} homeostasis in myocardial cells, and this process is thought to induce cardiac dysfunction resulting in ischemia-reperfusion, left ventricular remodeling, and HF [20]. Renal function impairment and cardiac hypertrophy have similar mechanisms in patients with T2DM. Our study suggests that T2DM patients also have albuminuria without significantly reduced GFR. This may be because albuminuria and decreased eGFR are hallmarks of different pathological processes [21]. Albuminuria could be a phenotypic expression of endothelial dysfunction, while decreased eGFR could be a renal manifestation of systemic atherosclerosis [22]. Currently, there is an increasing need to prevent renal and cardiac complications in T2DM patients.

In our study, the rate of LVH in patients with T2DM was 71.5%, which was slightly higher than that reported by Dawson et al. [3] and this may be due to differences in the diagnostic criteria for LVMI between studies. We adopted the diagnostic criteria of the 2013 ESC/ESH guidelines for the management of arterial hypertension: LVMI ≥ 115 g/m^2 (male) or LVMI ≥ 95 g/m^2 (female) by color Doppler ultrasound. Our study suggested a positive association between albuminuria and LVH in the absence of significant renal impairment (CKD 1–2). However, the results of this study differ from those of previous studies. Ren et al. suggested that there was no statistically significant difference between the prevalence of LVH in the microalbuminuria, macroalbuminuria, and normal albuminuria groups [9]. This may be due to the different definitions of LVH. In the ATTEND study, LVH was assessed using electrocardiograms which are less accurate than echocardiographic measurements [23]. Previous studies have shown that controlling BP and blood glucose is related to the low prevalence of albuminuria and LVH in T2DM patients [24–29]. However, in our study, fasting blood glucose and systolic BP were not significantly associated with LVMI. This may be because our patients were treated with antihypertensive and hypoglycemic medications. BMI is also an important factor that can affect the association between albuminuria and LVH. According to a previous study, increasing BMI is an independent risk factor for LVH [30, 31]. However, we failed to show a statistically significant association of BMI with LVMI, which may be because the majority of the patients included in our study had a low BMI of < 30 kg/m^2. In addition, BMI is not an accurate indicator of obesity and metabolic syndrome. ACE inhibitor (ACEi) treatment can significantly reduce albuminuria [32]. In multiple linear regression, lower LVMI was associated with the use of a GLP-1R agonist (p = 0.006), and this result was consistent with that of previous studies. GLP-1R agonists are new hypoglycemic drugs that can prevent macroalbuminuria and reduce cardiovascular mortality [33, 34]. GLP-1R agonists may improve CVD outcomes by affecting heart rate, BP, microvascular function, lipids, and inflammation [35]. Our study supports the observation that GLP-1R agonists can improve the cardiac and kidney function of patients with T2DM.

Our study has some limitations. First, this was a cross-sectional study that lacked follow-up data. Therefore, an accurate association between risk factors and disease development could not be determined. Second, our study was conducted in a medium-sized sample, and fewer patients with normal albuminuria were selected. Therefore, the results need to be verified using larger sample studies.

Conclusions

Our study showed that albuminuria increases the risk of LVH in T2DM patients with early renal insufficiency. In addition, a higher albuminuria level was associated with a higher prevalence of LVH and increased LVMI. These results suggest that albuminuria can be used as a predictor of CVD risk, and clinicians should pay more attention to the prevention of renal and cardiac complications in patients with T2DM. Further studies with a larger sample size or clinical trials are needed to enhance the reliability of these results.

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Author contribution Lei Liu: Conceptualization, methodology, acquisition of data, formal analysis, writing - original draft, writing - review and editing, project administration, and validation

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Data availability To get the data and material of the research, please contact correspondence by e-mail.

Declarations

Ethics approval The research was approved by Ethics Committee of the People's Hospital of Nanhai District, Foshan.

Consent to participate None declare.

Consent for publication All of the authors confirm the publication.

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Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Stewart (physicochemical) approach versus conventional anion gap approach for resolution of metabolic acidosis in diabetic ketoacidosis

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Abstract

Background Diabetic ketoacidosis (DKA) frequently requires emergency admission. The anion gap approach is conventionally used for the diagnosis and documenting the resolution of acidosis during treatment. However, it fails to detect hyperchloremic acidosis during the resolution and may result in the prolongation of treatment.

Objectives To determine the role of the Stewart approach of acid-base disorder during DKA management for the prediction of an earlier resolution.

Methods A prospective comparative study was conducted between January 2017 and December 2017 at a single academic hospital in north India. Patients aged above 12 years with a diagnosis of DKA were randomly divided into two groups—the conventional group and the Stewart group, according to the approach used for DKA resolution. The primary outcome was the time duration required for resolution. The secondary outcomes were the therapeutic requirement of intravenous fluid, insulin, and potassium, Acute Physiology and Chronic Health Evaluation II (APACHE II) score at the time of resolution, and hospital stay.

Results Forty-four DKA patients were equally distributed in the two groups with comparable baseline parameters. The Stewart group had early resolution of DKA (mean, 32.4±17.5 h versus 41.7±19.6 h; p value <0.001) at similar APACHE II scores. The duration of hospital stay was reduced but was not statistically significant (mean, 5.6±3.2 days versus 7.0±3.8 days; p value 0.16). The therapeutic requirement of fluid, insulin, and potassium was similar in groups.

Conclusion The Stewart approach may be a better alternative to the conventional anion gap approach for guiding the resolution of DKA.

Keywords Diabetic ketoacidosis · Metabolic acidosis · Acid-base disorder · Anion gap · Stewart · Physicochemical · Physiological

Introduction

Diabetic ketoacidosis (DKA) is an acute metabolic complication of diabetes mellitus (DM). It is characterized by hyperglycemia and metabolic acidosis due to the accumulation of serum ketones from free fatty acid oxidation. The management includes isotonic intravenous (IV) fluids, IV insulin infusion, identification and treatment of the precipitating factor (e.g., infection), and high-quality supportive care [1–4]. For the diagnosis,

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determination of severity, and resolution of metabolic acidemia during management, the anion gap (AG) approach is traditionally used [2–5].

The conventional AG approach also called the physiological approach is simple and rapid to analyze acid-base abnormalities at the bedside [6]. It is based on the carbonic acid-bicarbonate buffer system (Henderson–Hasselbalch equation) and uses serum bicarbonate level as a marker of metabolic disturbance in acid-base disorders [6, 7]. Stewart proposed an alternate approach—the physicochemical method. It describes the biological fluid (e.g., plasma) as a system containing multiple interacting constituents that control pH (H^+), and bicarbonate and pH are not independently determined [8–11]. Based on the principles of mass balance and electroneutrality, the Stewart approach identifies three independent determinants of acid-base state—strong ion difference (SID), circulating non-volatile weak acids and their dissociated ions, and partial pressure of carbon dioxide. Compared with the conventional approach, this approach involves complex calculations and is time-consuming; however, it helps determine the underlying specific mechanism producing the acid-base abnormality [11–14].

Despite having practical usefulness, the AG approach has few limitations in the management of DKA. It may underestimate the degree of acidemia in patients with hypoalbuminemia because albumin acts as a non-volatile weak acid in plasma [15–17]. Hyperchloremic metabolic acidosis, a typical example of strong ion acidosis, may occur during the resolution phase of DKA and remains unrecognized with the conventional approach, which precludes the stoppage of insulin infusion and may result in a more extended hospital stay and higher costs [2, 3, 18–20]. Case reports have demonstrated the Stewart approach's role in documenting DKA recovery and estimating the magnitude of hyperchloremic acidosis [21]. Based on this hypothesis, we conducted a prospective comparative study to determine whether using the Stewart approach to analyze metabolic acidosis during DKA management predicts an earlier resolution.

Material and methods

Study design

We conducted this prospective study between January 2017 and December 2017 at the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Consecutive patients were recruited from the adult medical emergency of the Department of Internal Medicine.

Participants

Individuals enrolled were above 12 years of age, with a diagnosis of DKA based on plasma glucose >250 mg/dL, arterial blood pH <7.30, and serum ketone >3.0 mmol/L, according to American Diabetes Association (ADA) criteria [22]. Patients with chronic kidney disease, chronic respiratory illnesses, congestive heart failure, and acute liver failure were excluded from the study.

Data collection

On enrolment, after a comprehensive clinical history and physical examination, all participants underwent the following routine investigations—serum electrolytes including sodium, potassium, chloride, magnesium, calcium, and phosphorus, renal functions with urea and creatinine, total protein and albumin, bilirubin and liver transaminases, complete blood count with hemoglobin, hematocrit, white blood cell count, and platelet count, and arterial blood gas analysis with pH, the partial pressure of oxygen (for the given fraction of inspired oxygen), the partial pressure of carbon dioxide, bicarbonate, and lactate. Further investigations, including radio-imaging, cultures of blood and body fluids, serum biomarkers (e.g., procalcitonin), and coagulation profile, were carried out in appropriate clinical settings. Acute Physiology and Chronic Health Evaluation II (APACHE II) score (on a scale of 0 to 71, with higher scores indicating greater disease severity) was calculated on admission and daily until the resolution of acidosis to assess the severity of illness [23]. After enrolment in the emergency room, the patients were shifted to a medical emergency high-dependency unit or an intensive care unit (ICU) and, after the resolution of DKA, to a general ward or step-down unit according to the availability of the bed. All patients were managed according to the standard guidelines of ADA [2, 22].

Study groups

Patients were randomly divided into a 1:1 ratio into two groups—the conventional group and the Stewart group. In the conventional group, the conventional bicarbonate and AG approach, and the Stewart group, the Stewart approach for metabolic acidosis was used to define DKA resolution. Blood gas analysis and serum electrolyte measurements were carried out at baseline and at least every 12 h to calculate AG in the conventional group and SID and strong ion gap (SIG) in the Stewart group. Criteria for resolution of DKA for the conventional group was defined as plasma glucose <200 mg/dL, blood pH >7.30, serum bicarbonate ≥18 mEq/L, and calculated AG value ≤12; and for Stewart group, plasma glucose <200 mg/dL, pH >7.30, and SIG value 0 ± 5. After the resolution of DKA, IV insulin infusion was changed to subcutaneous insulin.

Calculations

1. AG represents the total of all unmeasured charged species (predominantly albumin) in the serum. It was calculated as a difference between the measured anion and cation concentrations in the serum by using the following formula:

   $AG = (Na^+ + K^+) - (HCO_3^- + Cl^-)$ (all concentrations were measured in mEq/L).

   The normal value of AG is 12 ± 4.
2. SID is defined as the difference between total serum cations and total serum anions and is calculated with the following equation:

$$SID = \left(Na^+ + K^+ + Ca^{2+} + Mg^{2+}\right) - (Cl^- + \text{lactate})$$

(all concentrations in mEq/L).

A decrease in SID will increase water dissociation (to maintain electroneutrality), and pH will decrease. However, weak acids present in the blood (e.g., ketoacids and other organic anions, sulfate, etc.) are not considered in the above equation; therefore, it represents 'apparent' SID (SID_a).

The 'effective' SID (SID_e) can be calculated by using the following formula:

$$SID_e = \left[12.2 \times pCO_2 / \left(10^{-pH}\right)\right] + [(\text{albumin}) \times (0.123 \times pH - 0.631)] + [(\text{phosphate}) \times (0.309 \times pH - 0.469)]$$

(concentration of pCO_2 in mmHg, albumin in g/L, and phosphate in mmol/L).

The normal value of SID is 35 ± 5 mEq/L.

3. SIG represents the unmeasured anions present in the serum. It was calculated by using the following formula:

$SIG = SID_a - SID_e$.

The normal value of SIG is 0 ± 5.

Outcome measures

The primary outcome of the study was the time duration required for the resolution of DKA. The total amount of IV fluid therapy, IV insulin therapy, and potassium supplementation needed for the resolution of DKA, APACHE II score (to assess the severity of illness) at the time of DKA resolution, and the total duration of hospital stay were the secondary outcome measures.

Statistical methods

Discrete categorical data were represented in the number (n) and percentage (%). For continuous data, mean and standard deviation (SD) or median and interquartile range were used, as per the requirement. The normality of quantitative data was checked by measures of Kolmogorov-Smirnov tests of normality. For normally distributed data, the t test was applied for comparison of the two groups. Mann-Whitney U test was used for statistical analysis of skewed continuous variables. Proportions were compared using Chi-square or Fisher's exact test, depending on their applicability. Logistic regression analysis was applied to demonstrate which approach helped in the early prediction of acidosis resolution in DKA. All the statistical tests were two-sided and at a significance level of α=0.05. The analysis was conducted using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 22.0.

Results

Participants and baseline characteristics

A total of 44 patients (29 males and 15 females) were included in the study. The mean age of the study population was 24.9 ± 12.2 years (range, 12–55 years). The types of DM in this study were type 1 DM (n=35, 79.5%), type 2 DM (n=6, 13.6%), and latent autoimmune diabetes in adults (n=3, 6.8%). Six patients presented with DKA at the first time of their initial DM diagnosis. Dehydration was seen in the majority of the patients at presentation (86.4%). Other common presenting complaints were nausea and vomiting (75.0%), pain abdomen (47.7%), altered mental status (34.1%), and hypotension (34.1%). Poor compliance to antidiabetic treatment and infections were the usual precipitating factors of DKA (n=23, 52.3%; and n=21, 47.7%, respectively). The mean glycated hemoglobin of the study was 13.7 ± 2.7%.

The study patients were equally distributed in both conventional and Stewart groups (n=22, each). The baseline characteristics at admission were similar between the two groups (Table 1).

Metabolic parameters at the time of the resolution of DKA

At the DKA resolution, the mean values of plasma glucose in conventional and Stewart groups were 161.0 ± 32.8 mg/dL

Table 1 Baseline characteristics of the study population

Variable	Stewart group (*n*=22) (mean ± SD)	Conventional group (*n*=22) (mean ± SD)
Age (years)	21.2 ± 8.1	28.6 ± 14.5
Systolic blood pressure (mmHg)	103.0 ± 21.6	103.4 ± 22.8
Diastolic blood pressure (mmHg)	66.7 ± 11.1	65.9 ± 15.0
Mean arterial pressure (mmHg)	77.0 ± 16.8	78.4 ± 17.3
Pulse rate (per minute)	107.9 ± 22.8	108.6 ± 18.8
Respiratory rate (per minute)	25.1 ± 6.7	28.2 ± 7.8
Serum sodium (mEq/L)	137.9 ± 6.7	137.6 ± 6.7
Serum potassium (mEq/L)	3.8 ± 0.7	4.0 ± 1
Serum chloride (mEq/L)	107.9 ± 8.4	106.6 ± 6.8
Serum magnesium (mg/dL)	1.8 ± 0.4	1.8 ± 0.3
Serum calcium (mg/dL)	8.2 ± 0.6	8.4 ± 1.0
Serum phosphorus (mg/dL)	1.8 ± 1.0	1.8 ± 1.2
Blood urea (mg/dL)	30.9 ± 13.8	34.4 ±16.1
Serum creatinine (mg/dL)	1.0 ± 0.2	1.1 ± 0.6
Total serum protein (g/dL)	6.3 ± 0.6	6.2 ± 1.1
Serum albumin (g/dL)	3.6 ± 0.4	3.3 ± 0.8
Blood pH	7.13 ± 0.13	7.06 ± 0.12
Random plasma glucose (mg/dL)	437.5 ± 131.7	503.5 ± 115.3
Serum ketones (mmol/L)	5.6 ± 0.9	5.6 ± 1.1
Serum bicarbonate (mEq/L)	6.6 ± 3.0	5.4 ± 2.6
Anion gap	27.1 ± 5.8	29.0 ± 4.5
'Apparent' strong ion difference	35.1 ± 5.2	-
'Effective' strong ion difference	16.8 ± 3.9	-
Strong ion gap	15.6 ± 6.3	-
Glycated hemoglobin (%)	13.8 ± 3.0	13.6 ± 2.5
APACHE II score[a]	8.2 ± 3.4	11.5 ± 6.1

No significant differences between the groups

[a] Scores on the Acute Physiology and Chronic Health Evaluation II (APACHE II) range from 0 to 71, with higher scores indicating greater disease severity

and 147.0 ± 29.1 mg/dL, respectively, and pH were 7.42 ± 0.04, and 7.37 ± 0.06, respectively. In the conventional group, the mean bicarbonate and AG were 19.3 ± 1.1 mEq/L and 14.0 ± 3.7, respectively. In the Stewart group, the mean values of SIDa, SIDe, and SIG at resolution were 32.1 ± 4.4, 27.8 ± 3.9, and 0.8 ± 3.3, respectively.

Analysis of outcomes

The primary outcome of this study was time taken for the resolution of DKA, which was significantly earlier in the Stewart group (mean, 32.4 ± 17.5 hours versus 41.7 ± 19.6 hours; *p* value <0.001) (Table 2). The therapeutic requirement

Table 2 Primary and secondary outcome measures

Outcome parameters	Stewart group (*n*=22) (mean ± SD)	Conventional group (*n*=22) (mean ± SD)	*p* value
Time taken for resolution of DKA (hours)	32.4 ± 17.5	41.7 ± 19.6	<0.001
Intravenous fluid required (mL)	10091 ± 6673	9684 ± 6479	0.838
Intravenous insulin required (Units)	147.5 ± 69.5	175.6 ± 79.0	0.218
Potassium replacement required (mEq)	286.4 ± 42.6	236.4 ± 50.6	0.29
APACHE II score[a]	2.2 ± 1.9	3.2 ± 2.8	0.218
Total duration of hospital stay (days)	5.6 ± 3.2	7.0 ± 3.8	0.16

[a] Scores on the Acute Physiology and Chronic Health Evaluation II (APACHE II) range from 0 to 71, with higher scores indicating greater disease severity

of IV fluids, IV insulin, and potassium supplementation for DKA resolution was not different in both approaches. APACHE II scores, used to determine the illness severity at the time of metabolic acidosis recovery, were similar between the two groups. Use of Stewart approach for the recovery of DKA reduced duration of hospital stay, but could not reach a statistically significant difference (mean, 5.6 ± 3.2 days versus 7.0 ± 3.8 days; p value 0.16).

Overall, the mean length of hospitalization of the study was 6.3 ± 3.5 days. Out of 44 patients, 43 improved and discharged. One patient, complicated with pulmonary mucormycosis, died (in the conventional group).

Discussion

Improved ICU management has reduced mortality to <1% in DKA worldwide [2–4, 24, 25]. The current emphasis on cost containment, coupled with the scarcity of ICU beds, makes it critical to identify strategies that can speed recovery from DKA. The results of this prospective comparative study show that the use of the Stewart approach resulted in the early resolution of DKA during the management. Faster recovery of DKA at similar illness severity (APACHE II score) appeared to reduce hospital stay but without a statistical significance. Differences were not seen in the requirement of IV fluid, IV insulin, and potassium replacement.

The assessment of the majority of acid-base abnormalities can be done by applying the conventional approach, and the exact diagnostic and prognostic role of the Stewart approach in clinical disorders is yet to be defined [6, 11, 12, 16, 26]. However, increasing evidence suggests using both approaches to understand better acid-base disorders in a clinical setting [11, 26, 27]. We used the Stewart approach to document the resolution of DKA after the diagnosis was made with the conventional AG approach. Albeit more time-consuming, it was feasible to calculate Stewart equations at 8- to 12-h intervals in this study. A protocol with the AG approach for diagnosis and Stewart approach to follow treatment conveniently provided a correct and timely interpretation of metabolic acidosis in DKA. Redwan et al. also found that combining the two approaches resulted in more accurate quantification of acid-base status in ICU patients [27]. The conventional method was demonstrated to be useful in rapid diagnosis without complex calculations and determination of the body's compensatory mechanisms. Simultaneously, the Stewart approach was essential to reveal the individual pathogenesis of the acid-base disorders and their severity and resolution [27]. Besides time-consuming calculations, the Stewart equations require many laboratory parameters that may not be readily available on admission, making it less helpful in immediate recognition of DKA in the medical emergency setting.

Some ICU studies have found the equivalent performance of modified AG (albumin-corrected AG for hypoalbuminemia) or base-excess method when compared with the Stewart approach in evaluating acid-base abnormalities, mostly because hypoalbuminemia is very common and severe in critically ill patients [16, 17, 28–30]. Corrected AG is considered analogous to SIG, but the latter has the advantage of less unmeasured parameters [31]. Corrected AG adjusts for albumin fluctuation only, not allowing for other ions offsets [32–36]. Thus, it does not offer the benefit of unmasking hyperchloremic acidosis during the DKA resolution, the primary indication of applying the Stewart approach in this condition.

Our study does not necessarily contradict the results of the previous ICU reports that demonstrated no superiority of the Stewart approach over corrected anion gap because it had a different cohort (mean serum albumin level was near normal) and the specific application of the Stewart approach (only for DKA resolution rather than overall acid-base analysis). However, our results need validation in a larger and broader series.

Limitation

Our study's major limitations are the small sample size and single-center observation, which might explain the lack of statistically significant difference in the duration of hospital stay. We also did not calculate a separate high-dependency unit and the step-down unit stays to delineate further the isolated beneficial effect of the Stewart approach.

Conclusion

We conclude that the Stewart approach is a better alternative to the conventional AG approach for guiding therapy and resolving metabolic acidosis in DKA, which can facilitate safe early discharge from the emergency department or ICU and reduce the cost of hospitalization. A further larger multicenter study is needed to confirm and clarify the role of the physicochemical approach in the management of DKA.

Author contribution AKP: patient management, drafted and revised the manuscript.

RS: patient management, collected patient data, drafted the manuscript.

NS: patient management, drafted, and revised the manuscript.

JK: patient management, revised the manuscript.

RW: patient management, revised the manuscript.

SK: patient management, revised the manuscript.

Declarations

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

Ethical approval The study protocol was approved by the Institutional Ethics Committee (IEC No.:INT/IEC/2020/SPL-546), and written informed consent was obtained from all participants and the parents or relatives of the participants age below 16 years.

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ORIGINAL ARTICLE

L-lysine supplementation improved glycemic control, decreased protein glycation, and insulin resistance in type 2 diabetic patients

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Abstract

Background Lysine treatment decreased diabetic complications associated with type 2 diabetes in the rat models of diabetes and *in vitro*.

Aims/hypothesis Herein, in a single-evaluator–blinded, randomized clinical trial, we investigated the effect of L-Lysine (Lys) intervention in type 2 diabetic patients.

Methods Two groups of type 2 diabetic patients (15 females and 10 males in each group) who were under treatment with glibenclamide and metformin underwent a short term (3 months), trial. The test group was orally administered with 3 g/day of Lys. Clinical and biochemical parameters of all patients were measured prior to and after the experimental period and were statistically analyzed.

Results Among the serum parameters, FBS, insulin, HOMA-IR, HSP 70, LCAT, PON1, AOPP, and FRAP improved significantly in the test group. A significant decrease in HbA1c and urine glucose accompanying with positive correlations between HbA1c and FBS, fructosamine and both FBS and HbA1c, HOMA with both insulin and FBS, and FBS with urine glucose indicates that Lys prevents diabetes complications. There were no significant changes in the liver and kidney function tests indicating no toxicity of Lys for patients.

Conclusions/interpretation In conclusion, Lys inhibited protein glycation, improved glycemic control, and increased antioxidant markers in type 2 diabetic patients; thus, it could be suggested for combinatorial therapy of diabetes with oral hypoglycemic agents to protect against vascular risk factors and other diabetic complications.

Keywords Diabetes · HOMA-IR · HSP 70 · LCAT · PON1 · AOPP · FRAP

Abbreviations

Lys	L-lysine
NIDDM	Noninsulin-dependent diabetes mellitus
T2DM	Type 2 diabetes mellitus
HSP	Heat shock protein
ELISA	Enzyme-linked immune assay
Glc	Glucose
Alb	Albumin
TG	Triglyceride
BMI	Body mass index
TC	Total cholesterol
HbA1c	Hemoglobin A1c
LDL-c	Low-density lipoprotein-cholesterol
HDL-c	high-density lipoprotein-cholesterol
ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
LDH	Lactate dehydrogenase
GGT	Gamma-glutamyl transpeptidase
CPK	Creatine phosphokinase
CRP	C-reactive protein

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LPL Lipoprotein lipase
BUN Blood urine nitrogen

Introduction

Type 2 diabetes mellitus (T2DM) has been recognized as a metabolic disorder and characterized by hyperglycemia and chronically increased blood glucose (Glc). Due to chronic hyperglycemia, glycation of inter- and intracellular proteins occurs, which lead to conformational alterations, misfolding and subsequently, functional changes of many proteins [1–7]. As a result, a variety of diabetic complications are pursued [8]. Therefore, treatment of T2DM or noninsulin-dependent diabetes mellitus (NIDDM) is aimed at optimal glycemic control, prevention of protein misfolding, and prevention of secondary complications. Hypoglycemic agents alone have failed to achieve this destination, may be due to their low efficacy in controlling the oxidative stress resulting from chronic hyperglycemia or prevention of protein glycation and misfolding. Therefore, attempts have been made to enhance the capability of hypoglycemic therapy by supplementation with other agents as a complement or combination therapy [9, 10].

The *in vivo* protein folding is under control by molecular and chemical chaperones, especially in the endoplasmic reticulum (ER). The major class of molecular chaperones is heat shock proteins (HSPs), which have crucial roles in the appropriate folding of proteins and protecting them against misfolding and inappropriate aggregation in various stress conditions [11]. Chemical chaperones, as a group of small molecules, by mimicking the role of molecular chaperones are capable to maintain the native protein conformation under various stresses [12, 13]. Moreover, they are potential regulators for molecular chaperones, leading to enhanced chaperone activity [14]. Two main groups of chemical chaperones have been introduced [15]; among them, amino acids with the free amine group are considered as the predominant candidates for controlling the proteins structure-function [1, 3, 16, 17]. It has been shown that amino acids protect intracellular proteins and also overcome the chemical stress in diabetes [16, 17]. Additionally, they are compatible osmolytes that stabilize protein structure, in addition to induction of molecular chaperones [4]. One of the protective mechanisms of amino acids is their combination with free glucose in the solution and hence, prevention of Glc interaction with free amine groups in the amino acid side chain or N-terminal of proteins; a harmful process which increases during hyperglycemia and result in the cross-link formation [4].

As described in the literature, there might be some changes in the molecular chaperone activity, which are necessary for maintaining protein integrity, in some diseases like diabetes [18, 19]. Therefore, elevating chaperoning capacity would be of potential therapeutic benefits, resulting in the maintenance of the native structure and function of proteins in diabetic patients and finally protecting them against complications [16, 20]. A variety of studies have been conducted on the effectiveness of chemical chaperones or chemical chaperone-like molecules in animal models of diabetes [4, 5, 20, 21]. In continuing with our studies about the improvement effect of Lys in hyperglycemic conditions [1, 3, 22], we investigated here the therapeutic effect of Lys supplementation in T2DM patients in a single-evaluator–blinded, randomized clinical trial. Thus, diabetic patients receiving the same hypoglycemic drugs were randomly divided into two groups. The test group was supplemented, additionally, with Lys. Then, various parameters were evaluated and compared in their blood/serum and urine, after three months.

Subjects, materials, and methods

Subjects

The clinical part of the study was performed at the Department of Endocrinology and Metabolism Research Center, Vali-Asr Hospital, Tehran University of Medical Sciences (Tehran, Iran). Participants were recruited from Diabetologists referrals in this department. The study plan was explained to the potential subjects who were identified based on their medical history and lifestyle assessment. All patients provided informed consent before the study. Also, every patient was requested to fill out the Food Frequency Questionnaire (FFQ) under the supervision of a registered nutritionist before the beginning of the study. FFQs were used to measure participants' eating habits at the baseline. The study protocol was registered as No. 416.871 by the Ethics Committee of the Tehran University of Medical Sciences and IRCT2014120220184N1.

In this interventional single-evaluator–blinded, randomized clinical trial, a total of 50 patients (age 41-75 years, diabetes duration <3 years) of either sex were enrolled. Patients who fulfilled the following criteria were included: (1) T2DM according to the American Diabetes Association (ADA) criteria [23]; (2) glycated hemoglobin (HbA1c) ≥ 7%; (3) normal serum insulin level; (4) no history of ketoacidosis, cancer, hyper- or hypothyroidism, diabetic ulcer, chronic renal failure, liver cirrhosis, feverish diseases, and consumption of corticosteroids with serum creatinine ≥2 mg/dl; and 5) no symptom of hypoinsulinemia such as ketonuria, weight loss, and serum glucose level more than 400 mg/dl. Patients were excluded or deprived from the study due to the following conditions: (1) if they didn't like to continue the therapy, (2) no response to oral agents, (3) need for insulin therapy, and (4) appearance of diabetic ulcer or feverish diseases during treatment. All participants received glibenclamide (10 mg/day) and metformin (1000 mg/day) for 3 months. They were

randomly divided into two main groups (control and treatment), each consisting of 15 females and 10 males. The treatment group was additionally supplemented with oral Lys monohydrochloride 1.5 g (one sachet) twice a day, for three months. The sachets were packaged by the Osveh Pharmaceutical Company, Karaj, Iran. Sampling, clinical assessment, and laboratory evaluation were carried out before and after the trial.

Clinical assessment

A detailed history and physical examination were obtained from all participants. Weight and height were measured in light clothing and without shoes by a calibrated electronic weighing scale and measuring inflexible bars. The constant tension tape was used for the measurement of waist and hip circumference at the end of a normal expiration. Body mass index (BMI) was measured using the Quetelet formula (weight in kilograms divided by height in square meters). Systolic and diastolic blood pressures were measured by calibrated Omron M7 sphygmomanometer (HEM-780-E). Subjects were asked to rest in a sitting position for at least 10 minutes in a calm environment. Three measures with 5 minute intervals were taken and the average was recorded. Systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, and consumption of antihypertensive drugs were considered as hypertension.

Laboratory measurements

All samples were obtained before and after the trial. Morning venous blood samples and urine samples were withdrawn after 12 hours of fasting. Consequently, there were 4 groups of serum samples belonging to two tests (1 and 2) and control (3 and 4) groups, before and after intervention, respectively. Serum quantities of insulin and heat shock protein 70 (HSP70) were measured by insulin enzyme-linked immune assay (ELISA) kit (BioSource Co, Nivelles, Belgium) and HSP70 ELISA kit (Cusabio Biotech Co., LTD, China), respectively. Measurements were assayed by Mindary Elisa Reader Model MR-96A (Germany, Hamburg). The high-performance liquid chromatography (HPLC) apparatus model DS5 (DREW Co, England) was applied for determining serum percentage of glycated hemoglobin (HbA1c). Fasting blood sugar (FBS) was measured according to the enzymatic calorimetric method using glucose oxidase (GOD) test. Fructosamine was measured in ethylene diamine tetra-acetic acid (EDTA) plasma with a Cobus Bio centrifugal analysis (Hoffman La Roche, Basel, Switzerland) by applying the technique previously described [24]. Concentrations of blood urea nitrogen (BUN), creatinine, albumin (Alb), triglyceride (TG), total cholesterol (TC), LDL-c, HDL-c, ALT, AST, ALP, LDH, GGT, CPK, CRP, LPL and lipase of serum and BUN, and creatinine in urine were determined according to enzymatic calorimetric methods (Pars Azmoon commercial kits, Karaj, Iran) which were applied by BT-3000 plus Auto Analyzer (Biotechnica Co, Italy). Urine analysis of Glc was performed by Medi-Test Combi 11 (test strip) (Macherey-Nagel Co, Germany). Lipoprotein lipase (LPL) activity assay kit (Roar Co, USA), Lecithin: cholesterol acyl transferase (LCAT) activity assay kit (Calbiochem Co, USA), and spectrofluorometer model Floustar (BMG Co, Germany) were used for analysis of the two named enzymes. Fully automated paraoxonase activity measurement kit (Rel Assay Diagnostics Co, Turkey) and ELISA microplate reader (Sunrise, Tecan Co, Austria) were applied for assessment of paraxonase-1 (PON1) function. Concentration of advanced oxidation protein products (AOPP) was calculated by the spectrophotometer model UV-3100 (Shimadzu Co, Japan) based on spectrophotometric analysis described by Kalousová et al. [25]; briefly, 200 μl serum consisting of phosphate-buffered saline (PBS) on the basis of 200 μl of chloramine T (0-100 μmol/l), as calibrator, and 200 μl of PBS, as blank, was prepared on Microtiter plate wells. At the end, a mixture of 20 μl acetic acid together with 10 μl of KI 1.16 M was poured into the wells, and absorption in chloramine T units was estimated at 340 nm as previously described [4].

The same spectrophotometer was used for evaluating ferritin reduction ability of plasma (FRAP) based on the method of Benzie et al. assay [26]. FRAP reagent included 300 mmol/l of acetate buffer (pH 3.6), 10 mmol/l of tripyridyl-triazine (TPTZ) in 40 mmol/l HCl and 20 mmol/l $FeCl_3.6H_2O$. After adding 25 μl serum to 750 μl of FRAP reagent, absorbance change (expressed in μmol/l) at 593 nm was analyzed. The observed absorbance results from the establishment of Fe II–TPTZ complex (blue color) from oxidized Fe III (colorless) by electron-donating antioxidants of serum.

The homeostasis model of assessment of insulin resistance (HOMA-IR) was calculated by applying the formula of Matthews et al. (fasting insulin (μU/ml) multiplied by FBS (mg/dl), divided by 405) [27].

Statistical analysis

All the obtained data were statistically analyzed by applying one-way ANOVA (analysis of variance) method followed by post hoc Tukey's test using the SPSS (Statistical Package for the Social Science) software, version 16.0 (SPSS Inc., Chicago, IL, USA), with a 0.05 significance level. The linear correlation between variables was studied by applying Pearson's correlation coefficient. Data are presented as mean ± SD (standard deviation).

Table 1 Demographic and anthropometric data of all patients, before and after three months' intervention

Groups	Treatment (# 25)		Control (# 25)		p value between groups after treatment
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
Age (year)	54.4 ± 8.7	No change	57.3 ± 9.3	No change	Not significant (NS)
Height (cm)	163.7 ± 8.9	No change	163.9 ± 9.4	No change	NS
Weight (kg)	71.6 ± 12.2	71.1 ± 12.2	74.2 ± 9.8	74.5 ± 9.5	NS
BMI (kg/m^2)	26.7 ± 3.4	26.5 ± 3.4	27.7 ± 3.7	27.8 ± 3.5	NS
Waist circumference (cm)	93.0 ± 9.5	92.7 ± 9.8	93.6 ± 8.9	93.9 ± 9.1	NS
Hip circumference (cm)	98.3 ± 8.3	97.7 ± 9.1	97.9 ± 9.6	98.4 ± 8.7	NS
Systolic blood pressure (mmHg)	122.4 ± 9.6	120.4 ± 10.1	121.6 ± 11.4	123.6 ± 9.0	NS
Diastolic blood pressure (mmHg)	74.8 ± 5.2	72.6 ± 8.3	73.4 ± 8.5	74.2 ± 7.5	NS

Treatment group: patients receiving metformin, glibenclamide, and Lys; control group: patients receiving only metformin and glibenclamide

Results

Clinical variables

Data collected from demographic and anthropometric assessment of all patients are illustrated in Table 1. No statistically meaningful changes were observed after supplementation.

Metabolic variables

Tables 2 and 3 show the effect of Lys supplementation on metabolic parameters and the lipid profile of the patients. The data indicated a significant difference in FBS, insulin, and HOMA-IR, between the test group received Lys in comparison with the control group. Table 4 shows the effect of Lys on non-enzymatic glycation of proteins. These data indicated a significant reduction in the HbA1c, fructosamine, and AGEs in the test group after three months of Lys supplementation. In addition, significant differences ($p < 0.05$) were observed between the values of HSP70, LCAT, LPL, PON 1, AOPP, and FRAP after the trial in the treatment group in comparison with the control group. No significant improvement was observed in these parameters among the control group after the trial period.

Urine parameters before and after the intervention are illustrated in Table 5. While urine urea nitrogen and creatinine levels in the control group showed a tendency to increase, there was a reverse tendency in the test group. Only, a significant change was observed in the urine glucose, which decreased significantly in the treatment group.

Various markers (Table 6) are indicating the non-toxic effect of Lys supplementation in the liver, kidney, and heart of the patients. There were no significant differences between these parameters before and after the trial and between two groups under the study.

The correlation between some parameters was also investigated. FBS and HBA1c (Fig. 1a) values were positively correlated ($p < 0.001$). Fructosamine with FBS and HbA1c has also positively correlated ($p < 0.05$, Figure 1b and c). HOMA also showed a positive correlation with both insulin and FBS values ($p < 0.001$ and $p < 0.05$, respectively) (Figure 2a and b).

Serum creatinine and BUN levels were significantly correlated with the same measures in the urine samples ($p < 0.05$ and $p < 0.01$, respectively). FBS and urine glucose after treatment were also positively correlated ($p < 0.001$).

In all tables, in the case of significant differences, the p value of the difference between the data before and after treatment is shown in a row below the data, and the p value of the

Table 2 Serum metabolic parameters of all patients, before and after three months' intervention

Groups	Treatment (# 25)		Control (# 25)		p value between groups before (B) and after (A) treatment
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
FBS (mg/dl)	184.3 ± 38.9 $p = 0.000$	138.4 ± 30.9	187.9 ± 41.9	171.2 ± 39.2	$P_B = 0.752$ $P_A = 0.002$
Insulin (μU/ml)	15.1 ± 1.8 $p = 0.016$	12.6 ± 3.6	15.2 ± 1.4	14.9 ± 2.6	$P_B = 0.898$ $P_A = 0.041$
HOMA-IR (U)	6.8 ± 1.5 $p = 0.000$	4.3 ± 1.1	7.0 ± 1.3	6.3 ± 1.6	$P_B = 0.772$ $P_A = 0.000$

Treatment group: patients receiving metformin, glibenclamide, and Lys; control group: patients receiving only metformin and glibenclamide

Table 3 The lipid profile of all patients, before and after three months' intervention

Groups	Treatment (# 25)		Control (# 25)		*p* value between groups
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
TG (mg/dl)	135.3 ± 35.9	126.7 ± 31.1	148.0 ± 39.7	146.6 ± 37.2	Not significant (NS)
TC (mg/dl)	223.3 ± 38.6	219.8 ± 45.3	217.4 ± 28.3	215.9 ± 47.0	NS
LDL-c (mg/dl)	103.4 ± 15.4	99.6 ± 18.6	104.28 ± 15.6	102.2 ± 15.5	NS
HDL-c (mg/dl)	42.8 ± 4.1	45.1 ± 5.8	43.8 ± 4.3	44.4 ± 6.1	NS

Treatment group: patients receiving metformin, glibenclamide, and Lys; control group: patients receiving metformin and glibenclamide

difference between two groups at the end of the experiment is shown in the right column.

Discussion

The results of the present study indicate the improving effect of Lys supplementation in T2DM. In fact, it is a combination therapy of Lys with usual medicine used in these adult patients. Herein, the patients were assigned in a case-control, simple randomized method to two groups of treatment and control. All patients received glibenclamide and metformin; however, the treatment group was additionally supplemented with Lys, 1.5 g sachets, twice a day. As indicated in the results, there were no significant changes in the demographic and anthropometric parameters, before and after intervention, while significant changes were observed in some markers indicating inhibition of protein glycation, and improvement in biochemical and metabolic parameters due to Lys supplementation.

In DM, glycation of proteins induces some changes in the cellular function and metabolism, which lead to the organ dysfunction [4, 16, 17]; hence, maintaining the structure and function of proteins prevents the development of diabetes

Table 4 The activity or concentration of some proteins under the effect of glycation/oxidation pathway as a result of diabetic complication, before and after three months' intervention

Groups	Treatment (# 25)		Control (# 25)		*p* value between groups before (B) and after (A) treatment
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
HbA1c % (mmol/ml)	9.1 ± 1.5 (75 ± 16) ($p = 0.005$)	7.7 ± 1.7 (62 ± 19)	9.2 ± 1.5 (78 ± 17)	9.0 ± 1.5 (75 ± 17)	$P_B = 0.714$ $P_A = 0.011$
AGEs (FI %)	69.3 ± 4.5 ($p = 0.039$)	63.9 ± 8.4	70.8 ± 5.1	69.2 ± 9.8	$P_B = 0.303$ $P_A = 0.045$
Fructosamine (μmol/l)	408.2 ± 67.9 ($p = 0.000$)	317.2 ± 100.1	409.1 ± 57.6	395.1 ± 87.3	$P_B = 0.963$ $P_A = 0.005$
HSP70 (ng/ml)	26.7 ± 3.4 ($p = 0.000$)	30.6 ± 4.7	25.4 ± 2.5	26.0 ± 2.4	$P_B = 0.126$ $P_A = 0.000$
Lipase (U/l)	39.3 ± 6.8 Not significant (NS)	38.1 ± 6.9	39.3 ± 8.0	37.8 ± 6.8	NS
LPL (pmol/ml/h)	18.7 ± 1.5 ($p = 0.000$)	24.4 ± 4.4	19.7 ± 1.4	20.0 ± 2.9	$P_B = 0.649$ $P_A = 0.000$
LCAT (nmol/ml/h)	30.6 ± 1.1 ($p = 0.000$)	34.5 ± 4.2	31.4 ± 1.1	32.3 ± 3.9	$P_B = 0.820$ $P_A = 0.041$
PON 1 (U/ml)	31.0 ± 8.8 ($p = 0.000$)	42.8 ± 12.4	32.7 ± 4.5	34.1 ± 8.9	$P_B = 0.388$ $P_A = 0.006$
AOPP (μmol/l)	133.9 ± 22.1 ($p = 0.041$)	113.1 ± 15.6	136.7 ± 19.0	134.5 ± 19.3	$P_B = 0.626$ $P_A = 0.025$
FRAP (μmol/l)	1088.0 ± 163.5 ($p = 0.000$)	1309.9 ± 252.9	1048.8 ± 146.1	1105.0 ± 214.1	$P_B = 0.377$ $P_A = 0.014$

Treatment group: patients receiving metformin, glibenclamide, and Lys; control group: patients receiving only metformin and glibenclamide

Table 5 Urine metabolic data of all patients, before and after three months' intervention

Groups	Treatment (# 25)		Control (# 25)		*p* value between groups before (B) and after (A) treatment
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
Creatinine (mg/dl)	14.6 ± 3.1	14.6 ± 2.7	14.6 ± 3.3	15.2 ± 2.9	Not significant (NS)
Urine nitrogen (mg/dl)	17.0 ± 1.9	16.5 ± 2.5	15.7 ± 2.6	16.2 ± 2.5	NS
Glucose	2.48 ± 0.71 $p = 0.000$	1.5 ± 0.88	2.68 ± 0.47	2.16 ± 0.8	$P_B = 0.768$ $P_A = 0.008$

Treatment group: patients receiving metformin, glibenclamide, and L-Lys; control group: patients receiving only metformin and glibenclamide

complications [28]. Chemical chaperones, as a class of small molecules, have been known for their potential to maintain the structure and function of proteins. They are classified into two main groups of osmolytes and hydrophobic compounds [15]. Amino acids are a class of osmolytes that because of their major pharmacological activity has been categorized as pharmacological chaperones [13, 15]. Among amino acids, Lys administration has gained more attention because of having two free amine groups, relatively high safety and nontoxicity, in addition to its chaperoning activity and potential to induce molecular chaperones, like HSP70 [1, 3–5, 29].

Both L- and D-lysine have been introduced as effective inhibitors of the AGEs formation [4, 30]. Furthermore, Lys can potentially lead to Schiff-base linkage with Amadori products, post-Amadori products, carbonyls of open chain sugar, and dicarbonyl fragments [4, 30]. However, among the two mentioned isomers, the L-isomer that is familiar with the body is more preferred. D-Lys as a foreign compound, has induced some degrees of nephrotoxicity in rat [30]. Therefore, L-lysine was administered here as a chemical chaperone and a glycation inhibitor [4, 31, 32].

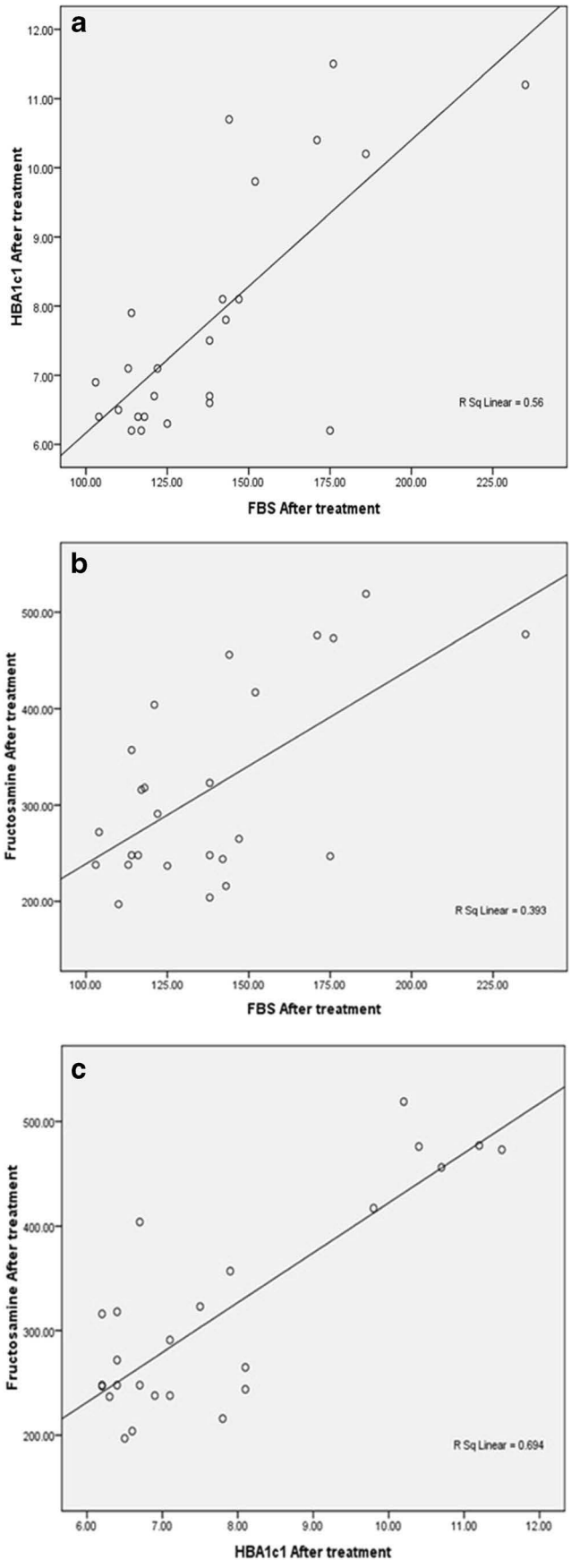
The results indicate the inhibitory effect of Lys on AGEs formation in diabetic patients. The data show both fructosamine (glycated serum protein) and HbA1c, which were significantly decreased after Lys supplementation. The efficacy of Lys in decreasing HbA1c in diabetic rats have also been shown, previously [1, 4, 30]. Although the effectiveness of metformin and glibenclamide administration on cutting down the HbA1c levels in T2DM has been demonstrated [33], the data presented here indicated that Lys increases the effectiveness of this usual treatment. Since, the role of HbA1c, as a risk factor for cardiovascular diseases, has been shown [34]; Lys could be considered as a suitable preventive strategy to control this risk factor.

Lys significantly improved Glc metabolism; as indicated by a significant improvement in FBS, insulin, and HOMA-IR in the treated group in comparison with the level of these parameters before treatment and with the control group without treatment. The latter (HOMA-IR) has been defined as an indicator of insulin resistance and is a marker of NIDDM. Interestingly, Lys increased insulin secretion in type 1 diabetic rats, which may be due to the improvement in the β-cells [4], but here, the reverse effect was observed. The decreased blood glucose, accompanying with a reduced HOMA-IR has been reported previously due to administration of dipeptidyl peptidase-4 inhibitors in diabetic patients [35]. Glueck et al.

Table 6 Parameters related to liver and kidney function, before and after three months of intervention

Groups	Treatment (# 25)		Control (# 25)		*p* value between groups before (B) and after (A) treatment
Parameter (mean ± S.D.)	Before treatment	After treatment	Before treatment	After treatment	
Albumin (g/dl)	4.1 ± 0.5	4.1 ± 0.4	4.2 ± 0.5	4.3 ± 0.3	Not significant (NS)
Creatinine (mg/dl)	0.95 ± 0.17	0.89 ± 0.15	0.95 ± 0.18	0.98 ± 0.16	NS
BUN (mg/dl)	15.5 ± 2.8	14.7 ± 2.2	14.0 ± 2.3	14.3 ± 2.3	NS
AST (U/l)	21.1 ± 3.7	19.4 ± 3.8	19.7 ± 4.6	21.16 ± 3.3	NS
ALT (U/l)	20.2 ± 3.8	18.4 ± 3.5	17.7 ± 3.0	19.6 ± 3.7	NS
GGT (U/l)	24.6 ± 3.3	22.2 ± 4.3	25.4 ± 3.8	26.6 ± 4.7	NS
ALP (U/l)	164.1 ± 27.7	157.9 ± 24.4	151.7 ± 26.9	154.3 ± 26.5	NS
LDH (U/l)	262.9 ± 38.4	239.3 ± 27.3	236.2 ± 24.6	253.0 ± 33.8	NS
CPK (U/l)	126.0 ± 21.8	119.9 ± 23.6	111.0 ± 24.3	114.9 ± 24.1	NS
CRP (mg/l)	5.2 ± 1.1	4.6 ± 1.0	5.4 ± 1.2	6.2 ± 1.2	$P_B = 0.749$ $P_A = 0.075$

Treatment group: patients receiving metformin, glibenclamide, and Lys; control group: patients receiving metformin and glibenclamide



◀ **Fig. 1** Statistical correlation between FBS and glycated proteins. **a** Correlation between HbA1c and FBS. **b** Correlation between fructosamine and FBS. **c** Correlation between HbA1c and fructosamine

have indicated a decrease in HOMA-IR, after metformin therapy and dieting after one year [36]. In this study, the combination of oral hypoglycemic therapy and Lys after three months yielded a significant outcome in terms of HOMA-IR reduction.

Statistical analysis showed the positive correlation of FBS with HBA1c ($p < 0.001$); HOMA-IR, with both insulin and FBS ($p < 0.001$ and $p < 0.05$, respectively), and serum fructosamine level with both FBS and HbA1c ($p < 0.001$). These interesting findings are in accordance with the previously reported data indicating the effectiveness of Lys to reduce the FBS and protein glycation in rat [1, 4]. All of these data indicated that the improvement effect of Lys is through decreasing serum Glc, inhibition of protein glycation, and maintaining the structure and activity of proteins [1, 2, 31, 32].

The induction of oxidative stress has been known as a major complication of diabetes and FRAP has decreased and AOPP has elevated in DM [37]. Moreover, Lys compensated the decreased FRAP level and prevented protein oxidation in diabetic rats [4]. A recently published paper also showed the antioxidative effect of Lys in the C2C12 Myocytes and 3T3L1 adipocytes [3]. Similar results proved in the present study and confirming the anti-oxidative property of Lys, possibly through its antiglycating activity. It means that the decreased glycated products result in a decreased formation of reactive oxygen species intermediates, which is shown by a significant decrease in AOPP and significant increase in FRAP and PON1 as two important parameters for removing and neutralizing the oxidants.

The role of lipid abnormalities, including hypertriglyceridemia, hypercholesterolemia, and low level of HDL [7], as well as the reduced HDL functionality [4, 38], has been considered as the risk factors of cardiovasculopathy in DM [39]. It has been postulated that lipoprotein abnormality in DM is due to the non-enzymatic glycation of receptors, enzymes, and apoproteins involved in their structure and metabolism [12, 40–42]. Lys supplementation has improved the lipid profile and increased the enzymatic activity of PON1 and LCAT enzymes in diabetic rats [4]. Those data showed the increased HDL level and improved HDL function. In the present study, the increased PON1 activity accompanying with the increased serum anti-oxidant capacity delays the LDL oxidation and prevents the atherosclerosis. The increased LCAT activity is also another reason for increasing the HDL functionality and decreasing the risk of atherosclerosis. The unchanged lipid profile in this study was due to our criteria for patient selection. As mentioned above, these parameters were not so high in both groups of patients entered into the study.

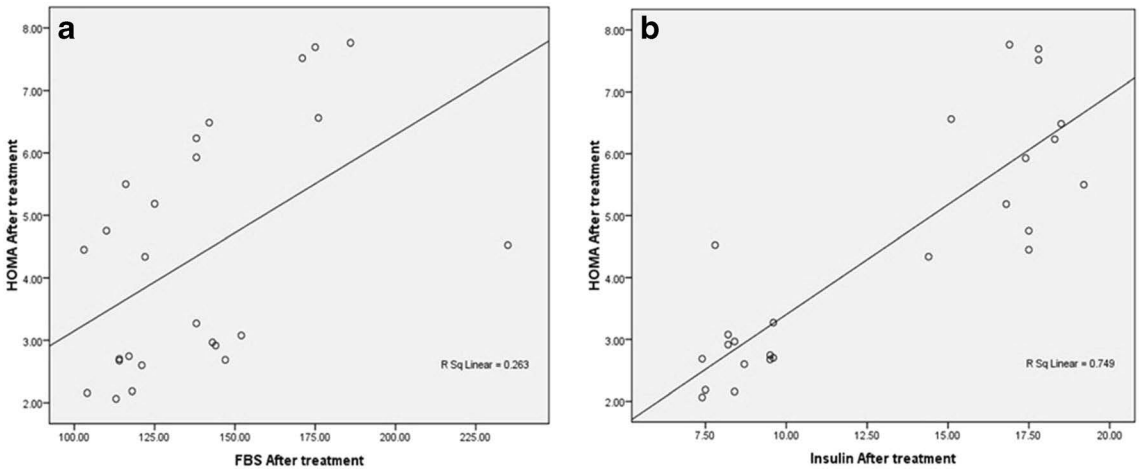


Fig. 2 Statistical correlation between metabolic parameters of glucose. **a** Correlation between HOMA-IR and FBS. **b** correlation between HOMA-IR and insulin

Since the animal study showed that Lys therapy for a longer period of time improved significantly the lipid profile in diabetic rats [4], it is predicted that Lys administration in a long term can prevent the changes in the lipid profile of diabetic patients too, the subject that should be studied in the near future in patients with abnormal lipid profile.

As the results indicated, Lys administration led to a significant increase of serum HSP70. HSP70 has been considered as an important molecular chaperone that exists in both intra- and inter-cellular space, and exhibits various roles including maintaining the structure of proteins and immunomodulation [16, 18]. Our previous study indicated a non-significant decrease in the HSP70 after diabetes induction in rat that significantly increased after treatment [4, 16, 20]. Proteomics analysis also indicated a decrease in this protein in the high Glc condition [43]. Our *in vitro* study confirmed the loss of the activity of glycated HSP70 to refold the denatured leuciferase [44]. It means that both glycation and decreased expression of HSP70 result in the loss of its chaperone activity, which has been reversed in the presence of antiglycating agents [4, 16, 20]. In the present study, we also observed a significant increase in the serum HSP70 after Lys administration in diabetic patient. This may cause the refolding of misfolded proteins and immunomodulation in these patients.

Since the patients who entered in this study had no diabetic complications, and as it is seen in the results, their biochemical parameters related to some organ functions such as liver and kidney were in the normal range; we could not examine the beneficial effect of Lys on these organs. However, the safety and nontoxicity of Lys was confirmed by no significant alterations in both kidney and liver function tests at the end of the experimental period. Jyothirmayi et al. have reported the reduction in the glomerular basement membrane collagen glycation and albuminuria after Lys administration in diabetic rats [5]. The significant decrease in the urine glucose of the supplemented group with Lys here and its positive correlation with FBS are affirmative reasons for the beneficial effect of this amino acid on the renal function of the diabetic patients.

Increased serum CRP has been known as an indicator of inflammation, and indicates a higher risk of cardiovascular diseases, especially in diabetic patients [45]. As indicated in the results, although the CRP level increased in the control group after three months, it showed a trend ($p = 0.07$) to decrease due to Lys supplementation. The parameters that should be studied in the near future in a long term and large population of diabetic patients.

A decrease in insulin secretion, decrease in HOMA-IR, and along with a decrease in HbA1c, fructosamine, and AGEs, accompanying with the improvement in the function of some enzymes and proteins (LPL, LCAT, and HSP70), are indicative of sustained protein structure along with the homeostasis of serum glucose. Increase in the PON1 activity, accompanied by improvement in AOPP and FRAP levels, supports the hypothesis that Lys protects the antioxidant system through inhibiting glycation of the proteins involved. Although patients in the present study were selected with no diabetic complications, at the end of the study, there were no side effects due to Lys administration, and some risk factors of diabetic complication as named above were decreased in the Lys treated group in comparison with the control group. The next step is continuing this study in a large population of diabetic patients with some complications, using Lys for a longer period of time.

In conclusion, daily supplementation with Lys in patients with T2DM who are on standard diabetic oral therapy with hypoglycemic agents proves beneficial effects on glycemic control, and therefore, Lys could be suggested as an effective combination therapy for the management of this entity and protection against various risk factors of diabetic complications, including cardiovascular diseases.

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Declarations

Conflict of interest The authors disclose there is no conflict of interest.

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ORIGINAL ARTICLE

A study on diabetes-related distress among type 2 diabetes mellitus patients using the diabetes distress scale in a tertiary care center in Telangana

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Abstract

Background and objectives Diabetes-related distress refers to the emotional burden and worries that are part of the spectrum of the patient experience, when managing a severe, demanding chronic disease, like diabetes. We aimed to study the association of diabetes-related distress and glycemic control in a cohort of our patients.

Methods We conducted a cross-sectional study among adults with type 2 diabetes in the endocrinology outpatient clinic of a tertiary care center in Telangana, India, during a three-month period between September 2019 and November 2019. We used the diabetes distress scale-17 items (DDS-17) to assess the presence of diabetes-related distress. A score of >2 was considered moderate distress and ≥3 was classified as high distress.

Results We recruited 142 patients. 63 (44.36%) were females and 79 (55.63%) were males. Mean HbA1c of total patients was 61 mmol/mol (7.72 ± 1.62%). The mean age of patients was 53.94 ± 12.25 years. The mean duration of diabetes was 6.56 ± 5.27 years. 12 patients (6 males (7.59%) and 6 females (9.52%)) had severe distress (score > 3). Severe emotional burden was found in 20 patients (14%), interpersonal distress among 6 patients (4%), and regimen-related distress among 19 patients (13.38%). There was a significant increase in the total DDS scores and the subscores with increasing diabetes duration. There was a significant positive correlation between HbA1c and total DDS scores. There was no correlation of higher DDS with older age groups.

Conclusions Diabetes distress and glycemic control have a strong correlation with each other, and both need to be addressed during management of type 2 DM.

Keywords Diabetes-related distress · Emotional burden · Glycemic control

Introduction

Diabetes is one of the most common metabolic disorders globally, of which India is one of the greatest contributors. It is estimated that there will be more than 123 million diabetic population in India by 2040 [1]. Not just the numbers but the economic impact of diabetes is so high that 12% of global health expenditure is being spent on diabetes [2]. It is the chronicity and the associated complications of this condition which impart a significant burden on the patient, society, and the nation as a whole, both economically and emotionally [3].

Once the patient is diagnosed to have diabetes, many complex changes have to be brought about in the lifestyle from strict adherence to medication, diet, physical activity, to frequent blood glucose monitoring. Diabetes-related distress refers to the unique and hidden emotional burdens that are part of the patient experience, while managing severe, demanding chronic diseases, like diabetes [4]. It is defined as "patient

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concerns about disease management, support, emotional burden, and access to care." Previous reports have suggested that most patients with highly depressive affect are not necessarily clinically depressed, but may rather be suffering from high levels of diabetes-related distress [5].

The prevalence of diabetes-related distress in previous studies ranges from 18 to 35% [3, 6]. This was also associated with negative acceptance of insulin therapy [3]. Many previously published studies suggest that moderate and severe diabetes distress may be associated with poor glycemic control, low self-care, and poor quality of life even in the absence of clinical depression or after it is controlled [7]. Clinical depression, depressive affect, and anxiety disorders all have been linked with poor disease management, days of missed work, significantly higher healthcare expenses, and mortality in diabetes. This could be the result of not only an underlying psychiatric illness but also high levels of diabetes-related distress or even coexisting diabetes-related distress with some degree of depressive affect [8].

As there is limited literature published concerning this issue in the south Indian population coupled with a high prevalence of diabetes in this population, this study aims to look at the association of diabetes-related distress among patients with type 2 diabetes in the south Indian state of Telangana as measured by the DDS and glycemic control as measured by HbA1c.

Materials and methods

This was conducted as a cross-sectional study over a period of 3 months from 1st September to 30th November 2019 in the endocrinology outpatient clinic of a tertiary care center in Hyderabad, India. The inclusion criteria consisted of patients above 18 years of age who had type 2 diabetes mellitus irrespective of their duration of illness or diabetic treatment. Patients with chronic medical or surgical illness other than diabetes, patients on long-term treatment for other medical illness, patients who were terminally ill, patients who required immediate hospitalization for a serious illness, patients who were on corticosteroids or any psychotropic drug, those with known comorbid psychiatric illnesses, and those who were not willing to participate in the study were excluded from the study.

The sample size was calculated by the formula $n = Z^2pq/d^2$, where Z is 1.96 with a confidence interval of 95%, p is the prevalence of 18% based on the prevalence reported by Fischer et al. [3], q is $1-p$, and d is the precision limit or proportion of sampling error of 7%. The sample size was calculated to be 115.7 patients. A total of 142 patients were enrolled in the study.

Informed consent of the patient and ethics committee approval were taken. The patients' demographic data and medical history including drug history were documented. Laboratory results were collected from the patients' records when they came for their routine follow-up visits to the clinic. HbA1c was measured using the HPLC method. At the same visit, the diabetes distress scale (DDS) consisting of 17 questions was used to measure distress among diabetes patients across four domains: physician-related distress, emotional burden, interpersonal distress, and regimen distress. The DDS was administered in either the local language or in English, as per patient preference. The responses of the patients were recorded using a 6-point Likert scale with the following grading: 1 or 2—not a problem, 3 or 4—moderate problem, and 5 or 6—serious problem. The patients' responses for each of the subgroups were added and then divided by the number of items in that particular subgroup. Study subjects with a total score of <2.0 were considered to have little or no distress, those with a score between 2.0 and 2.9 were considered to have moderate distress, and those with ≥3.0 were considered to have high distress.

The collected data was entered in Microsoft Excel. The statistical analysis was carried out by using the GraphPad Prism, version 4.0. The statistical data were described by using mean (S.D.) for the continuous variables and proportions for the categorical variables. The t test was used to assess the statistical significance to compare continuous variables. Spearman's rank correlation coefficient (r) which is a non-parametric measure of rank correlation was used to assess the relationship between glycemic control (HbA1c) and the diabetes distress scale. A p value less than 0.05 was considered to be statistically significant.

Results

A total of 142 patients were recruited, out of which 63 (44.36%) were females and 79 (55.63%) were males. Mean HbA1c of all the patients was 61 mmol/mol (7.72 ± 1.62 %), and the mean age of patients was 53.94 ± 12.25 years. The mean duration of diabetes in this study was 6.56 ± 5.27 years.

A total of 12 patients (6 males (7.59%) and 6 females (9.52%)) had severe distress (score > 3), while 8 patients had a DDS score between 2 and 3. Analysis of the DDS score results indicated that 14% of patients in this study had moderate to severe diabetes-related distress based on the total score of the questionnaire. Severe emotional burden was found in 20 patients (14%), while 11 (7.7%) patients had a score between 2 and 3. Severe interpersonal distress was found among 6 patients (4%) while 5 patients (3.5%) had a score between 2 and 3. 19 patients (13.3%) had a severe regimen-related distress while 5 patients (3.5%) had a score between 2 and 3. There was 1 patient (0.7%)

Table 1 DDS scores and subscores in the study population

Distress score (*n*/%)	Score < 2	Score 2–3	Score > 3
Total diabetes distress score	122 (85.92)	8 (5.63)	12 (8.45)
Emotional burden	11 (78.17)	11 (7.75)	20 (14.08)
Interpersonal distress	13 (92.25)	5 (3.52)	6 (4.23)
Physician-related distress	138 (97.18)	3 (2.11)	1 (0.70)
Regimen-related distress	118 (83.10)	5 (3.52)	19 (13.38)

Table 3 Correlation between age and diabetes distress scores

Age group	<60 yrs	>60 yrs	*p* value
Total diabetes distress	22.56±11.6	26.85±15.87	0.0712
Emotional burden	7.69±5.36	9.17±7.10	NS
Interpersonal distress	3.44±1.47	4.04±2.43	0.0706
Physician-related distress	4.35±1.26	4.54±1.29	NS
Regimen-related distress	7.19±5.00	8.43±6.15	NS

with severe physician-related distress and 3 patients (2.1%) with a score of 2 to 3 (Table 1).

Patients with higher DDS scores (>2) were more likely to be female (DDS score 2–3, 11.11% vs. 1.26%) (DDS score > 3, 9.52 % vs. 7.59%) (Fig. 1). In the patients with a DDS score less than 2, male patients predominated (91.1% vs. 79.3%). Patients with higher DDS scores (>2) were more likely to be older (DDS score 2–3, 28.26% vs. 20%) (DDS score > 3, 6.52% vs. 1.05 %). There was a significant increase in the total DDS scores and the subscores with increasing diabetes duration, with the highest DDS found in the longest duration of diabetes (Table 2). In the patients with a DDS score less than 2, younger patients predominated (78.95% vs. 65.22%). There was no significant correlation between DDS or subsets of DDS and older age group (Table 3). There was a significant positive correlation between higher HbA1c values and higher total DDS scores, regimen-related distress, and emotional burden (Fig. 2). In males, those with higher DDS scores had higher HbA1c which was statistically significant. In females, those with DDS scores > 3 had significantly higher HbA1c compared to those with DDS scores < 2.

Discussion

To assess the significance of diabetes-related emotional and behavioral issues, various scales have been designed and tested on patient population such as ATT39 [9], Questionnaire on Stress in Patients with Diabetes-Revised (QSD-R) [10], and Problem Areas in Diabetes (PAID) scale [11]. All these measures had some limitations for general clinical use. ATT39 and QSD-R are not brief and may not be suitable for use in centers with high patient loads due to time constraints. The PAID scale, though brief, does not have subscales and hence may not differentiate the different types of diabetes-related emotional distress. These lacunae in assessment and the growing need for a comprehensive and simple scale to assess the diabetes-related distress led to the development of the diabetes distress scale-17 items [12]. The DDS has certain advantages over the other scales. The subscales in DDS, by comparing four different types of distress, aid the clinician in making appropriate interventions. It is also more comprehensible to the patients compared to the other instruments.

By using the DDS 17-item scale, this study showed that moderate to severe diabetes-related distress was present in 14% of the studied population. Previous studies have shown a high degree of heterogeneity in prevalence of diabetic distress. A study from Saudi Arabia showed that around 25% of the patients with diabetes had moderate to high diabetes-related distress [8]. A study from the USA showed that 51.3% had moderate to high diabetic distress [13]. A study from Malaysia revealed that 49.2% of their diabetic population had moderate distress [14].

Table 2 Correlation between diabetes duration and diabetes distress scores

Distress type	Diabetes duration			*p* value vs. 1–5 yrs
	1-5 yrs (mean ± SD)	5-10 yrs (mean ± SD)	>10 yrs (mean ± SD)	
Diabetes distress	19.98±8.39	26.72±15.86*	29.9±14.24**	*p=0.0027, **p=0.0002
Emotional burden	6.37±3.94	9.23±6.99*	11.45±6.73**	*p=0.0044, **p=0.0001
Physician-related distress	4.20±0.77	4.47±1.43	4.9±1.80*	*p=0.0134
Interpersonal distress	3.14±0.54	4.0±2.39*	4.2±2.35**	*p=0.0033, **p=0.0009
Regimen-related distress	6.40±4.48	8.55±6.04*	9.15±5.57**	*p=0.0227, **p=0.0253

* 5–10 years vs. 1–5 years of diabetes duration; ** >10 years vs. 1–5 years of diabetes duration

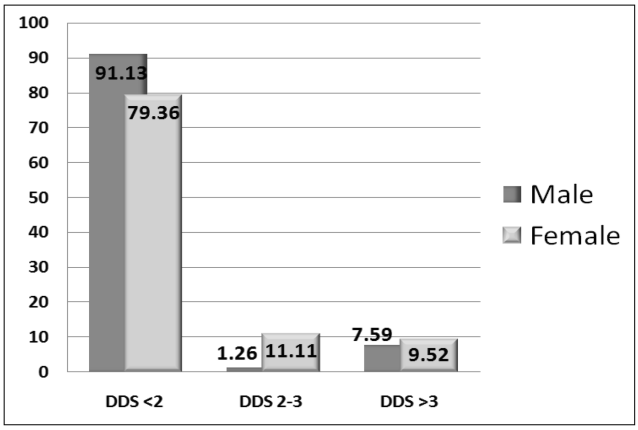

Fig. 1 Bar diagram depicting gender-wise difference in the DDS scores (percentage distribution)

Previous studies have also shown that diabetes-related distress, which is distinct from clinical depression, is associated with poorer treatment outcomes [15]. Diabetes-related distress has been found to be more prevalent than depression in these patients. The prevalence of diabetes-related distress in our study was 14% compared to studies conducted in Bangladesh, Malaysia, and Turkey which showed prevalence of 48.5%, 39%, and 24%, respectively [3]. Such a variation in prevalence might be due to comorbid depressive symptoms and the heterogeneity of the study population. Diabetes-related distress and physician-related distress were lower in our study compared to studies conducted in other developing countries, which might be due to the tertiary care nature of our center, where the study was conducted.

Emotion-related distress was very high among patients, which might be due to the chronicity of the disease that poses difficulty in self-management and care. These findings were consistent with the study done by Deepak et al. where the prevalence of diabetes distress was 18%, of which 16.1% of the patients had emotion-related distress and only 1.2 % of the patients had physician-related burden [16]. Similar to the previous studies, our study showed that the duration of diabetes was significantly associated with diabetes-related distress and two of its domains, namely, emotional burden and regimen-related distress.

Although depression and diabetes-related distress are similar conditions, a distinct line has been drawn between the two with respect to the prevalence as well as the clinical and biochemical features. Research has shown that there is a notable effect of diabetes-related distress on HbA1c whereas the effect of depression is equivocal. A study by Fisher et al. showed that diabetes-related distress, and not clinical depression, had significant cross-sectional and longitudinal relationship with HbA1c [3].

Similar findings have been recorded in our study where we have shown a significant correlation between HbA1c and the degree of diabetes-related distress. Nevertheless, this correlation could be bidirectional, i.e., poor glycemic control leads to high distress and similarly excessive distress could further accentuate hyperglycemia due to poor self-care, thereby perpetuating the cycle. These findings however highlight the importance of addressing diabetes-related distress to achieve a good glycemic control and vice versa. The higher DDS scores

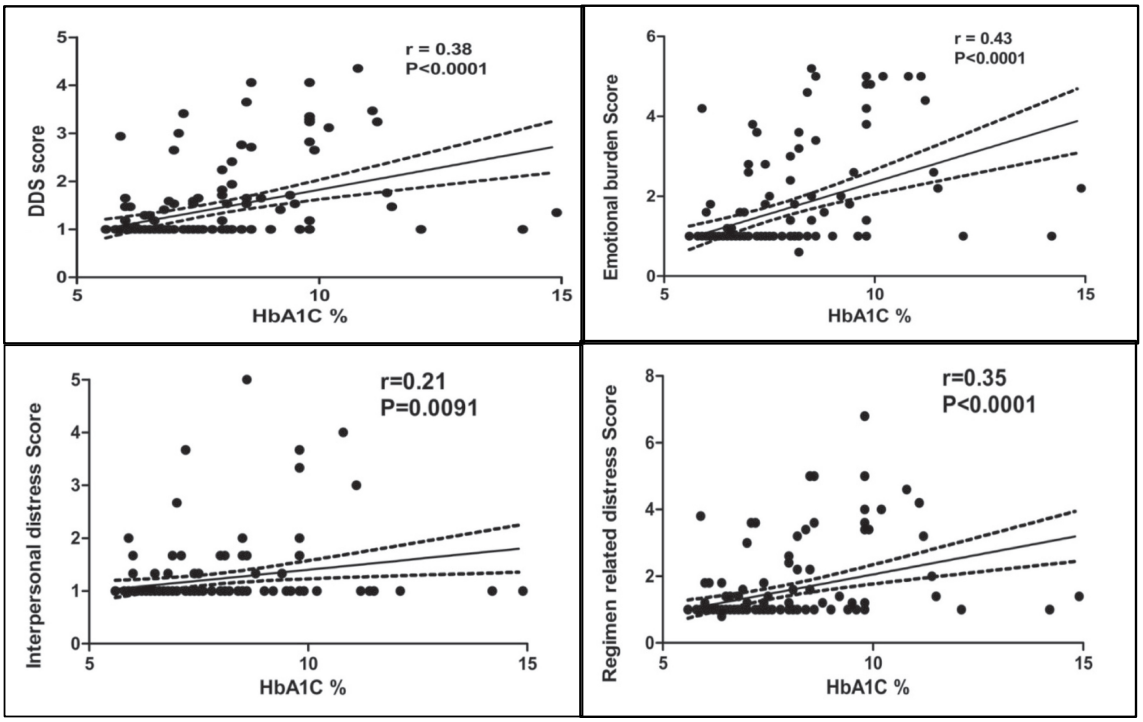

Fig. 2 Scatter plot showing correlation between HbA1c and the DDS scores. *r*, correlation coefficient

may pose a great challenge in achieving adequate glycemic control by virtue of poor self-care that ensues. In converse, inadequate glycemic control can lead to complications of diabetes which predispose to a higher DDS score.

This vicious cycle has to be broken by addressing both the diabetes-related distress and the poor glycemic status. In a study by Kumar et al. from Mangalore, it was shown that participants with low regimen-related distress, physician distress, and low interpersonal distress were found to have good adherence to antidiabetic medication [17]. This suggests that achieving adequate glycemic control by virtue of compliance to treatment may help in decreasing or reversing DDS.

Although we have not specifically studied treatment adherence in this study, better glycemic control, as reflected by HbA1c, was seen in those with lower scores on the DDS. This is important when addressing the poor glycemic status of a particular patient in spite of a reasonable regimen for treating diabetes. We may be overlooking the regimen-related distress, which may interfere with the adherence to medications.

Our study showed significant correlation with diabetes duration and female sex, but not with increasing age. Distress was seen more among females compared to males. This might be due to gender-related confounders like pregnancy, menstrual changes, social responsibilities, educational status, and the general idea that males are more physically and emotionally strong. In a developing country like India, this could also be contributed by the economic dependence of females on the males in the household. Another possible explanation for this discrepancy between males and females could be due to the likely possibility that men are less likely to expose their distress due to fear of emasculation or the need to appear capable, especially in patriarchal societies. The absence of a correlation between diabetes-related distress and age may highlight that this is a separate entity from depressive affect, which is commonly encountered in the geriatric age group [18]. Previous studies however have shown correlation between diabetes-related distress, especially certain subgroups such as regimen-related distress and older age groups [8].

Once identified, the management plan for diabetes-related distress should include optimization of self-care practices and coping skills coupled with a strict watch on the glycemic control. Regular follow-up and adherence to treatment are paramount. Repeat screening for glycemic control as well as diabetes-related distress every 3 to 6 months may also be appropriate after the initial intervention [8].

Limitations

With our study being a cross-sectional one, the results cannot be interpreted for long-term conclusions. This study has a small sample size and was conducted at a tertiary center; therefore, the results cannot be extrapolated to the entire population. Further research is needed to look at the diabetes-related distress and its association with socioeconomic status, educational status, drug regimens, and long-term complications. We were also unable to simultaneously screen for any underlying undiagnosed psychiatric disorder which might have confounded the results. Interventional studies tackling the specific domain may go a long way in achieving good glycemic control, and this may translate into a reduced incidence of diabetes-related complications. Clinicians need to be aware and address this issue of diabetes-related distress and its ensuing complications to reduce the disease burden as well as the economic burden of dealing with the diabetes-related complications, especially because of the galactic increase in the prevalence of diabetes in our country.

Conclusions

Our study reiterates the findings from previous studies that diabetes-related distress is one of the important factors that determine glycemic control in patients with diabetes. We have observed a significant positive correlation between the total DDS scores and subscores with factors like HbA1c and duration of diabetes. However, this correlation could be bidirectional. These findings highlight a very important but often neglected parameter that needs to be addressed in caring for patients with diabetes. However, in centers catering to a large number of patients, it may not always be possible to screen for diabetes distress. Hence, we recommend screening for diabetes distress using the DDS scale at least in patients with poor glycemic control so that the scores in the different domains will guide us to tackle the issue.

Declarations

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Effects of comprehensive nursing intervention on maternal and infant outcomes for gestational diabetes mellitus patients

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Abstract

Background Gestational diabetes mellitus (GDM) is a disease which seriously affects the maternal and infant outcomes. With the progress of the medical technology, more attention has been paid on the nursing intervention for GDM patients. This study aimed to investigate the effects of comprehensive nursing intervention on maternal and infant outcomes for GDM patients.

Methods Ninety-three GDM patients were randomly divided into intervention group (45 cases) and control group (48 cases). During the perinatal period, the control group received the routine nursing (one-off health education and nutrition and exercise guidance, regular pregnancy monitoring, regular postpartum care, etc.). The intervention group received the comprehensive nursing intervention including psychological intervention, health education, diet control, exercise intervention, pregnancy monitoring, and prevention of postpartum complications. The pregnancy indexes, anxiety and depression scores, pregnancy outcome, perinatal infant condition, and nursing satisfaction degree were compared between two groups.

Results After nursing, compared with control group, in intervention group, the pregnancy weight gain, amniotic fluid index, fasting blood glucose, 2-h postprandial blood glucose, and glycosylated hemoglobin were significantly decreased; the Self-Rating Anxiety Scale and Self-Rating Depression Scale scores were significantly decreased ($p < 0.01$); the incidences of gestational hypertension, premature birth, hydramnion, cesarean section, fetal distress, urinary tract infection, and postpartum hemorrhage were significantly decreased ($p < 0.05$); and the incidences of neonatal hypoglycemia, asphyxia neonatorum, and hyperbilirubinemia in intervention group were significantly decreased ($p < 0.05$). The nursing satisfaction rate in intervention group was significantly higher than that in control group ($p < 0.05$).

Conclusion For patients with GDM, the appropriate comprehensive nursing intervention can effectively improve the maternal and infant outcomes.

Keywords Comprehensive nursing intervention · Gestational diabetes mellitus · Outcomes

Introduction

Gestational diabetes mellitus (GDM) is a kind of transient diabetes caused by poor carbohydrate tolerance after the onset of pregnancy. It is accompanied by obvious metabolic changes and is one of the common complications during perinatal period. GDM usually occurs in the middle and late stages of pregnancy [1]. According to the guidelines issued by the American Diabetes Association (ADA) in January 2011, the pregnancy with diabetes occurring before pregnancy beginning, namely, diabetes combined with pregnancy, is excluded from GDM [2]. According to statistics, the amount of GDM patients shows a growth trend in recent years, and the incidence rate is as high as 0.15–13.8% [3]. This rapidly rising trend is closely related to modernization of life pattern, changes in dietary structure, accelerated urbanization degree, increased life pressure, lack of physical activities, obesity, environment and social factors, and improvement of diagnosis technology [4]. It is found that the occurrence of GDM often leads to complications such as hypertension and hydramnion, which reduces the natural childbirth rate, and leads to abnormal fetal development, huge fetal, and dyslipidemia, seriously threatening the health and safety of the fetus [5]. Diabetes mellitus along with hypertension and dyslipidemia is a major risk factor for cardiovascular diseases [6–9]. Their incidence is also rising in pregnant women as well. However, many

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pregnant women and their family members do not have a clear understanding of the dangers of GDM, so the compliance during pregnancy is poor, which leads to worse maternal and fetal prognosis. Therefore, more attention has been paid on the nursing intervention for GDM patients. With the transformation of modern medical mode, the clinical nursing is no longer a simple nursing on disease itself. The psychological, physiological, and social support factors of patients also cause widespread concern among medical staff. Research has shown that the comprehensive nursing intervention on the psychological, physiological, and social support aspects can not only reduce the severity of psychological disorders in patients but also reduce the incidence of disease complications, thus improving the treatment compliance and outcome [10–13]. This study investigated the effects of comprehensive nursing intervention on the maternal and infant outcome of GDM patients. The objective was to provide a reference for the further application of comprehensive nursing intervention to improving the maternal and infant prognosis for GDM patients.

Subjects and methods

Subjects

Ninety-three GDM patients treated in author's affiliation from January 2017 to September 2017 were enrolled in this study. The age of patients was 23–35 years, with average of 26.88 ± 3.15 years. The pregnancy frequency was 1–4 times, with average of 1.82 ± 0.34 times. The pregnancy duration was 20–28 weeks, with average of 24.12 ± 3.68 weeks. The inclusion criteria were as follows: (i) the patients met the diagnostic criteria of GDM, (ii) Chinese Han nationality, (iii) single live fetus, (iv) regular menstrual cycle, (v) no smoking and drinking history, and (vi) no placental abnormality by ultrasonography. The exclusion criteria were as follows: (i) multiple pregnancy; (ii) threatened abortion; (iii) threatened premature labor; (iv) old diabetes; (v) liver, kidney, or thyroid dysfunction; and (vi) the patients had poor compliance and could not complete the study. This study was approved by the ethics committee of author's affiliation. Written informed consent was obtained from all participants.

Nursing methods

The patients were divided into the control group (48 cases) and intervention group (45 cases). During the perinatal period, the control group received the routine nursing (one-off health education and nutrition and exercise guidance, regular pregnancy monitoring, regular postpartum care, etc.). The intervention group received the comprehensive nursing intervention. According to the gestational age of pregnant women, the intervention measures were divided into 3 stages: first stage (< 28 gestational weeks), second stage (28–36 gestational weeks), and third stage (from 36 gestational weeks to discharge). At the first stage, the nursing was focused on the psychological intervention and health education, combined with diet control and exercise intervention. At the second stage, the nursing was focused on pregnancy monitoring, combined with diet control and exercise intervention. At the third stage, the nursing was focused on psychological intervention, perinatal care, and prevention of postpartum complications.

Psychological intervention At the first stage, the patients were easy to produce negative emotions such as tension, anxiety, and fear. The nursing staff communicated timely with patients to make them correctly understand GDM. The patient confidence to treatment was enhanced, and the compliance of treatment was improved through the explanation of successful cases. At the second stage, the mental state of pregnant women is relatively stable. The nursing staff kept the communication with patients to make them always in a relatively good mental state. At the third stage, the patients were prone to negative feelings of childbirth. The nursing staff explained the childbirth-related experience to them and provided a quiet, comfortable, and warm parturient room to keep the pregnant women in a good state of mind.

Health education At the first stage, the education was focused on the GDM-related knowledge. At the second and third stages, the education was focused on exercise regulation, diet control, hypoglycemia and hyperglycemia management, emotional control, compliance with the doctor's orders, drug taking, and so on. The specific implementation methods included multimedia teaching, WeChat, QQ, health education short film, health manual reading, discussion, and so on.

Diet control The diet control was performed by the professional nutritionist based on the data of prenatal weight, weight gain during pregnancy, and exercise intensity consumption. The appropriate amount of protein and energy was supplemented at different stages. The patients were guided to rationally choose food and were taught to record the dietary diary.

Exercise intervention The nursing staff guided the patients to perform different exercise at different stages, which included walking, yoga, swimming, and family-based upper limb exercise. The exercise should be accompanied by family members, and the fatigue signals were closely observed. In addition, the regular follow-up was made to guide patients to carry out proper exercise and supervise the patients to daily record the exercise.

Pregnancy monitoring The blood glucose of patients was daily monitored, and the diet and exercise were adjusted

according to the blood glucose condition. The regular production examination was performed to monitor the fetal intrauterine condition. The weight, uterine height, and abdominal circumference were weekly measured. The blood pressure was daily monitored. The fetal heart sound was regularly monitored. The patients were hospitalized for delivery at about 35 weeks of gestation. At 37–38 weeks, the pregnancy was terminated after the comprehensive understanding of the fetal maturity.

Prevention of postpartum complications The prevention of postpartum complications was mainly performed at the third stage. The nursing staff guided the patients to make good personal hygiene management and do the cleaning and washing in time. During the nursing, the nursing staff ensured the strict implementation of aseptic operation. The infection or cross infection during the hospitalization was avoided. The ventilation was daily kept in the ward.

Observation indexes

On the day of delivering, the pregnancy indexes including pregnancy weight gain, amniotic fluid index, fasting blood glucose (FBG), 2-h postprandial blood glucose (2 h-PBG), and glycosylated hemoglobin (HbA1c) were measured. The anxiety and depression indexes including Self-Rating Depression Scale (SAS) [14] and Self-Rating Anxiety Scale (SDS) [15] were scored. In addition, the maternal pregnancy outcomes including gestational hypertension, placenta previa, premature birth, hydramnion, cesarean section, fetal distress, premature rupture of fetal membranes, urinary tract infection, postpartum hemorrhage and postpartum dominant diabetes, and perinatal infant health conditions including neonatal hypoglycemia, asphyxia neonatorum, neonatal hypoxic-ischemic encephalopathy, hyperbilirubinemia, macrosomia, fetal malformation, and neonatal pneumonia were evaluated. Using the self-made nursing satisfaction questionnaire, the working attitude and nursing quality were evaluated by the patients in two groups. The scores ≥ 90 points, 60–89 points, and < 60 points presented the very satisfied, satisfied, and dissatisfied, respectively. The satisfaction rate was calculated as follows:

$$\text{satisfaction rate}\ (\%) = ([\text{very satisfied number} + \text{satisfied number}]/\text{total number}) \times 100.$$

Statistical analysis

All statistical analysis was carried out using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The enumeration data were presented as number and were compared using χ^2 test. The measurement data were presented as means ± SD and were compared using paired t test. $p < 0.05$ was considered as statistically significant.

Results

General information of patients in two groups

General information of patients in two groups was shown in Table 1. There was no significant difference of age, education degree, occupation, pregnancy frequency, or pregnancy duration between intervention and control groups ($p > 0.05$).

Comparison of pregnancy indexes between two groups

As shown in Table 2, after comprehensive nursing intervention, the pregnancy weight gain, amniotic fluid index, FBG, 2 h-PBG, and HbA1c in the intervention group were significantly lower than those in control group, respectively ($p < 0.05$).

Comparison of SAS and SDS scores between two groups

Table 3 showed that, during the nursing period, the SAS score in intervention group was 41.78 ± 8.33 points, which was significantly lower than 49.72 ± 9.21 points in control group ($p < 0.01$). The SDS score in intervention group was 42.83 ± 7.03 points, which was also significantly lower than 52.31 ± 8.49 points in control group ($p < 0.01$).

Comparison of maternal pregnancy outcome between two groups

The maternal pregnancy outcomes in two groups were shown in Table 4. The incidences of gestational hypertension, premature birth, hydramnion, cesarean section, fetal distress, urinary tract infection, and postpartum hemorrhage in intervention group were significantly lower than those in control group, respectively ($p < 0.05$). There was no significance of each index between two groups ($p > 0.05$).

Comparison of perinatal infant condition between two groups

The perinatal infant conditions in two groups were shown in Table 5. The incidences of neonatal hypoglycemia, asphyxia neonatorum, and hyperbilirubinemia in intervention group were significantly lower than those in control group, respectively ($p < 0.05$). There was no significance of each index between two groups ($p > 0.05$).

Table 1 General information of patients in two groups

Parameter	Intervention group	Control group	t/χ^2	p
n	45	48		
Age (years)	26.33 ± 2.02	27.03 ± 2.44	− 1.609	0.111
Education degree [n(%)]			0.866	0.649
College and above	29 (64.45)	34 (70.83)		
High school	11 (24.44)	8 (16.67)		
Junior high school and below	5 (11.11)	6 (12.50)		
Occupation [n(%)]			0.205	0.651
Farmer	5 (11.11)	4 (8.33)		
Non-farmer	40 (88.89)	44 (91.67)		
Pregnancy frequency (times)	1.75 ± 0. 52	1.94 ± 0.62	1.596	0.114
Pregnancy duration (weeks)	25.21 ± 4.08	23.75 ± 3.33	1.896	0.061

Table 2 Comparison of pregnancy indexes between two groups

Pregnancy index	Intervention group	Control group	t	p
n	45	48		
Pregnancy weight gain (kg)	15.44 ± 3.22	16.88 ± 2.45	2.436	0.017
Amniotic fluid index (mm)	107.57 ± 24.67	122.34 ± 26.12	2.799	0.006
FBG (mmol/L)	4.36 ± 0.78	5.68 ± 0.94	7.343	< 0.001
2 h-PBG (mmol/L)	6.67 ± 0.98	8.12 ± 0.85	7.600	< 0.001
HbA1c (%)	7.10 ± 0.91	7.81 ± 0.82	3.888	< 0.001

BMI, body mass index; FBG, fasting blood glucose; 2 h-PBG, 2-h postprandial blood glucose; HbA1c, glycosylated hemoglobin

Comparison of patient satisfaction with nursing between two groups

In intervention group, there were 21 (46.67%) and 22 (48.89%) patients very satisfied and satisfied with the nursing. In control group, there were 13 (27.08%) and 20 (41.67%) patients satisfied and very satisfied with the nursing. There was significant difference of satisfaction rate between two groups ($\chi^2 = 6.222$, $p = 0.012$) (Table 6).

Table 3 Changes of SAS and SDS score before and after nursing in two groups (points)

Index	Intervention group	Control group	t	p
n	45	48		
SAS	41.78 ± 8.33	49.72 ± 9.21	4.351	< 0.001
SDS	42.83 ± 7.03	52.31 ± 8.49	5.844	< 0.001

SAS, Self-Rating Anxiety Scale; SDS, Self-Rating Depression Scale

Discussion

GDM is a common disease in the middle and late pregnancy. There are differences in the incidence of GDM among different regions, but the understanding on serious GDM harm to mother and infant life and health is clear [16]. In addition, due to the lack of typical symptoms of GDM in clinical manifestations and the special and complex physiological metabolism of pregnant women, the difficulty of preventing and treating GDM is significantly increased, and the fetal death, premature birth, neonatal respiratory distress, cesarean section, and other symptoms often occur due to GDM [17]. In terms of current conditions, the most effective means of treating diabetes are the insulin injection and oral administration of hypoglycemic agents. However, this is not acceptable for GDM patients, based on safety and patient acceptability. Therefore, the GDM patients can only be treated through comprehensive nursing intervention such as psychological intervention, health education, diet control, exercise intervention, and so on.

Studies [18, 19] have shown that the need of health education in GDM patients and family members is urgent and multifaceted. Through health education intervention, the diet, exercise, and personal hygiene habits of GDM patients can be

Table 4 Comparison of maternal pregnancy outcome between two groups [n(%)]

Index	Intervention group	Control group	χ^2	p
n	45	48		
Gestational hypertension	1	7	4.514	0.034
Placenta previa	1	1	0.0021	0.963
Premature birth	2	9	4.558	0.033
Hydramnion	2	11	6.591	0.010
Cesarean section	15	30	7.912	0.005
Fetal distress	1	8	5.544	0.019
Premature rupture of fetal membranes	2	3	0.149	0.670
Urinary tract infection	2	10	5.551	0.018
Postpartum hemorrhage	0	4	3.919	0.048
Postpartum dominant diabetes	0	3	2.906	0.088

Table 5 Comparison of perinatal infant condition between two groups [n(%)]

Parameter	Intervention group	Control group	t	p
n	45	48		
Neonatal hypoglycemia	1	10	7.714	0.005
Asphyxia neonatorum	2	12	7.674	0.006
Neonatal HIE	0	1	0.927	0.336
Hyperbilirubinemia	16	32	9.002	0.003
Macrosomia	3	5	0.415	0.519
Fetal malformation	0	1	0.948	0.330
Neonatal pneumonia	0	2	1.916	0.166

HIE, hypoxic-ischemic encephalopathy

effectively improved, and the compliance of patients to treatment is enhanced; thus, the pregnancy outcome is significantly improved. The psychological intervention can establish a good relationship between nurses and patients and alleviate the patient's bad mood state by using the scientific nursing methods [20]. Pengpid et al. [21] find that the psychological intervention can keep the patient emotionally stable and prevent the patient from blood glucose increase due to emotional excitement. Dietary control is the basis and the most important factor in the treatment of GDM. Through diet control, the weight gain during pregnancy and delivery weight of pregnant women can be effectively managed and controlled, so the pregnancy outcome can be effectively improved [22]. In addition, it is believed that the reasonable diet plus exercise therapy is the first choice for the treatment of GDM. In 75–80% GDM pregnant women, the blood glucose can be controlled in the ideal range by adjusting the lifestyles including diet and exercise [23, 24]. In this study, the effects of comprehensive nursing intervention on GDM patients were investigated. Results showed that after comprehensive nursing intervention, the pregnancy weight gain, amniotic fluid index, FBG, 2 h-PBG, and HbA1c in the intervention group were significantly lower than those in control group, respectively ($p < 0.05$), and the SAS and SDS scores in intervention group were significantly lower than those in control group, respectively ($p < 0.01$). In addition, the incidences of gestational

Table 6 Comparison of patient satisfaction to nursing between two groups [n(%)]

Group	Very satisfied	Satisfied	Dissatisfied	Satisfaction rate
Intervention ($n = 45$)	21 (46.67)	22 (48.89)	2 (4.44)	95.55
Control ($n = 48$)	13 (27.08)	20 (41.67)	15 (31.25)	68.75
χ^2				11.172
P				< 0.001

hypertension, premature birth, hydramnion, cesarean section, fetal distress, urinary tract infection, and postpartum hemorrhage in intervention group were significantly lower than those in control group, respectively ($p < 0.05$). This indicates that the comprehensive nursing intervention can obviously improve the pregnancy process quality and pregnancy outcome of GDM patients.

If the condition of GDM cannot be effectively controlled in time, the newborn is prone to fetal distress, neonatal hypoglycemia, neonatal hyperbilirubinemia, neonatal asphyxia, and even maternal and infant death [25]. Due to the lack of diabetes awareness, the GDM patients worry about the serious impact of diabetes on the health of pregnancy and development of fetus. They are more likely to appear in mental health, such as irritability, anxiety, and other bad state, which ultimately affects the growth and development of the fetus [26]. In this study, after comprehensive nursing intervention, the incidences of neonatal hypoglycemia, asphyxia neonatorum, and hyperbilirubinemia in intervention group were significantly lower than those in control group, respectively ($p < 0.05$). This indicates that the comprehensive nursing intervention can obviously improve the perinatal infant condition of GDM patients.

In conclusion, for GDM patients, the appropriate comprehensive nursing intervention can effectively improve the pregnancy process quality, pregnancy outcome, and perinatal infant condition of GDM patients. This study has provided a reference for further application of comprehensive nursing intervention to improving the maternal and infant prognosis for GDM patients. This study still has some limitations. The sample size of this study is relatively small. In further study, larger sample size will make the results more convincing.

Availability of data and material The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The author declares that she/he has no conflict of interest.

Ethical approval This study was approved by the ethics committee of The First Affiliated Hospital of Chongqing Medical University.

Consent for publication Written informed consent was obtained from all participants.

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ORIGINAL ARTICLE

The effects of in-water and on-land aerobic training on postural sway and balance in patients with type 2 diabetes

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Abstract

Background Balance and maintaining posture are essential requirements for the daily activities of diabetic patients. The purpose of this study was to investigate the effect of in-water and on-land aerobic training on balance of type 2 diabetic patients.
Research question Is there a difference in the balance of patients between aerobic training environments in water and on land?
Methods For this, 24 participants were assigned randomly in three eight-person groups of control, aerobics on land, and aerobics in water. Patients performed aerobic protocol in 12 weeks (2 sessions per week) and total of twenty-four 60-min training sessions of progressive challenging with one completely identical protocol. Before and after the training period, patients' postural sway and balance were measured and recorded in three components (anterior-posterior, lateral, and overall) by Biodex balance system. Data were analyzed using the dependent *t*, multivariate, and Tukey post hoc tests.
Results The results showed that patients who performed in-water and on-land aerobic training, compared to the control group, had a significant effect on their balance ($p \leq 0.05$). Although this significant effect was not observed between in-water and on-land aerobic groups just in the posterior-posterior component ($p \geq 0.05$), there was a significant difference in lateral and overall components between these two experimental groups ($p \leq 0.05$).
Significance The results showed that in-water and on-land aerobic training led to increased balance and decreased postural sways of diabetic patients. Also, in-water aerobics training compared to on-land aerobic training led to better effects on stability, consistency, and balance of patients due to increased stimulation of proprioception.

Keywords Aerobic · Balance · Diabetes 2 · Neuropathy · Postural sway

Background

Hypertension, smoking, diabetes, physical inactivity, and obesity are the five major causes of mortality worldwide [1]. Globally in 2010, only 23% of adults 18 years and above were insufficiently active, with South-East Asia having the physically inactive prevalence of 15% [1]. This fact is indeed worrying as physical inactivity accounts for a large number of comorbidities worldwide including causing more than 80% of cardiovascular disease, more than 20% of the cases of diabetes, and cancers of the breast and colon as well as killing about 3.2 million healthy individuals yearly [1]. Diabetes is a metabolic disorder in the body that results in metabolic disorders that result in damage to blood vessels and nerves [2]. Balance and stature are among the basic needs for the daily activities of diabetics, which play an essential role in static and dynamic activities. Decreased proprioception, reflexes, lower limb strength, and balance disorders are other complications of diabetes [3]. Destruction of the lower limbs or neuropathy destroys the precise feedback of the proprioception of the lower limbs and thus leads to situational instability in these individuals [4]; one of the problems of diabetics is the increase in instability and state control disorder that is caused by dysfunction of the proprioception system [5]. Studies have shown that

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there is a linear relationship between the severity of neuropathy due to diabetes and static instability, because the main source for maintaining and regulating posture control is proprioception, which originates in the lower limbs, especially the structures around the ankle. The imbalance of these individuals is 15 times greater than that of healthy individuals and diabetic patients without neuropathy [6]. A state of imbalance in diabetic individuals with sensory neuropathy is associated with impaired sensory feedback from the lower extremities [7]. People with peripheral neuropathy due to diabetes have a relative impairment of balance [8] and significant impairment of their sole, which reduces patients' ability to maintain balance during daily activities [9]. People with diabetes have neuropathy, even with their eyes open, and have a high level of control disorder and are more likely to fall [10]. Due to the dependence of stature control on the musculoskeletal and sensory skeletal system in case of reduction or discontinuation of one of these inputs, body fluctuations increase and as a result, muscle activities increase to maintain balance [11]. A group of researchers examined the balance of patients with diabetic peripheral neuropathy, and their results showed that static and dynamic balance was significantly reduced in people with diabetic neuropathy [12]. Another study of 24 diabetic patients with and without diabetic peripheral neuropathy also found that people with peripheral diabetic peripheral neuropathy were more unstable [13] because they slowed down the transmission of nerve messages and increased reaction time, reducing neural control. Muscle in these people affects their balance [14]. Sa et al. (2015) stated that exercise in water caused a significant increase in patient balance and decreased fear of falling compared to the controlled group [15].

Foot ulceration is the main precursor to lower limb amputation in patients with diabetes worldwide [16]. Estimates of the prevalence of diabetic foot ulcers (DFUs) in the United States range between 4 and 12% [16]. The annual and lifetime incidence of DFUs has been estimated as approximately 4 and 25%, respectively [16]. In Queensland, Australia, 24,917 hospital admissions were used for the principal management of a diabetes-related foot complication between 2005 and 2010 resulting in the use of 260,085 hospital occupied bed days [17]. Treatment of DFUs account for significant health care costs in Australia and foot complications are the second leading cause of diabetes-related mortality, second only to cardiovascular disease [18]. Most foot ulcers are triggered by diabetes associated peripheral neuropathy (DPN) [19, 20]. DPN, which is believed to be a consequence of uncontrolled hyperglycemia, prompts gradual loss of sensory, motor, and autonomic function of the lower limb which leads to foot deformities, abnormal gait, and higher plantar pressures which promote skin trauma and subsequent DFUs [19, 21, 22]. Although biomechanical studies have helped to identify potential triggers of ulceration, whether such triggers change and by how much when DFUs are healing remains largely unknown [23, 24]. It is unknown whether patients with DPN are capable of adjusting their gait and plantar pressure (i.e., reducing pressure) to account for plantar wounds, as is the case for healthy controls with normal peripheral sensation [23]. It can be hypothesized that due to DPN, patients with active plantar DFU should continue to demonstrate similar abnormal lower limb biomechanical characteristics as displayed prior to the DFU formation [24]. Biomechanical factors associated with DFUs include reduced temporal spatial parameters (TSPs), such as speed of walking and stride length; restricted kinematics (movement patterns); delayed muscle activations, and altered forces (kinetics), which may contribute to elevated plantar pressures during gait [23]. It may be possible that the patients who achieve successful DFU healing are able to compensate for the DFU through changes to these biomechanical factors, irrespective of external devices used to offload ulcers. Finding from a recent meta-analysis suggested that patients with active DFUs have reduced plantar pressure compared to those with DPN without ulcers, contrary to what was previously thought [23].

Research on diabetic patients has shown improvement in balance and stability as a result of aerobic exercise and proprioception, possibly as a result of increased sensory nerve input, which reduces the likelihood of sensory impairment-related falls. Aerobic exercise is also effective in reducing the risk of developing or exacerbating patients' peripheral neuropathy [10]. Exercise in the water is believed to provide an environment that reduces the fear of performing possible movements and falls, as well as increases the transmission of neurotransmitters, and by improving the accuracy of deep sensation in the joints of the lower limbs, the possibility of muscle arousal and provides the body with more ease of balance. According to Baldusi et al. (2006), proper physical activity is effective in improving balance and can reduce the progression of neuropathy, because aerobic exercise delays the onset of neuropathy or modulates its progression [25].

According to current guidelines, patients with type 2 diabetes should do aerobic exercise at least 150 min a week. Aerobic exercise can be an ideal exercise for these patients because exercise and activities increase intraocular pressure (e.g., exercises associated with pressure); walking and swimming are some of the activities offered to these people. In this regard, water is very useful as an environment that puts the necessary resistance in proportion to the needs of each person on his body and causes both upper and lower limbs to engage with the appropriate range of motion. Exercise in the water maintains a more balanced state, and water makes patients physically and mentally refreshed. Water sports can increase a person's ability to balance and thus reduce the risk of falling. Now, considering the mentioned cases and the lack of research on comparing aerobic exercises in land and aerobics in water on situational fluctuations and balance of type 2 diabetic male patients, the present study examines the effect of aerobic exercises in both water and land environments on

static fluctuations, and equilibrium of type 2 diabetic patients is discussed: to answer the question of whether there is a difference between the two aerobic exercise environments in water and on land in patient balance? Or which training method creates more stability?

Methods

The present study is a quasi-experimental with a pre-test-post-test design. The statistical population of the present study consisted of all type 2 diabetic men in IRAN-Tehran with an age range of 35 to 60 years' people should have no history of regular aerobic exercise and their diabetes rate should be controlled between 170 and 220 (mg/dl). All patients used metformin and Glibenclamide pills and did not inject insulin. And most importantly, they should not have high blood pressure as prescribed by a specialist. In order to determine the sample size based on similar research and to determine the sample size of G-Power semi-experimental research, among the people with the conditions to participate in the research, 24 people participated in the tests voluntarily and consciously. Stability was normal and they participated in the study without canes, walkers, and assistive devices. All subjects were fully trained before the start of the study in a justification class and all important cases of the test. First, they completed questionnaires for collecting personal information and also a questionnaire for conscious consent to participate in the test and then entered the research process. Subsequently, the subjects were randomly divided into three groups of eight and the control groups formed aerobic exercises in water and aerobic exercises in land. The pre-test was used for all groups with the same conditions and to measure static fluctuations using the Biodex balancing device. Validity and reliability have been confirmed by the American company Biodox (ICC ≥ 0/79) due to its standardization [26] and in other researches, it has been confirmed again by using gold standard and comparison with Force Plate device [27]. The screen of this device includes four areas for the placement of the right toe in the first quarter, the left toe in the second quarter, the heel of the left foot in the third quarter, and the heel of the right foot in the fourth quarter. To measure total, lateral-internal, and anterior-posterior posterior oscillations, a static stability test and a slip level of 6 were used to measure the slip level at a moderate level and to record equilibrium changes and static fluctuations. Also, the duration of each test was 20 s (three repetitions with 10 s interval between repetitions). The control group is non-training and the aerobic group is in the water and the aerobic group on land is by a male trainer with an aerobic coaching degree with the same training protocol for 12 weeks and 2 training sessions per week. Performed at each meeting for 60 min, aerobic exercise was divided into three main sections, which included: 10 min of basic warm-up movements; 40 min of main aerobic exercises; 10 min of stretching and cooling movements. In the main exercises section, rhythmic exercises were performed with fixed chain and variable chain patterns (Table 1). Finally, after the training sessions, the post-test was recorded exactly according to the pre-test conditions.

Statistics

In order to check the normality of data distribution, Shapiro-Wilk test was used and then to study the effect of exercise on dependent variables and also to examine the differences between groups, multivariate analysis of variance test and Toki test were used to determine the effect. Intervention dependent *t*-test was used; in all tests, the confidence level was 95% and the error rate was 5%. All statistical calculations and graphs were performed using SPSS 21.0 software.

Results

The following are the findings of different groups in the general characteristics shown in Table 2.

Also, the results of differences in postural sway of different groups are shown in Table 3.

As shown in Table 3, a significant difference was observed between the experimental groups and the control group in all three components of equilibrium (anterior-posterior, lateral, and general) ($p \leq 0/05$). This means that aerobic exercise increases balance and reduces patients' situational changes and side fluctuations ($p \leq 0/05$).

In the following, the difference between two groups of aerobic exercise in water and aerobic in land is stated to determine whether there is a significant difference between the two training groups in terms of balance changes. Tables 4 and 5 show the multivariate analysis of variance analysis in relation to differences between different groups:

According to the information in the table above, there is a significant difference between the groups ($p \leq 0/05$), which is used to determine the exact difference of the Toki test; see Table 6.

According to the above table, it can be stated that there was a significant difference between aerobic exercises in water and aerobic exercises on land in the two components of lateral fluctuations and general fluctuations between water aerobics and aerobics on land ($p \leq 0/05$), and there is no significant difference in the anterior-posterior balance component ($p \geq 0/05$).

Table 1 Protocol of aerobic exercise in water and land

Family 1	Raising 4&8 beat (variable)	Family 2	Raising 4&8 beat (variable)	Family 3	Raising 4&8 beat (constant)
Easy walk	Knee 1,3	Grapevine 4 beat	Knee 2,4	Heel dig Front heel	Knee 2,4
V walk	Side 1,3	Grapevine 8 beat	Side 2,4	Heel dig Back toe	Side 2,4
8 Walk	Kick 1,3	Chasse 2	Kick 2,4	Degage back toe	Kick 2,4
Mambo front/side/back	Heel 1,3	Mambo chasse	Heel 2,4	Degage front heel	Heel 2,4
Baby mambo		Mambo chasse front			
Chasse1		Mambo chasse side			
Pivot					
Jaz square					
Box step					

Discussion

The most common cause of peripheral nerve involvement is about 50% of cases of neuropathy due to diabetes. Neuropathy exacerbates the foot by causing numbness in the foot and impaired perception of the proprioception; what further endangers the health of patients is the reduction in the transmission of sensory messages and the lack of accurate feedback of the proprioception of the lower limbs, which reduces the accuracy and efficiency of the balancing control response strategies. According to the findings of the present study, the effect of aerobic exercise in both aquatic and terrestrial environments and their possible effects on the balance of diabetic patients can be stated that aerobic exercise increases balance and ultimately reduces changes in patients' static fluctuations compared to the group. Controlled means that exercise in water and land is significantly different from the control group on static fluctuations in the anterior-posterior, lateral, and general directions. In this regard, Taheri (2015) reported that participation in water training can affect neuromuscular function and balance and significantly reduce the likelihood of falling. Matthias (2013) also obtained similar results in a recent study [28, 29]; however, the results of Fakhari and Mahdieh's findings (2014) showed that physical activity does not increase the balance in diabetic patients, because after 2 months of training and selected physical activity, there was no significant difference in balance, which is consistent with the present study. Perhaps the reason for this is the type of training and the duration of training, because their training is selected aerobic training and has been held for 2 months. But aerobic exercise may have improved balance and decreased oscillations, perhaps due to static and dynamic changes and movements, and subtler contractions in muscles and limbs, and increased proprioception.

Also, according to the above findings (Tables 4, 5, and 6), there was a significant difference between the experimental and control groups in the anterior-posterior oscillation variable, but there was no significant difference between the two experimental groups, is drought and water. This means that both experimental groups had fewer anterior-posterior oscillations than the control group, which led to a significant effect of crying; however, there was no significant difference between the two groups of drought and water in anterior-posterior oscillations and both methods were not superior to each other in this variable. Although the post-aerobic score on the water was better, it was not statistically significant. There was also a significant difference between the groups in terms of lateral fluctuations and general fluctuations. This means that both aerobic water and land groups have a significant advantage over the control group, and there is also a

Table 2 General characteristics of subjects (average ± standard deviation)

Group	Age	Weight	Height	BMI (kg/m^2)	Duration of diabetes (years)	FBS (mg/dl)
Total	44/9 ± 6/7	79/7 ± 6/8	174/6 ± 4/8	26/0 ± 1/4	12/2 ± 2/6	193/9 ± 14/0
Control	42/7 ± 3/8	77/1 ± 4/8	173/8 ± 5/0	25/4 ± 1/6	12/0 ± 2/3	193/3 ± 15/8
Water	45/1 ± 8/6	80/6 ± 8/6	174/7 ± 5/1	26/3 ± 1/1	12/7 ± 3/2	193/3 ± 13/7
Land	46/8 ± 7/0	81/5 ± 7/9	175/9 ± 4/8	26/4 ± 1/4	12/1 ± 2/5	195/0 ± 14/4

Table 3 Results of postural sway in water and land training group

Component	Group	Count	Pre-test	Post-test	df	*t*	Sig
Posterior-anterior	Control	8	1/87 ± 0/04	1/88 ± 0/04	7	− 0/12	0/901
	Water	8	1/86 ± 0/05	1/31 ± 0/10	7	6/37	0/000*
	Land	8	1/81 ± 0/11	1/41 ± 0/10	7	4/36	0/003*
Lateral	Control	8	1/56 ± 0/05	1/59 ± 0/05	7	− 1/59	0/156
	Water	8	1/52 ± 0/03	1/04 ± 0/01	7	14/31	0/000*
	Land	8	1/52 ± 0/03	1/24 ± 0/06	7	4/48	0/003*
Overall	Control	8	2/41 ± 0/09	2/44 ± 0/07	7	− 0/69	0/508
	Water	8	2/50 ± 0/09	1/91 ± 0/05	7	5/34	0/000*
	Land	8	2/50 ± 0/08	2/20 ± 0/04	7	6/89	0/000*

*Significant. Significance level $p < 0.05$

significant difference between the two groups of dry and water training, and this shows that the aerobic training group in water is superior to the aerobic training group. On land, it is superior to situational fluctuations in lateral and general changes. This means that aerobic exercise in water can create a more stable condition for diabetics in the lateral direction and reduce their lateral postural sway.

Overall, aerobic exercise has been shown to be beneficial in increasing the balance of diabetic patients. In this regard, Douris et al. (2003) found that a 3-month hydrotherapy exercise program, by emphasizing height control and resistance training and walking, can improve people's balance when standing [30] in line with the present study. Lazzarini et al. (2015) also showed in a study that 12 weeks of water training in the shallow part of the pool increase the strength of the lower torso muscles and also improve static and dynamic balance in inactive people. He also concluded that changes in body composition and body mass index, which was associated with increased muscle mass and decreased fat mass, delayed the traction line and reduced the risk of falling [18], which could be true for the present study, which led to an improvement in posture oscillations. On the other hand, the combination of repetition and speed of movement may increase strength and endurance, as well as improve flexibility and reaction time [31], which in both experimental groups have been able to improve performance compared to the control group. The researchers said that the loss of balance was exacerbated by a lack of activity, and this could be true of the findings, as after 12 weeks of high-energy activity both in water and on land, the balance and fluctuations of diabetic patients in experimental groups improved significantly.

Previous studies have reported that physical activity improves posture control and falls. Among these activities, water exercise has a significant effect on people with diabetes due to creating a safe and diverse environment [32] because water exercise has a double effect due to more variety of training, vitality, and excitement [33]. The research that was done in the shallow part has increased the morale and motivated us to perform the exercises as well as possible. It can be said that perhaps the reason for the improvement in controlling the body's position in the two dimensions of lateral and general fluctuations is due to water activity, which has had better effects on balance than the land-based training group, because in-water environmental conditions allow people to perform a wide range of movements without increasing the risk of falling or injury [34]. At the same time, the water protection environment allows the person to maintain a straight and straight stature independently [35]. Stabilizing and water-balancing forces also provide a good environment for balancing activities and challenging systems involved in balance, all of which are included in aerobic exercise in the water of diabetic patients and help water to reduce fluctuations. And creating a better balance and more stability is more effective. Also, due to the

Table 4 Results of MANOVA in postural sway

Effect		Value	*F*	Freedom degree error	Sig
Different					
Group	Hoteling's trace	12/93	38/81	36	0/000*

*Significant. Significance level $p < 0.05$

Table 5 Multivariate analysis of variance test results (subscales)

Variable	Component	Sum of squares	df	Average of squares	*F*	Sig
Swing	Posterior anterior	1/45	2	0/729	11/62	0/000*
	Lateral	1/22	2	0/613	33/12	0/000*
	Overall	1/15	2	0/577	19/72	0/000*

*Significant. Significance level $p < 0.05$

Table 6 The results of the Toki test postural sway

Variable	Component	The difference in averages	Std. E	Sig
Posterior-anterior	Control–Land	0/468	0/12	0/003*
	Control–Water	0/563	0/12	0/001*
	Land–Water	0/095	0/12	0/732
Lateral	Control–Land	0/315	0/06	0/000*
	Control–Water	0/546	0/06	0/000*
	Land–Water	0/195	0/06	0/024*
Overall	Control–Land	0/238	0/08	0/028*
	Control–Water	0/536	0/08	0/000*
	Land–Water	0/297	0/08	0/006*

*Significant. Significance level $p < 0.05$

increase in reaction time in water, these exercises are suitable for people with balance disorders, because due to the water viscosity property, slower movements are performed and as a result, people have more time to respond and react [34] which can eventually lead to better balance. One of the factors that seems to be related to improving balance is the combination of exercises to stimulate the atrial system [32], because water exercise facilitates atrial input and the proprioception of pressure in water [34]. One of the factors that may have been the reason for the improvement in patients' water balance was the strengthening of the atrial system and the proprioception. Another reason that is likely to be effective in improving static balance is stimulation of neurotransmitters in water, exercise and being in water can increase neurotransmitter stimulation using data from skin inputs [32]. Therefore, these exercises can probably be used to increase the transmission of sensory messages to the specific level of the central nervous system. In general, the results of the research have shown an improvement in the balance, which is in line with the results of the present study.

Conclusion

Overall, aerobic exercise in water and land generally improves the balance in diabetic patients and can be one of the most effective and practical exercises in strengthening the muscles in the lower limbs (large and delicate) and increasing the balance of patients exposed to neuropathy. They are environmentally friendly. Even according to the above results, it can be said that aerobic exercise in water has a better and more effective impact on reducing changes and situational fluctuations of patients compared to the same aerobic exercise on land; therefore, it is recommended that all patients and treatment working groups use aerobic exercise as a useful exercise to increase balance and control posture.

Limitations

The most important limitation of the present study was the difficulty in finding eligible diabetic patients who met the inclusion and exclusion criteria.

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Author contribution All authors made substantial contributions to the conception and design of the study, or acquisition of data, or analysis and interpretation of data and contributions to drafting the article. Each of the authors has read and concurs with the content in the final manuscript. The material within has not been and will not be submitted for publication elsewhere except as an abstract.

Declarations

Conflict of interest This research was approved by the ethics committee of Iran University of Medical Sciences with the code IR.IUMS.REC.1398.405 and obtaining the ethical codes related to the research. The subjects were informed about obtaining informed consent, confidentiality, non-compliance with religious and professional rights, and non-physical-psychological harm.

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ORIGINAL ARTICLE

The efficacy of negative pressure wound therapy compared with conventional dressing in treating infected diabetic foot ulcers: a randomized controlled trial

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Abstract

Introduction Despite advances made in diagnosing and controlling diabetes mellitus (DM), treatment of diabetic foot ulcers (DFUs) is still among challenges faced by physicians. Negative pressure wound therapy (NPWT) is one of newer modalities proposed in the treatment of DFUs. However, there is lack of evidence to support its mileage in this regard. This study was conducted with the aim of assessing the efficacy of NPWT in healing process of DFUs.

Materials and methods Sixty patients with DM were randomly allocated into two groups consisting of 30 patients each: the intervention group received sub-atmospheric pressure of − 75 to − 100 mmHg (5 min on, 2 min off) with dressings changed every 48 h, and the control group was treated with silver sulfadiazine dressings, changed twice daily. Patients were followed up until complete closure of ulcers, with a mean duration of 3 months.

Results Of the total 60 patients, 27 patients (45%) were females. Most of the patients in both groups had DFUs of grade 2 according to Wegner's classification. Rate of healing of the ulcers was significantly higher by using NPWT (p-value 0.01). NPWT also caused a significant reduction in ulcer surface area, depth, size, major and minor amputations, and disability duration (p-values 0.008, 0.002, 0.02, 0.03, and 0.01, respectively). No significant decrease in occurrence of complications was seen with NPWT.

Conclusion NPWT seems to be more efficacious than conventional dressing in treating infected DFUs.

Keywords Negative pressure wound therapy · Diabetic foot ulcer · Conventional dressing

Introduction

Encountering DFUs is often troublesome both for the patient and physician. The prevalence and lifetime incidence of this pernicious complication of DM is estimated to be 4–10% and 10–25%, respectively [1]. Ulcers of the lower extremity are accountable for 20% of hospital admissions of diabetic patients, and 40–70% of all nontraumatic amputations in this population [2]. Diabetic foot wounds, even superficial ones, are often associated with poor treatment responses and high rates of complications [3].

General approach to DFUs consist of glucose control and adequate nutrition, infection control, debridement, pressure off-loading, and dressings. However, asperities brought about by DFUs have drawn attention of many researchers to come along with novel efficacious and safe treatment options. Negative pressure wound therapy (NPWT), also called vacuum-assisted wound closure, is among recently developed, noninvasive modalities proposed to be utilized in treatment of DFUs. It is based on vacuum-sealing drainage and vacuum-assisted closure which results in a localized controlled negative pressure environment [4]. Although this modality has been accompanied by convincing results, remarkable heterogeneity in primary and secondary endpoints of available randomized trials has made delicate comparisons challenging and

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limited the ability to generalize their results. The aim of this study was to appraise the efficacy of NBWT in healing of infected foot ulcers encountered in patients with DM.

Materials and methods

Patients with nonischemic infected DFUs admitted to our hospital from January 2017 to December 2018 were considered for enrollment in this controlled trial. There were no limitations on age, gender, location of DFU (dorsal, calcaneal, plantar), grade of DFU (based on Wagener's classification), duration of ulcers, size of ulcers, or type of DM (type 1 or type 2 DM). Exclusion criteria were as follows: (a) positive medical history of coagulopathies; (b) ulcers resulted from venous insufficiency, as well as those from chemical, electrical, or radiation burns; (c) peripheral vascular disease; (d) malignancy in the foot ulcer; (e) treatment with growth factors or normothermic/hyperbaric oxygen therapy during the study period or within 30 days; (i) inadequate lower extremity perfusion; (f) inadequate glycemic control (HbA1C > 8%); (g) concomitant use of corticosteroids, immunosuppressive, or chemotherapy medications.

Based on Bland-Altman plot and similar studies [5], a total number of 60 patients were recruited and, after being matched for age and gender, were randomly allocated to a treatment at a 1:1 ratio of study patients to controls, using randomized block design.

Ulcers had been investigated for debris or sloughs prior to initiation of treatment, and if necessary, surgical debridement was performed until exposure of viable tissue (Fig. 1a). DFUs were classified using Wagner system [6]. Perfusion of the foot was evaluated by using ankle brachial index (ABI) with a handheld 5- or 10-mHz Doppler instrument, and ABI < 0.8 was excluded.

Patients in the control group were treated using silver sulfadiazine ointment dressing, which was changed twice daily. NPWT systems consist of open-pore polyurethane ether foam sponge, semi-occlusive adhesive cover, fluid collection system, and portable suction pump which is connected to the suction tubing, applying − 50 to − 125 mmHg of intermittent or constant suction [7, 8]. Our patients in the treatment group received − 75 to − 100 mmHg sub-atmospheric pressure intermittently (5 min on, 2 min off) through the VAC system (Vacuumed V.A.C., FAPSCO, Tehran, Iran) (Fig. 1b, c) with dressings changed every 48 h according to standardized treatment guidelines. The precise time and date of initiating therapy were recorded to calculate the healing time.

In both groups, the required data was recorded twice weekly. All the patients were followed up until complete closure of foot ulcers. There were no withdrawn or lost to follow-up patients.

The primary endpoint of this study was to evaluate the efficacy of NPWT versus conventional silver sulfadiazine dressings by comparing the healing rate (defined as time to formation of 70–100% of granulation tissue on the ulcer bed) between these two groups. Secondary endpoints were impact of NPWT on surface area and depth of DFUs (using disposable paper rulers), need for major (above the ankle level) and minor (below the ankle level) amputations, and disability period (defined as time needed to resume daily activities) and complications experienced by the patient.

All the procedures in both groups were carried out by a single third-year surgical resident. Due to the nature of the study, neither patients nor investigators could be blinded to the treatment assignment. But to ascertain the accuracy of information provided in this study, data was provided independently by two other third-year surgical residents. We used Scientific Package for Social Sciences (version 25.0, SPSS, Chicago, IL, USA) to analyze the data. Kolmogorov-Smirnov test and other appropriate tests were selected based on data distribution status. Continuous variables were expressed using mean ± standard (Std.) deviation, and categorical variables were presented using absolute and relative frequencies. To compare the intergroup results, independent *t*-test and chi-square were used. The significance level (α) was considered 0.05 (Table 1).

Results

A total of 60 patients with DFUs were enrolled in this study between January 2017 and December 2018. They were allocated into intervention group (13 females, 17 males) and control group (14 females, 16 males). Table 2 depicts patients' demographics. In both groups, most DFUs were in grade 2 according to Wagner's classification [6] (82% in group A, and

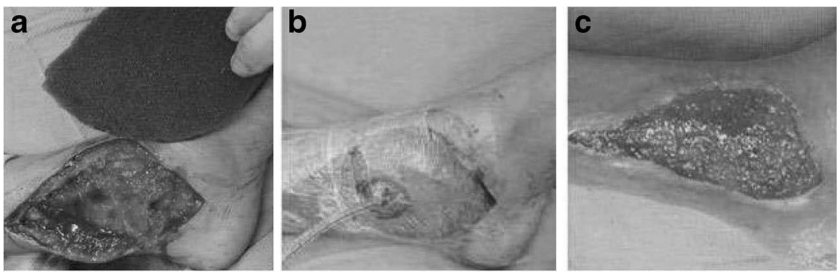

Fig. 1 Grade 2 DFU. **a** On the first day of NPWT, the ulcer has been debrided (open-pore polyurethane ether foam sponge can also be seen in this image). **b** Ulcer undergoing NPWT. **c** The same ulcer after 3 weeks of NPWT

Table 1 Wagner's [6] classification of DFUs

Grade	Definition
0	No open ulcers (cellulitis or deformity may be present)
1	Superficial ulcer (partial or full thickness)
2	Ulcer extension to ligament, joint capsule, tendon, or deep fascia (no abscess or osteomyelitis present)
3	Deep ulcer with abscess, osteomyelitis, or joint sepsis
4	Gangrene localized to portion of forefoot or heel

87.8% in group B, *p*-value 0.18). The mean duration of follow-up was 3 months.

Rate of healing

In group A, about 49% of the granulation tissue was formed within 49 days, and in group B, about 79% of this tissue was formed after 75 days. Rate of ulcer healing was significantly higher in intervention group (*p*-value 0.01).

Ulcer surface area

After the treatment, the mean (± SD) was 7.05 (± 1.65) and 10.8 (± 1.09) cm2 in intervention and control group, respectively. This shows a significant reduction in surface area of ulcers after using NPWT (*p*-value 0.008).

Ulcer depth

Following treatment, the mean (± SD) depth of ulcers was 10.5 (± 2.3) and 15.2 (± 1.9) in the intervention and control groups, respectively. *P*-value of 0.002 indicates a significantly lower depth of ulcer after using NPWT.

Ulcer size

Ulcer size (surface area and depth) significantly reduced in the intervention group (*p*-value*:* 0.02).

Number of amputations

Patients in the intervention group did not undergo major amputations. Seven patients (23%) of this group underwent minor amputations. In the control group, major and minor amputations were performed on 5 (16.5%) and 7 (23%) of the patients, respectively. The reason for final amputation was clinical alarm signs in favor of sepsis. Minor amputations include disarticulation of the toe (removal of the toe at level of MTP joint, ray amputation (a toe and part of the corresponding metatarsal bone are amputated), transmetatarsal amputation (amputation of the forefoot at the mid-metatarsal level), Lisfranc disarticulation (hindfoot disarticulation at the tarsometatarsal articulation), Pirogoff's amputation (amputation of the foot through the talotibial articulation with preservation of part of the calcaneus), and Syme's amputation (amputation of the foot through the talotibial articulation with removal of the malleoli of the tibia and fibula) In this regard, there was a significant difference between the two treatments (*p*-value 0.01).

Disability duration

On an average, patients in the control group were unable to perform their daily activities for 8 days, while this period was decreased to 3 days in the intervention group. NPWT significantly reduced the post-treatment disability period in patients (*p*-value 0.01).

Treatment-related complications

Seven patients (23%) in the intervention group and 9 patients (30%) in the control group experienced osteomyelitis, with no significant difference between the two groups. Treatment was complicated by sepsis in one case of each group.

Table 2 Patients' demographics

Characteristics	Intervention group (n = 30)	Control group (n = 30)	p-value*
Age (years)	70.31 ± 5.92	71.80 ± 6.32	0.483
Mean duration of DM (years)	15.66 ± 4.86	15.97 ± 5.79	0.724
Baseline ulcer depth (mm)	17.01± 6.03	20.04 ± 8.07	0.506
Mean of baseline ulcer surface area (cm^2)	15.07 ± 2.92	14.09 ± 2.81	0.951

*Independent *T* test

Discussion

In this RCT, we have evaluated the efficacy of NPWT in healing process of infected DFUs and shown NPWT to be an efficacious treatment option for DFUs as it significantly reduced amount of time to reach 70–100% of granulation tissue, as well as surface area and depth of ulcer, need for major and minor amputations, and disability duration.

The main mechanism of NPWT in healing ulcers is believed to be the sub-atmospheric pressure created by a vacuum-assisted closure (VAC) device which results in further removing fluid from wounds; reducing edema; promoting formation and fusion of granulation tissue; and hence, preparing the wound bed for closure [9]. It has also been proposed that NPWT has anti-inflammatory effect, presumably via suppression of proinflammatory cytokines and enzymes resulting from nuclear factor-κB downregulation and activating transcription factor-3 upregulation [10].

A number of RCTs have been conducted to assess the efficacy of NPWT in healing process of DFUs [11–21]. The total study size of these studies varies between 10 and 342 patients, and the population studied had been equally or almost equally allocated into experimental and control groups. We used a total population of 60 patients, which is larger than most of prior RCTs in this regard. In this RCT, we did not set up a fixed follow-up period for our patients. Rather, we followed up the patients until the time a complete closure of the wounds was achieved. Relatively similar to this protocol, Sepúlveda et al. [16] and Vaidhya et al. [17] have evaluated the percentage of granulation tissue formed to decide on continuation or termination of their intervention. Other RCTs have generally used a fixed follow-up period, ranging from 14 to 112 days [11, 12, 14, 15, 18–21].

Various features of VAC have been used in studies. In some of them, only negative pressure had been used, without dressing [12]. In other studies, which had utilized VAC along with dressing, dressing had been changed every 2 days [11, 21], every 3 days [19], 3 times a week or more [14, 20], or when needed [18]. We changed dressing once every 48 h in patients undergoing VAC, similar to the design of study conducted by Armstrong et al. [11] and McCallon et al. [21].

Indicators utilized in prior works to determine the efficacy of NPWT in treating DFUs include the rate of ulcer healing, amount of time until granulation tissue formation, adverse events, amputation, the rate of 76–100% granulation tissue formation, time and/or the rate of reaching 90% granulation tissue formation, amount of time until healing of ulcer, reduction of ulcer area and/or depth, major, and minor amputation; patient satisfaction; number of dressings applied, and total cost of dressings [11–21]. Most of these factors were covered in our study, too. In addition, we also evaluated disability duration in our patients, which was not considered in prior studies. According to our study, treating DFUs using NPWT significantly reduces disability period in patients (p-value 0.01).

Although most of available literature in this regard have reported NPWT to be efficacious [11, 12, 14, 18, 22, 23], they are not united in regard to NPWT being superior in safety [22, 23]. Adverse events reported with NPWT include infection, pain, edema, and bleeding [23]. Armstrong et al. [11] stated a similar frequency of adverse effects in their control group and patients who underwent NPWT. Our study echoes this finding since there was no significant reduction in complications experienced by patients receiving NPWT.

There are some points which distinguish our study from previous ones. The first point is that we continued the follow-up of our patients until the complete healing of ulcers, which has resulted in a relatively longer term of follow-up. The next point is that we have considered duration of DM of our patients, a factor which has been overlooked in most of the previous works. Longer duration of DM can potentially have a negative influence on response to therapy, since it can lead to higher degrees of neuropathy, but there was no meaningful difference between our case and the control group, regarding duration of DM affliction [24]. We have also assessed the duration of disability in our patients; an important clinical outcome which is easy to evaluate. Another point is classification of DFUs in our study, while details of classification of ulcers were not provided in most of the previous studies

We are also aware of our study's limitations, the first of which is the relatively small population of patients enrolled (although the population size in our study is larger than most of prior works). The second point is that we did not consider the location of DFUs, although it can have a close relationship with the prognosis. The reason for this is that matching patients based on location of ulcers is a complex process and could result in further narrowing the study size. Cost-effectiveness of NPWT was not covered in this study, either.

It seems that further studies are still required to be conducted to generate adequate evidence for the safety, efficacy, and cost-effectiveness of NPWT in treating DFUs.

Conclusion

Our study supports the efficacy of NPWT in treating infected DFUs as NPWT proved to increase healing rate of ulcers, along with reducing ulcer size, disability duration following treatment, and reducing major and minor amputations. In terms of complications, however, our study failed to show the superiority of NPWT over conventional dressing. Further studies are yet to be conducted to put an end to the dilemma of choosing NPWT as the optimal treatment modality for DFUs.

Declarations

Ethical consideration This study has been approved by clinical trial review board (IRCT20160803029181N6), and an informed written consent has been obtained from all patients prior to participating in this trial.

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ORIGINAL ARTICLE

Evaluation of ultrasound examination combined with intensive injection technique education on insulin-induced lipohypertrophy (LH) management: a prospective cohort study in China

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Abstract

Aim This study was aimed to assess the impact of ultrasound examination combined with intensive injection technique education for LH management.

Method A total of 120 patients with diabetes who were identified as existing clinical LH were enrolled into the study. All patients underwent ultrasound examination and received intensive injection technique education based on ultrasound detection results and Chinese guideline for diabetic injection technology in a prospective single study in China with a follow-up of 3 months. Injection Technique Questionnaire (ITQ) and assessment of glycemic control were used to assess the impact of ultrasound examination combined with intensive injection technique education for LH management.

Results As expected, after 3 months of intensive injection technique education based on ultrasound examination, the proportion of patients who had mastered insulin injection technique was significantly increased ($p<0.05$). The mean HbA1c was decreased by 0.60 (0.28)% from 7.61 (0.34)% to 7.01 (0.23)%, mean FBG was decreased by 1.20 (1.05) mmol/L from 8.10 (1.02) mmol/L to 6.90 (0.71) mmol/L, mean 2hPG was decreased by 1.70 (1.31) mmol/L from 11.49 (1.25) mmol/L to 9.79 (0.87) mmol/L without increasing the insulin dosage. The glycemic variability (GV) indicators (LAGE, MBG, SDBG, and PPGE), hypoglycemic, hyperglycemic, and IM injection events were markedly decreased ($p<0.05$).

Conclusions Ultrasound examination combined with intensive injection technique education markedly improved glycemic control in diabetes with LH without increasing the insulin dosage, as well as reducing the occurrence of adverse blood glucose events. It can be a new effective approach for LH management.

Keywords Lipohypertrophy management · Ultrasound examination · Intensive injection technique education · Glycemic control

Introduction

Lipohypertrophy (LH) is a common complication of long-term insulin therapy in patients with diabetes characterized by rubber-like or scar-like lesions at the injection sites [1]. Cross-sectional studies from different countries such as Italy, Spain, and China reported prevalence between 49% and 72% in patients with both Type 1 and Type 2 diabetes [2–4]. LH has clinically significant sequelae, including a greater risk of glycemic variability and higher insulin dose requirement. Extrapolated to 9 million insulin-injecting patients in China and adjusted for therapy adherence, LH-related excess annual insulin consumption cost is estimated at nearly $297 million (RMB 2 billion) [4, 5]. Correct injection technique can reduce insulin absorption variation and achieve optimal treatment results, and it is essential to achieving good diabetes management [6, 7]. Epidemiological data have shown that not all patients with diabetes have received relevant injection technique education [8]. Therefore, intensive injection technique training in subjects who inject into LH lesions is necessary.

Clinical studies showed that LH may have characteristic ultrasound manifestations such as nodular shape lesion with a hypoechoic halo or hyperechoic foci being well circumscribed in the subcutaneous layer, with distortion of surrounding connective tissue, absence of vascularity and

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capsule, heterogeneous in echotexture compared with surrounding tissue, and seems to have more sensitive and specific than physical examination, especially for LH without visual and palpable changes [9, 10]. Despite ultrasound has been used in a small number of studies, but its exact use for LH management is still being explored. Based on the above studies, it is hypothesized that ultrasound examination combined with intensive injection technique education can improve glycemic control for diabetes with LH. Therefore, we designed a prospective study to assess the effect of ultrasound examination combined with intensive injection technique education on HbA1c, total daily insulin dose, glycemic variability, and adverse blood glucose events in diabetes with LH, further exploring the exact use of ultrasound for LH management.

Research design and methods

Study design

This prospective study was conducted with individuals from the Department of Endocrinology at our hospital who fulfilled the study criteria between March 2020 and May 2020. Study inclusion criteria were as follows: (1) diagnosed Type 1 or Type 2 diabetes with basal-bolus insulin therapy currently; (2) with inadequate glycemic control (current HbA1c>7.0%), and 3) confirmed with LH at the injection sites by a single team of nurses certified as diabetes educators. Clinical LH was defined as discrete palpable rubbery, inelastic nodules, or visible pigmentation and hair changes at insulin injection sites. Participants were excluded if they were the following: (1) evidence of dermatitis and cutaneous disease; (2) allergic to ultrasound coupling agent; (3) pregnancy and lactation, history of malignant tumor, serious heart, lung, kidney, and liver diseases. All patients were confirmed by a single team of nurses certified as diabetes educators for the presence of LH at the injection sites through inspection and palpation. Demographic information, assessment of glycemic control, and insulin injection technique were evaluated by a physician or research nurse. And then, the patients were further detected by ultrasound.

Ultrasound assessment

Ultrasound examination was conducted by a portable GE LOGIQe machine with a L8-18i-D probe (8-18 MHz; GE Health care, Frankfurt, Germany) by ultrasonographer who had been engaged in superficial tissue for a long time. The set of ultrasound was the same for all the patients. The inspection areas were the abdomen, upper part of the gluteus, and arms. Ultrasonographer demarcated the area containing the following ultrasound manifestations such as (1) a nodular shape lesion with a hypoechoic halo or hyperechoic foci was well circumscribed in the subcutaneous layer, (2) with distortion of surrounding connective tissue, (3) absence of vascularity and capsule, and (4) heterogeneous in echotexture compared with surrounding tissue (Fig. 1A). The subcutaneous fat thickness (SFT) in the normal area (Fig. 1B) was measured, and the area with SFT less than 4, 6, and 8 mm was marked. Insulin injection sites that matched the above characteristic ultrasound manifestations were also identified as lipohypertrophy.

Intensive injection technique education

Insulin injection technique questionnaire (ITQ) was used to assess whether patients have mastered standardized or complete injection technique education previously. The various language versions of the questionnaire (nurse and patient forms) can be found at http://www.fitter4diabetes.com. All patients received intensive injection technique education based on ultrasound detection results and Chinese guideline for diabetic injection technology [11]. The main intensive injection technique education includes (1) making necessary psychological adjustments for patients with injection fear, (2) choosing the right injection regimen, (3) selecting the appropriate insulin injection device to ensure patients can accurately extract and inject the insulin dose, and (4) insulin injection sites with LH and SFT less than 4, 6, and 8 mm were marked. According to ultrasound detection results, the optimal injection sites were selected for patients to avoid injecting into LH and muscle, (5) learning self-examination and nursing, (6) educating patients to rotate the injection sites correctly and cautioned about the risks of reusing needles to minimize the risk of injection site complications, (7) recommending patients to use 4-mm pen needles to minimize accidental IM risk, and (8) educating patients to avoid reusing pen needles. Patients were followed up by telephone every two weeks and received intensive injection technique education again.

Insulin dose titration

Insulin dose was titrated twice a week by patients, and a treat-to-target approach was used to ensure optimum titration throughout the study. The basal and bolus insulin doses were titrated with the aim of achieving a fasting plasma glucose (FBG) less than 7.0 mmol and 2hPG less than 10.0 mmol according to a titration algorithm (Appendix 1).

Glucose measurements

All patients were provided with a blood glucose meter and instructed on how to use the device. Mean of 3 consecutive days 7-point self-measured blood glucose (SMBG) was used to evaluate indicators of glycemic variability (GV) at T-0 (1 week before intensive injection technique education) and T-3

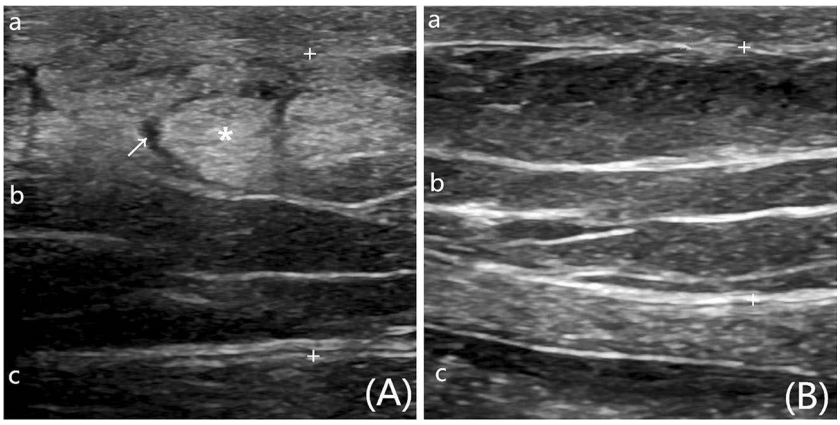

Fig. 1 Characteristic ultrasound manifestations of lipohypertrophy (A) and normal subcutaneous fat tissue (B). **a** Epidermal and dermal layers. **b** subcutaneous layer. **c** Muscular layer. *: lipohypertrophy with a hyperechoic foci and hypoechoic halo (↗)

(3 months after intensive injection technique education). Time points included in the 7-point SMBG profile were breakfast, 2 h after breakfast, lunch, 2 h after lunch, dinner, 2 h after dinner, and bedtime. The indicators of GV were the following:(1) standard deviation of blood glucose (SDBG) was defined as the standard deviation of blood glucose values within 1 day. (2) The largest amplitude of glycemic excursion (LAGE) was defined as the maximum value of blood glucose within 1 day. (3) Postprandial glucose excursion (PPGE) was defined as the average value of the difference between postprandial blood glucose and corresponding pre-prandial blood glucose. (4) Mean blood glucose (MBG) was defined as the average value of 7-point SMBG. HbA1c and total daily insulin dose (TDD) was measured at T-0 and T-3. ITQ was used to assess whether patients have mastered standardized or complete injection technique education at T-3. Adverse blood glucose events like symptomatic hypoglycemia, hypoglycemia, hyperglycemia, and IM injection were recorded at T-0 and T-3. Symptomatic hypoglycemia was defined as the occurrence of low sugar symptoms (e.g., tiredness, sweating, and strong hunger) with a confirmed blood glucose meter recording less than 3.9 mmol/L at 1 week. Hypoglycemia was defined as patients measured blood glucose meter recording less than 3.9 mmol/L with or without low sugar symptoms at 1 week. Hyperglycemia was defined as the occurrence of blood glucose more than 13.9 mmol/L at 1 week. IM or intradermal injection was defined as the occurrence of severe pain, bleeding, bruising at injection sites, or accompanied by hypoglycemia and increased GV.

Statistical analysis

Data were analyzed by using the SPSS 23.0 software (SPSS Inc.IBM). Descriptive data are expressed as means ± standard deviation (SD). Discrete variables were summarized in frequency tables (*N*, %). Differences in HbA1c, SMPG, insulin dose, and GV indicators between T-0 and T-3 were analyzed using a paired Student's t-test for normal continuous variables and a non-parametric Wilcoxon test for non-normal data. Contingency tables were evaluated using chi-square. Values of $p<0.05$ were considered statistically significant.

Results

A total of 120 patients meeting the inclusion and exclusion criteria were selected for the study. 25.0% of patients were diagnosed with Type 1 diabetes, and 75% of patients were diagnosed with Type 2 diabetes. The majority were men (56.7%), the mean age of the patients was 59.4 ± 11.6 years, mean duration of insulin used was 7.1 ± 4.4 years, and mean BMI was 25.3 ± 3.5 kg/m^2. The proportion of patients who had mastered insulin injection technique at T-0 and T-3 was shown in Table 1. After intensive injection technique education, the proportion of patients who reuses needles, reuses a single needle > 3 times, skips injections, and leaves needle under skin < 10 s were lower in T-3 than T-0 (all $p<0.05$). Moreover, the proportion of patients who rotates the injection site, masters skin lift, uses 4- or 6-mm pen, checks injection site, and puts used needles into specific trash can were higher in T-3 than T-0 (all $p<0.05$).

Inspection and palpation identified 184 lesions consistent with LH present in 120 patients by a single team of nurses certified as diabetes educators. Ultrasound examination identified 296 lesions meeting ultrasound criteria in 120 patients. All inspection and palpation identified lesions meet the ultrasound criteria. Ultrasound examination detected 61% more LH lesions than inspection and palpation($p<0.05$).

Ultrasound examination combined with intensive injection technique education also optimized glycemic control. The mean 7-point SMBG profile was significantly decreased at T-3 than at T-0 (Fig. 2a). As expected, the mean HbA1c was lower at T-3 with a 0.60 (0.28)% mean decrease than at T-0 from 7.61 (0.34)% to 7.01 (0.23)% (Fig. 2c). The mean FBG was also lower at T-3 with a 1.20 (1.05) mmol/L mean decrease than at T-0 from 8.10 (1.02) mmol/L to 6.90 (0.71) mmol/L (Fig. 2b). The mean 2hPG also lower at T-3 with a 1.70 (1.31) mmol/L mean decrease than at T-0 from 11.49

Table 1 Proportion of patients mastered injection technique at T-0 and T-3

Characteristics	T-0 (n = 120)	T-3 (n = 120)	P value
Reuse needles	98 (81.6)	72 (60.0)	0.009
Reuse a single needle>3 times	76 (63.3)	42 (35.0)	0.002
Rotate the injection site	68 (56.6)	112 (93.3)	0.000
Use 4mm pen	66 (0.55)	116 (96.7)	0.000
Master skin lift	74 (61.6)	114 (95.0)	0.000
Skip injections	38 (31.6)	8 (6.7)	0.001
Check injection site	54 (45.0)	104 (86.7)	0.000
Leave needle under skin <10 s	52 (43.3)	10 (8.3)	0.000
Put used needles into specific trash can	44 (36.7)	98 (81.6)	0.000

Data are number (%). Pearson chi-square test was used to analyze the data. T-0:1 week before intensive injection technique education; T-3:3 month after intensive injection technique education

(1.25) mmol/L to 9.79 (0.87) mmol/L (Fig. 2d). Ultrasound examination combined with intensive injection technique education also improved glycemic variability (GV). As shown in Fig. 3, the indicators of GV (LAGE, MBG, SDBG, and PPGE) were also significantly decreased at T-3 than T-0. Although the mean basal insulin dose decreased by 0.38 IU, the mean bolus insulin and total insulin dose increased by 0.56 IU and 0.18 IU, while there were no significant differences between T-0 and T-3 (all p>0.05) (Fig. 4).

Adverse blood glucose events were significantly decreased at T-3 than at T-0, the total hypoglycemia events were decreased by nearly three quarters (18 vs. 75; p<0.001), the proportion of patients with symptomatic hypoglycemia was decreased from 31.6% to 6.7%, hyperglycemia was decreased from 58.3% to 15.3%, and IM injection was decreased from 11.6% to 1.7% (Table 2).

Discussion

Lipohypertrophy (LH) is a common complication of long-term insulin therapy, and all currently used types of insulin

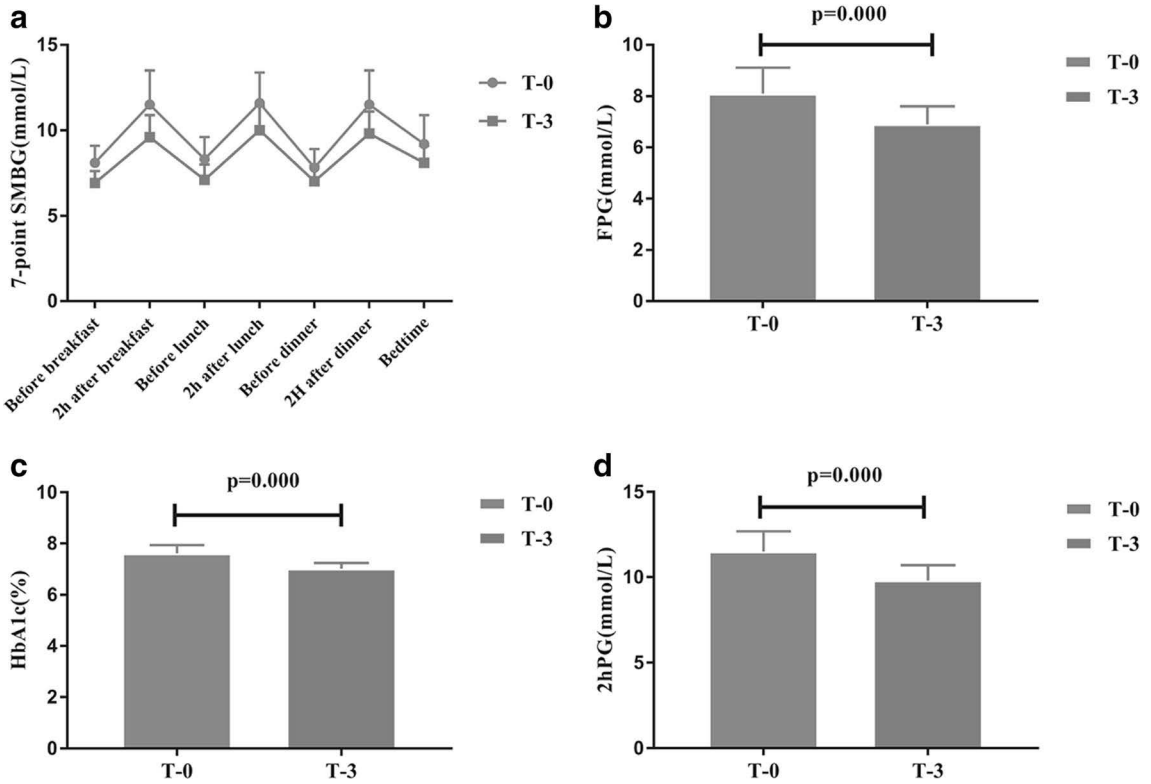

Fig. 2 Effects of ultrasound examination combined with intensive injection technique education on 7-point SMBG (**a**), FBG (**b**), HbA1c (**c**), and 2hPG (**d**). T-0: 1 week before intensive injection technique education. T-3: 3 months after intensive injection technique education

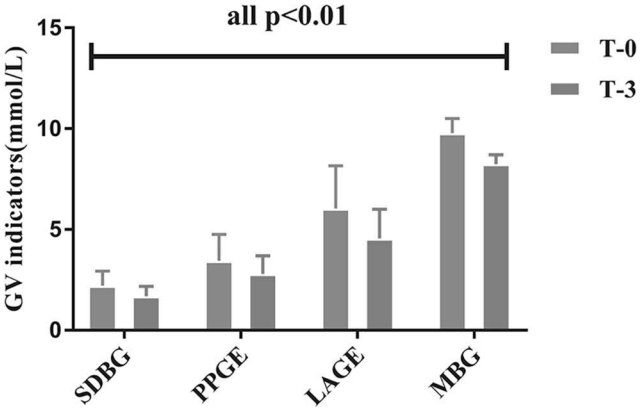

Fig. 3 Effects of ultrasound examination combined with intensive injection technique education on GV indicators. *GV* glycemic variability, *SDBG* standard deviation of blood glucose, *PPGE* postprandial glucose excursion, *LAGE* largest amplitude of glycemic excursion, *MBG* mean blood glucose

are associated with the risk of LH. Due to the high potential harm to patients, management of LH should rank higher among the priorities of healthcare professionals (HCP). However, the assessment of LH presents technical difficulties currently. Ultrasound examination seems to have more sensitivity and specificity than physical examination, especially for LH lesions without visual and palpable changes, but its exact use for LH management is still being explored. Thus, we established a study to explore the benefits of ultrasound examination combined with intensive injection technique education for LH management. In the present study, the data showed that ultrasound examination combined with intensive injection technique education markedly reduced the 7-point SMBG, FBG, HbA1c, and 2hPG without increasing the insulin dosage. As well as reduced occurrence of adverse blood glucose events. The proportion of patients who mastered the correct injection technique was significantly improved.

Insulin Injection Technique Survey in China showed that patients apparently do not realize they have LH or are unaware of the hazards of injecting into LH [12]. Duration of insulin

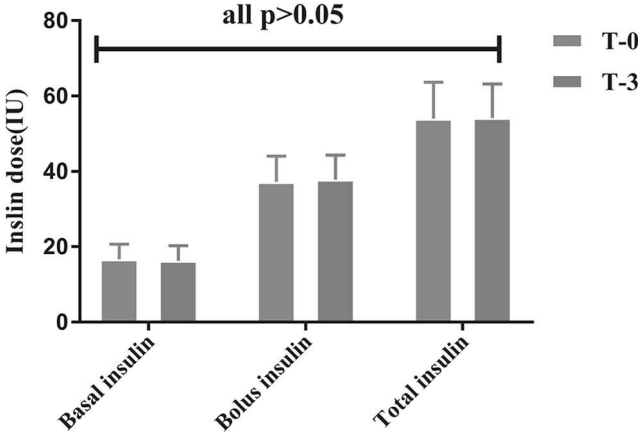

Fig. 4 Effects of ultrasound examination combined with intensive injection technique education on basal, bolus, and total insulin

Table 2 Proportion of patients with adverse blood glucose events at T-0 and T-3

Characteristics	T-0 (n = 120)	T-3 (n = 120)	P value
Symptomatic hypoglycemia	38 (31.6)	8 (6.7)	0.001
Hyperglycemia	70 (58.3)	26 (15.3)	0.000
IM injection	14 (11.6)	2 (1.7)	0.028

Symptomatic hypoglycemia was defined as the occurrence of low sugar symptoms (e.g., tiredness, sweating, and strong hunger) with a confirmed blood glucose meter recording less than 3.9 mmol/L at 1 week. Hyperglycemia was defined as the occurrence of blood glucose more than 13.9 mmol/L at 1 week. IM injection was defined as the occurrence of severe pain, bleeding, and bruising at the injection area or accompanied by hypoglycemia and increased GV

use, frequency of needle reuse, and site rotation are three independent risk factors for the formation of LH [13]. Although the Chinese Diabetes Society has released guidelines in 2017 to address these issues, the ITQ results in our study that showed the proportion of patients who mastered the correct injection technique are sub-optimal. Therefore, it is very necessary to strengthen the injection technique education. In this study, we achieved the purpose of intensive injection technique education by giving patients a face-to-face insulin injection technical guidance based on ultrasound detection results and Chinese guideline for diabetic injection technology and conducting telephone follow-up education every two weeks. The ITQ results at T-3 are encouraging, the proportion of patients with the correct injection technique was greatly improved, and the proportion of patients with incorrect injection habits was also markedly decreased after intensive injection technique education. Despite the proportion of patients reused needles was decreased, there still have nearly 60.0% patients reuse needles. Injection needles are not covered by the medical insurance reimbursement, that is the main reason for this situation. As a result, patients are less willing to replace needles, especially in patients with multiple injections per day. In this study, we found that 11.6% of patients had a recent history of IM injection, which may be related to vertical injection of a longer needle into injection sites with thinner SFT. Palpation and visual examination alone cannot accurately assess SFT, which may result in IM injection. Ultrasound can provide an accurate way to measure SFT at injection sites. The survey of SFT at insulin injection sites in Chinese diabetes showed that the mean (SD) SFT ranged from 7.23 (3.58) mm in the arm to 12.14 (4.90) mm in the abdomen; the proportion of patients with STF less than 4, 6, and 8 mm were 0.4%, 5.7%, and 15.3% [14]; and there still have a certain IM injection risk for patients using 4-mm needles with thinner SFT. In the present study, we accurately measured SFT and marked the sites with SFT less than 4, 6, and 8 mm by ultrasound to select optimal injection sites for patients to minimize the risk of IM injection. Our present data showed that

ultrasound can help patients choose optimal injection sites to reduce the risk of IM injection.

LH is associated with worse glycemic control, greater insulin consumption, and large cost implications [15, 16]; a sensitive detection method is urgently needed for clinical diagnosis. In the present study, the data showed that ultrasound examination detected 61% more LH lesions than inspection and palpation. The results suggested that palpation and visual examination alone may misdiagnose some LH lesions, especially for LH without visual and palpable changes. Volkova et al. [17] have reported that LH without visual and palpable changes could also worsen compensation of glycemic control and result in hypoglycemia and chronic Somogyi rebound. Thus, LH without visual and palpable changes is as important and clinically significant as clinical LH that should be paid more attention in clinical practice. Prospective Multicenter Study of the intervention for lipohypertrophy in France, China, and the UK that showed HbA1c was decreased by ~0.5% and total daily insulin (TDD) was decreased by ~5 IU at 6 months after intervention [18–20]. Compared to the above studies, we adopted intensive injection technique education based on ultrasound examination to intervene for LH, the present data showed that mean HbA1c seemed to decrease more in a shorter period (0.6% vs 0.5%) with a lower baseline HbA1c level. The results in our study may indicate us that ultrasound examination combined with intensive injection technique education can improve glycemic control for diabetes with LH better than traditional intervention for LH. Unlike the above research results, despite the mean bolus insulin showed a decreased trend, there were no statistical differences in mean insulin dose between T-0 and T-3, it may be related to the insulin titration in our study to reach target blood glucose in a shorter period of time. Based on the above studies and the findings in our study, a decrease in the total insulin dose is expected with the extension of follow-up time.

The increase of glycemic variability (GV) can bring many harms to patients with diabetes, which activates oxidative stress pathways, damages endothelial cells, exacerbates chronic inflammation, and finally leading to the increased risk of diabetic complications [21]. LH can blunt insulin absorption and action, thereby increasing glycemic variability and leading to profound deterioration in postprandial glucose control [22]. Clinical cases have reported that hypoglycemic and hyperglycemic events were also related to LH [23–25]. In the present study, the data showed that GV indicators like LAGE, MBG, SDBG, and PPGE were significantly decreased and the proportion of patients with adverse blood glucose events was also significantly decreased. It indicates us that diabetes with insulin injection accompanied by unexpected hypoglycemic and hyperglycemic events should undergo ultrasound examination and intensive injection technique education to manage LH.

To our knowledge, this is the first study in China that used ultrasound examination combined with an intensive injection technique education approach to manage LH. There still are some limitations in our study. First, this study was limited by manpower and financial resources, only 120 patients were rolled into this study and carried out in a single care center, and multiple testing comparisons were not performed across various parameters. Despite the results in our study showed a greater improvement of glycemic control, randomized controlled trials compared with intensive education alone should be conducted to further confirm the results. Another limitation of this study was GV indicator that was calculated based on 7-point SMBG rather than Continuous Glucose Monitoring (CGM), while studies have shown that SMBG can also accurately estimate GV in diabetes by 7 to 8 times SMBG, and had a good correlation with GCM [26].

In conclusion, ultrasound examination can detect more LH than physical examination and provide an accurate way to measure SFT to select optimal injection sites for diabetes with LH. Ultrasound examination combined with intensive injection technique education also markedly improved glycemic control in diabetes with LH without increasing the insulin dosage, as well as reducing the occurrence of adverse blood glucose events. It can be a new effective approach for managing LH.

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Declarations

Ethical Statements The study was approved by the Ethical Committee of First Hospital of Longyan. Written informed consent from each patient was waived.

Conflicts of interest We declared that all authors have read and approved the manuscript and there is no conflict of interest existing in the submission of this manuscript, and the manuscript is approved by all authors for publication.

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ORIGINAL ARTICLE

The self-efficacy improvement in adolescent girls with type 1 diabetes mellitus with self-care education through mobile-based social networking

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Abstract

Introduction Type 1 diabetes mellitus (T1DM) is the most common chronic metabolic disease in childhood and adolescence. The increasing use of social networks among adolescents provides a unique opportunity to implement self-care educational programs for improving self-efficacy in diabetic adolescents in terms of awareness, attitude, and performance.

Objective This study aimed to assess the effectiveness of a self-care educational program provided by mobile-based social networking (MBSN) on the self-efficacy of diabetic adolescent girls.

Methods A quasi-experimental study with 76 Iranian 14- to 18-year-old girls with T1DM was conducted in 2018–2019. The subjects were selected with aconvenient sampling method and randomly assigned to the experimental and control groups. The MBSN was applied to implement a self-care educational program in the experimental group for 12 weeks. The Diabetes Management Self-Efficacy Scale (DMSES) questionnaire with the main domains of blood sugar control (BSC), diet control (DC), medical care (MC), and exercise was used to evaluate the self-efficacy level before and 4 weeks after the intervention.

Results The mean age and T1DM duration of participants were 15.9 ± 1.5 and 7.24 ± 3.9 years, respectively. No significant difference in the self-efficacy of the two groups before the intervention was found. The self-efficacy level in the experimental group was increased after the intervention so that there was a significant difference in this parameter between the experimental and control groups ($p < 0.0001$). Three DMSES domains of BSC ($p = 0.003$), DC ($p = 0.011$), and MC ($p = 0.016$) after the intervention significantly differed between the two groups.

Conclusion As the self-care education with MBSN significantly increased the self-efficacy level of girls with T1DM, this technique would be a complementary of health care in educating and supporting diabetic patients in other age groups.

Keywords Self-care · Self-efficacy · Education · Learning · Social networking · Diabetes mellitus

Introduction

Type 1 diabetes mellitus (T1DM) is one of the most common metabolic endocrine diseases among children and adolescents. The prevalence of T1DM is increasing among children around the world so that this disease affected 0.2–0.3% of children or adolescents under the age of 18 years. About 1% of 5–7 million of the diabetic population in Iran are children and adolescents [1]. A variety of complications affecting the patients' health and families' lives are associated with this high-burden disease. The main pathogenesis of uncontrolled T1DM occurs as a result of the onset of vascular changes. Hyperglycemia, hypoglycemia, and ketoacidosis are common causes of hospitalization among children with T1DM. Other devastating complications include retinopathy, nephropathy, neuropathy, cardiovascular diseases, and even amputation

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which usually occur as a result of the poor control of blood sugar levels [2–4].

Adolescents are exposed to many personality and physiological changes. Adolescent patients with diabetes are more afraid of being rejected by their friends due to the fear of difference and peer pressure. Accordingly, they may become isolated and consequently may not follow their treatment plan as a result of the desire for independence [5]. Despite the same treatment, adolescents often suffer from more complications than adults due to higher levels of hemoglobin A1c [6]. Therefore, the growing prevalence of this chronic disease and its related complications reduplicate the necessity of long-term care, daily blood sugar control (BSC), lifestyle modification, and knowledge acquisition to improve self-efficacy and self-care behaviors [7]. It was demonstrated that the lack of self-care is the major reason for the development of diabetic complications. Self-care refers to the fact that the patients have a fundamental role in their health and well-being and provides a type of health care in which the patient plays a pivotal role in health promotion, as well as in the prevention and control of diseases and consequence complications [8]. Some other advantages of self-care behaviors are the life expectancy increase, healthier and more active life, quality improvement of life, better control of blood sugar levels and adverse outcomes, and the reduction of treatment costs and hospitalization time [9].

Self-efficacy is one of the best strategies to improve self-care behaviors in diabetic patients [10]. Self-efficacy is a significant concept referring to a person's belief and judgment about his/her abilities in performing duties and responsibilities. According to Albert Bandura's theory, there is a system of self-control and self-regulation to manage feelings and behaviors in each person, which have a great impact on his/her destiny. This self-sustaining trait makes us more successful by giving us the adventitious ability to use skills in dealing with problems. In another word, self-efficacy affects the person's motivation so that the stronger a person's self-belief, the more behavioral persistence in his/her efforts to achieve the goals [11]. Self-efficacy also plays an important role in disease management and adaptation and reduces anxiety and other psycho-mental complications of the disease [12, 13]. This parameter can potentially predict adherence to regular blood sugar tests, dietary patterns, insulin injections, and exercise [14]. However, most patients with diabetes are unsuccessful in accomplishing the aforementioned actions and other key behaviors to manage their disease, indicating a weakness in their self-efficacy [15]. Therefore, the education of patients to potentiate their self-care behaviors and the optimal BSC can ultimately lead to an improvement in their life quality as one of the important determinants of health care in diabetes [16].

Nowadays, the most important approaches for nurse's educational interventions on diabetes self-care are the Internet, computer, training camps, telephone, individual and group training, group counseling, and group discussion training [17]. Meanwhile, the most acceptable educational interventions are considered learning through the Internet and group counseling. Peer-based groups via virtual network platforms such as "membership in social networks" are interesting approaches for education [18]. Social networking as one of the most important and successful Internet applications provides a good platform for people with diabetes to exchange up-to-date, scientific information related to their disease. Among social networks, Telegram has recently become more popular among people in a short period of time as it has over 40 million members in Iran and more than 400 million members worldwide [19]. There is a possibility to send text, voice and video messages, various stickers, and documents (e.g., photos, videos, and text files) to one or more people in this social network at a high speed. In Telegram, several groups or channels can be created, and people can be included as members to exchange information and opinions with each other. These appropriate features and capabilities of interactive communications and rapid feedbacks make it a good platform to share important and attractive content that is preferred by many Iranian teenagers.

Few studies have been conducted on the self-efficiency promotion of diabetic people in this age group, and hence, there is an urgent need to implement a low-cost and accessible educational program for improving awareness, attitudes, and performance in these vulnerable patients. Thus, this study aimed to assess the effect of mobile-based social networking (MBSN) education on adolescent patients with T1DM in terms of healthcare programs to improve their self-efficacy.

Methods

Study design and participants

This quasi-experimental study was conducted with participating 90 girl adolescents aged 14–18 years with T1DM. In the study, a convenient sampling method was used to select participants referred to the Iranian Diabetes Society (Tehran, Iran). Then, they were randomly divided into two groups of experimental (n = 45) and control (n = 45). However, seven patients from each group were dropped out due to unwillingness, failure to complete the questionnaire, and corrupted cell phone. Therefore, the study was finally completed with 76 adolescent girls with T1DM (38 patients in each group).

Inclusion and exclusion criteria

Inclusion criteria were female adolescents aged 14–18 years, having T1DM (diagnosed by a doctor) for at least 6 months, having a fault-free cellphone with the installation ability of Telegram social network application, and having no

devastating chronic disease except diabetes. T1DM was diagnosed based on the following criteria: (i) fasting plasma glucose (FPG, no caloric intake for at least 8 h) ≥ 126 mg/dL (7.0 mmol/L), (ii) 2-h PG ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT), and (iii) A1c ≥ 6.5% (48 mmol/L). In general, there is a random PG ≥ 200 mg/dL (11.1 mmol/L) in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis [20]. Exclusion criteria in this study included unwilling to complete participation, being hospitalized, and not responding to messages sent by the researcher for at least a week during the intervention.

Implementation of MBSN-assisted educational intervention

Two separated Telegram groups were initially created for the experimental and control participants. The intervention group daily received a text or a video message for 12 weeks with self-care based-educational contents including T1DM self-care principles in the fields of diet, physical activity, blood sugar self-monitoring, insulin preparation and injection, insulin unit regulation, blood glucose testing steps, insulin storage location, and insulin absorption speed. Moreover, members of this group discussed their problems by presenting some suggestions, comments, and questions and offered solutions to each other under a healthcare professional's supervision (HPS). However, members of the control group received messages with non-diabetic content. The last visit time of members in both groups was recorded by the researcher. If people who were not online for at least 7 days and did not check the messages to respond, they were first notified with a text message, and subsequently, the phone call was made to find their unresponsiveness reason in case of no response. Also, another mobile number was asked under the necessary condition, and messages were sent to the new number. Otherwise, they were removed from the study as mentioned earlier. The diabetes management self-efficacy scale (DMSES) questionnaires were sent to each participant in both groups using Telegram to be completed at the beginning of the intervention and also 4 weeks after the intervention end.

Data collection

A self-developed questionnaire was designed to answer a set of demographic characteristics including age, diabetes history, participant's education level, parents' education level, history of diabetes in the family, and daily social media usage. The DMSES questionnaire was also used to assess the self-efficiency of patients with T1DM. This questionnaire, with an internal consistency of 0.93, was first developed by Van Der et al. [21] in Australia to assess self-efficacy behaviors in diabetic patients. In Iran, Sabet et al. [22] translated and validated this tool using a panel of experts after the cross-cultural adaptation in which the questionnaire reliability based on Cronbach's alpha was 0.84. The DMSES consists of 20 questions which scored according to a Likert scale ranging from 0 (can't at all) to 10 (completely capable) within 4 domains including (i) nutritional specific and weight (diet control (DC)) with 5 questions, (ii) nutritional general and medical treatment (medical care (MC)) with 9 questions, and (iii) physical activity (exercise) with 3 questions, and blood sugar control (BSC) with 3 questions. Accordingly, the total score can be obtained between 0 and 200 so that a higher score indicates more self-efficacy [23].

Data analysis

The data were statistically analyzed using the SPSS software package version 16.0 (SPSS Inc., Chicago, IL, USA) at a significant level of $p < 0.05$. The Kolmogorov-Smirnov test was used to examine the normality of data distribution. Chi-square or Fisher's exact tests for categorical variables were applied to compare the two groups, while the analysis of continuous variables was performed using the independent-sample *t*-tests.

Results

There were no significant differences in all the studied demographic characteristics of participants between the two groups. The mean age of the participants was 15.9 ± 1.5 years, whereas the mean duration of diabetes was 7.24 ± 3.90 years. Forty-one patients (53.9%) were studying in high schools. More than one third (42%) of people used social media for over 2 h per day, while only 11 participants (14.5%) applied social media for less than 30 min per day (Table 1).

Cronbach's alpha coefficient of the DMSES questionnaire in this study was estimated to be 0.90. Before the intervention, there was no significant difference in self-efficacy between the experimental and control groups ($p = 0.505$). After the intervention, the self-efficacy level in the experimental group significantly increased ($p < 0.0001$) so that a significant difference was found between the two groups ($p < 0.0001$). In the control group, there was no remarkable difference between the mean scores of self-efficacy before and after the intervention ($p = 0.065$; Fig. 1). The study of self-efficacy domains after the intervention showed that there were significant differences between the two groups in BSC ($p = 0.003$), DC ($p = 0.011$), and MC ($p = 0.016$) domains, while the exercise domain had no significant difference between the two groups ($p = 0.054$; Fig. 2).

Table 1 A summary of demographic characteristics of experimental and control groups

Demographic variables		Experimental group Mean ± SD	Control group	p-value
Age (year)		15.89 ± 1.43	15.92 ± 1.57	0.939
Diabetes duration (year)		7.18 ± 4.10	7.31 ± 3.83	0.885
Number (%)				
Educational level	Guidence school	12 (31.6)	15 (36.4)	0.358
	High school	20 (52.6)	21 (55.3)	
	University student	6 (15.8)	2 (5.3)	
Father's educational level	Under diploma	7 (18.4)	9 (23.7)	0.841
	Diploma	22 (57.9)	20 (52.6)	
	Academic	9 (23.7)	9 (23.7)	
Mather's educational level	Under diploma	24 (36.8)	16 (42.1)	0.710
	Diploma	20 (52.7)	13 (34.2)	
	Academic	4 (10.5)	9 (23.7)	
History of diabetes in the family	Yes	28 (73.7)	22 (57.9)	0.147
	No	10 (26.3)	16 (42.1)	
Daily social media usage	≥ 30 min	6 (15.8)	5 (13.2)	0.510
	30–60 min	11 (28.9)	6 (15.8)	
	1–2 h	7 (15.8)	9 (23.7)	
	>2 h	14 (36.9)	18 (47.3)	

Discussion

This study was aimed to investigate the effect of MBSN-assisted self-care education on the self-efficacy of adolescent girls with T1DM. The poor baseline self-efficacy score in both studied groups was consistent with the findings of Belsabneh et al. [6] and Bernal et al. [24]. A similar result in Iran was reported by Kermansarovi et al. [25] on the self-efficacy score in adolescent patients with T1MD. Most recently, Chen et al. [26] demonstrated that the intensity and frequency of online interactions had a positive effect on the improvement of self-management of diabetes and patients' self-efficacy. Also, diabetics with high self-efficacy positively tended to have online interactions through different communication ways. We found that the experimental group after the intervention obtained a significantly higher self-efficacy score compared with the control one due to the effectiveness of the implemented program. A review of studies showed that the patients' education, especially in self-care behaviors, was an effective strategy in promoting their self-efficacy. Heidari et al. [27] by evaluating the effect of a self-management teaching program on patients with chronic obstructive pulmonary disease (COPD) reported a significant improvement in self-efficacy after the intervention implementation. A clinical trial conducted in Bangladesh also showed that the self-management program could significantly improve the self-efficacy of patients with COPD [28]. In the study of Kennedy et al. [29], the self-care support program increased the self-efficacy level in 609 subjects with chronic illnesses. Hejazi et al. [30] also showed that patients with diabetes after the education presented high self-efficacy scores. Mohamadinejad et al. [31] also evaluated the effect of a self-care educational program on the self-efficacy of patients with type 2 diabetes mellitus (T2DM) and found that the self-care training can considerably improve self-efficacy [31]. The results of the abovementioned studies are consistent with our findings and show that the development of an educational program, especially a self-care program, can be effective in promoting the patient's self-efficacy. In contrast, the results of Naderipour et al. [32] reported that there was no significant effect of self-care education on the self-efficacy of patients undergoing coronary artery surgery. Also, Hamnes et al. [33] proved that self-management training did not significantly affect the self-efficacy of patients with fibromyalgia syndrome. This discrepancy may be attributed to the difference in

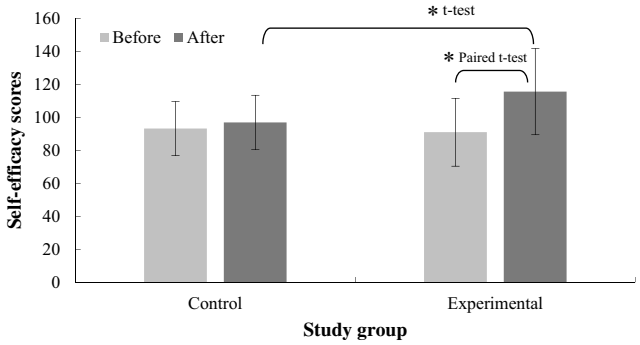

Fig. 1 The mean self-efficacy scores in control and experimental groups before and after the intervention (*$p < 0.0001$)

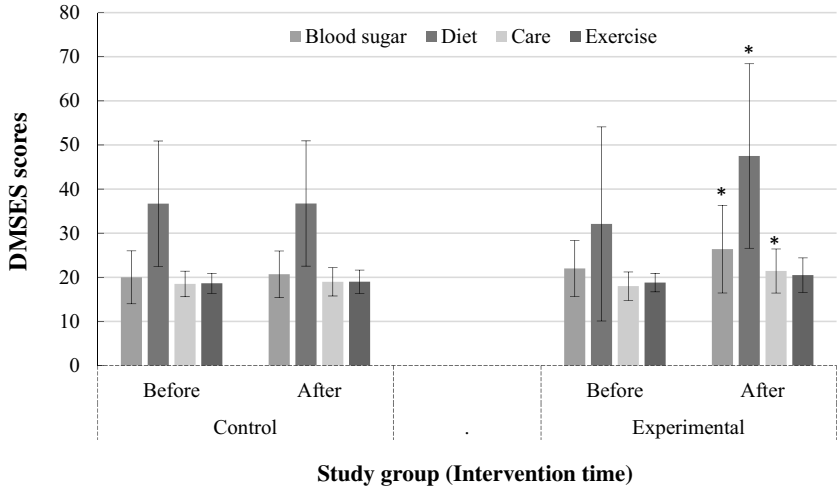

Fig. 2 Mean DMSES scores for four self-efficacy domains before and after the intervention in control and experimental groups (*$p < 0.05$ compared with the control group after the intervention)

implementation methods, measurement tools, and target groups [33].

Another different strategy used in this study was the membership in MBSN. Vorderstrasse et al. [34] showed that metabolic control could be improved by participating in a virtual environment through providing synchronous diabetes self-management training and supporting with peer and provider interactions. Other studies have also shown that telephone service is an effective, cost-saving method for education and follow-up in managing chronic diseases [35]. Mobile communications provide an opportunity for care not to be limited to clinics and hospitals but to transfer patients' care to their homes. This result was in line with the findings of Shaya et al. [36] who showed that the care intervention through social networks improved BSN, weight control, quality of life, and self-efficacy in patients with T2DM. Lorig et al. [37] also investigated the effect of an online training program of self-care on patients with T2DM, in which patients in the experimental group than the control one revealed lower hemoglobin A1c levels as well as higher scores of self-efficacy, mobility, and physical activity [38]. The mobile-and Internet-based educational interventions also were effective in controlling the metabolic status and reducing the treatment cost of patients with T1MD [38]. Zhang et al. [39] explained that the use of training packages related to the self-care program using short messages reduced the level of hemoglobin A1c and improved the care status of patients with T1DM [39]. Also, an improvement in the self-efficacy of women with T2DM was obtained after the telephone-based education and follow-up [40]. A mobile-based education also increased the self-efficacy level in patients with arthroplasty and prostatectomy [41, 42].

There was no study on the effect of self-care education through membership in MBSN on the self-efficacy of adolescent girls with T1DM. The distinguishing feature of this study was in how to apply virtual social networks with the presence of peers and their communications together via a user-friendly and cost-effective platform. Social networks allow people to use the educational space and to expand their social interactions at lower costs and without any limitation in time and place [40]. In addition to the self-care education provided by researchers, members in this study were allowed to participate in discussions, to share their experiences, and to encourage each other for choosing appropriate health behaviors. Studies showed that the membership in social networks and the presence of similar people with a common disease in groups provide a suitable and reliable educational environment, allowing them to express their problems without any fear of judgment and to receive some solutions from other patients, which may not be provided by healthcare professionals [41]. The presence of similar patients with diabetes in groups reduced stress scores by increasing the understanding of social support and improving the positive belief about their disease and medication adherence [42].

Conclusion

This study showed that virtual networks had a notable capacity to be used in educating diabetic adolescent patients to improve their self-efficacy. The patient education through virtual networks not only had any time and space limitation but also could be a useful way to support and answer patients' questions as easy and cost-effective access to information. Therefore, it is recommended to encourage adolescents with T1DM for creating these virtual groups in social media under HPS, as a part of their treatment programs to reduce the disease-related problems, to increase their self-care and self-efficacy knowledge, to get in touch with other similar patients, and finally to receive useful information and support with no extra cost. The most important limitations of the present study are the low sample size, the absence of male adolescents with T1MD, as well as individual and cultural differences of Iranian Diabetes Society clients. Accordingly, the results cannot be generalized to all adolescents. Other similar studies

with a higher sample size should be carried out in diabetic patients (in both sexes) in other cultural contexts and also for patients with other chronic diseases.

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Author contributions All authors contributed substantially to the research design and/or data acquisition and analysis were involved in drafting the manuscript, approved the final version, and agree to be accountable for addressing any questions relating to the accuracy or integrity of the work.

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Availability of data and materials The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate The study protocol followed the requirements of the Declaration of Helsinki. The present study was approved by the ethics committee of Iran University of Medical Sciences (IUMS) with the code of IR.IUMS.REC.1394.9311687001. Informed consent was obtained from all voluntary participants with a full elucidation of the study objectives. The verbal and written informed consent from all the participants and their parents was obtained before performing this research. A single code number was assigned to each of the participants to maintain the confidentiality of their personal and medical information. After completing the study, the self-care training content was provided to the control group as well.

Consent for publication Not applicable.

Competing interests The authors declare that they have no conflict of interest.

Abbreviations BSC, blood sugar control; COPD, chronic obstructive pulmonary disease; DC, diet control; DMSES, diabetes management self-efficacy scale; HPS, healthcare professional's supervision; MBSN, mobile-based social networking; MC, medical care; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

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CASE REPORT

Secondary diabetes mellitus in Prader-Willi syndrome

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Abstract
Objective Prader-Willi Syndrome (PWS) is a rare autosomal dominant disorder on the 15q11.2-q13 chromosome. PWS may impact metabolic, endocrine, and neurologic systems and combine with hypotonia, sleep disturbances, cognitive disability, central adrenal insufficiency, hypothyroidism, hypogonadism, short stature, and other diseases.
Methods Here ,we report a PWS patient with secondary diabetes mellitus. Clinical and laboratory features are summarized.
Results The patient had typical manifestations of PWS including almond-shaped eyes, narrow temples, narrow nasal bridge, and short hands and feet. Lab test revealed hypogonadism and growth hormone deficiency. SNP array revealed one copy of chromosome 15 q11.2q13.1 was missing, with a length of about 6.4 mbp.
Conclusions This case emphasizes the importance of considering PWS in the differential diagnosis of an adolescent with diabetes. Antenatal history of neonatal hypotonia and childhood history of feeding disorder are the keys to the diagnosis of PWS.

Keywords Prader-Willi syndrome · Secondary diabetes mellitus · Growth hormone deficiency · Hypogonadism · Case report

Introduction

Prader-Willi syndrome (PWS) is an autosomal dominant genetic disorder caused by the absence of paternally expressed, imprinted genes on chromosome 15q11–13. The genetic mechanisms include microdeletion of the paternally inherited chromosome, maternal uniparental disomy subsequent to trisomic rescue, or mutation/epimutation of the PWS/Angelman syndrome (PWS/AS) [1] . PWS was first reported by Prader Labhart and Willi in 1956 [2]. Clinical manifestations include facial dysmorphism, mental retardation, hypotonia, hyperphagia, short stature, obesity, hypogonadism, strabismus, and sleep disturbances [3, 4]. Most patients with PWS are diagnosed in childhood. We report an adolescent with PWS who was first diagnosed on account of hyperglycemia.

Case report

A 14-year-old female was referred to us for polydipsia and polyuria for 1 year. Her parents are genetically unrelated. Her grandmother and father had type 2 diabetes, and her grandmother had schizophrenia. She was born at full term by cesarean section for pregnancy. The birth weight was 2.0 kg with cord round neck and asphyxia. She presented with growth retardation and was not sensitive to sound, light, and other stimulation since she was 3 months old. She was weak because of poor suck. She could walk but was easy to fall, could not run, jump, and could say simple words such as "dad, mom" when she was 3 years old. She ate 2–3 times than other children at the same age, but her height was significantly lower than theirs. Intellectual disabilities were generally evident by the time she reached school age. One year ago, polyphagia and polyuria appeared. The parents did not care and measure blood glucose. She had not menstruated yet.

Physical examination showed distinct features suggestive of PWS, such as the almond-shaped eyes, narrow temples, narrow nasal bridge, and short hands and feet. Her axillary hair was sparse; pubic hair was absent. She had bilateral breast Tanner staging (B2), small nipple, and faint areola. External genitalia were immature; clitoris and labia were undeveloped. Her height was 140 cm, and her weight was 66 kg, with a body mass index (BMI) of 33.6 kg/m^2, indicating severe obesity. The systemic examination was unremarkable.

Laboratory investigations (Table 1) were remarkable for central hypogonadism. Gonadotropin-releasing hormone (GnRH) excitation test (Table 2) showed the pituitary gland responded to GnRH, and hypogonadism was considered to be

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Table 1 Laboratory findings on admission of the patient

Laboratory Test	Patient's Values	Reference Range
Blood sample		
RBC	5.2	3.8–5.1 × 10^{12}/L
Hb	141	115 − 150 g/L
WBC	8.76	3.5–9.5 × 10^{9}/L
PLT	398	×10^{9}/L
HbA1c	12.1	4–6%
E2	27.3	45.2-854 pmol/L
Prog	0.16	0.181–2.84 nmol/L
PRL	17.12	4.79–23.3 ng/ml
LH	1.11	2.4–12.6mIU/ml
FSH	5.79	3.5–12.5mIU/ml
T	0.382	0.29–1.67 nmol/L
DHEA-S	2.72	1.77–9.99 μmol/L
GH	<1.70	<10 μg/L
ACTH	9.3	7.2–63.3 pg/ml
CORT (8 am)	29.0	5–28 μg/dl
T4	11.4	4.2–13.5 μg/dl
T3	0.871	0.78–2.2 ng/ml
FT4	20.9	9.05–25.5 pmol/L
FT3	4.78	2.91–9.08 pmol/L
TSH	1.39	0.25–5 μIU/ml
TPO	<15	<15 u/ml
TGAB	2.31	<30%
TMAB	1.76	<20%
Urine sample		
KET	N	N
GLU	2+	N
24hU-TP	0.03	0–0.15 g/24 h
24hmALB	28.6	0–30 mg/24 h

mainly caused by hypothalamic lesions. Both basal and post-exercise growth hormone (GH) were < 1.70ug/L, which meant growth hormone deficiency. Fasting blood glucose was 10.93 mmol/L, and glycosylated hemoglobin was 12.1%. Adrenocorticotropic hormone (ACTH) and cortisol and thyroid function were within the normal range. Urinalysis showed ketone (-) and glucose (++). Dual-energy X-ray absorptiometry was consistent with osteopenia (Z-score − 2.0). Left tibiofibular and inferior femur epiphysis were not closed. Ultrasound showed small uterus and fatty liver. SNP array revealed one copy of chromosome 15 q11.2q13.1 was missing, with a length of about 6.4 mbp consistent with the diagnosis of PWS.

The diagnosis was PWS and secondary diabetes mellitus. Metformin (0.5 g tid po) and intense insulin therapy (premeal insulin aspart, respectively, 16 IU, 16 IU, and 18 IU+ long-acting insulin detemir 18 U qn ih) were initiated. Her parents refused the treatment of GnRH pump and GH replacement. After 3 years, her height was 145 cm, and her weight was 60 kg. The treatment was only metformin (0.5 g tid). Fasting blood glucose was around 7 mmol/L. An informed consent from the parents was obtained for the publication of the case report.

Discussion

PWS is a rare multisystem genetic disorder demonstrating great variability with changing clinical features during patient's life. Here, we report a PWS patient with secondary diabetes mellitus. Different from previous cases which were children or newborns, this patient was diagnosed at her adolescence. She had typical manifestations of PWS including almond-shaped eyes, narrow temples, narrow nasal bridge, and short hands and feet. Lab test revealed hypogonadism and growth hormone deficiency. Motor milestones and

Table 2 GnRH excitation test of the patient

Time	-15′	0	25′	45′	90′	180′
LH(mIU/ml)	1.53	5.82	12.16	12.41	9.76	10.90
FSH(mIU/ml)	7.45	8.63	13.17	15.94	15.47	18.07

language development are delayed. Learning disability was noticed since early childhood, and intellectual disability was suspected. Consensus clinical diagnostic criteria for PWS using a numerical scale, developed in 1993 [4] before the availability of diagnostic testing, have proven to be accurate. Clinical diagnosis requires five points (at least four of them major) at age < 3 years and eight points (at least five of them major) at age 3 years or older. This patient gets 8.5 points, clinical conformity with PWS diagnosis.

PWS occurs as the result of absence of expression of paternal genes from chromosome 15q11.2-q13. This lack of expression occurs by three primary mechanisms [3]: (1) deletion of a region(5–6 Mb) from the paternally contributed chromosome 15; (2) maternal uniparental disomy 15; and (3) a defect in the genomic region that controls the imprinting process. This patient has a deletion of 15 q11.2q13.1.

Since the first study evaluating the prevalence of DM and PWS in 1963 [5], accumulating reports have documented the association of PWS and DM. Previous literature showed the prevalence of DM in PWS at 7–25% [6–10]. A large French cohort study with PWS revealed 4% patients younger than 20 years of age had impaired glucose tolerance and nobody had diabetes mellitus [11]. A multicenter Italian cohort study [9] and a retrospective cohort study in Korean, [12] both showed BMI and HOMA-IR were strong predictive factors for the development of DM in PWS. And DM is usually avoided if obesity is avoided [3]. GH therapy may affect the occurrence of DM. Fasting insulin levels may be slightly elevated in children with PWS during GH therapy, but this is temporary and does not eventually progress to diabetes [9, 13–16]. Extreme obesity (BMI>26–28 kg/m^2) facilitates insulin secretion leading to overt diabetes via insulin resistance (IR) regardless of GH therapy, in spite of the effect of PWS [12, 14, 17]. Treatment for DM or IR with PWS should follow routine guidelines for the general patients [18]. The first-line therapy should be intensive dietary counseling and regular exercise. Metformin had positive effects in some children and adolescents and can be added as adjunctive therapy [19]. Insulin may be necessary in case of insulin deficiency (ketoacidosis, unexplained weight loss) and should be chosen by the level of HbA1c [20, 21]. Treatment with GLP-1 receptor agonists (liraglutide, exenatide), dipeptidyl peptidase-4 (DPP4) inhibitors, and glucose-lowering SGLT2 inhibitor has beneficial effects on glycemic control, satiety, and weight in PWS patients with DM [22–29]. In serious obesity-related comorbidities, rapid weight loss is needed, and surgery is an alternative approach to managing PWS [20, 21]. Strict control over access to food, dietary restriction, and regular exercise are the only available options to prevent obesity and diabetes, and are the potential difficulties in achieving glycemic control because of the intellectual disability [30]. In animal and clinical trials, oxytocin (OXT) and cannabidiol (CBD), AZP-531(a cyclic 8 amino-acid peptide), setmelanotide, diazoxide choline controlled release, RM-853 (orally available ghrelin O-acyltransferase inhibitor), and JD5037 (peripherally restricted cannabinoid type 1 receptors antagonist) have shown the effectiveness for reduction of food intake, and body weight and their potential for the treatment of obesity are being investigated in patients with PWS [31]. We expect these new drugs to help control weight and blood glucose.

Although this patient refused, GH replacement therapy can be started at any age of PWS to normalize height, decrease fat mass, and increase mobility, activity level, and mental development [13, 32–33]. The earlier GH replacement therapy starts, the sooner the patient will benefit. No age is too early for GH replacement therapy. The recommended dosage for GH is 0.5–1 mg/m^2/day. GH replacement therapy should not be started while combining adenotonsillar hypertrophy, DM, severe obesity, and obstructive sleep apnea syndrome. GH replacement treatment should be started in conjunction with dietary and lifestyle measures [32].

There is no systematic study of sex hormone treatment in adolescents or adults with PWS. If accepted, sex hormone treatment should have been started at around age 11–12 years for females and age 12–13 for males. Females with PWS receive low doses of estrogen therapy, gradually increasing the dose for 2 years or until menstruum, when they can transition to estrogen-progestin oral contraceptives or percutaneous patches. Males with PWS can use a low dose of percutaneous testosterone patch or increase the dose every 3 to 6 months of gel, to make the testosterone levels reached the age of the normal range or HCG therapy treatment. With HCG can increase the production of endogenous testosterone, and thus increase the testicular volume and lean body mass, and won't cause parents reported occasionally testosterone treatment own mood and aggressive [34]. Notice, sex hormone treatment had a negative effect on bone size and strength. This patient refused estrogen therapy.

In summary, this case emphasized the importance of considering PWS in the differential diagnosis of an adolescent with diabetes. Antenatal history of neonatal hypotonia and childhood history of feeding disorder remain the key to the diagnosis of PWS. Family and social support are as important as medical treatment.

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Declarations

Informed consent Has been signed.

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Conflict of interest Authors declare that they have no conflict of interest.

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CASE REPORT

Doege-Potter syndrome: a case report

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Abstract

Introduction Doege-Potter syndrome is a rare paraneoplastic syndrome presenting as hypoinsulinemic hypoglycemia due to ectopic secretion of prohormone: insulin-like growth factor-2 (IGF-2) from a solitary fibrous tumor (SFT). It is essentially a non-islet cell tumor hypoglycemia (NICTH) or in more recent terms, an 'IGF-2oma'. In case of delay in diagnosis, tumor-induced hypoglycemia could cause hypoxic cerebral injury or death. We hereby present a case of 45 years old male who presented with hypoglycemia due to pleural SFT, which is a very rare and unique presentation.

Results Upon biochemical and radiological investigations, Doege-Potter syndrome was diagnosed and the patient was planned for surgical resection of the tumor.

Conclusion This case stresses upon thorough evaluation of patients presenting with non-diabetic hypoglycemia to rule out underlying malignancy.

Keywords Doege-Potter syndrome · Non-islet cell hypoglycemia · IGF-2oma · Case report · Paraneoplastic

Introduction

Doege-Potter syndrome (DPS) is a paraneoplastic syndrome associated with solitary fibrous tumors (SFTs) characterized by occurrence of hypoglycemia. It was described in 1930, by Doege and Potter independently as a non-islet cell tumor hypoglycemia (NICTH) [1]. Solitary fibrous tumors are mesenchymal origin tumors that can be benign or malignant [2]. SFTs of pleura are very rare and constitute <5% of pleural neoplasms. Most of the pleural SFTs are small, delineated, and pedunculated masses arising out of visceral or parietal layer of pleura which can be managed via thoracoscopic wedge resection, while some large tumors may require thoracotomy with pulmonary resection [3]. About 13% of them are histologically malignant [4]. Furthermore, DPS in SFTs is also a rare occurrence as less than a hundred cases have been reported until date [1]. Here we describe the case of a 45-year-old male who was diagnosed to have a pleural SFT associated with DPS.

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Case report

A 45-year-old male farmer, chronic smoker, presented with complaints of generalized weakness and weight loss for previous 6 months. The total weight loss was estimated to be around 10 kg over past 6 months ascribed mostly to the loss of appetite. He also had a dry cough with mild right-sided chest pain for past 3 months. Two days before admission, he had two episodes of confusion and sudden loss of consciousness requiring hospitalization locally. He was found to have low blood sugar levels during these events and consequently improved upon administration of 10% continuous dextrose infusions and intermittent intravenous bolus administration of 25% dextrose. He did not have a history of diabetes mellitus and was not on any glucose lowering medications. The family history and genetic history were unremarkable. Physical examination revealed pallor and asymmetrical chest movements on respiration. Right side chest movements were found to be decreased along with decreased air entry, dullness on percussion, and decreased vocal fremitus over right middle and lower lung fields. Neurological, cardiovascular, and abdominal examination was unremarkable. Routine investigations revealed a normochromic normocytic anemia. Serum tumor markers carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), and prostate-specific antigen (PSA) were not elevated. His 8 AM serum cortisol (5.53μg/dl; normal value 4.3-22.4μg/dl on IMMULITE™ 2000, Siemens healthcare

diagnostics, USA) and growth hormone (GH) levels (0.068ng/ml; normal value <1ng/ml for males on IMMULITE™ 2000, Siemens healthcare diagnostics, USA) were normal. Serum procalcitonin was within normal limits. Blood and urine cultures were sterile. Glycated hemoglobin (HbA1c) was 4.4% indicating chronic hypoglycemia. Despite marked hypoglycemia (39 mg/dl), the concurrent serum insulin level was low (0.24μIU/ml; normal 2.6 to 37.6 μIU/ml), and C peptide level was also very low 0.00 ng/ml (normal 0.9–7.1), confirming hypoinsulinemic hypoglycemia. Serum IGF-1 level was 23.8 ng/ml (normal 55–424 ng/ml), and serum IGF-2 level was 107 ng/ml (normal 267–616 ng/ml) with IGF-2/IGF-1 ratio as 4.49 (normal up to 3:1) [5]. Ultrasound (US) examination of whole abdomen and pelvis was normal. Chest X-ray revealed a massive pleural effusion on the right side (Fig. 1A). Pleural fluid aspirated was hemorrhagic and exudative in character. Contrast-enhanced computer tomographic (CECT) scan of thorax showed a large heterogeneous density lesion abutting right pleura in basal region of right lung of size 129*112*153 mm suspected of malignant nature (Fig. 1B). US-guided biopsy was done from the pleural mass which showed benign spindle cells with mild pleomorphism. On performing IHC staining, tumor cells showed diffuse positivity for CD34 and were negative for S-100, favoring the diagnosis of a solitary fibrous tumor (SFT). A diagnosis of DPS was made, and the patient was started on steroids, i.e., prednisolone 40mg/day orally. His symptoms resolved without any further episodes of hypoglycemia. For definitive management, he was offered surgical excision of the tumor, to which he refused, and 3 months later after having discharged from the hospital, he expired.

Discussion

SFTs are mesenchymal neoplasm of CD34-positive fibroblasts which frequently originate in the pleural cavity but also arise from the pelvis, liver, kidney, and mediastinum, etc. The UK's pathologic criteria (hypercellularity, >4 mitotic figures/10 high-power fields (HPFs), pleomorphism/atypia, infiltrative growth pattern, necrosis, and hemorrhage) are used to classify SFT as either benign or malignant [1]. Doege-Potter

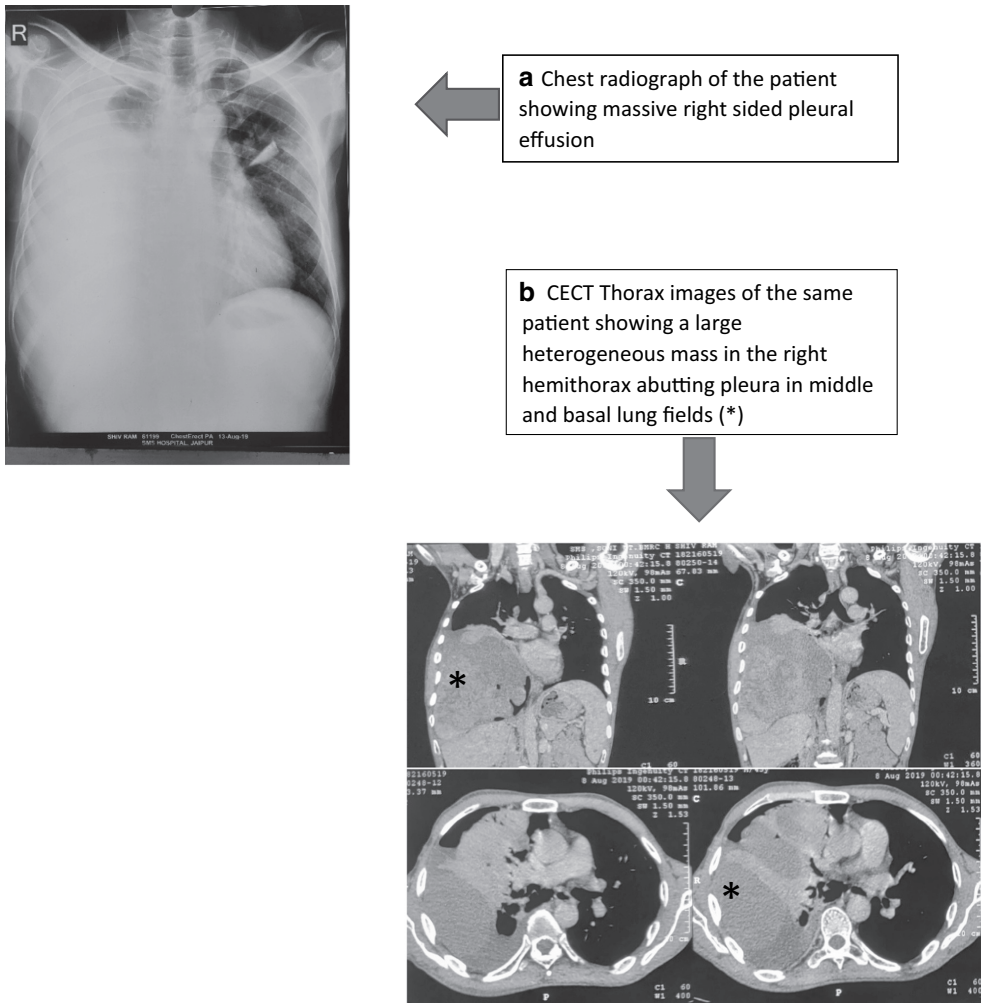

Fig. 1 Various imaging findings of the case

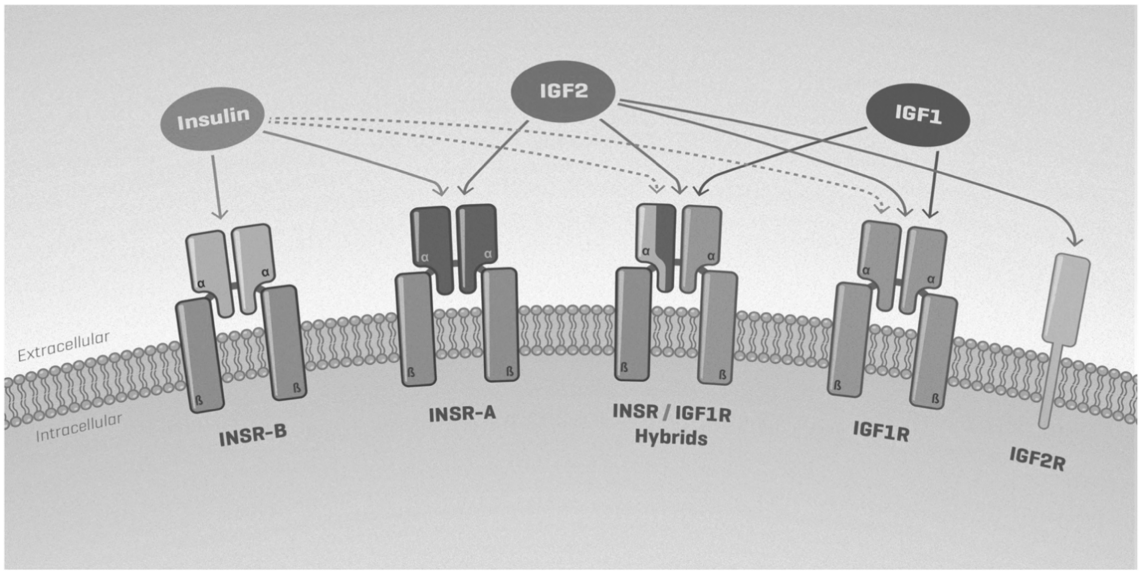

Fig. 2 Interaction between insulin and insulin-like growth factors (IGF) with their receptors causing various metabolic and mitogenic effects. (INSR, insulin receptor; IGFR, IGF receptor) (from open access article: Neirijnck Y, Papaioannou MD, Nef S. The insulin/IGF system in mammalian sexual development and reproduction. Int J Mol Sci. 2019 Sep 9;20(18):4440)

syndrome (DPS) is a paraneoplastic syndrome where hypoglycemia occurs due to secretion of precursor of insulin-like growth factor 2 (pro IGF-2 or big IGF-2) by tumor cells [3]. It is a very rare syndrome, and around 76 cases have been published all over the world. The peak incidence is seen in sixth to eighth decade of life, with similar incidence across genders [1]. SFTs which are located in right hemithorax, and/or having large size (>20 cm) are more likely to cause hypoglycemia. Ectopic overexpression of IGF-2 in these tumors promotes malignant transformation, mitogenesis, and differentiation of tumor cells by binding the IGF-1 receptor. Malignant SFTs are more prone to develop hypoglycemia and carry a poor prognosis [1].

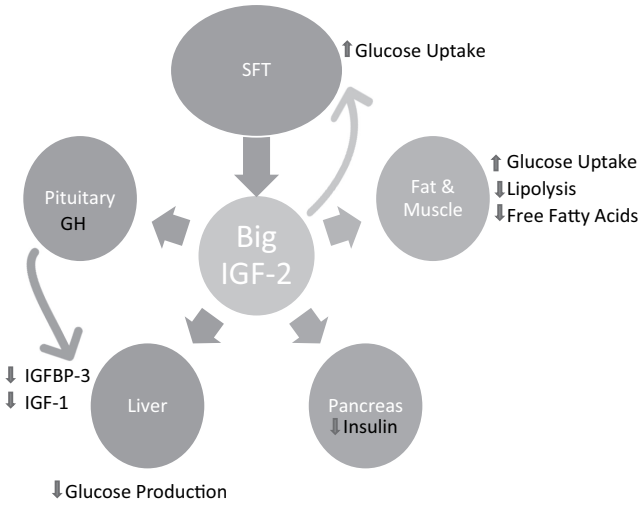

Fig. 3 Various mechanisms of IGF-2-mediated tumor hypoglycemia

Hypoglycemia in DPS is typically associated with decreased levels of serum insulin and c-peptide. An IGF-2/IGF-1 ratio of more than ten typically favors the diagnosis of NICTH, though many cases of DPS have been reported to have IGF-2/IGF-1 ratios less than 10 but higher than normal (up to 3:1) with IGF-2 levels being normal and IGF-1 levels significantly suppressed. Hypoglycemia, as seen in the present case, typically presents as diaphoresis, anxiety, tremors, loss of consciousness, as well as may be asymptomatic.

The Pierre Marie-Bamberg syndrome is a paraneoplastic syndrome associated with bronchogenic carcinoma and rarely with SFTs characterized by development of hypertrophic osteoarthropathy. It is attributed to abnormal hyaluronic acid production by tumor cells. Very rarely both DPS and Pierre Marie-Bamberg can occur together [1].

CT and MRI imaging of tumor is helpful in detection of extent of tumor and invasion of surrounding structures as well as metastasis. SFTs are typically smooth, well-circumscribed, and homogenous masses. Heterogeneity can be seen in necrotic areas of tumor. Pathologically, tumors are well circumscribed, with lobular, firm, and gray white cut surface. Microscopically, spindle cells are seen arranged in fascicle-like pattern, separated by thick and thin collagen bundles forming a definitive diagnosis of SFT [6]. Malignancy is associated with areas hemorrhage and necrosis along with high cellularity and pleomorphism. Immunohistochemical analysis shows CD34, Bcl-2, and vimentin positivity and negative S-100 expression [1].

Potential mechanism of NICTH can be explained by IGF system which is composed of IGF-1 and IGF-2. IGF-1and IGF-2 both are structurally related to insulin. Their actions

are mediated through IGF-1 receptor (IGF-1R). IGF-2 can also interact directly with insulin receptor. Both IGFs compete for binding at IGF-1R, IGF-2/mannose-6-phosphate receptor, circulating IGF-binding proteins (IGFBP) as well as for insulin receptor (IR-A) (Fig. 2).

Normally, essentially all IGF-2 in the circulation are complexed to binding proteins. About 20% is in a 50-kDa binary complex with IGFBP-3 and the rest 80% as 150-kDa ternary complex of IGF-2, IGFBP-3, and ALS (acid labile subunit). "Big" IGF-2 has relatively higher affinity for insulin receptor than for its binding proteins leading to hypoglycemia. Excessive IGF-2 production by tumor cells, low ALS production, and stearic hindrance lead to elevated levels of 50-kDa complexes in DPS. These 50-kDa complexes can easily cross capillaries and mediate their effect through IGF-1R, IR-A, and IR-B receptors in peripheral tissues, causing hypoglycemia (Fig. 3) [5].

Definitive treatment of DPS is complete tumor resection. Completeness of initial resection is the key to prevent recurrence of SFTs and DPS [7]. Neoadjuvant therapy involves chemoradiation, selective embolization of feeding vessels, etc. SFTs are considered relatively chemoresistant. IGF-2 levels fall after complete tumor resection and hypoglycemia also resolves. Awaiting tumor resection, or in cases of malignant tumors or metastasis where surgical resection is not possible, pharmacologic management of hypoglycemia is sought via use of glucocorticoids, GH, and glucagon. Glucocorticoids suppress tumor-specific secretion of "big IGF-2" and thereby reduce hypoglycemia. Serum ALS levels also increase significantly. Prednisolone with a dose of >25 mg/day, dexamethasone at 2mg/day, and methyl prednisolone at 32mg/day could effectively reduce hypoglycemia. GH by stimulating hepatic gluconeogenesis and glycogenolysis alleviates hypoglycemia. Supraphysiological doses of recombinant human growth hormone (rhGH) also increase IGFBP-3 and ALS promoting ternary 150-kDa complexes, in turn, reducing bioavailability of IGF-2. Glucagon is less commonly used via infusion route in patients of tumor hypoglycemia, but its effectiveness still remains controversial [1].

Conclusion

This unique case stresses upon expanding the etiologic considerations of hypoglycemia [8]. NICTH or in more recent terms "IGF-2oma" constitute the main clinical characteristic of DPS [5]. SFTs with hypoglycemia are frequently malignant and carry poor prognosis. Reduced serum insulin and c-peptide levels in settings of hypoglycemia, with raised IGF-2/IGF-1 ratio in setting of a SFT, indicate towards a diagnosis of Doege-Potter syndrome. The effects of adjuvant and neoadjuvant therapies have been controversial, but complete tumor resection along with pharmacologic management is a definitive treatment option [1].

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CASE REPORT

An ultra-rare case of 47,XXY/48,XXXY/49,XXXXY mosaic Klinefelter syndrome associated with diabetic ketosis and foot ulcer

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Abstract

Objective Klinefelter syndrome (KS) is the most common chromosome aneuploidy and a common cause of infertility in males. KS patients have at least one extra X chromosome, and about 80% of patients have the karyotype 47,XXY. Twenty percent of KS patients have higher-grade aneuploidies (48,XXXY, 48,XXYY, 49,XXXXY) and exert some clinical manifestation different from those with 47,XXY.

Methods Here we report the case of a young man who initially presented with diabetic ketosis and foot ulcer and was later diagnosed as having an extremely rare mosaic variant of KS (47,XXY/48,XXXY/49,XXXXY). Clinical and laboratory features are summarized.

Results The patient developed a severe foot ulcer with an 18-month history of diabetes. Learning disability was noted since early childhood. The patient had typical manifestations of KS, including increased height, sparse beard and body hair, and small penis. He also had some characteristic features of patients with additional sex chromosomes, including epicanthal folds, narrow palpebral fissures, prominent elbows, and undescended testicles. Severe myopia and myopic macular degeneration was also found. Lab tests revealed low testosterone and elevated FSH but normal LH levels. Chromosome analysis on lymphocytes revealed an aberration of 47,XXY/48,XXXY/49,XXXXY.

Conclusions This case provides information on KS patients with the extremely rare karyotype 47,XXY/48,XXXY/49,XXXXY, and also emphasizes the importance of considering KS in the differential diagnosis of a sterile male with diabetes.

Keywords Klinefelter syndrome · Karyotyping · Type 2 diabetes mellitus · Insulin resistance · Foot ulcer

Introduction

Klinefelter syndrome (KS) is a congenital disease resulting from additional X chromosome(s) and is also the most common sex chromosome disorder causing primary hypogonadism in males with an incidence of 1–2 per 1000 [1, 2]. The typical description of KS patients emphasizes a tall eunuchoid body proportion, sparse facial and pubic hair, and small testicles and penis. Laboratory tests are characterized by hypergonadotropic hypogonadism. The classical karyotype 47,XXY occurs in 80% of KS patients, while other karyotypes including 48,XXYY, 48,XXXY, 49,XXXXY, and the mosaic forms occur occasionally in the remaining 20% of cases [3]. Besides typical features of classic KS, variants with more extra sex chromosomes have additional congenital malformations and medical problems. In light of the very low frequency (1:18,000–1:40,000 for 48,XXYY, 1:50,000 for 48,XXXY, and 1:85,000–1:100,000 for 49,XXXXY) [4, 5], studies of these variants are largely carried out as case reports or case series. New cases for the rare karyotypes are very important for providing additional information. As a very rare mosaic, few KS cases with 47,XXY/48,XXXY/49,XXXXY have been reported to date, among which most were newborns or children [6–9]. Here we report a 47,XXY/48,XXXY/49,XXXXY adult with diabetic ketosis, foot ulcer, and severe myopia, in order to provide information regarding KS patients with this extremely rare karyotype.

Case

A 30-year-old man was admitted to the Endocrinology Department of the First Affiliated Hospital of Xi'an Jiaotong University for a large expanding ulcer on his left

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foot which had been present for 8 weeks. He was previously admitted to a hospital for local wound care, and sulfadiazine cream had been ineffective. Eighteen months earlier he had been diagnosed with type 2 diabetes (T2DM), and his mother had been helping him inject insulin since then. Fasting blood glucose fluctuated from 6.3 to 14.5 mmol/l. The patient was single without children. He had mental retardation since childhood. He was also found to have severe nearsightedness since early adolescence. No similar cases were found in his family.

Physical examination

On examination, the patient had impaired intelligence, and the history was presented by his mother. The patient was 180 cm tall, 73 kg, with body mass index (BMI) of 22.5 kg/m^2. The upper segment-to-lower segment (86 cm and 94 cm, respectively) ratio equaled 0.91. He had an immature face characterized by extended interpupillary distance, protruding lip, and faintly visible beard. Epicanthal folds, narrow palpebral fissures, and flattened occiput were also noted. Small prominentia laryngea and sparse body hair but no gynecomastia were found. Cardiovascular, pulmonary, and abdominal exams were unremarkable. He was noted to have a small penis and unpalpable testicles. Examination of the lower extremities showed foul, purulent drainage from an ulcer extending deep to muscles and bones on the left foot, along with bilateral dispersed hyperpigmentation. No varicose veins were evident. Left dorsalis pedis pulses were unpalpable, and elevated temperature of the left foot was noted. Prominent elbows and limited elbow joint motion were also observed.

Laboratory, imaging, and other findings

A complete blood count was characterized by strikingly elevated white blood cells (WBC), increased neutrophil count, and moderate anemia. Random capillary blood glucose (about 3 h after breakfast) was 27.5 mmol/l, and glycosylated hemoglobin was 11.8%. Urinalysis showed ketone (+++) and glucose (++++). Liver function revealed decreased albumin. Twenty-four-hour urine protein and albumin were within the normal range. In light of the facial features, sparse body hair, small penis, and unpalpable testicles, KS was suspected. Further examination including evaluation of endocrine glands, ultrasound of male genitals, and chromosome analysis were consequently applied. Low testosterone and dehydroepiandrosterone sulfate with elevated follicle-stimulating hormone (FSH) was noted, but with normal luteinizing hormone (LH). Growth hormone, adrenocorticotropic hormone (ACTH), and cortisol were within normal range. Thyroid function revealed decreased TT3 and FT3, with normal TT4, FT4, and TSH. The results of the lab tests are summarized in Table 1. No bone destruction was detected by X-ray for the left foot. Ultrasound revealed bilateral undescended testes in the inguinal canals. Color Doppler flow imaging (CDFI) showed atherosclerosis plaques in the bilateral superficial femoral and posterior tibial artery and right anterior tibial artery.

Table 1 Laboratory findings on admission of the patient

Laboratory test	Patient's values	Reference range
Blood sample		
RBC	3.61	$\times 10^{12}$/L
Hb	104	120-172 g/L
WBC	20.56	$4–10 \times 10^{9}$/L
NEUT%	88.14	50–70%
Albumin	25.1	35-55 g/L
HbA1c	11.8	4–6%
E2	195.9	28–156 pmol/L
Prog	1.54	0.7–4.3 nmol/L
PRL	11.56	4.04–15.2 ng/ml
LH	6.62	1.7–8.6 mIU/ml
FSH	14.85	1.5–12.4 mIU/ml
T	2.96	9.9–27.8 nmol/L
DHEA-S	2.46	4.34–12.2 μmol/L
GH	3.42	<10 μg/L
ACTH	46.53	5–60 pg/ml
COR (8 a.m.)	13.4	5–28 μg/dl
T4	5.14	4.2–13.5 μg/dl
T3	<0.5	0.8–2.2 ng/ml
FT4	11.3	9.05–25.5 pmol/L
FT3	1.57	2.91–9.08 pmol/L
TSH	2.14	0.25–5 μIU/ml
TPOAB	<0.84	<15 u/ml
TGAB	3.90	<30%
TMAB	2.41	<20%
Urine sample		
KET	≥3+	N
GLU	3+	N
24hU-TP	0.13	0–0.15 g/24 h
24hmALB	6.91	0–30 mg/24 h

RBC, red blood cell count; Hb, haemoglobin; WBC, white blood cell count; Neut%, percentage of neutrophils; HbA1c, Glycosylated Hemoglobin; E2, oestradiol; Prog, progesterone; PRL, prolactin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; DHEAS, dehydroepiandrosterone sulfate; GH, growth hormone; ACTH, adrenocorticotropic hormone; COR, cortisol; T4, thyroxine; T3, triiodothyronine; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibody; TGAB, thyroglobulin antibody; TMAB, thyroid microsomal antibody; KET, ketone; GLU, glucose; 24hU-TP, 24-hour urine protein; 24hmALB, 24-hour urine microalbumin

Electrocardiogram (ECG) showed ST-T changes in V4–V6. Chromosome analysis of lymphocytes revealed an aberration of 47,XXY/48,XXXY/49,XXXXY. Ophthalmology consultation found severe myopia and myopic macular degeneration without visual field defects. In light of the elbow deformity and abnormal ECG, X-ray for elbow and echocardiography were suggested but refused by the family. Other tests including dual-energy X-ray absorptiometry for bone mass density, CDFI for veins of the lower extremities, and IQ test were also recommended but refused by the family for financial reasons.

Diagnosis and treatment

The primary diagnosis was diabetic ketosis and diabetic foot. Fluid replacement and low-dose intravenous insulin (0.1u/kg*h) was given on admission. Intense insulin therapy (multiple daily injections) was later initiated. Besides debridement dressing, antibiotics were given empirically initially and later adjusted according to the bacterial culture. The diagnosis of diabetic peripheral vasculopathy was made based on CDFI, and atorvastatin together with aspirin was prescribed. Testosterone replacement was initiated on diagnosis of KS.

Discussion

Although KS is the most common sex chromosome disorder causing primary hypogonadism in males, variants with more extra sex chromosomes (48,XXYY, 48,XXXY, 49,XXXXY) have very low incidences. Here, we reported a KS patient with an extremely rare mosaic karyotype of 47,XXY/48,XXXY/49,XXXXY. Different from previous cases which were children or newborns, this patient was diagnosed in his adulthood. He had typical manifestations of KS, including increased height, sparse beard and body hair, and small penis. He also had some characteristic features of patients with additional sex chromosomes, including epicanthal folds, narrow palpebral fissures, prominent elbows, and undescended testicles [10]. Lab tests revealed low testosterone and elevated FSH but normal LH levels, in accordance with some reported cases [11–13]. Learning disability was noted since early childhood, and intellectual disability was suspected. Severe myopia and associated fundus lesions were also found in this patient.

Since the first study evaluating the prevalence of T2DM and KS in 1958 [14], accumulating reports have documented the association between KS and T2DM [15]. Abnormality in oral glucose tolerance tests can be detected in more than one-third [16] and overt diabetes in more than 10% of KS patients [15]. Epidemiological studies have revealed a threefold increase in both morbidity and mortality associated with diabetes in KS patients [17, 18]. Although there have not been adequate explanations for the concomitant occurrence of T2DM and KS, hypogonadism has been presumed to play a vital role [19, 20]. A hypothesis of a vicious cycle including hypogonadism, abdominal obesity, and insulin resistance was raised years ago [21]. Hypogonadism causes accumulation of abdominal adipose, which results in insulin resistance and consequently leads to impaired LH-induced testosterone production, deteriorating the preexisting hypogonadism. Hyperinsulinemia and insulin resistance in KS have been demonstrated both by the measurement of HOMA-IR [homeostatic model assessment for insulin resistance] and with the use of a hyperinsulinemic euglycemic clamp [22]. Hypogonadism has been identified as an independent predictor of truncal adiposity, insulin resistance, and metabolic syndrome in KS [23]. Insulin resistance of Leydig cells has also been confirmed. Based on the vicious circle hypothesis, testosterone replacement should improve body composition and insulin sensitivity in KS patients. Although this benefit has been confirmed in men with hypogonadal states [24, 25], the effect of testosterone treatment on HOMA-IR of KS patients is controversial [22]. Additionally, testosterone therapy does not change the prevalence of T2DM [22]. In fact, higher rates of metabolic syndrome have even been observed in several studies in treated KS patients, although the difference was not statistically significant [26–28]. The report of insulin resistance and metabolic syndrome in KS boys as young as 4–12 years also suggests contributing factors other than hypogonadism [29]. The above findings indicate a more complicated underlying mechanism. In a longitudinal observation study, a higher risk of diabetes was found in KS patients with more copies of the X chromosome, and a dosage effect of genes located on the X chromosome was speculated [28]. Unfavorable socioeconomic status is also considered a contributing factor. Further research is needed to investigate the exact contribution of genetic or epigenetic alteration, hypogonadism, and socioeconomic conditions. The T2DM in our patient was characterized by early onset and lower associated BMI, consistent with a previous study [30]. He developed a foot ulcer with a remarkably shorter history of diabetes than other patients with T2DM, suggesting a mechanism other than diabetic peripheral vasculopathy and neuropathy. As testosterone exerts an antiatherogenic effect via anti-inflammatory mechanisms [31], a long period of hypogonadism results in the susceptibility of KS patients to arteriosclerotic cardiovascular disease (ASCVD). A decrease in endothelial progenitor cells is also considered to lead to the impairment of endothelial function in KS patients. A more prominent disturbance lies in the hemostatic balance. The prevailing hypothesis is that increased plasminogen activator

inhibitor-1 (PAI-1) results in the decreased fibrinolytic capacity [32] and the consequent increase in venous thrombosis (VTE) in KS. Autoimmune disorders are also involved in some cases. Thus, for KS patients with a foot or leg ulcer, both arteries and veins should be evaluated.

There is no solid evidence as to which antidiabetic treatment is better for KS patients with T2DM. However, in light of the abdominal adipose accumulation and insulin resistance in KS, drugs targeting truncal obesity and insulin resistance rather than insulin should be preferable. For those with established ASCVD, according to the consensus report by the American Diabetes Association and the European Association for the Study of Diabetes, SGLT2 inhibitors or GLP-1 receptor agonists (GLP-1 RA) with proven cardiovascular benefit are recommended as part of glycemic management [33]. In consideration of compromised bone health in patients with KS, GLP-1 RA seems to be the more reasonable choice. Future research is needed to provide evidence for optimizing the antidiabetic approach for KS patients with T2DM.

The low diagnosis rate and delay in diagnosis remain an issue for KS. Only 25–40% of expected KS males are ever diagnosed [34–36]. High stature is not a specific sign for distinguishing KS patients from normal individuals. Persistent gynecomastia is more characteristic, but occurs in only about one-third of adult KS patients [37–40]. Small testes are undetected in many cases, since palpation of the scrotum is not routine for patients, for example, with diabetes. Sometimes the missed diagnosis is due to insufficient awareness. There is also no universal agreement on the necessary clinical signs leading to karyotyping [22]. In preadolescent and adolescent KS, absence of overt clinical signs results in an even lower rate of diagnosis. Another issue to address is the importance of multidisciplinary cooperation for comprehensive assessment and treatment of KS.

In summary, this case emphasizes the importance of considering KS in the differential diagnosis of a sterile male with diabetes and, far more importantly, provides information regarding KS patients with this very rare karyotype.

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Compliance with ethical standards

Disclosure of potential conflicts of interest The authors have nothing to disclose, and no conflict of interest exists in the submission of this manuscript.

Research involving human participants Informed consent has been signed.

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CASE REPORT

Mauriac syndrome: a rare cause of massive hepatomegaly

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Abstract

Mauriac syndrome or glycogen hepatopathy (GH) is an uncommon complication of poorly controlled type 1 diabetes mellitus commonly occurring in adolescents. It has become even less common after the emergence of advances on diabetes treatment, but still exists. It manifests as growth failure, hepatomegaly, elevated liver enzymes, and accumulation of glycogen in hepatocytes related to poorly controlled diabetes and a glucose trap in the liver. GH has got good prognosis and fast resolution after adequate glycemic control, with no progression to end-stage liver disease.

We report a 10-year-old boy with poorly controlled type I diabetes mellitus (on pre-mix insulin), hepatomegaly, and extremely high levels of liver transaminases. He underwent a liver biopsy elsewhere which showed glycogen deposits. He was referred to our institution for confirmation of diagnosis of glycogen storage disorder. Clinical examination revealed short stature, moon facies, and protuberant abdomen with hepatomegaly of 8 cm below the right costal margin. After exclusion of other causes of hepatitis, we controlled his sugars using basal bolus insulin regimen. Clinical exome sequencing revealed no abnormal mutations in the enzymes linked to glycogen metabolism. In an about 8 weeks, his transaminases normalized and hepatomegaly regressed.

Keywords Mauriac syndrome · Massive hepatomegaly · type 1 diabetes mellitus · Premix insulin · glycogen hepatopathy

Background

Mauriac syndrome manifests as short stature, glycogen laden enlarged liver, limited joint mobility, tight waxy skin, growth delay, moon facies, protuberant abdomen, and muscle wasting in children with poorly controlled type 1 diabetes mellitus (T1DM). It was first described by Leonard Pierre Mauriac in 1930 [1]. It is frequently associated with retinopathy and nephropathy [2]. Since the advent of the long and intermediate-acting insulin, various manifestations of Mauriac syndrome are rarely encountered [3]. Most of the cases occur in adolescence with an equal gender distribution.

Learning points • Mauriac syndrome is a rare complication of poorly controlled type 1 DM related to glucose trap in the liver.
• Use of pre-mix insulin is a risk factor for development of this condition.
• Mauriac syndrome can present with extremely high levels of transaminases.
• Basal bolus insulin regimen and tight control of glucose is the key for resolution.

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Case presentation

A 10-year-old boy was referred to us to rule out glycogen storage disorder as his liver biopsy was suggestive of glycogen hepatopathy. He appeared to have growth failure, was obese with protuberant abdomen, and had proximal myopathy. He was born out of a non-consanguineous marriage with a birth weight of 3 kg. He was apparently normal until the age of 5 years when he was noticed to have polyuria and weight loss and was diagnosed with T1DM. His blood sugars were managed with pre-mix insulin twice daily, and he would get admitted intermittently for poorly controlled diabetes and would be given intravenous insulin in the hospital for 1–2 days every month. At the time of current clinical presentation, he had poor glycemic

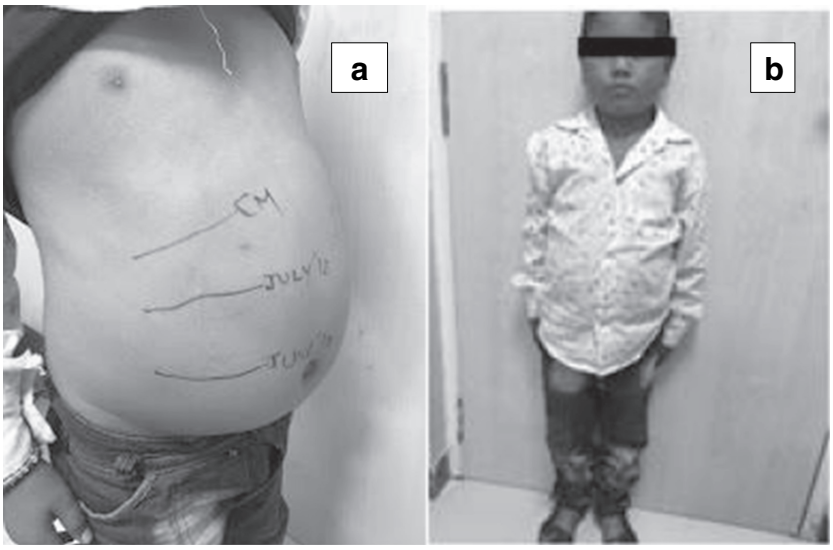

Fig. 1 **a**) Hepatomegaly, **b**) Patient at diagnosis with cushingoid face [CM: Costal margin]

control with HbA1c of 11% and was on a pre-mix insulin regimen. On examination, he had moon facies and stunted growth (Fig. 1b). His height was 114 cm (< 3rd percentile) and weight was 19 kg (< 10th percentile). His bone age was delayed (between 5 and 6 years). He had massive hepatomegaly measuring 8 cm below the right costal margin (liver span 14 cm), non-tender, smooth surface and rounded margins (Fig. 1a). All other systemic examination was normal.

Investigations revealed AST of 1625 IU/L [normal 10–36 IU/L], ALT of 961 IU/L [normal 24–49 IU/L], total cholesterol of 278 mg/dl [normal < 170 mg/dl], and serum triglycerides of 908 mg/dl [normal < 150 mg/dl], and serum lactate was 9.0 mg/dL [normal < 2 mg/dl]. Urine routine showed mild proteinuria (1+). Review of liver biopsy slides showed glycogen deposition in hepatocytes. Markers of autoimmune hepatitis and viral hepatitis were negative. Clinical exome sequencing was done which did not show any mutation in the enzymes linked to glycogen metabolism. We managed his poor glycemic control by changing his insulin regimen to a basal bolus regimen and educating the parents on compliance to treatment and regular follow-up. Nearly 2 months on the new regime, his glycemic control improved, hepatomegaly regressed, and repeat LFT showed remarkable improvement (AST 33 IU/L; ALT 42 IU/L), and subsequently have remained normal for the past 1 year of follow-up.

Discussion

Mauriac syndrome is a rare disorder and was more common before long-acting insulins were introduced for the treatment

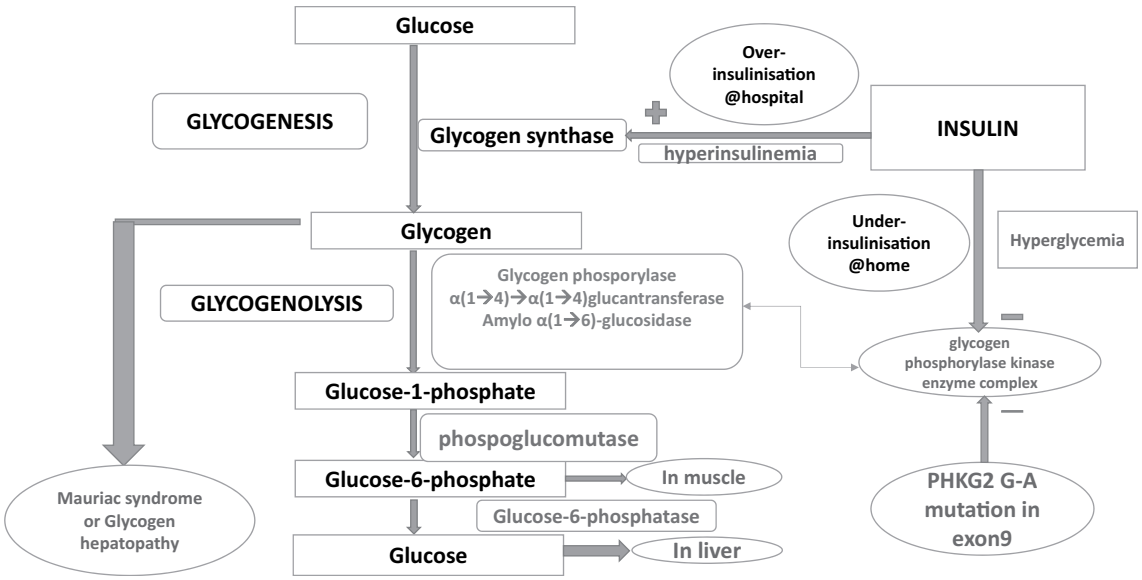

Fig. 2 Pathogenesis of Glycogen Hepatopathy

of T1DM. Our patient had the classic features of Mauriac syndrome including short stature, delayed sexual maturity, moon facies, and hepatomegaly. Two different types of Mauriac syndrome have been described in literature based on the presence or absence of obesity. In the obese subtype, poor glycemic control involves wide fluctuation between hyper and hypoglycemia suggestive of a pattern of over and under-utilization of glucose, respectively. Non-obese subtype occurs in patients who are inadequately insulinized, without a history of alternating hypoglycemia and ketoacidosis [4, 5]. Our case fits into the former phenotype with periods of uncontrolled hyperglycemia at home with the pre-mix insulins and rapid correction of glucose with intravenous insulin infusion in the hospital driving the glucose into the hepatocyte (stimulation of glycogen synthase) which gets converted into glycogen and deposited in the liver. Once he is discharged back home, underinsulinization inhibits glycogenolysis (inhibition of glycogen phosphorylase), and the hepatocyte glycogen content cumulatively increases resulting in glycogen hepatopathy (Fig. 2). Poor glycemic control due to hypoinsulinemia leads to lipolysis and ketone liberation. Ketosis activates cortisol synthesis, promoting the release of fatty acids and hyperglycemia [6].

For the first time, a genetic association was reported in a patient with Mauriac syndrome in 2016 [7]. They described a mutation in the catalytic subunit of liver glycogen phosphorylase kinase enzyme complex [PHKG2 G-A mutation in exon 9] in a patient with Mauriac syndrome who developed growth failure and massive hepatomegaly. This enzyme complex activates glycogen phosphorylase which catalyzes the first step in glycogen breakdown [4]. It was shown that the mutant subunit acts in a dominant manner to completely inhibit glycogen phosphorylase kinase enzyme activity and that this interferes with glycogenolysis causing increased levels of glycogen in human liver cells [4].

One interesting aspect in our case was the degree of elevation of liver enzymes. Transaminases can be elevated up to 1000s in children with poorly controlled diabetes having Mauriac syndrome [8]. Mauriac syndrome should be highlighted as a differential along with the other causes of extremely high transaminases including autoimmune hepatitis, acute viral hepatitis in a child with diabetes mellitus.

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Send in your Research proposals by email to the RSSDI Secy/ Chairman research committee by email/ apply directly on web site.

Research proposal should have following proofs-

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Proposals will be reviewed by the research committee of the RSSDI.

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A minimum of 50% of the grant amount will be disbursed initially. Further disbursement will be done annually based on submission of progress reports on the work done and utilisation of sanctioned amount. These reports must be filed to the secretary of the RSSDI

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All grant awardees are expected to present their work at RSSDI Annual Conference during research presentation's session. Failure to file progress reports annually and when requested by the RSSDI and failure to present progress at RSSDI Annual conf may result in the forfeiture of the grant.

All awardees are expected to follow the tenets of responsible and ethical conduct of research. Unethical or fraudulent use of RSSDI research funds will warrant adverse action from the society including

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The proposals may of clinical or translational research importance. A maximum grant amount of INR 5 Lakhs will be sanctioned. All grants will be reviewed by the research committee.

The detailed proposals should include the following:
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Brief biodata of principal investigator and other co-investigators.

Importance of work
Detailed Budget sought along with full justification/ proposed utilization, of funding sought from RSSDI

Whether the project is being partly funded from any other source? If yes, please mention the source and the amount received.

Ethics Committee clearance of the Institution or other bonafide body.

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All applications should be addressed to:
1. The Secretary , RSSDI
2. Soft copy of the research proposal should be sent to Secretary, RSSDI

When to apply
Proposals will be accepted Twice a year. Once between 1st Jan - 31st April & then July 1st to 30th Nov.

All research proposals will be reviewed by Research committee over a period of 4-6 weeks & approved proposals will be provided Research Grant after fulfilling all documentation by 30th June & then 31st December of each year.

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ADVANCED CERTIFICATE COURSE IN DIABETOLOGY

(IN ASSOCIATION WITH JAIPUR NATIONAL UNIVERSITY)

Research Society for the Study of Diabetes in India (RSSDI) was founded by Prof. M.M.S. Ahuja in 1972. RSSDI is the largest body of professional doctors and researchers in Asia, working in the area of Diabetes & is the National Body recognized by IDF (International Diabetes Federation). One of the key areas of focus is to train doctors at all levels to better manage Diabetes and its complications. RSSDI recognizes this problem and runs a well-structured, full time, residential "Advanced Certificate Course in Diabetology". This two-year course is like any other post graduate course and has immensely helped doctors to practice better diabetes care. RSSDI has

List of RSSDI Accredited Centres

Sl. No	Institute Name	Institute Location
1.	Diacon Hospital	Bangalore, Karnataka
2.	North Delhi Diabetes Centre	New Delhi, Delhi
3.	Prithvi Hospital	Tumkur, Karnataka
4.	Total Diabetes Hormone Institute	Indore, Madhya Pradesh
5.	Dia Care - A Complete Diabetes Care Centre	Ahemdabad, Gujarat
6.	Sonal Diabetes Hospital	Surat, Gujarat
7.	Jothydev's Diabetes and Research Center	Trivandrum, Kerala
8.	Advanced Endocrine & Diabetes Hospital	Hyderabad, Telangana
9.	Sunil's Diabetes Care N' Research Centre	Nagpur, Maharashtra
10.	Marwari Hospital and Research Centre	Guwahati, Assam
11.	Down Town Hospital	Guwahati, Assam
12.	St.Theresa's Hospital	Hyderabad, Telangana
13.	Aegle Clinic	Pune, Maharashtra
14.	Lilavati Hospital & Research Centre	Bandra West, Mumbai
15.	Srajan Hospital	Udaipur, Rajasthan
16.	Endeavour Clinics & Dr. Sambit's Centre of Diabetes and Endocrinology	Bhubaneswar, Odisha
17.	ILS Hospital, Salt Lake	Salt Lake City, Kolkata
18.	Belle Vue Clinic	Dr. U N Brahmacahri Sreet, Kolkata
19.	Arthur Asirvatham Hospital	Mdurai, Tamil Nadu
20.	M V Hospital for Diabetes	Chennai, Tamilnadu
21.	Sarvodaya Hospital and Research Centre	Faridabad, Uttar Pradesh
22.	Galaxy Speciality Centre	Sodala, Jaipur

carefully looked into all aspects of this course & has accredited & recognized 22 centres across India at present and more centers are being inspected for accreditation. National Faculties and experts of RSSDI chosen from Academia visit these centers from time to time to ensure high standards. Now this Advanced Certificate Course has Dual Accreditation from RSSDI and Jaipur National University.

COURSE DETAILS

Name of the Course: Advanced Certificate Course in Diabetology

Duration: 2 Years – Post MBBS & 1 Year - Post MD / DNB (Gen - Medicine)* (Full Time) Educational.

Qualification: A candidate must possess MBBS degree from ANY of the recognized university approved by Medical Council of India (*The duration of the course is 1 Year for those with MD/ DNB in Internal Medicine. Candidates having MD degree in other specialties will have to do the course over 2 Years).

Number of seats: 2 seats per year for every eligible teacher as per rules of Medical Council of India (MCI).

Selection of Candidates: Selection for the Certificate course is through a performance evaluation by Theory test for 90 marks (90 minutes duration) which is conducted at all accredited centres. The result is displayed WITHIN 3 days on the Web site of JNU and RSSDI. Post MD (Internal Medicine) will be given !

COURSE FEES:

- Rs 30000/- (for post MD/DNB (internal medicine), 1 year program)
- Rs. 50000/- (for post MBBS, MD in other branches, 2 years program)

Session: Two sessions are run annually, in January and in July. Prospectus details are available on the RSSDI website. All applications must be sent to Jaipur National University.

ANNOUNCEMENTS

Dear Member,

Please update your Membership details like Complete Postal Address, Email Id, Pan No. & Mobile no. after log in your membership area on our website www.rssdi.in under sub heading Membership corner, so that we can send you RSSDI Newsletter & Journals.

1. RSSDI Research Retreat 2022
26th & 27th March 2022 in Pune

2. RSSDI 50th Golden Jubilee Year Celebrations
(look out for more details on our website)

3. Advance Course in Diabetology Important Dates for January 2022 batch:

Last date of application - 31st December 2021
Screening interview date - 7th January 2022
Screening result date - 10th January 2022
Last date for fee submission - 15th January 2022
Course starting date - 16th January 2022

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