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EDITORIAL

Post COVID-19 diabetes care—lessons and challenges

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The outbreak of COVID-19 all over the world this year has posed several new challenges to diabetes care and has also provided an opportunity for new lessons to be learnt in what seems to be transforming the way we treat diabetic patients.

Some of the issues and challenges that have emerged include the reported high incidence [1, 2] of COVID-19 infections among patients with diabetes mellitus (DM), the greater severity, and higher rates of progression resulting in a higher number of ICU admissions [3] and higher mortality reported in them [1, 2]. The increased predisposition of patients with diabetes to COVID-19 infections particularly severe infections with acute respiratory distress is believed to be related to a compromised innate immunity that accompanies uncontrolled DM and an exaggerated proinflammatory cytokine response involving IL-6 and TNF-alpha [4]. The situation would be worse if there are other comorbidities such as hypertension, chronic kidney disease, or coronary artery disease which often coexist with diabetes and are also associated with more aggressive COVID-19 infections. This calls for early and aggressive management of COVID-19 in diabetic patients to prevent adverse outcomes.

It is equally important to detect fresh cases of diabetes early and pick up hyperglycemic spikes in those known to be diabetic without delay—something which is very difficult at a time when the medical fraternity is struggling to manage the COVID-19 pandemic. A high degree of alertness with frequent self-monitoring of blood glucose levels is certainly advisable in known diabetic patients particularly insulin users to reduce the risk of being affected with COVID-19 infection and avoid any undesirable consequence should they get infected. As physicians involved in diabetes care, we need to advise our patients accordingly besides telling them to follow with greater intensity all the general measures to prevent exposure to COVID-19 infection.

Just as it is important that we target and make every effort to achieve good glycemic control during these COVID times, it is equally important that we identify diabetes comorbidities and complications early and effectively manage them. It is well-known that several of these including hypertension, heart disease including heart failure, and chronic kidney disease are all associated with higher mortality in COVID-19 infections.

Challenges in diabetes management begin with difficulties in effective implementation of lifestyle measures particularly with respect to physical activity and exercise. These are surmountable and can be largely met by advising indoor exercise routines, yoga, and other innovative ways of enhancing physical activity. Fortunately, limited access to fast foods and other unhealthy dietary choices in restaurants should help, but one should still be advised to judiciously choose from available homemade and packaged dietary choices. Poor access to alcohol and limited opportunities for smoking during lockdown can also become a unique opportunity to seriously consider giving up these habits which can help prevent long-term complications of diabetes. Stress and the higher levels of anxiety and depression that can occur during periods of lockdown not only affect the mental health and sleep of the diabetic patient but also adversely affect glycemic control. Appropriate measures to relieve stress are also important to maintain glucose control.

Another important challenge in the management of diabetes mellitus has been regarding the appropriate use of various antidiabetic agents. This has been an area of concern as well as an area of intense speculation and debate. Advisories, not always supported by well-designed studies, are often confusing the treating physician. Overall, insulin appears to be a good option for glycemic control and is safe. It is advised that anti-hyperglycemic drugs that cause volume depletion or hypoglycemia are best avoided or used in small dosages. Metformin may show a higher propensity to lactic acidosis especially in those with volume depletion. SGLT2 inhibitors are contraindicated not only because they are associated with fluid loss but these groups of agents have also been found to be associated with a higher incidence of diabetic ketoacidosis

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and euglycemic ketoacidosis. Hence, SGLT2 inhibitors may need to be stopped in patients developing COVID-19 infections especially if these are moderate or severe [5, 6]. On the contrary, the DARE-19 trial is also ongoing to examine the role of dapagliflozin in preventing multiorgan failure in severe COVID-19 disease based on earlier reports of its beneficial effects in preventing heart and kidney failure.

DPP4 inhibitors are another class of antidiabetic agents which are believed to have a potential to reduce the incidence and severity of complications associated with COVID-19 infections particularly in diabetic patients. This is based on the belief and earlier observations in MERS-COV-infected diabetic patients that a DPP4-mediated immune dysregulation could result in higher rates of mortality and complications in diabetic patients. However, there is no direct evidence so far that the same is true of COVID-19 infections and also the beneficial effects of DPP4 inhibitors are yet to be proven in randomized controlled trials. Until then, this will remain a potential strategy only, particularly to prevent progression to the hyperinflammatory state in severe COVID infections [5, 6].

It has also been hypothesized that insulin resistance promotes inflammation and has been shown to be associated with increase in several inflammatory markers such as C-reactive protein, IL-6, and TNF-alpha and procoagulant markers such as fibrinogen and PAI-1. Pioglitazone, a potent insulin-sensitizing agent, would therefore be expected to significantly reduce insulin resistance-related inflammation and favorably affect the diabetic patient's response to COVID-19 infection [7]. While this appears to be a promising approach, we have to wait for clinical trials to provide necessary evidence for its use.

Hydroxychloroquine (HCQ) is an approved drug by DGCI for use in DM. Ever since the pandemic of COVID-19 unfolded, there has been a lot of interest in some of its properties which could be very useful in the treatment and prophylaxis of this novel infection. It is believed that HCQ lowers the acidity in endosomes and prevents the release of coronavirus from them into the cytoplasm [8]. While its activity against this virus has been shown in vitro, these have not clearly translated to significant clinical benefit in in vivo studies particularly in humans yet. A recent systematic search and narrative review with a special reference to India and other developing countries [8] has reported that 2 small human trials have found improvements in some parameters such as viral load. It has recommended that larger randomized clinical trials (RCTs) should be done urgently to confirm benefit especially in diabetic patients. Currently, HCQ given together with azithromycin is being evaluated for treatment of mild to moderate cases of COVID-19 to see if hospitalizations and deaths can be prevented. India is also testing HCQ for COVID-19 as one of four drugs being evaluated as part of the WHO Solidarity trial. As of now, we need to be cautious in recommending its use in the prevention or treatment of COVID-19 and should wait for the results of these large trials. Since the drug is already approved for use in DM, we may consider its use for glycemic control more often in the current scenario in view of possible collateral benefits.

Statins and ACE inhibitors are 2 groups of drugs very commonly prescribed to diabetic patients, and it is necessary to critically evaluate the current evidence regarding the benefit or risk associated with their continued use. As for statins, there are several reasons to believe that they could benefit patients with COVID-19 coinfection. They could promote innate immune responses to CoV respiratory infections, lead to fewer severe viral pneumonias, and also help in preventing some of the cardiovascular complications of COVID-19. However, there is as yet no clinical evidence that these benefits actually accrue although there are compelling reasons to undertake appropriate clinical trials [9]. Since statins are cheap and are widely prescribed in patients with diabetes, a prudent approach may be to continue statins if already prescribed and consider starting statins without delay in those in whom a recommended indication presents itself.

The relationship of ACE and COVID-19 infections particularly pneumonias and ARDS is complex. While reduced ACE2 expression in DM is believed to predispose COVID patients to severe pneumonias and ARDS, it has also been postulated that ACE2 expression promotes entry of SARS CoV-2 virus into host pneumocytes [4, 5]. There is no clear evidence that would suggest withdrawing ACE inhibitors or ARBs in these patients, and hence, most international societies do not recommend stopping them.

The medical fraternity is eagerly awaiting the development of a successful vaccine against COVID-19, and there has been significant progress. Meanwhile, it should be emphasized that existing influenza and pneumococcal vaccines should be taken as recommended by diabetic patients.

Last but not the least, during these times of lockdown and even post lockdown, all diabetes physicians will have to deliver diabetes care more innovatively and increasingly use telemedicine and other novel approaches to be in touch with the diabetic patient while keeping face-to-face consultations to a minimum. This will ensure maximum benefit with minimum risk.

Let us prepare for all the new post COVID challenges in diabetes care even as we remain alert to newer lessons that are constantly emerging and will help us organize our practice.

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REVIEW ARTICLE

A systematic review of the effect of sleep apnea syndrome and its therapy on HbA1c in type 2 diabetes

Cornelia Bala[1,2] · Gabriela Roman[1,2] · Dana Ciobanu[1] · Adriana Rusu[1]

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Abstract

Background Sleep apnea syndrome (SAS) emerged as a risk factor for alterations of glucose metabolism independent of other risk factors. In persons with type 2 diabetes, clinical studies suggested that SAS may be associated with a worse glycemic control. The aim of this review was to summarize the effect of SAS and its therapy with continuous positive airway pressure (CPAP) on HbA1c in persons with type 2 diabetes.

Methods A systematic literature search was conducted in Embase, PubMed, and Web of Science for studies published from database inception until January 24, 2019. Two reviewers assessed identified studies and performed data extraction. Any disagreement was resolved by a third reviewer.

Results Most of the cross-sectional studies reported no statistically significant difference in HbA1c between those with and without SAS and no linear relation between AHI and HbA1c. Statistically significant higher HbA1c levels in those with than in those without SAS (8.4% vs. 7.6%, $p = 0.04$) were reported only when the analysis was adjusted for potential confounders. An inverse linear association between lowest oxygen saturation, oxygen desaturation index, and HbA1c, independent of other confounders, was reported. Of the 13 non-randomized studies and randomized clinical trials included, 7 reported no statistically significant change of HbA1c following CPAP therapy. The results were similar when we analyzed separately non-randomized and randomized clinical trials.

Conclusion Current findings suggest an effect of hypoxemia during apnea/hypopnea episodes on glycemic control and do not support any effect of CPAP on glycemic control in type 2 diabetes.

PROSPERO register number CRD42019123097

Keywords Sleep apnea · Type 2 diabetes · Glycemic control · HbA1c · CPAP therapy

Introduction

Diabetes has reached epidemic proportions worldwide. According to World Health Organization, the number of persons with diabetes increased from 188 million in 1980 to 422 million in 2014 and is estimated to reach 693 million in 2045 [1, 2]. Large efforts have been made in the past decades to better understand the drivers of this epidemic, and among identified risk factors are genes, epigenetic changes, and an unhealthy lifestyle [3].

In the past 10 years, sleep apnea syndrome (SAS), a sleep-disordered breathing characterized by repetitive episodes of apnea and hypopnea during sleep associated with oxygen desaturation, has emerged as a risk factor for alterations of glucose metabolism independent of other risk factors [4]. In persons without diabetes, it has been shown that SAS is associated with increased insulin resistance and a higher risk of developing type 2 diabetes (T2DM) [5–7]. In persons with T2DM, the results of individual studies have been conflicting, but it has been suggested that the presence of SAS may be associated with a worse glycemic control and that a dose-response relationship may exist between the severity of SAS and glycated hemoglobin (HbA1c) [8, 9]. The mechanisms by

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which SAS impairs glucose metabolism are not completely understood, but it has been postulated that repeated episodes of apnea/hypopnea, associated hypoxemia, and sleep disruption increase the insulin resistance by activation of the sympathetic nervous system, hypothalamic-pituitary-adrenal axis, oxidative stress, and release of proinflammatory cytokines [10].

The prevalence of SAS in patients with T2DM is alarming, with figures ranging between 20.0% and 86.6% of the screened adults [11, 12]. Starting from the high prevalence, the observed effect of SAS on glycemic control and its association with a decreased quality of life, high blood pressure, and an increased risk of cardiovascular diseases, it has been hypothesized that the therapy of SAS may have a beneficial effect on glucose metabolism and on the development of diabetes chronic complications. Currently, multiple methods are available for the therapy of SAS. Among them, continuous positive airway pressure (CPAP) is the one most commonly used. Previous individual studies investigating the effect of CPAP on HbA1c in patients with T2DM showed conflicting results, with either no effect or with an improvement of glycemic control. Systematic reviews and meta-analyses of observational and randomized clinical trials found that CPAP improved insulin resistance, quality of life, and blood pressure in these patients but had no effect on glycemic control [13–16].

Although systematic reviews are available on the effect of CPAP on glycemic control in patients with T2DM, no such approach is available on the effect of SAS on HbA1c. Thus, the aim of this systematic review was to assess the effect of SAS and its therapy with CPAP on glycemic control, as assessed by HbA1c, in persons with T2DM and to identify potential methodological issues that may explain the conflicting results of individual studies.

Materials and methods

To fulfill the objectives above, we developed a protocol compliant with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol [17]. The full protocol is available in PROSPERO register (registration number: CRD42019123097).

Literature search strategy

A systematic literature search was conducted in Embase, PubMed, and Web of Science for studies published from database inception until January 24, 2019. An example of the search query used in one of the databases is provided below:

((“diabetes mellitus, type 2”[MeSH Terms] OR “type 2 diabetes mellitus”[All Fields] OR “type 2 diabetes”[All Fields]) AND (“sleep apnoea”[All Fields] OR “sleep apnea syndromes”[MeSH Terms] OR (“sleep”[All Fields] AND “apnea”[All Fields] AND “syndromes”[All Fields]) OR “sleep apnea syndromes”[All Fields] OR (“sleep”[All Fields] AND “apnea”[All Fields]) OR “sleep apnea”[All Fields])) AND (“glycated hemoglobin a”[MeSH Terms] OR “glycated hemoglobin a”[All Fields] OR “hba1c”[All Fields])

After the deletion of duplicates, two independent reviewers screened titles and abstracts to determine whether the studies met the eligibility criteria. Full-text copies of articles that were considered as potentially relevant were retrieved and reviewed by the two researchers to determine the inclusion in the review based on the below inclusion and exclusion criteria. Any disagreement between them was resolved by consulting a third reviewer.

Inclusion and exclusion criteria

Cross-sectional studies, prospective/retrospective observational studies, and interventional trials performed in adult (> 18 years of age) patients with T2DM and in which SAS was diagnosed by full night polysomnography or home sleep apnea testing devices (respiratory polygraphs) were included. Exclusion criteria were publications in other languages than English, case reports, commentaries, personal opinions, review articles, meta-analyses and book chapters, studies which enrolled children or adolescents, studies in which SAS was diagnosed by questionnaires, clinical tools, prediction algorithms, or pulse oximeters only.

Data extraction and synthesis

After an agreement was reached on all articles, data was extracted by the two independent reviewers using an excel-based data extraction form. The following information was extracted: author, year of publication, study design, method of sleep apnea syndrome diagnosis, sample size, age, gender, diabetes duration, apnea-hypopnea index (AHI), oxygen desaturation index (ODI), oxygen saturation during sleep, and HbA1c. For the latter four parameters, if the article described a CPAP intervention, data were collected before and after CPAP therapy.

Outcome measures

The main outcome measures of interest in the clinical studies included in this systematic review were:

- HbA1c levels according to AHI in untreated patients with T2DM and sleep apnea syndrome

- The effect of CPAP on HbA1c when AHI was the variable of interested that was used to evaluate the control of sleep apnea syndrome
- HbA1c levels according to other sleep parameters (i.e., ODI, oxygen saturation during sleep)
- The effect of CPAP on HbA1c when other sleep parameters (i.e., ODI, oxygen saturation during sleep) were the variables of interested that were used to evaluate the control of SAS

Quality assessment and risk of bias

Methodological index for non-randomized studies (MINORS) was used to assess the quality of prospective observational studies; studies with a score of ≥ 12 were rated as good methodological quality [18] and included in the systematic review. For randomized clinical trials, the risk of bias was assessed by Cochrane risk of bias tool and based on risk level were graded as "good quality" or "fair quality" or "poor quality" [19]. The randomized clinical trials assessed as fair or good quality were included in the systematic review. For the quality assessment of cross-sectional studies, we used the National Heart, Lung, and Blood Institute's Quality Assessment Tool [20], and those graded as fair or good quality were included in the review.

Results

Study selection

The initial search in the selected databases yielded 585 results. After removal of duplicates, and of those not fulfilling the inclusion/exclusion criteria, 143 underwent full-text screening. Following the full-text review, 120 were excluded for not fulfilling the inclusion/exclusion criteria, due to quality issues, not enough data provided, or duplication of the results provided in another manuscript. Thus, we included 23 studies in this systematic review (Fig. 1).

SAS and glycemic control

Apnea-hypopnea index and HbA1c

Table 1 summarizes the key characteristics and outcomes of the seven studies that fulfilled the quality criteria and were included. All studies were cross-sectional and included between 60 and 762 participants with a mean BMI > 30 kg/m^2 [8, 9, 21–25].

HbA1c levels in patients with SAS compared to those without SAS were reported in four studies [8, 9, 23, 25]. However, the statistically significant difference was reached in only one study [8]. In a sample consisting of 100 consecutive patients with T2DM, Rusu et al. [8] reported significantly higher HbA1c values in patients with SAS as compared to those without SAS (8.4% vs. 7.6%; $p = 0.04$) after adjustment for potential confounders.

Two studies reported a significant association between HbA1c and SAS severity [9, 22]. In a sample including 762 patients with T2DM, Priou et al. [22] reported a significant increase of HbA1c from 6.68% in the lowest AHI quartile to 7.2% in the highest AHI quartile ($p = 0.033$) only in untreated diabetic patients. No difference in glycemic control between AHI quartiles was observed in treated diabetic patients ($p = 0.589$). Similarly, in a smaller sample, Aronsohn et al. [9] reported that HbA1c was increased by 1.49% ($p = 0.0028$), 1.93% ($p = 0.0033$), and 3.69% ($p < 0.0001$) in patients with mild, moderate, and severe SAS, respectively, compared with those without SAS. Conversely, two studies reported no association between HbA1c and SAS severity [8, 21]. However, there was a difference in HbA1c levels between those without SAS and those with severe SAS in one study [21].

The association between HbA1c and AHI considered as a continuous variable was assessed in five studies. Grimaldi et al. [23] found that HbA1c was significantly associated with higher rapid eye movement (REM) AHI but not with non-rapid eye movement (NREM) AHI in all enrolled participants, while in another study, Priou et al. [22] reported that adjusted HbA1c was associated with AHI ($b = 0.093$; $p = 0.0007$) only in untreated diabetic patients. Conversely, HbA1c was not significantly associated with AHI in three studies [8, 24, 25].

Hypoxemia parameters and HbA1c

Table 2 summarizes the key outcomes of the eight studies that fulfilled the quality criteria and were included. All studies were cross-sectional and included between 52 and 1138 participants, and a mean BMI was > 30 kg/m^2 [8, 21–24, 26–28].

Four of the eight included studies assessed glycemic control according to ODI categories. Studies of Kent et al. [26] and Priou et al. [22], enrolling 1138 and 762 participants, respectively, found increasing HbA1c levels with increasing ODI. In another study, enrolling 115 patients, Grimaldi et al. [23] showed that HbA1c was significantly lower in the lowest REM ODI category (6.5%) than in the highest REM ODI category (7.5%, $p = 0.039$). No difference in HbA1c between NREM ODI categories was observed. In the study enrolling the lowest number of participants (only 52 participants), a plateau effect was noted in HbA1c levels in moderate and severe SAS, as defined by ODI [28].

When the linear association between glycemic control and ODI was tested, of the four studies that assessed this association [8, 22, 23, 27], two found no association after adjustment for potential confounders [8, 27]. Priou et al. [22] reported an association of ODI with HbA1c levels only in untreated

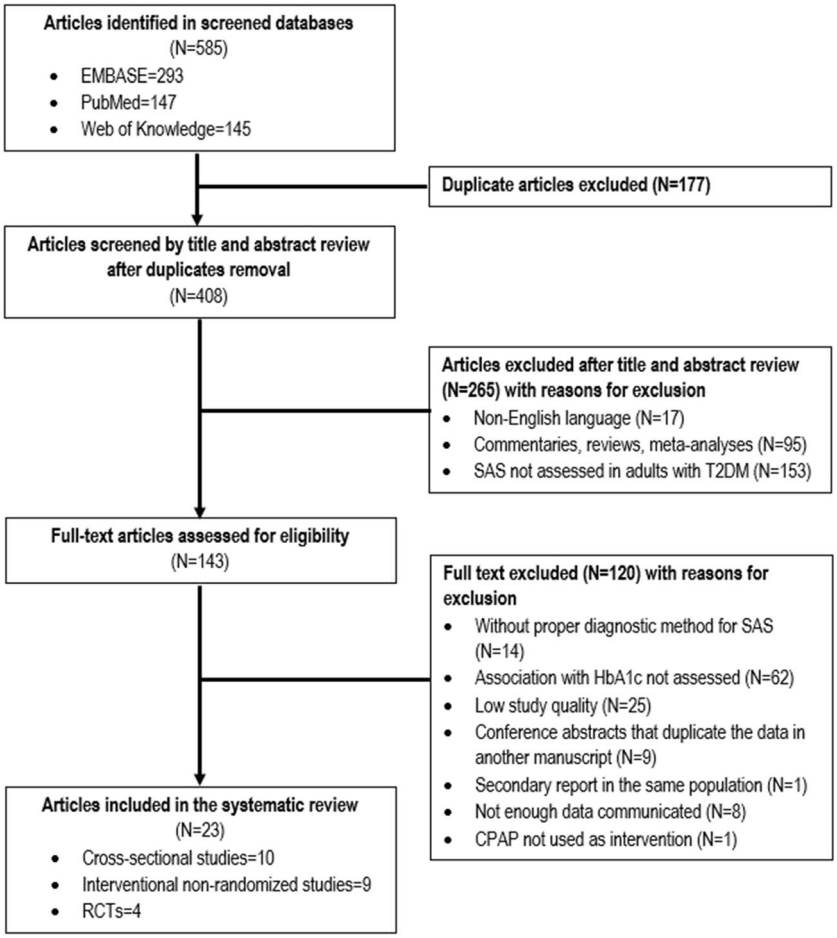

Fig. 1 PRISMA flow diagram of selected studies. *N*, number of studies; RCT, randomized clinical trial; T2DM, type 2 diabetes mellitus; CPAP, continuous positive airway pressure

diabetic patients, while Grimaldi et al. [23] reported the association only for REM ODI and not for NREM ODI.

For lowest oxygen saturation, the association with HbA1c was tested in two studies performed by Rusu et al. [8] and Leong et al. [24], and both showed a significant linear relation independent of other confounders. None of them found any association of mean oxygen saturation with HbA1c. Percentage of time spent with oxygen saturation < 90% was also correlated with HbA1c values [8, 21, 24]. However, as shown by Rusu et al., the relation was mediated by the lowest oxygen saturation [8].

Effect of CPAP on HbA1c

Thirteen studies which reported the effect of CPAP on HbA1c levels in patients with T2DM and fulfilled the quality criteria were identified. Of these, one was a retrospective observational study with a control group [29], eight were non-randomized, prospective, interventional studies (one with a control group) [30–35, 44, 45], and four were randomized controlled trials (of which only one had a double-blind design using therapeutic and placebo/sham CPAP) [36–39]. The number and main characteristics of subjects are presented in Tables 3 and 4.

Non-randomized studies

The retrospective study [29] included 300 participants (150 users of CPAP, 150 matched controls) and had the longest observation period (5 years). About 93% of patients continued to use CPAP in year 1 and 89% in year 5, with a mean usage of 5.8 ± 1.0 h/night (data available for 11 patients only). Subjects using CPAP had a significantly better glycemic control starting from year 2, and the difference remained significant through year 5, mainly due to an important deterioration of HbA1c in controls (Table 3).

The 8 prospective non-randomized studies included between 9 and 193 participants and had an intervention period ranging from 30 days (2 studies) to 12 months (3 studies) [30–35, 44, 45]. The reported mean average use of CPAP was of more than 4 h/night with only one study reporting a PAP use over 90 days of 3.5 ± 2.5 h/night [30]. A significant decrease of HbA1c levels was reported only in three prospective studies out of eight selected, after an intervention period of 30 days, 6 months, and 12 months, respectively [31–33].

Table 1 Summary of studies assessing the effect of sleep apnea syndrome and apnea-hypopnea syndrome on HbA1c

Author, year, country	Type of study	No of participants with T2DM included	Age (years)	Male sex *n* (%)	BMI (kg/m2)	Diabetes duration (years)	Sleep assessment method	SAS diagnosis criteria	SAS *n* (%)	Study results – HbA1c in groups (no SAS vs. SAS; severity of SAS); correlation coefficients, regression coefficients
Whitaker et al., 2018 [21]	Cross-sectional	369	69.3 ± 8.9	164 (44.4-%)	31.5 ± 5.8	Not disclosed	Compumedics Somte System, Compumedics, Abbottsville, Australia	AHI categories: < 5, 5–14.9 none/minimal; 15–29.9 mild, moderate ≥ 30 severe SAS	279 (75.6%)	No difference in adjusted HbA1c between SAS severity categories (7.1% in no SAS, 7.07% in mild SAS, 7.17% in moderate SAS and 7.25% in severe SAS, $p = 0.16$)
Rusu et al., 2017 [8]	Cross-sectional	100	55.4	61 (61%)	37.2	7.3	ApneaLink	No SAS: AHI < 5 events/h or AHI = 5–15 events/h, sleep without symptoms; mild SAS: AHI = 5–15 events/h recording plus symptoms; moderate SAS AHI = 15–30 events/h; severe SAS: AHI ≥ 30 events/h	64 (64%)	Adjusted HbA1c was higher in patients with SAS than in those without SAS (8.4% vs. 7.6%; $p = 0.024$) No difference in crude and adjusted HbA1c levels between SAS severity categories (8.8% in mild SAS, 8.1% in moderate SAS, 8.3% in severe SAS, $p = 0.08$) No association between HbA1c and AHI in the adjusted model that included hypoxemia parameters
Priou et al., 2015 [22]	Cross-sectional	762	63.5 ± 11.4	513 (67.3-%)	34.4 ± 6.9	Not disclosed	PSG	AHI ≥ 5 events/h	720 (94.4%)	HbA1c was associated with AHI ($b = 0.093$; $p = 0.0007$) and with SAS severity as assessed by the AHI ($b = 0.105$; $p = 0.0009$) in untreated diabetic patients in the adjusted model Adjusted HbA1c increased from 6.68% in the lowest quartile of AHI (< 17) to 7.20% in the highest quartile of AHI (> 61; $p = 0.033$) in untreated diabetic patients* HbA1c was not associated with AHI ($b = 0.002$) in treated patients with diabetes
Grimaldiet al., 2014 [23]	Cross-sectional	115	55.2 ± 9.8	61 (43.5-%)	34.5 ± 7.5	4 (1–12)	PSG (Neurofax EEG 1100 system, Nihon Kohden, Foothill Ranch, CA)	AHI ≥ 5 events/h	98 (85.2%)	No difference in unadjusted HbA1c between no SAS 6.7 ± 1.3% and SAS 7.4 ± 1.7, $p = 0.10$ Total AHI was significantly associated with higher HbA1c ($b = 0.046$, $p = 0.019$). REM AHI was significantly associated with HbA1c ($b = 0.063$, $p = 0.001$ and $b = 0.060$, $p = 0.008$) in the adjusted model Adjusted HbA1c increased from 6.3% in patients with REM AHI < 12.3 events per hour to 7.3% in subjects with

Table 1 (continued)

Author, year, country	Type of study	No of participants with T2DM included	Age (years)	Male sex *n* (%)	BMI (kg/m2)	Diabetes duration (years)	Sleep assessment method	SAS diagnosis criteria	SAS *n* (%)	Study results – HbA1c in groups (no SAS vs. SAS; severity of SAS); correlation coefficients, regression coefficients
										REM AHI > 47 events per hour ($p = 0.044$ for trend). No association between NREM AHI and HbA1c was found. No increase of HbA1c by NREM AHI quartiles was observed ($p = 0.819$).
Leong et al., 2014 [24]	Cross-sectional	161	51.1 ± 10.2	65 (40.4-%)	48.8 ± 9.1	Not disclosed	Embletta (Embla Systems)	AHI ≥ 5 events/h	129 (80.1%)	HbA1c was not significantly associated with AHI ($b = 0.0011$ [95%CI: − 0.0088 to 0.0109]) in adjusted linear regression analysis
Lam et al., 2010 [25]	Cross-sectional	165	Available only for the whole cohort	Available only for the whole cohort	Available only for the whole cohort	Not disclosed	PSG (Alice 5 Diagnostics System; Respironics; Murrysville, PA)	AHI ≥ 5 events/h	89 (53.9%)	No difference in unadjusted HbA1c levels between those with and without SAS (8.5% vs. 8.4%, $p = 0.685$) HbA1c did not correlate with AHI ($p = 0.310$)
Aronsohn et al., 2010 [9]	Cross-sectional	60	57.0 ± 9.2	27 (45%)	33.8 ± 7.7	9.6 ± 8.0	PSG	AHI ≥ 5 events/h	46 (77%)	No difference in unadjusted HbA1c between no SAS and SAS (7.2 ± 1.4% vs. 7.8 ± 1.9%; $p = 0.28$) Adjusted HbA1c was increased by 1.49% ($p = 0.0028$), 1.93% ($p = 0.0033$), 3.69% ($p < 0.0001$) in patients with mild, moderate and severe SAS, respectively, compared with those without SAS

AHI=apnea hypopnea index; BMI=body mass index; SAS=sleep apnea syndrome; PSG=polysomnography; REM= rapid-eye movement; HbA1c = glycated hemoglobin; NREM=non-rapid eye movement

Table 2 Summary of studies assessing the effect of hypoxemia parameters on HbA1c

Author, year, country	Type of study	No of participants with T2DM included	Age (years)	Male gender *n* (%)	BMI (kg/m2)	Diabetes duration (years)	Sleep assessment method	SAS diagnosis criteria	SAS *n* (%)	Study results – HbA1c in groups (no SAS vs. SAS; severity of SAS); correlation coefficients, regression coefficients
Whitaker et al., 2018 [21]	Cross-sectional	369	69.3 ± 8.9	164 (44.4-%)	31.5 ± 5.8	Not disclo-sed	Compumedics Somte system, Compumedics, Abbottsville, Australia	AHI categories: < 5, 5–14.9 none/minimal; 15–29.9 mild, moderate ≥ 30 severe SAS	279 (75.6-%)	Significant association between % time spent at oxygen saturation < 90% categories and HbA1c after adjustment ($p < 0.05$)
Rusu et al., 2017 [8]	Cross-sectional	100	55.4	61 (61%)	37.2	7.3	ApneaLink	No SAS: AHI < 5 events/h or AHI = 5–15 events/h, sleep without symptoms; mild SAS: AHI = 5–15 events/h recording plus symptoms; moderate SAS AHI = 15– 30 events/h; severe SAS: AHI ≥30 events/h	64 (64%)	No association was found between HbA1c and ODI in the adjusted model that included other hypoxemia parameters ($\beta = 0.17$, $p = 0.15$) No association was observed between HbA1c and mean saturation of arterial oxygen during sleep in the adjusted model that included other hypoxemia parameters ($\beta = -0.18$, $p = 0.12$) Significant negative association was observed between HbA1c and lowest saturation of arterial oxygen during sleep in the adjusted model that included other hypoxemia parameters ($\beta = -0.21$, $p = 0.05$)
Kent et al., 2014 [26]	Cross-sectional	1138	Available only for the whole cohort	Available only for the whole cohort	Available only for the whole cohort	Available only for the whole cohort	Cardiorespiratory polygraphy (PG) or full polysomnogra-phy (PSG)	ODI ≥ 5/h	not discl-osed	Adjusted HbA1c increased with increasing SAS severity, from 6.76% in those with no SAS to 6.70%, 6.88% and 7.48% in those with mild, moderate or severe SAS ($p < 0.001$)
Aurora et al., 2017 [27]	Cross-sectional	398	59.2 (53.1–6-6.5)	52% men	32.3 ± 5.6	Not disclo-sed	Apnealink Plus	Not used	Not discl-osed	No association between ODI and HbA1c in unadjusted or adjusted models
Priou et al., 2015 [22]	Cross-sectional	762	63.5 ± 11.4	513 (67.3-%)	34.4 ± 6.9	Not disclo-sed	PSG	AHI ≥ 5 events/h	720 (94.4-%)	HbA1c was associated with ODI (b = 0.097; $p = 0.0016$) in untreated diabetic patients HbA1c was associated with SAS severity as assessed by the ODI (b = 0.104; $p = 0.0034$) only in untreated patients with diabetes Adjusted HbA1c increased from 6.69% in the lowest quartile of ODI (< 17) to 7.16% in the highest quartile of ODI (> 57.3; $p = 0.042$) in untreated patients with diabetes HbA1c was not associated with ODI (b = 0.028) in treated patients with diabetes
Grimaldi et al., 2014 [23]	Cross-sectional	115	55.2 ± 9.8	61 (43.5-%)	34.5 ± 7.5	4 (1–12)	PSG (Neurofax EEG 1100 system, Nihon Kohden,	AHI ≥ 5 events/h	98 (85.2-%)	REM ODI was significantly associated with increasing levels of HbA1c, independent of age, sex, BMI, race risk, years of type 2 diabetes, insulin use, and NREM ODI.

Table 2 (continued)

Author, year, country	Type of study	No of participants with T2DM included	Age (years)	Male gender *n* (%)	BMI (kg/m2)	Diabetes duration (years)	Sleep assessment method	SAS diagnosis criteria	SAS *n* (%)	Study results – HbA1c in groups (no SAS vs. SAS; severity of SAS); correlation coefficients, regression coefficients
							Foothill Ranch, CA)			Mean adjusted HbA1c: 6.5% in the lowest quartile of REM ODI and 7.5% in the highest quartile ($p = 0.039$). NREM AHI and NREM ODI were not associated with HbA1c.
Leong et al., 2014 [24]	Cross-sectional	161	51.1 ± 10.2	65 (40.4-%)	48.8 ± 9.1	Not disclo-sed	Embletta (Embla Systems)	AHI ≥ 5 events/h	129 (80.1-%)	HbA1c were associated with worse %total saturation time < 90% ($b = 0.0177$ [95%CI 0.0043 to 0.0310]) and lowest oxygen saturation ($b = -0.0304$ [95%CI − 0.0595 to − 0.0013]) in adjusted linear regression analysis HbA1c was not significantly associated with mean oxygen saturation (b = − 0.0688 [95%CI − 0.1598 to 0.0221])
Pillai et al., 2011 [28]	Cross-sectional	52	54.4 with SA S vs. 53.7 without SAS		37.0 with SAS vs. 35.7 without SAS	9.6 with SAS vs. 9.2 ye-ars without SAS	VISI-3, Stowood Scientific Instruments, and oximetry	ODI ≥ 5 (severity also defined according to ODI)	30 (58%)	Patients with or without SAS had similar HbA1c levels (9.2% vs. 8.9%, $p = 0.46$) Adjusted HbA1c in each SAS category was 8.62% for none, 9.36% for mild, 10.61% for moderate, and 9.91% for severe SAS ($p = 0.014$)

SAS = sleep apnea syndrome; HbA1c = glycated hemoglobin; AHI = apnea hypopnea-index; ODI = oxygen desaturation index; SD = standard deviation; BMI=body mass index; PSG=polysomnography; REM=rapid-eye movement; NREM=nonrapid-eye movement

Table 3 Summary of non-randomized studies evaluating the effect of CPAP therapy on HbA1c

Author, year, country	Type of study	Control group	No of participants with T2DM included	Age (years)	Gender	BMI (kg/m^2)	Diabetes duration	Baseline mean AHI (episodes/h)	Treatment duration and adherence	Study results – HbA1c in CPAP vs. no intervention or baseline vs. post-intervention
Harsch, 2004, Germany [44]	Prospective, interventional	No	9	Mean ± SD 56.3 ± 8.2	7 men 2 women	Mean ± SD 37.3 ± 5.6	Range 1–48 months	43.1 ± 21.3	3 months CPAP use 39.2 ± 6.5 nights out of 42 nights; 5.8 ± 1.2 h/night	6.4 ± 0.7%% at baseline vs. 6.3 ± 0.6% after 3 months; p = NS
Babu, 2005, USA [35]	Prospective, interventional	No	24	Mean ± SD 50.7 ± 9.0	64% men	Mean ± SD 42.7 ± 8.7	Mean 8.3 ± 6.8 years	56 ± 37	30–90 days CPAP use 4.2 ± 2.9 h-/night	For the entire patient population 8.3% ± 2.2% at baseline vs. 7.9% ± 1.8% after CPAP treatment (p = 0.06) Pre-specified analysis (HbA1c > 7% at enrollment; 17 patients) 9.2% ± 2.0% at baseline vs. 8.6% ± 1.8% after CPAP treatment (p = 0.02)
Myhill, 2012, Australia [34]	Prospective, interventional	No	44 completers/59 included	Mean ± SD 66.1 ± 8.8	61.4% male	Mean ± SD 33.6 ± 5.5	Median (interquartile range) 10.1 (3.8–15.3) years	Median (interquartile range) 38 (27–58)	3 months, CPAP use 5.4 ± 1.6 h/night	Baseline 6.9 (6.1–7.3); 1 month 6.7 (6.1–7.4); 3 months 6.7 (6.1–7.4), p = 0.68
Guo, 2015, China [31]	Prospective, interventional	No	40	Mean ± SD 54.8 ± 9.8	28 males and 12 females	Mean ± SD 29.8 ± 3.5	Not disclosed	30.65 ± 18.56	30 days, CPAP use 5.57 ± 1.19 h/night	Unadjusted baseline HbA1c 8.70 (7.40, 10.40); 30 days HbA1c 6.95 (6.38, 7.52), p < 0.001
Wago, 2016, Japan [33]	Prospective, interventional	No	37	Not reported	Not reported	Mean ± SD 28.1 ± 1.3	Not reported	Not reported	12 months, CPAP use not reported	Baseline HbA1c: 6.9 ± 0.3%, 12 months HbA1c 6.3 ± 0.3%, p < 0.005
Donovan, 2017, USA [30]	prospective, interventional	Yes	193 (128 with PAP/ 65 controls)	Mean ± SD PAP 61.6 ± 11.-8 controls 61.0 ± 12.-3	PAP 52.3-% males controls 34.4-% males	Mean ± SD PAP 33.9 ± 7.1 controls 33.4 ± 7.3	Not reported	PAP 25.0 ± 23.-5 controls 14.0 ± 14.7	12 months, PAP use over 90 days 3.5 ± 2.5 h-/night	PAP group HbA1c at baseline 7.7%, 7.9% at 90 days (p = 0.04) and 7.9% at 1 year (p = 0.12 compared to baseline, delta HbA1c 0.20 ± 0.12). Control group delta HbA1c 0.05 ± 0.19 from baseline to 1 year Same results were seen in adjusted analyses, or when restricting the PAP group to those adherent to treatment (≥ 4 h/night on average).
Torrella, 2017, Spain [45]	Prospective, interventional	No	47	Median (interquartile range) 60 (53–65)	Not reported	Median (interquartile range)	14 (7–18) years in 78% of participants	68% had > 30 events/h	56 weeks, CPAP use: 10 not used CPAP 37 used CPAP 5.3 [0.5/6.6 h/night]	Unadjusted HbA1c baseline 8.9 (8–9.7)%; at 56 weeks: 8.8 (7.7–9.9)%, p = 0.526

Table 3 (continued)

Author, year, country	Type of study	Control group	No of participants with T2DM included	Age (years)	Gender	BMI (kg/m^2)	Diabetes duration	Baseline mean AHI (episodes/h)	Treatment duration and adherence	Study results – HbA1c in CPAP vs. no intervention or baseline vs. post-intervention
						35.6 (31.8–38.-5)				
Caseiro, 2018, Spain [32]	Prospective, interventional (CPAP and APAP)	No	31	Mean ± SD 56.7 ± 12.-5	58.1% males	32.5 ± 5	Not reported	Not reported	6 months, device use 6.14 ± 1.64 h-/night	Baseline HbA1c 8.25 ± 1.30%; at 6 months: 7.79 ± 1.33%, $p = 0.026$ There was also an improvement in HbA1c values in patients that used APAP compared to CPAP therapy
Guest, 2014, UK [29]	Retrospective, observational	Yes	300 (150 users of CPAP, 150 matched controls)	Mean ± SD 53.9 ± 0.9 in CPAP group 53.6 ± 1.0 in control group	82% men in CPAP group 83% men in control group	Mean ± SD 38.9 ± 0.6 in CPAP group 35.4 ± 0.5 in control group	Not disclosed	Not disclosed	5 years, CPAP use 93% in year 1 to 89% in year 5 5.8 ± 1.0 h/night (data available for 11 patients)	Unadjusted HbA1c in CPAP vs. control: baseline 7.5% vs. 7.4% year 1: 7.5 ± 2.6% vs. 7.9 ± 2.6, p = NS year 2: 8.8 ± 4.0% vs. 13.1 ± 4.3, $p < 0.05$ year 3: 8.1 ± 3.9 vs. 12.2 ± 4.2, $p < 0.05$ year 4: 7.8 ± 3.9 vs. 13.3 ± 4.3, $p < 0.03$ year 5: 8.2 ± 3.5 vs 12.1 ± 3.8, $p < 0.03$

SAS = sleep apnea syndrome; HbA1c = glycated hemoglobin; SD = standard deviation; PAP = positive airways pressure; CPAP = continuous positive airways pressure; APAP = automatic positive airways pressure; BMI=body mass index; NS=non-significant

Table 4 Summary of randomized clinical trials evaluating the effect of CPAP therapy on HbA1c

Author, year, country	Type of study	Control group	No of participants with T2DM included	Age (years)	Gender	BMI (kg/m^2)	Diabetes duration	Baseline mean AHI (episodes/h)	Treatment duration and adherence	Study results – HbA1c in CPAP vs. no intervention or baseline vs. post-intervention
West, 2007, UK [37]	Randomized, double-blind	Yes (20 CPAP vs. 22 sham)	42	Mean (range) CPAP 58 (29–74) years, sham 55 (24–66) years	All men	Mean (range) CPAP 36.6 (26.2–49-.2), sham 36.8 (29.2–47-.1)	7.3 years in the CPAP group and 6.5 years in the sham group	> 4% Sao2 dips/h CPAP 33.1 (11.0–87.-9), sham 39.1 (10.8–82.-2)	3 months CPAP use 3.3 (2.6) h/night in therapeutic 3.5 (2.8) h/night in placebo	Baseline HbA1c therapeutic 8.5 (6.5–12.1), placebo 8.4 (6.0–13.6) Change from baseline: therapeutic CPAP − 0.02 (1.5), placebo + 0.1 (0.7); $p = 0.7$ (unadjusted)
Shaw, 2016, Australia and North America [39]	Randomized (1:1), parallel--group, open trial	Yes	298	Mean ± SD PAP 62.4 ± 9.1 usual care 62.1 ± 9.0	PAP 65.-6% males usual care 63.-3% males	Mean ± SD PAP 33.4 ± 5.9 usual care 32.6 ± 4.9	PAP 8.4 ± 7.3 years usual care 7.9 ± 6.9 years	30.65 ± 18.56	6 months, PAP use 4.9 h/night	PAP group: baseline HbA1c 7.3 (0.5), 6 months 7.2 (0.8) usual care group: baseline HbA1c 7.3 (0.5), 6 months 7.1 (0.8) Unadjusted Difference (95% CI) − 0.0 (− 0.2 to 0.1) The change in HbA1c was also similar between groups after adjusting for the difference in BMI change over time
Martinez-Ceron, 2016, Spain [38]	Randomized (1:1), parallel groups, open trial	Yes	50	Mean ± SD 61 ± 9	60% males	Mean ± SD 32.5 ± 4.5	Median (interquartile range) 5 (3–15)	32.1 ± 20.9	6 months, CPAP use 5.2 ± 1.9 h-/night	*Intention-to-treat analysis* CPAP group: baseline HbA1c 7.6 ± 1.3; 6 months 7.3 ± 1.1 Control group: baseline HbA1c 7.6 ± 0.7; 6 months 7.6 ± 0.7 Intergroup crude difference at 6 months − 0.4 (− 0.7 to − 0.1), $p = 0.024$ Intergroup adjusted difference at 6 months − 0.4 (− 0.7 to − 0.04), $p = 0.029$ *Per-protocol analysis* (18 patients in the CPAP group and 24 patients in the control group) Adjusted treatment effect, − 0.5% [95% CI, − 0.9 to − 0.09%]; $p = 0.017$
Lam, 2017, Hong Kong, China [36]	Randomized, with two parallel treatment groups, open trial	Yes	64	Mean ± SD 55 ± 9	81% men	Mean ± SD 29.9 ± 5.3	8.8 ± 5.5 years in the intervention group 8.5 ± 6.4 years in controls	45.3 ± 23.2	3 months, CPAP usage 2.5 ± 2.3 h-/night	*Intention-to-treat analysis:* intervention group 8.1 ± 1.1 at baseline, 7.8 ± 1.2 at 3 months; control group 8.4 ± 1.6 at baseline and 8.2 ± 1.5 at 3 months; within group changes − 0.3 (− 0.6 to 0) for intervention group and − 0.2 (− 0.6 to 0.2) in control group; between groups differences in changes 0 (− 0.5 to 0.4) $p = 0.866$ *Per protocol analysis* (diet and medication changes excluded): within group changes − 0.3 (− 0.6 to − 0.1) for intervention group and 0.1 (− 0.2 to 0.3) in control group; between groups differences in changes − 0.4 (− 0.7 to − 0.1) $p = 0.027$

SAS = sleep apnea syndrome; HbA1c = glycated hemoglobin; SD = standard deviation; PAP = positive airways pressure; CPAP = continuous positive airways pressure; BMI=body mass index

One study was underpowered to detect a significant difference due to low enrolment as compared with the calculated sample size [34]. In the study of Babu et al. [35], a pre-specified analysis which included only patients with an HbA1c of more than 7% at baseline demonstrated a significant improvement of HbA1c levels from 9.2% ± 2.0% at baseline vs. 8.6% ± 1.8% after CPAP treatment ($p = 0.02$). In the same study, there was a highly significant correlation between HbA1c improvement and the number of days of CPAP use (Spearman correlation, $r = 0.74$, $p = 0.006$) in the high-compliance CPAP group (defined as average use, > 4 h/night; $n = 12$), while in the low-compliance CPAP group, no such correlation was seen (Table 3).

BMI change during the follow-up period may be an important confounding factor in the HbA1c change. Of the included studies [29–35, 44, 45], only three reported the BMI changes during the follow-up, but none of these changes were statistically significant at group level [31, 34, 44]. Also, none of these studies adjusted the HbA1c levels following the CPAP therapy for BMI changes [31, 34, 44].

Randomized clinical trials

Among the 4 randomized clinical trials, 3 were small-scale studies (among 42 and 64 patients included), and one had 298 subjects included, with a study duration of 3 months (2 studies) and 6 months (2 studies) [36–39] (Table 4). Characteristics of the patients included were similar to those from observational studies. Three studies, including the study of Shaw et al. which had the largest number of participants, did not report any benefit of CPAP therapy on HbA1c levels in the intention-to-treat analysis [36–39]. Nevertheless, two of these three studies reported a surprisingly low adherence to therapy of 2.5 to 3.3 h/night [36, 37]. In the study of Lam et al. [36], the per-protocol analysis (excluding subjects with diet and medication changes during the intervention) found within group changes of − 0.3 (− 0.6 to − 0.1) for HbA1c in the intervention group and 0.1 (− 0.2 to 0.3) in the control group with group differences in changes of − 0.4 (− 0.7 to − 0.1), $p = 0.027$. Only one RCT found significant benefits on glycemic control following CPAP therapy. The study of Martinez-Ceron et al. [38] included 50 patients with T2DM randomized 1:1 to CPAP therapy or no therapy for 6 months. The adherence to therapy was good, with a device usage of 5.2 ± 1.9 h/night. Intergroup HbA1c-adjusted difference at 6 months was − 0.4% (− 0.7 to − 0.04%), $p = 0.029$ in the intention-to-treat analysis, and − 0.5% [95% CI, − 0.9 to − 0.09%], $p = 0.017$ in the per-protocol analysis.

The change of BMI during the follow-up period was assessed in all RCTs [36–39], and in three of them, the change was not statistically significant compared to baseline and was not used in the adjustment [36–38]. In the study of Shaw et al. [39], the BMI decreased during the follow-up in the usual care group while remained stable in the PAP group. The adjustment for the BMI change did not change the results; the HbA1c change at 3- and 6-month follow-up remained similar after this adjustment [39].

Discussion

The aim of this systematic review was to summarize the existing literature on the effect of SAS and its therapy with CPAP on glycemic control in adult patients with T2DM. The accumulation of a higher number of studies of fair and good quality has allowed the analysis of the influence of AHI and parameters associated with hypoxemia on glycemic control in addition to the existing evidence on the effect of CPAP therapy in patients with T2DM.

SAS and glycemic control

In this review, we found conflicting results on the effect of SAS, as defined by AHI, and on the association between AHI and HbA1c levels. The only study in which the HbA1c was adjusted for potential confounders (gender, age, diabetes duration, diabetes treatment, BMI, and waist circumference) reported statistically significantly higher HbA1c levels in those with SAS as compared to those without SAS (8.4% vs. 7.6%, $p = 0.04$) [8]. For the deterioration of glycemic control in parallel with SAS severity, a number of two [8, 21] of the four studies that assessed this [8, 9, 21, 22] reported no increase of HbA1c in parallel with AHI categories, irrespective of the adjustment for other confounders. With regard to the linear relation of AHI with HbA1c, most of the cross-sectional studies analyzed reported no association [8, 24, 25]. However, an interesting finding was that of Grimaldi et al. [23] which showed that the severity of SAS was higher in REM sleep as compared to NREM sleep and that only AHI during REM sleep was associated with HbA1c ($b = 0.060$, $p = 0.008$). This latter observation may explain the conflicting results of individual studies with regard to the association of SAS and AHI with glycemic control. Normal sleep consists of cycles of NREM and REM sleep. The onset of REM sleep occurs normally after 90 min of sleep, and its duration increases with consecutive NREM/REM cycles [40]. A lower mean evaluation period during sleep (4 h or less), probably during the first part of the night, that was reported in several studies might have recorded a lower time spent during REM sleep and thus a lower number of AHI events during sleep offering false-negative results for both SAS severity and the association of SAS severity and AHI with glycemic control. One of the hypothesized mechanisms by which SAS impairs glycemic metabolism is sympathetic hyperactivity [10]. REM sleep is a stage associated with increased sympathetic activity

[41, 42], and this is more pronounced in patients with SAS during apneic episodes.

Despite the mixed results on the relation of AHI on glycemic control, we found that glycemic control in patients with T2DM is more strongly associated with parameters that describe hypoxemia. Two studies examined the effect of lowest oxygen saturation during sleep with glycemic control, and both reported an inverse linear association between this hypoxemia parameter and HbA1c, independent of sociodemographic and anthropometric parameters and diabetes medication [8, 24]. The mechanisms by which SAS impairs glucose metabolism are not yet completely understood. It has been postulated that intermittent hypoxemia and sleep fragmentation observed in SAS promote insulin resistance and impair glycemic control through inflammation, oxidative stress, sympathetic hyperactivity with increased catecholamine levels, and activation of the hypothalamic-pituitary-adrenal axis with consequent cortisol release [10]. Prospective studies showed that ODI and lowest oxygen saturation during sleep are the most important predictors of the development of T2DM during the follow-up [43]. In this systematic review, we also found reports on the association of ODI with HbA1c in patients with T2DM [8, 22, 23, 27]. The largest study that investigated the association of ODI with glycemic control was the ESADA study, which enrolled 1138 patients from European populations and which showed that adjusted HbA1c levels increased in parallel with SAS severity, as assessed by ODI, independent of sociodemographic characteristics, BMI, and comorbidities, supporting a dose-effect relationship [26]. Also, again an interesting observation is the one of Grimaldi et al. [23] which showed that HbA1c was associated only with ODI during REM sleep, independent of age, gender, body mass index, race risk, years since diagnosis of T2DM, insulin use, and NREM ODI. The authors also reported a deterioration of glycemic control with increasing REM ODI quartiles, supporting a dose-response relationship. As stated above, increased sympathetic activity during REM sleep is accentuated in SAS and thus may have a deleterious effect on glycemic control of patients with diabetes [41, 42].

Of note, of all studies that evaluated the effect of AHI and hypoxemia parameters on glycemic control, only one was rated as good quality and the rest fair quality. Although for most studies included the sample sizes were medium to large (> 100 participants), the calculation of sample size before study start was reported only for one study [8]. This quality issue may have influenced the results, either by the inclusion of a smaller sample than the one needed to identify a significant difference between groups when it existed or by the inclusion of large sample and identification of small and clinically not significant differences between groups. Previous research showed that SAS is associated with hypothyroidism [46] and insulin sensitivity may be impaired in patients with hypothyroidism [47]. Only one study included in this review assessed the presence of hypothyroidism and listed hypothyroidism as an exclusion criterion [31].

Effect of CPAP on HbA1c

The effect of CPAP therapy on glycemic control remains controversial. Of the 13 non-randomized interventional studies and randomized clinical trials included in this review, 7 reported no statistically significant change of HbA1c following CPAP therapy [29–35, 44, 45]. The results were similar when we analyzed separately non-randomized and randomized clinical trials: five of the nine observational studies and two of five RCTs reported no effect of CPAP on glycemic control. These results confirm previous systematic reviews and meta-analyses. The most recent meta-analysis is the one of Labarca et al. [16]. This meta-analysis included 6 randomized clinical trials and 581 participants with T2DM and SAS and showed that CPAP therapy for 12 to 24 weeks had no effect on HbA1c levels (mean difference between CPAP and sham CPAP = − 0.10; 95%CI: − 0.25; 0.04) [16]. A previous systematic review by Feng et al. [13] and meta-analysis that included observational studies and randomized clinical trials also reported no change of HbA1c following CPAP therapy. We have to reiterate several methodological issues of all of these studies that have been previously identified by others [16]. First, currently only a limited number of RCTs that evaluated the effect of CPAP are available. Additionally, most of the available studies have limited power to evaluate the effect of CPAP therapy due to their small sample size. All but one study had sample sizes below 100 and no formal sample size calculation [29–39, 44, 45]. Also, data on the change in diabetes medication and BMI was not always reported, and these are important confounding factors that should be accounted for in future clinical trials adequately powered to identify the effect of CPAP on HbA1c. Of the 13 longitudinal studies [29–39, 44, 45], only 7 reported the BMI changes [31, 34, 36–39, 44], and of these, only 1 included BMI change as a potential confounder in the analysis [39]. The last but not the least is the short duration of identified clinical studies that we included in the review. The majority of studies had a CPAP duration of up to 6 months. The largest observational study to date is the one published by Guest et al. [29] and in which were enrolled 300 patients with T2DM and SAS (150 treated with CPAP and 150 in control group). This study showed that HbA1c was lower in the CPAP group than in the control group starting from 1 year after CPAP initiation, but the difference became statistically significant only at 2 years following CPAP initiation.

Strengths and limitations

This systematic review has several strengths and limitations that should be discussed. To the best of our knowledge, this systematic review is the first to include studies that assessed

the effect of untreated SAS on glycemic control in adults with T2DM. However, as with other systematic reviews on this topic [13, 15, 16], due to its limitations, the findings of this review should be interpreted with caution. Although we performed a comprehensive search in the databases, the number of articles included is small. This is due to the low number of studies overall and to the low number of studies of good and fair quality. All these suggest the need for high-quality studies, powered to assess the effect of SAS on glycemic control or the effect of CPAP on glycemic control in patients with diabetes and SAS, properly controlled for potential confounders and with longer follow-up and an increased CPAP usage per night for those assessing the effect of CPAP.

Conclusion

Current findings suggest an effect of SAS and especially of hypoxemia during apnea/hypopnea episodes on glycemic control in T2DM. However, current evidence does not support any association between the apnea-hypopnea index and glycemic control or an effect of CPAP therapy on glycemic control in these patients.

Author contributions Rusu A and Roman G conceptualized and designed the systematic review; Rusu A, Ciobanu D, and Bala C carried out the search and performed data interpretation; Roman G, Rusu A, Ciobanu D, and Bala C drafted the manuscript; all authors reviewed and approved the final version of the manuscript.

Compliance with ethical standards

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

Ethical approval This was a systematic review and did not collect individual patient data. Thus, no ethics approval was required. The articles performed by any of the authors and included in this systematic review followed all applicable international, national, and/or institutional guidelines for the care of human participants.

Informed consent This was a systematic review of already published articles and did not collect individual patient data. Thus, informed consent of participants was not required.

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ORIGINAL ARTICLE

Assessment of risk for obstructive sleep apnea by using STOP-BANG questionnaire in type 2 diabetes mellitus

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Abstract

Background Obstructive sleep apnea (OSA) is a disorder of breathing during sleep. It typically presents as repetitive short episodes of partial or complete stoppage of breathing due to upper airway obstruction during sleep and awakens the person. The association between OSA, type 2 diabetes mellitus (T2DM), and obesity has been suggested in numerous studies. It is expected that the prevalence of OSA will increase with rising number of T2DM. Unfortunately, many of the patients are not aware about OSA and also remain undiagnosed. The questionnaire-based OSA screening for risk stratification is validated in many studies in diverse populations. This approach can help early detection of OSA in T2DM. Hence, the study was undertaken to find out prevalence of OSA in T2DM patients by validated screening questionnaire, STOP-BANG. Objective of the study is to assess the prevalence of OSA among the type 2 diabetic patients attending tertiary care hospital.

Methods This was a questionnaire-based cross-sectional study done in a tertiary care hospital affiliated to a medical college in Maharashtra. The number of respondents were 374 attending diabetic clinic, who were interviewed using STOP-BANG questionnaire and assessed on four symptoms and four signs to stratify the OSA risk.

Results 47.3% of type 2 diabetics had STOP-BANG score ≥ 3, which is high-risk score for OSA

Conclusion Type 2 diabetics are at risk of OSA and STOP-BANG questionnaire could detect nearly 50%. The screening is simple and can be used in primary care setting. These sub-set of patients can be subjected to gold standard polysomnography at home or in hospital for further management.

Keywords Obstructive sleep apnea · STOP-BANG · Type 2 diabetes

Introduction

Obstructive sleep apnea (OSA) affects about 4% of men and 2% of women of middle-aged population, as defined by apnea-hypopnea index (AHI) > 5 and daytime excessive sleepiness [1]. OSA is common in obese patients and also its prevalence increases with increasing body mass index (BMI). Type 2 diabetes mellitus (T2DM) is characterized by impaired glucose tolerance and insulin resistance, and both of these have causal relationship with central obesity. OSA is also associated with incremental insulin resistance independent of general obesity [2, 3]. Hence, OSA may therefore be present in patients with T2DM irrespective of obesity.

Sleep apnea is characterized by the interruption of airflow during sleep. Obstructive sleep apnea (OSA) is commonly due to obstruction to airflow in the oropharynx. Patients with OSA have repeated episodes of partial or complete upper airway obstruction during sleep, leading to poor inflow of air. This is usually followed by awakening from sleep. The clinical manifestations and consequences of OSA are a direct result of hypoxia that occurs due to repetitive short spells of upper airway blockade. In the Western countries, it is estimated that 2–4% of middle-aged adult population suffers from OSA (3.6% in India). OSA is an important risk factor in the development of diseases including cardiac, cerebrovascular, cognitive dysfunction and excessive daytime somnolence, and risk of road traffic accidents [4].

In obese people, deposition of excess fat around the chest reduces the functional capacity while increasing

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Table 1 Observations on STOP-BANG parameters

Parameters	Total 374	%	Males 200	%	Females 174	%
Snore	223	59.6	124	55.6	99	44.4
Tired	153	40.8	84	55.0	69	45.0
Observed-apnea	67	18.0	19	28.2	48	71.8
Blood pressure (high)	258	69.0	139	53.9	119	46.1
BMI > 35 kg/m	65	17.4	42	64.6	23	35.4
Age > 50 years	212	56.7	97	45.6	115	54.4
Neck circumference $M > 43$ $F > 41$ cm	208	55.6	119	57.0	89	43.0
Gender		$M = 200$	53.5	$F = 174$	46.5	

oxygen demand. In addition, increased fat deposition around esophagus also predisposes the lumen of airway for narrowing and poor airflow [5, 6] There are numerous studies on OSA and diabetes, showing a strong bilateral association. OSA is associated with impaired fasting glucose, glucose intolerance, and type 2 diabetes [4, 7–11]. However, OSA is likely to be underdiagnosed in primary care set-up in patients with T2DM than obese men with other comorbid chronic health conditions, because they are more likely to be screened for OSA. Hence, it is suggested that primary care providers should make efforts to implement OSA screening [12].

Compared to the general population, studies have reported a much higher prevalence of OSA in patients with T2DM, ranging from 53.9 to 86% from the studies in the USA, Hong Kong. and Japan [13]. The prevalence of OSA and type 2 diabetes will increase, as obesity is reaching epidemic proportions. The adverse effects of OSA on cardio-cerebrovascular, metabolic, and cognitive function make it imperative that OSA ought to be detected and treated early. Obesity is common risk factor both for T2DM and OSA. The evidence gathered so far supports the view that there is an independent association between T2DM and OSA. The prevalence of OSA in type 2 diabetics is reported to be over 50% [6, 12, 14].

Review of literature showed that the screening of type 2 diabetics for OSA is not a standard practice in primary care setting in India. The majority of affected individuals remain undiagnosed [1, 15–18]. There are scant data from India on the epidemiology of OSA [19–23] and rise of both T2DM and obesity warrants screening for OSA in India as well.

This study was therefore undertaken to screen diabetic patients for the risk of OSA attending outpatient clinic in a tertiary care hospital. This study will help to reassess the burden of OSA in diabetic patients who need definitive diagnosis by polysomnography in hospital or home with portable equipment. Hence, we undertook this questionnaire-based study with primary objective to assess the magnitude of obstructive sleep apnea (OSA) among the type 2 diabetic (T2DM) patients.

Methods

This was a cross-sectional study carried out in a tertiary care hospital affiliated to medical college in Western Maharashtra. The study was approved by Institutional Ethics Committee. Participants included all type 2 diabetic patients aged > 18 years attending diabetic clinic of the hospital. Participants who were too ill or having impaired cognitive ability to understand the questions were excluded from the study. The sample size was calculated assuming 60% prevalence of OSA among type 2 diabetics based on the range of 30–90% reported in literature. The confidence interval of 95%, the sample size calculated to be 340. Adding 10% as non-response error, final sample size calculated was 374. The patients were selected consecutively in the outpatient clinics. The scores derived were used to categorize the patients as "low risk" if score < 3 and "high risk" if score ≥ 3.

Study tool

The data was collected from study participants using a structured form (STOP BANG questionnaire) using interview technique and measurements as appropriate.

The STOP-BANG questionnaire used in this study included four yes/no questions:

- S- Snore loudly (louder than talking or loud enough to be heard through closed doors).
- T- Tired, fatigued, or sleepy during daytime.
- O- Observed apnea- you stop breathing during your sleep.
- P- Pressure-blood pressure high/treated?

Table 2 Number of patients according to the risk of OSA

	LR	%	HR	%	HR	%	Total
SB score	< 2		3–4		5 >		
Males	102	52.0	94	46.0	4	2.0	200
Females	95	54.6	74	42.5	5	2.9	174
	197	52.7	168	44.9	9	2.4	374

SB score STOP-BANG score, *LR* low risk, *HR* high risk

The BANG portion of the questionnaire

- B-BMI calculated from measured height and weight.
- A-Age.
- N-Neck circumference or collar size measured in cm.
- G-Gender.

Patients received an additional point toward their STOP-BANG scores for the presence of each of the following clinical characteristics:

BMI > 35 kg/m^2, age > 50 years, neck circumference > 40 cm, and male gender. Patients were classified as having high risk for OSA if they had a total STOP-BANG score ≥ 3 points; out of a possible 8 points as both self-reported and measured or observed values for BMI, age, neck circumference, and gender were collected, two sets of scores were calculated. All patients underwent general clinical examination, calculated BMI, and measured circumference of the neck.

Interpretation of questionnaire [24]

OSA - Low Risk: Yes to 0–2 questions.

OSA - High Risk: Yes to 3–8 questions.

or Yes to 2 or more of 4 STOP questions + male gender.

or Yes to 2 or more of 4 STOP questions + BMI > 35 kg/m^2.

or Yes to 2 or more of 4 STOP questions + neck circumference 17 in./43 cm in male or 16 in./41 cm in female.

STOP BANG questionnaire is a validated simple and easy to use screening tool for risk stratification for OSA. Total scores are interpreted in our study as: high risk of OSA ≥ 3; low risk of OSA < 3. The pooled mean sensitivity and specificity of STOP-BANG in diabetic population varies from 47%, 51.7%, and 56.1%, to 87.5%, 75%, and 67%, respectively for mild, moderate, and severe sleep apnea matched to AHI index of the respective class [25].

Table 3 Observations on various parameters of STOP-BANG questionnaire

Variables	Males (200)	Females (174)	*p* value
Age [years]	50.39 ± 9.97	42.53 ± 11.80	< 0.0001
BMI [kg/m^2]	28.63 ± 3.97	27.25 ± 3.54	0.0005
BMI > 28 kg/m^2, *n* (%)	85 (42.5)	59 (33.9)	0.1099
BMI > 30 kg/m^2, *n* (%)	65 (32.5)	45 (49.4)	0.1733
BMI > 35 kg/m^2, *n* (%)	45 (22.5)	23 (13.4)	0.0224
Neck circumference [cm]	39.16 ± 2.77	37.28 ± 2.85	< 0.0001
STOP-Bang score	3.91 ± 1.41	2.23 ± 1.00	< 0.0001

Statistical analysis

Data was analyzed using EpiInfo version 7.2 (CDC, Atlanta), and results obtained were described in percentages, mean, and standard deviation. Fisher Exact Test was used to test the association between categorical variables with level of significance, $p < 0.05$.

Results

A total of 374 participants were included in the study. The observations on STOP-BANG parameters are seen in Table 1.

It is revealed that age > 50 years, presence of snoring, high blood pressure, and increased neck circumference were present in more than 50% of diabetics as risk for OSA.

The number of patients according to the perceived risk of OSA based on the STOP-BANG score is illustrated in Table 2.

47.3% of diabetics are at high risk for OSA and these subset of patients may benefit from polysomnography.

Among these patients, 48% of males and 45.4% of females were found to be at high risk.

Table 3 shows the observations on various parameters of STOP-BANG questionnaire.

STOP-BANG parameters compared between males and females showed that the differences were statistically significant (Table 3).

Discussion

The results from this study highlight outcomes on the prevalence of the risk factors for OSA in diabetics. There is a male propensity for OSA risk and among the symptoms; snoring followed by day time sleepiness reflects an added risk. On physical assessment, hypertension and neck circumference (> 43 cm in males and 41 cm in females) are associated with high risk of OSA in diabetics. Secondly, the study revealed that nearly 50% of T2DM patients are at high risk of OSA. More than 50% of study participants had hypertension and high neck circumference which are simple physical measurements useful in the assessment of OSA risk. The BMI > 35 kg/m^2 is a high risk for OSA in males ($p = 0.0224$). Multiple studies across the globe have shown similar association with aforementioned risk

factors reflected in our study. The outcomes in our study suggest that screening for OSA identifies nearly 50% of diabetics at high risk. The recommendation by International Diabetes Federation's Task Force on Epidemiology and Prevention to undertake screening for OSA emphasizes the need for such practices [26].

Therefore, type 2 diabetics need to be screened for OSA, which can be actively implemented in primary health-care setting in developing countries.

Limitations

When using the STOP-BANG questionnaire, a likely selection bias is possible due to more prevalent OSA in the study populations, may affect interpretation of the predictive parameters. Secondly, the specificity and sensitivity of this questionnaire has shown variable consistency in specificity and sensitivity in different populations and comorbidities. Although the STOP-BANG questionnaire is validated in multiple populations, it was less useful in identifying OSA patients in patients with renal failure. Potential error due to variability in measuring neck circumference can affect accuracy of the STOP-BANG score [27].

Our study did not take into account all possible comorbidities for assessing the risk of OSA among diabetics, especially chronic kidney disease in diabetics.

Conclusion

There is limited data on the burden of OSA in India. Our study emphasized the need for screening of type 2 diabetics for OSA risk. Nearly half of T2DM patients included in the study are at high risk of developing OSA. We noticed that there is statistically significant association with snoring, age >50 years, neck circumference >41 cm, and male sex in addition to BMI >35 kg/m^2. Diabetes and OSA have important public health implications. Hence, there is a pressing need to screen for OSA among all T2DM patients in primary health facility; using validated tools like STOP-BANG quetionnaire and to search for simple, yet highly sensitive, specific and predictive test.

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Compliance with ethical standards

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

Ethical approval The study was approved by the Institutional Ethics Committee.

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ORIGINAL ARTICLE

Type 2 diabetes: epidemiological changes at Instituto Mexicano del Seguro Social—associated with complications in Mexico

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Abstract

Background Mexico is a country with a high frequency of type 2 diabetes mellitus (DM2). DM-related complications can increase the number of medical appointments and hospitalizations, impact patient quality of life, and exacerbate hospital care costs. The goals of this study were to analyze epidemiological data of DM2 over the last 10 years and to compare its comorbidities and mortality in individuals listed at the Instituto Mexicano del Seguro Social (IMSS) in 2013 and 2015. The database was compiled for doctors, validated by engineers in computer systems, and analyzed by statisticians. E-10-E-14 registered patients with DM2 (based on International Classification of Diseases (ICD-10)) from 2005, 2013, and 2015 were analyzed through the Family Medicine Units and Non-Communicable Diseases Analysis System (SANENT)® databases. Incidence, comorbidity, and mortality were described.
Results We included 29,525,905 individuals, including 3,395,389 DM2 patients, in 2013, and 31,389,711 individuals, including 3,547,006 DM2 patients, in 2015, in the IMSS. The incidences of DM2 in 2005 and 2015 were 124 and 120 per 100,000 IMSS members, respectively, without significant differences. The diabetes complications caused partial disability in 253 and 341 per 100,000 individuals in 2013 and 2015, respectively. Overall mortality decreased from 2005 to 2015 (rates of 594 vs. 350 per 100,000 persons; $p < 0.001$).
Conclusion Though the overall mortality and incidence of DM2 decreased from 2005 to 2015, the complications maintained the same prevalence rates in 2013 and 2015; however, disability complications increased in 2013 and 2015, emphasizing the need for proper control methods acting over this dual axis.

Keywords Type 2 diabetes · Epidemiology · Incidence · Complications · Mortality

Introduction

Diabetes *mellitus* (DM) is a group of chronic diseases characterized by the presence of persistent hyperglycemia and multiple metabolic abnormalities. Type 2 diabetes *mellitus* (DM2) is a subtype of DM characterized by insulin resistance and hyperinsulinism and is often non-insulin-dependent [1, 2]. The number of people with diabetes has increased due to population growth, urbanization, extended life expectancies, an elevated prevalence of obesity, and a lack of physical activity. The prevalence of DM2 is rapidly increasing in both developed and underdeveloped countries [3].

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The urban population in Latin America presents a prevalence of diabetes between 4 and 8%, but this rate is higher in countries or areas with low or middle socioeconomic status as well as in rural areas. The CARMELA study, which was performed in 7 cities in Latin America in 2005, reported that the prevalence of diabetes in Mexico City was 8.9% [4, 5]. In Mexico, according to the National Survey of Health and Nutrition in 2012, there was a significant increase of 10% points in the prevalence of overweight and obesity [6, 7] compared with the prevalence reported in 2000. Concomitant with the increased incidence of obesity among the adult population (aged 20 or older) from 61.8 to 71.3% from 2000 to 2012, the percentage of the adult population with diabetes also increased from 5.7 to 9.1%, which corresponds to an increase of almost 60% [8] and indicates increased morbidity and mortality due to DM2.

DM-related complications can increase the number of medical appointments and hospitalizations, impact patient quality of life, and exacerbate hospital care costs. Macrovascular complications include systemic hypertension, acute myocardial infarction (AMI), congestive heart failure (CHF), cerebrovascular accident (CVA), and peripheral artery disease (PAD). Patients with DM2 are at a 2- to 4-fold increased risk of suffering from either AMI or CVA [9]. Microvascular complications include neuropathy, retinopathy, and nephropathy, which are related to HbA1c levels, as was demonstrated in the UKPDS, but it is now known that the high glycemic variability is also important [10]. This is because HbA1c only analyzes glycemic averages, and the hyper- and hypoglycemic indexes can generate more complications over the long term [11]. One of the most common chronic complications associated with DM is diabetic foot, which is related to extended, inappropriate glycemic control of DM [12].

In Mexico, the Instituto Mexicano del Seguro Social (IMSS) is the health institute that assists the largest percentage of the population [11]. Since 2006, DM screening using capillary blood glucose has been performed in addition to campaigns to detect and prevent obesity and DM [13]. However, there is no updated epidemiological data regarding DM and the changes in the incidence and mortality of DM since the implementation of these prevention campaigns. Therefore, the objectives of this study are to compare the incidence, prevalence, morbidity, chronic complications, and disability in active workers among the Mexican population who were included in the IMSS in the years 2013 and 2015 and to compare the incidence and mortality of DM2 in 2005 and 2015.

Materials and methods

This was a cross-sectional study. The patients were clinically diagnosed by doctors at the IMSS. The data used in this study were obtained from the Non-Communicable Diseases Analysis System (SANENT)® and the databases of Family Medicine Units. At the IMSS, medical attention is classified as three levels: 1st level facilities perform preventive measures and treat acute and chronic pathologies without complications; 2nd level facilities address complicated pathologies, chirurgic interventions, and treatments that require hospitalization; and 3rd level facilities are equipped to treat individuals with complex and complicated diseases. The SANENT database contains information for every enrolled patient and for individuals who attended regular medical appointments at the IMSS. Diseases are classified using codes from the International Classification of Diseases (ICD-10).

The data to compose the database were compiled for doctors who attend and diagnose patients attending the hospitals affiliated with the IMSS; the data are validated by a team of engineers in computer systems and are analyzed by statisticians affiliated with specialized centers strategically located at regional and state levels.

For this study, information collected from patients aged 20 years or older (as of 2005, 2013, and 2015) was described using the following ICD-10 codes: E10 (insulin-dependent diabetes mellitus), E11 (non-insulin-dependent diabetes mellitus), E12 (malnutrition-related diabetes mellitus), E13 (other specified diabetes mellitus), and E14 (unspecified diabetes mellitus) [14].

Among all the patients with DM2 who were enrolled in the IMSS, active workers with partial disability due to DM2 were described (as of years 2013 and 2015), and individuals with major amputations of lower extremities were also included and described. The latter group was identified using the ICD-9 codes 8415-8419, and those who required amputation due to traumatic causes were excluded (CIE-10: S77, S78, S87, S88, S98, T053, T055, and T136). Based on the extracted partial disability cases, the partial disability rate out of every 100,000 active workers who were enrolled in IMSS was calculated. The prevalence of disease, number of new DM2 cases, and deaths were also calculated. The death rate per 100,000 DM2 patients by city and age group was estimated.

From the data encompassing all the enrolled patients, the data of those who attended regular medical appointments at first-, second-, and third-level care medical units were analyzed. It was considered that these patients had relatively appropriate control of their diabetes by measuring their fasting blood glucose levels, which should remain in a range of 70–130 mg/dl. Appropriate control of blood pressure was also defined as systolic blood pressure between 100 and 130 mmHg and diastolic blood pressure between 60 and 80 mmHg. The body mass index (BMI) was calculated using the somatometric variables of patients (e.g., weight and height), and obesity was classified as a BMI > 30 kg/m^2.

Among the DM2 patients who attended their regular medical appointments (as of years 2013 and 2015), complications due to DM2 were included based on the ICD-10 classification with the codes E-10-E-14; these complications include

disruptions in the following systems: peripheral circulation, kidney, ophthalmic, cerebrovascular, and ischemic heart. The number of registered cases with these codes was analyzed.

Mortality analysis included causes of death using the following ICD-10 codes: E10 (insulin-dependent diabetes mellitus), E11 (non-insulin-dependent diabetes mellitus), E12 (malnutrition-related diabetes mellitus), E13 (other specified diabetes mellitus), and E14 (unspecified diabetes mellitus) in years 2005 and 2015.

Statistical analyses

The media and standard deviations of quantitative variables and the ratios of the qualitative variables were calculated. To calculate the incidence, global mortality, and mortality by city and age in 2005, 2010, and 2015, p values were calculated using the chi-square test. A p value < 0.05 was considered statistically significant. SPSS v.17.0 was used for the statistical analyses.

According to the Helsinki Declaration, the protocol was evaluated and approved by the National Research and Health Ethics Committee of IMSS with registry number R-2014-785-024.

Results

Prevalence of DM2

In 2013, among 29,525,905 people registered in the IMSS, 3,395,389 patients had DM2 (prevalence of 11.4%), with an incidence of 3.8% ($n = 129,186$). For 2015, among 31,389,711 people registered in the IMSS, 3,547,006 patients with a record of DM2 according to ICD-10 codes were included; this corresponds to a prevalence of 11.3% within the studied population. The incidence of DM2 was 3.128% ($n = 110,983$), and females were predominant among patients (59.8%; $n = 2,121,109$).

Demographic and clinical characteristics of subjects with DM2

In 2013, among the 3,395,389 patients with DM2, only 1,017,360 (29.9%) attended regular medical appointments at first- and second-level care facilities. Control parameters showed that less than half of the patients had an appropriate fasting blood glucose level (44.3% $n = 450,984$), and appropriate hypertension control was observed in approximately 79.5% ($n = 809,667$) (Table 1).

For 2015, among the 3,547,006 patients with DM2, only 69.2% ($n = 2,453,795$) attended regular medical appointments at first- and second-level care facilities. The analysis of the results in this year based on the control parameters showed that almost half of the patients had an appropriate fasting blood glucose level (57.5%) and that 42.73% ($n = 1,048,600$) were obese. Appropriate hypertension control was observed in approximately 80% of the patients (Table 1).

DM2-associated chronic complications

The chronic complications were identified in 59,819 patients in 2013 and in 62,005 patients by 2015. When comparing the complication rates per 100,000 patients in 2013 and 2015, there were no significant differences. The most frequently observed complications involved the kidney (rates of 513 and 517 per 100,000 in 2013 and 2015, respectively) followed by ischemic heart disease (rates of 496 and 469 per 100,000 in 2013 and 2015, respectively) (Fig. 1).

Complications that cause partial disability in active workers for regions

In 2013, among all patients with DM2, 33.42% ($n = 1,134,886$) were registered as active workers. Within this subgroup, the presence of complications that caused partial disability totaled 2882 cases, which correspond to a rate of 253 cases per 100,000 workers nationwide. For 2015, among all patients with DM2, 39.76% ($n = 1,410,442$) were registered as active workers; the presence of complications that caused partial disability increased to 4822 cases, which correspond to a rate of 341 cases per 100,000 workers nationwide.

The state of Coahuila was identified as having the highest rate, with 827 cases per 100,000 workers, followed by the states of San Luis Potosí, Tabasco, Sinaloa, Chihuahua, Baja California Sur, and Tamaulipas, with rates between 480 to 423 cases per 100,000 workers. Among all the patients registered with a partial disability, 95% ($n = 4584$) were due to major amputation of a lower extremity. According to the data identified for 2013, the states of Coahuila and San Luis Potosí also had the highest rates of disability cases.

Comparison of the incidence and mortality rates of DM2

As shown in Fig. 2a, the incidence rates of DM2 in 2005 and 2015 were 124.8 and 120.7 per 100,000 people enrolled in IMSS, respectively, without statistical significance. In addition, global mortality also presented a decrease when comparing the rate in 2015 to that in 2005 (594 vs. 350 per 100,000 people in 2005 and 2015, respectively, $p < 0.001$) (Fig. 2a). However, when the mortality rates from 2005 and 2015 were stratified by age group, there were significant decreases in mortality rate in the age groups ranging from 55 to 79 years and from 35 to 79 years ($p < 0.001$) (Fig. 2b).

Table 1 General characteristics of patients registered with type 2 diabetes mellitus

.	Year 2013 *n* = 1,017,360 Media ± standard deviation	Year 2015 *n* = 2,453,795
Demographic characteristics		
Age	*62.3 ± 12.8*	*60.1 ± 13.2*
Sex female[a]	*622,624 (61.2)*	1,428,108 (58.2)
Clinical measurements		
Fasting blood glucose (mg/dl)	136.1 ± 69.8	142.2 ± 79.2
Systolic blood pressure (mmHg)	121.7 ± 12.4	122.2 ± 13.7
Diastolic blood pressure (mmHg)	76.9 ± 8.1	76.4 ± 8.4
Body mass index (kg/m^2)	28.6 ± 5.2	29.7 ± 5.5
Control parameters		
Appropriate fasting blood glucose level[a]	450,984 (44.3)	1,410,932 (57.5)
Appropriate hypertension control[a]	809,667 (79.5)	1,965,489 (80.1)
Obesity[a]	393,718 (38.7)	1,048,600 (42.73)

[a] Frequency (%)

The states with a DM2 population greater than 200,000 individuals correspond to the most populated cities in the country, including Mexico City, the State of Mexico, Jalisco, and Nuevo León. These cities correspond to the most important in Mexico and comprise almost 40% of all patients; the northern and southern states, where fewer than 50,000 patients with DM2 live, comprise the second and third largest concentrations of individuals with DM2.

A total of 22,516 DM2-related deaths were recorded in 2015, which correspond to a mortality rate of 120.7 per 100,000 people enrolled in the IMSS. According to the geographical distribution, cities with a higher mortality rate do not correspond to the cities with the highest percentage of patients with DM2. For instance, the southern states have less than 100,000 patients registered with DM2, but the mortality rate is greater than 150 per 100,000 people. The mortality rate was lowest in most of the northern states of the country (Fig. 3).

Discussion

In our study, we investigated the epidemiological data of patients with DM2 in the IMSS from Mexico, and the prevalence was 11.3%; however, only half of registered patients with DM2 attended regular medical appointments. Approximately half of the patients had an appropriate fasting blood glucose level, and chronic complications were present in 20.8%. Additionally, the incidence and mortality rates in 2015 had decreased compared with those in 2005.

The National Survey of Health and Nutrition 2012 (ENSANUT) reported a national prevalence of DM2 at 9.1%. However, there is a high percentage of the population which does not know they are diabetic [11]. Based on our report, the prevalence rates in 2013 and 2015 were 11.4% and 11.3%, respectively, which are higher than the rates observed in the previous report. ENSANUT [11] is a survey conducted throughout the general population, whereas our

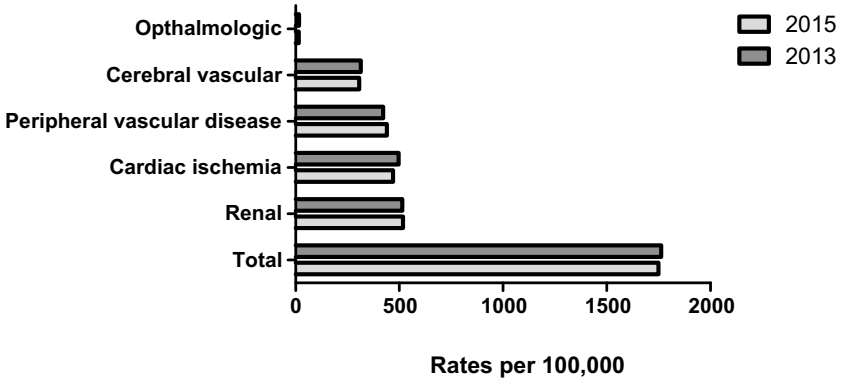
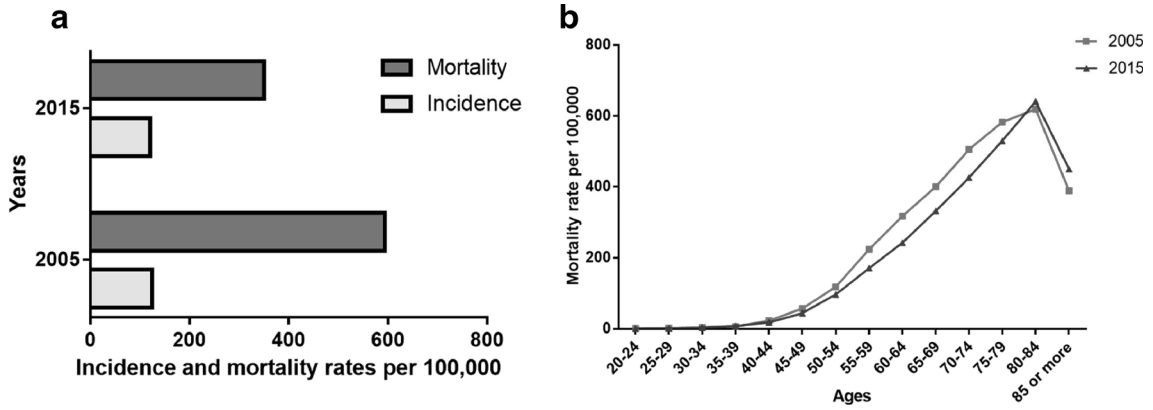


Fig. 1 Patients with DM2 and complications in based on the SANENT record in 2013 and 2015



a) Comparison of the global incidence and mortality of DM2

b) Comparison of the mortality rate of DM2 in 2005 and 2015 by age group

Fig. 2 Comparison of the global incidence and mortality of DM2 and the mortality rate of DM2 by age group in 2005 and 2015. **a** Comparison of the global incidence and mortality of DM2. **b** Comparison of the mortality rate of DM2 in 2005 and 2015 by age group

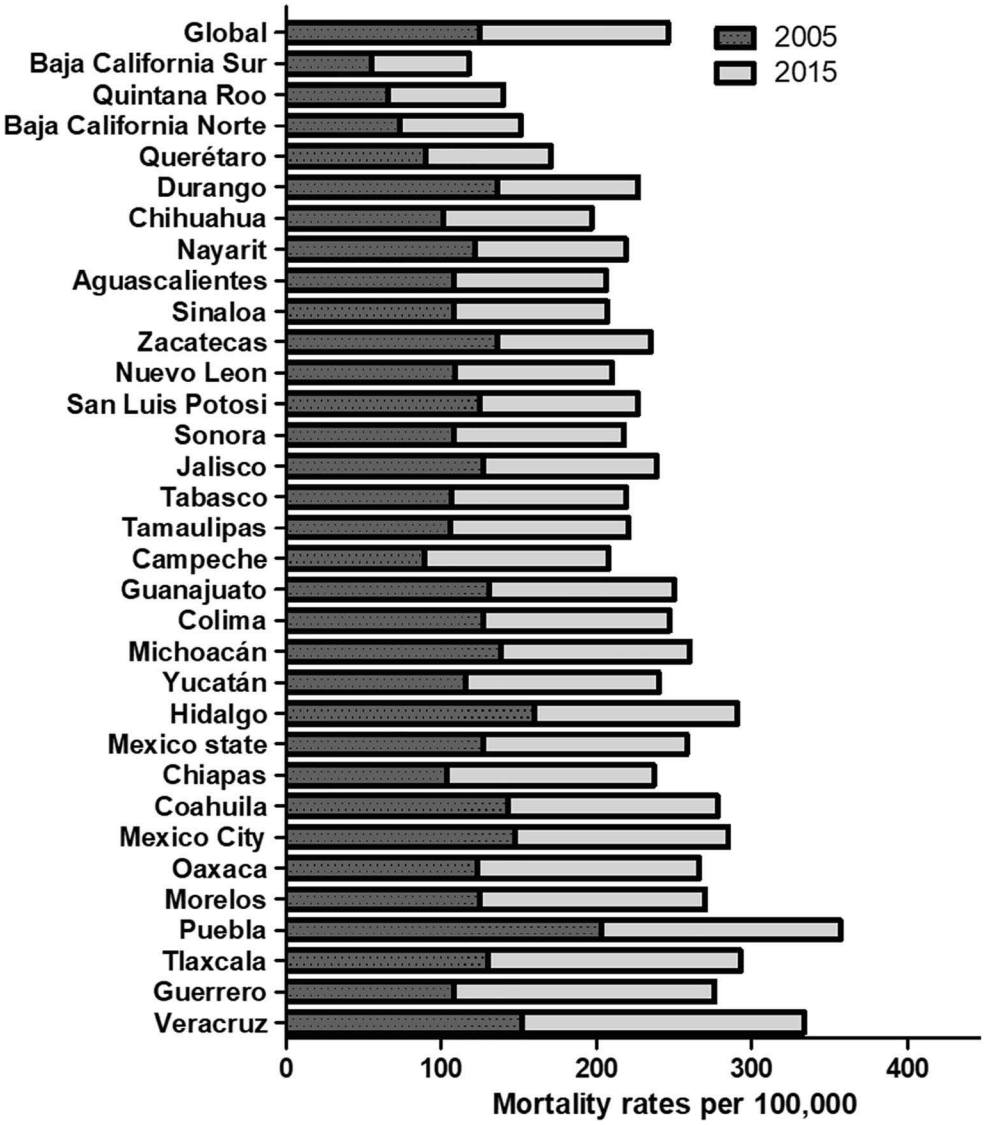


Fig. 3 Mortality rate in 2005 and 2015 among individuals with DM2

study used data extracted from existing medical records, which produced more reliable results. In recent years, multiple health campaigns have been implemented for the timely detection of chronic diseases, including DM2. When comparing the prevalence of DM2 with that in other countries where DM2 is considered one of the primary public health problems, the prevalence is similar; for instance, the prevalence in Korea was 11% in 2013 [15].

By analyzing the incidence calculated in 2015 and comparing it to reports from 10 years ago, there was an observed increase over time. This increase is likely attributed to the timely detection of the disease due to health campaigns initiated in 2006 [13]. In China, an incidence of 15.8 per 1000 person-years [16] was reported between 2006 and 2010. In 2015, we found a rate of 120 per 100,000 subjects, which is higher in our population, and a similar rate was maintained over the last 10 years.

In a study testing the safety of two medications, the prevalence of obesity was also analyzed. The average BMI in the cohort of 9340 patients was 32.5 ± 6.3 kg/m^2, and only 9.1% of the patients had a BMI < 25 kg/m^2. Although there is a higher prevalence of overweight and obesity among individuals with DM2, selection bias can arise, and this should not be taken as a representative sample of patients with DM2 [17]. According to ENSANUT 2012, Mexican individuals aged 20 years or older had a prevalence of either overweight or obesity of 71.3%, which is slightly lower than that observed in our population (42.73% with obesity) however as patients with DM2, are expected to have a higher prevalence of this condition [18]. Nonetheless, a significant increase in the medical care coverage of patients with DM2 between 2013 and 2015 was observed (from 29.9 to 69.2%), which emphasizes the favorable results of health campaigns for sensitizing patients to receiving appropriate medical attention and avoiding chronic complications. Although this is an encouraging start, it is necessary to continue to encourage continued medical care and lifestyle modifications, regardless of whether increased coverage is significant; indeed, for subjects controlled through fasting glucose, an increase of only 44.3 to 57.3% was observed from 2013 to 2015. However, this result does not indicate that the glycemic control was adequate because we could not evaluate these patients with HbA1c; this parameter is important to confirm regarding this issue and represents a weakness in our study.

The decrease in the mortality rate reflects the effects of the medical care that has been implemented in the past decade. This is due to the prevention of severe complications and the early detection of chronic complications as well as the implementation of timely treatments to delay DM2 progression. Specifically, the decrease in mortality rate in individuals between the ages of 40 and 70 can be translated into increased availability, access, and quality of medical care. The availability of alternatives that can replace damaged renal function has led to lower mortality rates and higher survival of renal patients. Although we have observed an improvement in recent years, it has not been sufficient. The mortality rate in Mexico is higher than that in other countries; for instance, Taiwan [19] has a mortality rate of 77.8 (1000 person-year) for people older than 75 years of age, Korea [20] has a mortality rate ranging from 14 to 104 for people aged 50 to 79 years old, and the global mortality rate was 238 (1000 person-years). However, the mortality rate in Mexico ranged from 96 to 529, with a global rate of 350 (per 100,000 people).

The northern states of Mexico receive higher income than those registered in the central and southern states, coinciding with a higher degree of development and higher rates of schooling performance; this is because the northern area has a concentration of industries with the best salaries in Mexico. With better salary and education, patients have better control of their DM2; consequently, the northern states of the country had lower mortality rates [21].

Mexico is a country with a high frequency of DM, and according to our results, the annual incidence of this disease has not changed in the last 10 years. Although health programs have been aimed at detecting DM2 and preventing its complications, these efforts have not been sufficient and require a rethinking of the strategy to promote preventive measures from childhood, since a child with obesity and a sedentary lifestyle has a high probability of having DM with complications in adulthood.

Despite the limitations of this study (i.e., the extracted data were based on secondary sources and glycemic control was determined using fasting plasma glucose levels), this epidemiological study encompasses one of the largest cohorts to date. These data support the notion that the results accurately reflect the current conditions of this pathology in a country whose population is highly prone to suffering from DM2.

With increased awareness of DM2, both demand and costs due to hospitalization and medical care will likely increase. Strict metabolic control is still the best preventive measure to decrease or (at least) delay the development of chronic complications, which will consequently reduce the demand of hospital care. However, effective measures to prevent the occurrence of DM2 would be ideal under these circumstances.

The results of this study support research on multidisciplinary interventions aimed at vulnerable populations, where salaries and education levels are low and the mortality rate due to DM is high.

Conclusions

We conclude that despite identifying a decrease in overall mortality and in the incidence of DM2 from 2005 to 2015, complications maintained the same prevalence rate in 2015 and 2013, while disability complications increased. Our study also shows a higher prevalence of obesity in females than in

males, and complications involved the kidney and ischemic heart disease, which might be due to various lifestyle and environmental factors, as Mexico is a developing country, and the majority of the population live with limitations, including an absence of medical appointments, which would enable better control of their diabetes; furthermore, many cannot afford adequate treatment, medicine, and healthy food.

Perspective

Future research studies are needed on clinical practices in different regions to clarify the perceived reasons for the control and optimal medical treatment of patients with DM2 in the IMSS, which could result in a decrease in their chronic complications.

Author's contribution All authors (Jessie N. Zurita-Cruz, Leticia Manuel-Apolinar, María Luisa Arellano-Flores, Alejandro Gutierrez-Gonzalez, Aleida de Jesus Rivera Hernandez, Rosa Angelica Carranza-Muleiro, Victor Hugo Borja-Aburto, Nelly Cisneros-González) made substantial contributions to the study design, acquisition, analysis or interpretation of data, and writing of the article, critically reviewing it for important intellectual content or final approval of the version to be published.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Research involving human participants and/or animals According to the Helsinki Declaration, this protocol was evaluated and approved by the National Research and Health Ethics Committee of IMSS, with the registry number R-2014-785-024.

Informed consent The data were analyzed from a database, so authorization was requested from National Research and Health Ethics Committee of IMSS, with the registry number R-2014-785-024.

Abbreviations DM2, type 2 diabetes *mellitus*; ICD-10, International Classification of Diseases; AMI, acute myocardial infarction; CHF, congestive heart failure; CVA, cerebrovascular accident; PAD, peripheral artery disease; HbA1c, glycosylated hemoglobin; IMSS, Instituto Mexicano del Seguro Social; SANENT, Non-Communicable Diseases Analysis System; BMI, body mass index

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Association of Cornell product with metabolic syndrome in middle-aged people in China

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Abstract

Background Metabolic syndrome (MS) has attracted much attention worldwide for its harmful effects. Although the Cornell product (CP) and Sokolow-Lyon (SL) voltage are independent and strong risk factors of cardiovascular disease, their associations with MS and the relative strength of these associations are unknown. Therefore, we studied these aspects in a community of middle-aged people.

Methods A total of 1112 community residents aged 40–65 years, among which 169 had IDF (2005)–defined MS (MS group) and 943 did not have MS (NMS group), underwent electrocardiography (ECG) at baseline. A questionnaire survey, physical measurements, laboratory biochemical assessments, urine test, ECG, body fat analysis, and abdominal MRI examination were performed subsequently. Fifty-eight participants had developed MS (new-onset MS group) at the follow-up, while 472 residents had not developed MS over the next year (MS-free group). We compared the CP and SL voltage between these two groups.

Results In the overall analysis, CP values were higher in the MS group than in the NMS group ($p < 0.05$), but SL voltage values were not significantly different ($p > 0.05$). In the gender-stratified multivariate analysis, CP was significantly different in the male and female populations ($p < 0.05$), but SL voltage was only different in the male population ($p = 0.042$). After removing hypertension from MS and NMS groups, there was still significant difference in CP value (< 0.05). Thus, CP showed a more pronounced influence. The new-onset MS group showed significantly higher CP values and the changes in CP values than did the MS-free group at baseline and follow-up ($p < 0.05$), but the changes in SL values were not significant ($p > 0.05$).

Conclusions CP values differed significantly between the MS and NMS groups. The new-onset MS group showed higher baseline CP values. Thus, CP is more advantageous than SL voltage in presaging the incidence of MS-related cardiovascular risk.

Keywords Cornell product · Sokolow-Lyon voltage · Electrocardiogram · Metabolic syndrome

Introduction

Metabolic syndrome (MS) is linked to a cluster of cardiovascular risk factors and has attracted and continues to attract a lot of attention worldwide. The prevalence of cardiovascular disease and the mortality risk in patients with MS are two to three times higher than those in patients without MS. Previous research has demonstrated that MS is associated with abnormal cardiac structures [1]. High-risk factors for cardiovascular disease associated with MS have been studied [2, 3]. The Cornell product (CP) and Sokolow-Lyon (SL) voltage are common electrocardiography (ECG) indicators of left ventricular hypertrophy (LVH). The values of these parameters are associated with significantly increased risks of cardiovascular- or stroke-related morbidity and mortality, are often used as indicators of a high risk of cardiovascular disease in epidemiology studies, and are mainly related to cardiac hypertrophy and cardiac structural changes. A recent study proved that CP and SL voltage are powerful and independent predictors of cardiovascular morbidity and mortality [4–6]. However, previous studies have not elucidated whether CP is associated with MS, whether CP and SL voltage could be indicators of cardiovascular risk in patients with MS, and which of the two

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is a more appropriate indicator of MS. Furthermore, recent studies on CP and SL voltage and their association with MS are rare. Therefore, in this study, we investigated CP values in community residents. A previous study showed that participants with abnormal glucose and lipid metabolism had higher CP levels than in those without, that CP is a risk factor for metabolic abnormalities, and that changes in CP values are associated with the risk of new-onset diabetes [5, 7]. Therefore, we compared baseline CP values and changes in CP values in patients with new-onset MS after 1 year to determine whether the changes in the CP values are related to newly developed MS.

Patients and methods

Populations

We analyzed 1168 subjects aged 40 to 65 years from a community in Hangzhou, China, for the baseline examination, after excluding 56 subjects with a complete right bundle branch block or a history of thoracic surgery, which can influence the chest wall thickness. People with missing data were excluded, and finally, a total of 1112 participants were included. Six hundred sixty-two cohort members underwent the follow-up examination (450 were reluctant to participate in follow-up), of which 132 were excluded for missing data and MS population at baseline. Eventually, 530 participants were included. The examination included a questionnaire survey, physical examination, laboratory biochemical assessments, urine test, electrocardiography, body fat analysis, and abdominal MRI examination. According to the diagnostic criteria for MS proposed by the International Diabetes Federation (IDF, 2005), the subjects were divided into MS group and a non-MS group (NMS group) to explore the correlation between CP and MS. Fifty-eight of the 530 participants who underwent the follow-up examination (underwent the same examination except MRI) developed MS (the new-onset MS group). The remaining 472 participants were classified as the MS-free group. CP values in these two groups were compared. The study was approved by the ethics committee of our department (Department of Endocrinology, Sir Run Run Shaw Hospital).

Diagnostic criteria

ECG-LVH was defined by one of the following two criteria: (1) CP-LVH was defined as sex-adjusted CP ((RaVL + SV3 (+8 in women)) × QRS duration > 2440 mm·ms) [8]. (2) SL-LVH was defined as (SV1 + RV5) ≥ 3.8 mV (38 mm) or RV6 ≥ 3.8 mV (38 mm). Patients were classified based on the presence or absence of MS as per the 2005 IDF guidelines.

Sample collection and evaluation methods

Participant data, including height, weight, waist circumference, and blood pressure, were collected. Medical history was collected using a questionnaire survey. Fasting blood samples were obtained to determine the levels of hemoglobin, fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), fasting insulin (FINS), blood lipids, uric acid (UA), creatinine (Scr), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (γ-GT). The 30-min and 2-h postprandial blood glucose (2-h PPG) and 30-min and 2-h post-meal insulin level data were collected by performing the oral glucose tolerance test. The morning urine specimens were collected for urine microalbumin (MA) and urinary creatinine/albumin (ACR) testing. The C1600 automatic biochemical analyzer was used to analyze blood lipid, blood sugar, UA, ALT, and AST levels. Blood sugar levels were determined by the glucose oxidase method; triglyceride (TG) levels, by the glycerol phosphate oxidation method; total cholesterol (TC) levels, by PAP; and high-density lipoprotein triglyceride (HDL-C) and low-density lipoprotein (LDL-C) levels, by the clearance assay. HbA1c was determined using high-performance liquid chromatography with BIO-RAD. Linco's radioimmunoassay was used to determine insulin levels.

A 12-lead standard ECG (10 mm = 1 mV, 25 mm/s; standard sensitivity was 10 mm/mV; frequency was 500 Hz; QRS duration was about accurate to the millisecond, QRS-wave amplitude was about accurate to μV) was acquired in a quiet environment. QRS duration, R-wave amplitudes in leads aVL, V5, V6, and S-wave amplitudes in leads V1 and V3 were collected. QRS duration was automatically reported by the ECG unit. Three or more cardiac cycles of each lead were collected, the average was obtained, and the data were analyzed by the same doctor to reduce the artificial error. All examinations were accurately measured and recorded by workers with specialized training.

Statistics

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software. Quantitative data are expressed as mean ± SD ($\overline{x} \pm s$). The *t* test was used to compare two groups' data, which showed normal distribution. Qualitative data were represented by frequency and composition, and the composition of each group was analyzed by chi-square test. Binary logistic regression was used for analysis, the values of CP and SL voltage were used as independent variables, and the data of MS were used as the dependent variable. $p < 0.05$ was considered statistically significant.

Table 1 Clinical characteristics of the MS and NMS groups

Variable	Baseline MS group	NMS group	*Z*	*p*
Sex, male (%)	61.8%	59.5%	0.339*	0.560
Age (years)	55.3 ± 6.5	52.8 ± 6.8	− 4.399	0.000
Waist/height	0.6 ± 0.0	0.5 ± 0.0	− 17.856	0.000
Height (cm)	163.0 ± 7.9	162.1 ± 7.8	− 1.431	0.152
Weight (kg)	72.0 ± 9.3	60.0 ± 9.0	− 13.626	0.000
BMI (kg/m^2)	27.1 ± 2.6	22.8 ± 2.6	− 16.240	0.000
Waist circumference (cm)	90.1 ± 6.7	76.4 ± 7.9	− 16.977	0.000
Heart rate (beats/min)	79.8 ± 7.9	77.2 ± 7.5	− 4.287	0.000
SBP (mmHg)	131.2 ± 15.8	119.7 ± 15.2	− 8.599	0.000
DBP (mmHg)	85.0 ± 9.2	79.1 ± 9.3	− 7.583	0.000
Fat mass (kg)	26.3 ± 7.4	17.2 ± 8.6	− 16.700	0.000
Body fat content (%)	36.1 ± 7.0	27.8 ± 6.6	− 13.257	0.000
Fat-free block (kg)	46.0 ± 8.4	43.2 ± 7.0	− 4.084	0.000
Subcutaneous fat (cm^2)	216.1 ± 57.1	153.5 ± 62.6	− 10.743	0.000
Abdominal fat (cm^2)	129.0 ± 42.7	72.4 ± 41.1	− 12.635	0.000
Hemoglobin (g/L)	14.7 ± 1.5	14.3 ± 1.5	− 3.059	0.002
FPG (mg/dL)	104.4 ± 30.6	90.0 ± 19.8	− 9.062	0.000
30-min PPG (mg/dL)	183.6 ± 48.6	151.2 ± 41.4	− 8.785	0.000
2-h PPG (mg/dL)	149.4 ± 73.8	108.0 ± 55.8	− 8.541	0.000
HbA1c (%)	6.1 ± 0.9	5.7 ± 0.7	− 6.650	0.000
FINS (mIU/L)	26.9 ± 12.2	18.0 ± 10.4	− 11.204	0.000
30-min insulin (mIU/L)	99.6 ± 59.6	85.4 ± 47.6	− 2.849	0.004
2-h insulin (mIU/L)	100.5 ± 62.1	64.0 ± 42.0	− 8.212	0.000
HOMA-IR	6.9 ± 3.6	4.1 ± 4.8	− 13.005	0.000
TC (mg/dL)	229.2 ± 39.1	213.5 ± 40.4	− 4.614	0.000
LDL-C (mg/dL)	98.2 ± 24.4	92.4 ± 22.2	− 3.184	0.001
HDL-C (mg/dL)	48.9 ± 11.4	57.6 ± 14.2	− 7.865	0.000
TG (mg/dL)	234.3 ± 235.8	128.9 ± 102.5	− 12.358	0.000
Scr (mg/dL)	0.8 ± 0.6	0.8 ± 0.3	− 0.450	0.653
BUN (mg/dL)	16.7 ± 4.2	16.3 ± 4.0	− 1.026	0.305
UA (mg/dL)	5.5 ± 1.5	4.6 ± 1.4	− 7.176	0.000
ALT (U/L)	30.1 ± 23.8	20.8 ± 12.6	− 8.030	0.000
AST (U/L)	24.2 ± 15.6	20.9 ± 7.6	− 4.252	0.000
γ-GT (U/L)	39.6 ± 38.1	24.2 ± 22.0	− 9.368	0.000
MA (mg/dL)	2.4 ± 5.9	1.0 ± 3.4	− 4.900	0.000
ACR (mg/g creatinine)	0.0 ± 0.1	0.0 ± 0.1	− 6.241	0.000
CP	1406.9 ± 506.4	1193.6 ± 485.5	− 5.183	0.000
SL	2.0 ± 0.6	2.1 ± 0.6	− 1.893	0.058

*By χ^2 test

Results

Analysis of baseline clinical characteristics in the MS and NMS groups

The study population included 458 males (41.2%) and 654 females (58.8%) with an average age of 53.2 ± 6.9 years. The MS and NMS groups contained 169 and 943 members, respectively. The physical and metabolic characteristics, CP, and SL voltage values of the two groups are listed in Table 1. Single-factor analysis showed significant intergroup differences ($p < 0.05$) in baseline values for almost all parameters except the gender composition, height, Scr, BUN, and SL voltage.

Table 2 shows significant differences in the CP values in the MS and NMS groups ($p < 0.05$ in total and $p < 0.001$ in the

Table 2 Comparison of baseline CP and SL voltage values in the MS and NMS groups

	Group	*N*	Mean	SD	*t*	*p*
CP (total)	NMS	943	1193.59	485.46	− 5.27	< 0.001
	MS	169	1406.86	506.38		
SL (total)	NMS	943	2.13	0.63	1.95	0.052
	MS	169	2.02	0.59		
CP (male)	NMS	382	981.24	521.90	− 2.88	0.004
	MS	62	1184.26	570.42		
SL (male)	NMS	382	2.37	.70	2.04	0.042
	MS	62	2.18	.69		
CP (female)	NMS	561	1338.60	398.93	− 4.86	< 0.001
	MS	107	1544.16	408.26		
SL (female)	NMS	561	1.964	.52	0.66	0.512
	MS	107	1.93	.50		

female population). However, SL voltage showed a significant intergroup difference only in the male population ($p = 0.042$) and did not show intergroup differences in the total and female population.

SL, CP, mean arterial pressure (MAP), high density lipoprotein, triglyceride, and fasting blood glucose were taken as independent variables, MS as dependent variables, and binary logistic regression was performed. The results are shown in Table 3.

CP, sex, FPG, MAP, age, and blood lipid were correlated with MS, but SL had no significant correlation with MS ($p > 0.05$). The risk of MS increased by 1.001 times for every unit of CP.

The difference of CP between MS and NMS after removing hypertension

In order to know whether the correlation between CP and MS is only caused by hypertension, age and sex were included in the regression analysis after removing the hypertension population from baseline MS and NMS.

Table 4 shows there were significant differences in CP between the two groups after considering age and gender ($p < 0.05$). CP was still associated with MS after removing hypertension.

Comparison of CP values between the new-onset MS and MS-free groups

The follow-up examinations involved 530 participants and contained all the tests performed at baseline except MRI. Participants who had no MS at baseline but had developed MS at the follow-up were classified into the new-onset MS group ($n = 58$), while those in the NMS group at baseline who had not developed MS were classified into the MS-free group ($n = 472$). We compared the new-onset MS group and MS-free group's CP and SL voltage values at baseline and follow-up and analyzed the differences in the changes in CP values. Table 5 shows that the CP value of the new-onset MS group was significantly higher than that of the MS-free group at baseline ($p = 0.027$); increase of CP value is also significantly different ($p = 0.015$) after taking age and gender into account. However, the SL voltage value was not different at baseline and the follow-up examination ($p > 0.05$).

Discussion

Increase of CP value was associated with development of MS in this study, and statistical analysis showed significant differences in the CP value between the MS and NMS groups. The CP value in the MS group was significantly higher than that in the NMS group in both male and female participants, indicating that the development of the MS is associated with an increase in the CP value. Although SL voltage showed a significant difference in the male population, it did not show any difference in the total and female population. Thus, CP was not affected by sex when used as a risk factor in predicting the

Table 3 Multivariate analysis of population characteristics in baseline MS and NMS

Variable	*B*	Wald	*p*	Exp(*B*)	95.0% CI for EXP(*B*)	
					Lower	Upper
HDL-C (mg/dL)	− 0.060	38.260	0.000	0.941	0.923	0.960
MAP (mmHg)	0.068	48.819	0.000	1.070	1.050	1.091
FPG (mg/dL)	0.318	27.275	0.000	1.375	1.220	1.549
TG (mg/dL)	0.003	19.676	0.000	1.003	1.002	1.005
Age (years)	0.052	12.269	0.000	1.053	1.023	1.084
Sex, male (%)	1.426	38.331	0.000	4.164	2.651	6.540
CP	0.001	23.777	0.000	1.001	1.000	1.001
SL	− 0.281	3.650	0.056	0.755	0.566	1.007

Table 4 The difference of CP between MS and NMS after removing hypertension

Variable	*B*	Wald	*p*	Exp(*B*)	95.0% CI for EXP(*B*)	
					Lower	Upper
Sex, male (%)	− 0.221	0.775	0.379	0.802	0.490	1.312
Age (years)	0.000	0.006	0.940	0.999	0.977	1.022
MAP	0.066	17.952	0.0000	1.068	1.036	1.101
CP	0.000	4.875	0.027	1.000	1.000	1.001

cardiovascular risk in MS and was better than SL voltage in MS patients.

CP, sex, FPG, MAP, age, and blood lipid were correlated with MS. After removing hypertension, CP values were still significantly different between MS and NMS groups. The CP value and the increase of CP of the new-onset MS group was significantly higher than that of the MS-free group, indicating that residents with higher baseline CP values were more likely to develop MS.

A previous study has shown that insulin resistance and hyperinsulinemia, independent of the common cardiovascular risk factors, are underlying factors for an increased risk of MS [9] and could direct influence myocardial function. Insulin resistance is related to a chronic proinflammatory and prothrombotic state that leads to hypertrophy and myocardial fibrosis. Some reports have noted that protein glycation may be a common pathway to myocardial damage in diabetes. Advanced glycation products bind to their receptor, which induces nuclear factor and proinflammatory cytokines, inflammation, growth factor release, and fibrosis. In contrast, hyperinsulinemia can activate the sympathetic nervous system or elevate sodium retention and cause fluid retention and increase the cardiac afterload. Insulin can increase ventricular mass as a cardiac growth factor in laboratory rats with hyperinsulinemia [10, 11]. All of the abovementioned factors can aggravate LVH. A previous branch of our study also involved 1168 community members and found that participants with higher CP values had higher fasting and 30-min and 2-h postprandial insulin levels. Thus, individuals with high CP levels may have cardiac hypertrophy because of the influence of hyperinsulinism, which may be related to the correlation of MS with CP, and may be the reason for the stronger correlation of MS with CP than with SL voltage. SL voltage is affected by the chest wall thickness, resulting in an underestimation of the prevalence of LVH in overweight MS patients or obese people. In contrast, CP provides a more accurate measure of LVH in obese and type 2 diabetes patients [12, 13]; therefore, we think CP is more suitable for MS patients.

Studies have shown that CP is more sensitive and specific than SL voltage, particularly in obese people and in patients with metabolic diseases [14]. Considering these advantages of CP, it is worth considering whether it may be possible to use CP alone to predict cardiovascular risk in MS populations. However, the obesity rate in Asian individuals is lower than those in European and American individuals, and some studies have suggested that CP shows reduced diagnostic value for LVH in Asian individuals [15]. Nevertheless, previous studies have shown CP and SL voltage have high specificity and a low sensitivity index, and the two parameters can be used together to improve the detection rate of LVH [16]. Therefore, we recommend the use of both CP and SL voltage together to identify more cardiovascular risk factors in the Chinese population.

However, this study still had some limitations. ECG-LVH and echocardiographic LVH have been shown to predict mortality independently of each other and may, therefore, assess different aspects of the underlying pathology [17]. Another study showed that the changes obtained by ECG could precede the morphological changes in the myocardium observed by echocardiography [18]. Echocardiography, which is the most accurate diagnostic method for assessment of LVH, was not performed in this study, and both CP and SL voltage show poor sensitivity [19]. Furthermore, the LIFE study that lasted for 5 years [20]; but only 1 year of follow-up was

Table 5 CP and SL values between the new-onset MS and MS-free group at follow-up

Variable	Unstandardized coefficients		Standardized coefficients	*t*	*p*
	B	Std. error	Beta		
CP baseline	145.445	65.714	0.088	2.213	0.027
Increase in CP	111.273	45.391	0.107	2.451	0.015
SL baseline	− 0.004	0.088	− 0.002	− 0.049	0.961
Increase in SL	− 0.293	0.412	− 0.031	− 0.712	0.477

conducted in our study, and we do not have enough new new-onset MS samples.

Conclusions

CP at baseline and the change in the CP value were predicted for development of MS in the future. CP is recommended for use as an ECG indicator for screening for a high risk of cardiovascular disease in patients with MS.

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Compliance with ethical standards

The study was approved by the ethics committee of Sir Run Run Shaw Hospital.

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ORIGINAL ARTICLE

The relationship between insulin resistance, obesity, and endotrophin

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Abstract

Background Endotrophin is the cleavage product COL6. Recent findings indicate that endotrophin is actively involved in inflammation, angiogenesis, and fibrosis. It also seems to play a critical role in obesity-induced systemic insulin resistance by increasing chronic inflammation and fibrosis in adipose tissues. In this study, we aimed to reveal the relationship between human adipose tissue, inflammation, and insulin resistance (IR).

Methods The study included 51 IR patients and 37 healthy controls. The presence of IR was based on Homeostatic model of assessment-insulin resistance (HOMA-IR) level of 2, 7, or higher. Fasting plasma samples were obtained from the patients and the control group.

Results The IR patients had a higher HOMA-IR, waist circumference, fasting plasma glucose (FPG), fasting insulin (FI), and triglyceride levels than the control subjects ($p < 0.05$). Endotrophin and CRP levels were significantly higher in patients with IR than controls (23.12 ± 18.32 ng/mL; 11.20 ± 6.60 ng/mL $p < 0{,}001$; 5.5 ± 3.37 ng/L; 1.97 ± 0.89 ng/L $p < 0.001$). The serum endotrophin level showed a positive correlation with HOMA-IR ($r = 0.359$; $p < 0.001$), waist circumference ($r = 0.214$; $p < 0.05$), FI ($r = 0.386$; $p < 0.001$), and CRP level ($r = 0.251$; $p < 0.05$)

Conclusion The serum endotrophin level was higher in patients with IR, independently of BMI, fasting glucose, and all other factors. Therefore, we foresee that high levels of endotrophin may be a new biomarker for early diagnosis of metabolic worsening in overweight and obesity. We also found a positive correlation between endotrophin and CRP. This indicates that endotrophin is closely related to inflammatory processes.

Keywords Endotrophin · Insulin resistance · Obesity · Inflammation

Highlights
- Determination of inflammation-associated biomarkers will help to understand the pathophysiology of IR.
- Endotrophin, the alpha 3 chain of type VI collagen, plays a critical role in obesity-induced systemic insulin resistance by increasing chronic inflammation and fibrosis in adipose tissues.
- Endotrophin level was higher in patients with IR, independently of BMI, fasting glucose, and all other factors.
- Endotrophin can be used either during the follow-up or in treatment of inflammation and IR.

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Introduction

In the past two decades, the number of overweight and obese people has increased substantially [1]. Excessive calorie intake more than the energy spent is the main cause of this [2, 3].

Obesity is closely associated with a chronic, low-grade inflammatory state characterized by macrophage infiltration of adipose tissue (AT) and subsequent proinflammatory adipokine expression. These cause various metabolic disorders, including insulin resistance (IR) [4, 5]. Insulin resistance occurs as a result of increased levels of proinflammatory cytokines and reactive oxygen species (ROS) [6].

Adipose tissue is a crucial organ for the maintenance of whole body energy homeostasis and a major source of collagen VI (COL6) [7]. In obese human adipose tissue, there are areas of fibrosis which have elevated levels of COL6 and M1 macrophages [8, 9]. COL6 is one of the major extracellular

matrix (ECM) proteins secreted from adipocytes. It is composed of three major polypeptide chains, α1, α2, and α3, which are encoded by distinct genes (COL6A1, COL6A2, and COL6A3, respectively) [10, 11].

Endotrophin is the C-terminal cleavage product, involving C5 domain of COL6α3 [12]. Recent findings indicate that endotrophin is actively involved in various biological processes, such as inflammation, angiogenesis, fibrosis, and epithelial–mesenchymal transition (EMT) in the context of cancer. Additionally, it seems to play a critical role in obesity-induced systemic insulin resistance by increasing chronic inflammation and fibrosis in adipose tissues [11–13]. Although there are many animal experiments which clearly demonstrated the relationship between endotrophin and insulin resistance, there are not many clinical trials in humans.

In this study, our primary objective was to investigate whether there is a relationship between endotrophin induced human adipose tissue inflammation and IR. Our secondary goal in the study was to determine the predictive role of endotrophin levels on insulin resistance.

Patients and methods

Patient selection

This cross-sectional study was performed at outpatient department of internal medicine, Okmeydanı Education and Research Hospital, Istanbul, Turkey. The study included 51 non-diabetic patients (24 male and 27 female) who had insulin resistance (IR) and 37 individuals (18 male, 19 female) with similar baseline characteristics as control group. The diagnosis of IR was based on the Homeostatic model of assessment-insulin resistance (HOMA-IR) level. Those patients with 2.7 or above were considered to have IR. The presence of diabetes has excluded with OGTT. None of the control subjects were taking any medication known to alter glucose tolerance. Exclusion criteria were the presence of systemic, inflammatory or infectious disease, autoimmune disease, chronic renal failure, and malignancies. All participants have normal blood pressure (BP). Informed written consent was obtained from all the participants (patients and controls) after they received full explanation about the study and its purpose. The study was conducted in accordance with the Helsinki Declaration and rules of Good Clinical Practice. Local ethics committee approved the investigational protocol described herein.

Measurements

Body mass index (BMI) was obtained using the formula weight (kg)/height $(m)^2$: overweight (defined as a body mass index [BMI] of 25 to 29.9 kg/m^2) and obesity (BMI ≥ 30 kg/m^2)

Blood samples were obtained after an overnight fasting. Biochemical measurements were done for serum cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glucose, blood HbA1c; "Creactive" protein (CRP) to reflect systemic inflammation; insulin and glucose for quantifying HOMA-IR, and endotrophin. IR was estimated via the HOMA-IR. HOMA-IR was calculated with the following equation: HOMA-IR = insulin (uU/mL) × glucose (mg/dL)/405. HOMA-IR value ≥ 2.7 was considered as insulin resistance and level < 2.7 was considered as insulin sensitive [14]. Endotrophin levels were measured by the enzyme-linked immunosorbent assay (ELISA) method using a Humanendotrophin kit (catalog no. 201-12-9305, manufacturer name of the kit is SunRed bio). For the endotrophin assay kit, the analytical (linear) detection range was 1.5–300 ng/mL, the minimal detection limit was 1.398 ng/mL, and the reported intraassay and interassay coefficient of variances (CVs) were %10 and %12, respectively.

Statistical analyses

SPSS version 12.0 was used for statistical analyses; descriptive statistical methods (mean, standard deviation, frequency, ratio, minimum, maximum) were used to evaluate the study data. The data obtained in the study are expressed as the mean ± SD. Student's *t* test was used for normally distributed quantitative parameters. Independent-sample *t* test was used to compare groups. Spearman's correlation coefficients were employed for bivariate associations of endotrophin and nonparametric covariates, and Pearson's correlation was employed for others. Multivariable logistic regression analyses were used to analyze the association of endotrophin with other variable. Values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were accepted as statistically significant.

Results

Clinical characteristics of the study subjects

The clinical characteristics of the study subjects are shown in Table 1. There were no significant differences in the age, BMI, HbA1c, total cholesterol, or LDL-C levels between the IR patients and control subjects. The IR patients had higher HOMA-IR, waist circumference, fasting plasma glucose (FPG), fasting insulin (FI), and triglyceride levels than those of the control subjects ($p < 0.05$). IR patients had a lower HDL levels than those of the control subjects ($p < 0.05$). Endotrophin and CRP levels were significantly higher in patients with IR than controls (23.1 ± 18.3 ng/mL; 11.2 ± 6.6 ng/mL $p < 0.001$; 5.5 ± 3.3 mg/L; 1.9 ± 0.8 mg/L $p < 0.001$).

Table 1 Baseline characteristic of patients and control group

	Patient group ($n = 51$) Mean ± SD	Control group ($n = 37$) Mean ± SD	p
Age (years)	44.80 ± 10.64	42.14 ± 9.34	0.231
BMI (kg/m^2)	31.09 ± 4.37	29.26 ± 5.64	0.092
HbA1c (%)	5.73 ± 0.39	5.60 ± 0.37	0.114
HOMA-IR	5.48 ± 3.31	1.55 ± 0.53	*0.000**
Waist circumference (cm)	90.71 ± 9.6	80.07 ± 13.4	*0.001**
Fasting insulin (μU/mL)	22.65 ± 12.48	7.56 ± 2.80	*0.000***
Fasting plasma glucose(mg/dL)	99.10 ± 12.14	87.34 ± 9.03	*0.000**
Total cholesterol (mg/dL)	196.03 ± 42.38	193.86 ± 43.84	0.815
LDL-C (mg/dL)	119.98 ± 37.28	114.20 ± 47.89	0.526
HDL-C (mg/dL)	43.84 ± 10.62	50.10 ± 10.62	*0.007**
Triglyceride (mg/dL)	160.45 ± 94.95	118.62 ± 62.64	*0.022**
CRP (mg/L)	5.50 ± 3.37	1.97 ± 0.89	*0.000***
Endotrophin (ng/mL)	23.12 ± 18.32	11.20 ± 6.60	*0.000***

Student's t test, Yates' continuity correction test, and Mann-Whitney U test

Statistical significance: *$p < 0.05$, **$p < 0.001$

BMI body mass index, *HOMA-IR* homeostasis model assessment of insulin resistance, *LDL-C* low-density lipoprotein-cholesterol, *HDL-C* high-density lipoprotein-cholesterol, *CRP* C-reactive protein

Relationship between serum endotrophin level and other variables

The serum endotrophin level showed a positive correlation with HOMA-IR ($r = 0.359$; $p = 0.001$), waist circumference ($r = 0.214$; $p = 0.045$), FI($r = 0.38$; $p = 0.001$), and CRP level ($r = 0.251$; $p < 0.05$) as shown in Table 2.

There was no correlation between endotrophin levels and BMI, TG, and FPG ($p > 0.05$).

Table 2 Relationship between HOMA-IR, waist cir, BMI, FI, FPG, CRP, triglyceride, and HDL with endotrophin levels (univariate correlation and multivariate linear regression analysis of endotrophin)

	Univariate correlation		Multivariate regression	
	r	p	OR (95% CI)	p
HOMA-IR	0.359	*0.001***	1.507 (0.57–2.44)	*0.002*
Waist cir	0.214	*0.045**	–	–
BMI	0.074	0.495	–	–
FI	0.386	*0.001***	–	–
FPG	0.209	0.067	–	–
CRP	0.251	*0.018**	–	–
Triglyceride	− 0.182	0.099	–	–
HDL	− 0.173	0.110	–	–

Statistical significance: *$p < 0.05$, **$p < 0.001$

HOMA-IR homeostasis model assessment of insulin resistance, *Waist cir* waist circumference, *BMI* body mass index, *FI* fasting insulin, *CRP* C-reactive protein, HOMA-IR with nonparametric Spearman's correlation, and others with Pearson's correlation. Model R2 0.11 and $p < 0.002$. Regression equation for serum endotrophin on the basis of this model was 11.08 + HOMA-IR × 1.507

Serum endotrophin levels of IR patients

When the patients with IR were divided into two groups as patients with normal glucose tolerance and impaired glucose tolerance, there was no significant difference in endotrophin levels between the two groups ($p = 0.67$). Changes in fasting plasma glucose levels did not affect endotrophin levels.

Endotrophin was significantly higher in all individuals with BMI > 25 in the study (IR and healthy group), and the difference between the two groups was statistically significant. However, there was no correlation between endotrophin levels and BMI ($p > 0.05$).

Known important confounding factors (e.g., HOMA-IR, WAISTC, FPI, CRP, and BMI) were included in multivariable regression analysis with a forward inclusion strategy. Regression analysis showed that none of the parameters except HOMA-IR did explain the serum endorphin levels (Table 2).

In the IR group with BMI > 25 (49 of 75 with BMI > 25), endotrophin levels were significantly higher than the insulin sensitive group whose BMI > 25 (23.31 ± 18.61 ng/mL vs 11.8 ± 7.04 ng/mL, respectively; $p = 0.001$).

Discussion

Obesity is characterized by abnormal production of proinflammatory cytokines and acute phase reactants. Various cell types, especially adipocytes in the fatty tissue, are responsible for this abnormal cytokine production [15]. Previous studies reported that macrophages in adipose tissues of obese mice

and humans showed the expression of several proinflammatory cytokines such as TNF-α and IL-6 [4, 16, 17].

Since macrophages in adipose tissues are identified as the primary source of many cytokines, much interest was shown to the relationship between insulin resistance, extracellular matrix (ECM), and adipose tissue. Previous studies showed the presence of both subcutaneous and omental adipose tissue fibrosis in obese individuals. Meanwhile, the mechanisms of adipose tissue fibrosis and its metabolic impact on the pathophysiology of obesity and insulin resistance are still unknown. It is suggested that the excessive deposition of ECM components, such as collagens and osteopontin, in adipose tissues triggers the necrosis of adipocytes, which later attracts classically activated proinflammatory macrophages. As a consequence, tissue inflammation and metabolic dysfunction occur.

Endotrophin is col6-alpha-3 subunit of col6. It is related to variety of biological processes such as inflammation, fibrosis, and angiogenesis [11–13]. The overexpression of endotrophin in mice stimulates fibrotic collagen deposition in adipose tissues and triggers adipose inflammation and insulin resistance [12]. Endotrophin seems to play a critical role in systemic insulin resistance which is induced by obesity. This is the result of increased chronic inflammation and fibrosis in adipose tissues.

In this study, our objective was to determine the relationship between endotrophin and insulin resistance. Endotrophin levels were significantly higher in the IR group than the controls.

Sun et al. performed endotrophin immunostaining studies on human mesenteric adipose tissue from BMI-matched individuals with either normal insulin sensitivity (HOMA-IR < 2.6) and insulin resistance (HOMA-IR > 2.6). The groups were comparable for BMI, age, and fasting glucose levels. The group of individuals with HOMA-IR > 2.6 displayed higher levels of endotrophin. The authors claimed that the increase in endotrophin signal was more likely to be associated with the reduced insulin sensitivity, rather than fasting glucose levels which were comparable between the two groups [11].

We did not find any significant difference in endotrophin levels between the two groups (impaired fasting glucose and normal glucose tolerance). Similarly, we did not find any correlation between endotrophin levels and FPG either in the study or the healthy control group. Based on these results, it can be concluded that endotrophin levels are high in IR patients irrespective of fasting plasma glucose levels, which is similar to the findings of previous studies.

Human studies have provided evidence that there is a relationship between adipose tissue fibrosis and obesity and insulin resistance. There are wide fibrotic areas in fat tissue of clinically obese people. These fibrotic areas contain classically activated macrophages (M1) and express collagen VI [6]. Consistent with the literature, endotrophin levels were significantly higher in overweight individuals with a BMI > 25 than those with a BMI < 25.

Lawler et al. [18] found out that obese individuals with insulin resistance have more fibrosis in their fat tissue than obese individuals with insulin sensitivity. Likewise, other studies showed the correlation between chronic inflammation and systemic insulin resistance, and tissue endotrophin levels in obese individuals [12].

A recent human study has demonstrated that endotrophin mRNA in adipocytes isolated directly from fresh human adipose tissue biopsies expression correlate positively with BMI [20]. In this study, all samples used were from IR (+) individuals. In our study, despite higher endotrophin levels in the group with higher BMI, there was no correlation between endotrophin levels and BMI. These conflicting outcomes may be due to the inclusion of different patient groups.

One of the main tasks of adipocytes is to store energy in the form of the triglyceride (TG). It is known that endotrophin-mediated changes in adipose tissue trigger an increase in fat mass and total weight as it reduces energy consumption without affecting food intake. It has been observed that the adipose tissue with elevated endotrophin levels in transgenic mice leads rapidly to dysregulation of circulating triglycerides and free fatty acids, and eventually to hepatic steatosis [11]. Although triglyceride levels were significantly higher in our patient group, it did not correlate with endotrophin levels.

Hotamisligil et al. [15] showed that the proinflammatory cytokine TNF-α was expressed in adipose tissue of obese mice and this was linked to insulin resistance. This was the first time a functional link between obesity, insulin resistance, and inflammation was suggested. Over the years, this association has evolved into the concept of metabolic inflammation. Several studies have reported that moderate elevations in CRP, an indicator of inflammation, might predict the development of insulin resistance [21–23]. Moreover, Tanigaki et al. showed that moderate elevations in CRP caused insulin resistance in the mice [24].

In our study, CRP levels were significantly higher in the IR group, just as expected. Furthermore, we found a positive correlation between CRP and the endotrophin level. Endotrophin mediates the systemic elevation of proinflammatory cytokines and insulin resistance in many tissues. Khan [19] T. found that genetically altered collagen VI null mice had fewer macrophage infiltration and were less insulin resistant although they were obese.

The both obesity and insulin resistance are chronic inflammatory processes. We know that most individuals develop insulin resistance while BMI (overweight and obese patients) increases. However, there are also individuals with BMI > 25 and without insulin resistance. In our study, we have selected similar BMI for the groups with/without insulin resistance. In this way, we have tried to explain the relationship between insulin resistance and endotrophin independent of overweight and obesity.

In conclusion, endotrophin level was higher in patients with IR, independently of BMI, fasting glucose, and all other factors. We observed that the high levels of endotrophin seen in overweight and obesity do not correlate with the increase in BMI. Nevertheless it is actually correlated with IR which is related with obesity. Therefore, we foresee that high levels of endotrophin may be a new biomarker for early diagnosis of metabolic worsening in overweight and obesity. Secondly, the positive correlation between endotrophin and CRP suggests that the inflammatory processes and endotrophin level are closely related. This relationship suggests that endotrophin can be used either during the follow-up or in treatment of inflammation and IR. Further studies with larger sample sizes are needed in order to determine the effects in different metabolic inflammatory scenarios.

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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ORIGINAL ARTICLE

Irisin in elderly people with hypertension, diabetes mellitus type 2, and overweight and obesity

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Abstract

Objectives This study aimed to investigate the irisin levels in elderly patients with hypertension, diabetes mellitus type 2, and overweight and obesity, to investigate the possible association between irisin levels and anthropometric and biochemical parameters, and also to assess the irisin levels associated with a risk of hypertension, diabetes mellitus type 2, and overweight and obesity.

Methods A nested case-control study was conducted, with hypertension, diabetes mellitus type 2, and overweight and obesity being set as the case group; 71 elderly patients from the cohort were enrolled in each case group, and 71 healthy elderly from the cohort were included in the control group. The anthropometric and biochemical parameters were measured in all elderly, and irisin levels were measured using enzyme-linked immunosorbent assay.

Results The irisin levels were significantly lower in patients with hypertension, diabetes mellitus type 2, and overweight and obesity than in controls ($p < 0.001$). Irisin levels were negatively correlated with body mass index, systolic blood pressure, diastolic blood pressure, fasting blood glucose, cholesterol, triglyceride, and alanine aminotransferase. Moreover, the model adjusted for body mass index, systolic blood pressure, diastolic blood pressure, and fasting blood glucose showed that increasing irisin levels were associated with a reduced risk of hypertension, diabetes mellitus type 2, and overweight and obesity. Receiver operating characteristic curve analysis showed that the area under the curve was 0.779 for hypertension, 0.976 for diabetes mellitus type 2, and 0.957 for overweight and obesity.

Conclusion Irisin may thus play a role in blood pressure and blood glucose regulation.

Keywords Irisin · T2DM · Hypertension · Overweight and obesity · Cardiovascular

Introduction

According to the Fifth National Health Services Survey Analysis Report in China released in 2016 [1], the top five chronic diseases in the elderly population are hypertension, diabetes, cerebrovascular disease, ischemic heart disease, and chronic obstructive pulmonary disease. Moreover, the latest Cardiovascular Disease Report in China (2017) showed that cardiovascular disease (CVD) accounts for more than 40% of all deaths in the population, ranking first, above

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cancer, and other diseases. Hence, it appears that the number of CVD patients will continue to grow rapidly in the next 10 years, and indeed, the burden of CVD has been getting heavier [2]. Yang et al. [3], who had the same opinion as that put forth by the CVD report in China, conducted a study in 2016 and indicated that clustering of CVD risk factors led to a high health burden in the Chinese elderly population. Modifiable risk factors for CVD mainly include hypertension, smoking, abdominal obesity, and diabetes, which were all included in the list of the top five chronic diseases. China is currently experiencing rapid economic, social, and cultural changes, including accelerated changes in nutrition pattern and lifestyle, which may contribute to a greatly increased health burden. Therefore, there is an urgent need to manage risk factors of CVD, for example, by reducing the prevalence of hypertension, diabetes, and obesity.

Recent studies have confirmed that sarcopenia is a risk factor for CVD [4, 5]; this concept has led to the hypothesis that some myokines might confer cardioprotection and/or vascular protection [6]. Irisin is one such myokine that can increase energy consumption, decrease body mass, and improve insulin sensitivity [7, 8]. At present, many functional studies of irisin are conducted in mice, but related research studies in humans are limited, and there are several controversies surrounding these studies. Additionally, to our knowledge, the research on irisin and hypertension is limited.

Considering that hypertension, diabetes mellitus type 2 (T2DM), and overweight and obesity are risk factors for CVD, we conducted a nested case-control study and aimed to investigate irisin levels in elderly patients with hypertension, T2DM, and overweight and obesity, to investigate the possible association between irisin levels and anthropometric and biochemical parameters, and also to assess the irisin levels associated with a risk for hypertension, T2DM, and overweight and obesity.

Materials and methods

Ethics statement

All patients were informed of the purpose of the study in advance and signed a consent form. The study was conducted in compliance with the World Medical Association Declaration Helsinki, and the procedures were approved by the ethics committee of the school of public health, Xiamen University.

Patients

We conducted a nested case-control study among people with aged 60 year or older in Guankou Town, Xiamen, China. From March 2013, 3386 subjects were enrolled into our cohorts. A standardized procedure of follow-up was performed yearly until May 2016. A total of 213 elderly patients, including 71 hypertension patients, 71 T2DM patients, and 71 patients with overweight and obesity, were selected as three case groups. Following the rule of a case-control study, a 1:1 matched method was adopted according to the age. The control group consisted of 71 people from the same cohort who did not have hypertension, T2DM, overweight and obesity, or any metabolic disorders. All cases were first diagnosed during physical examination, and there was no related drug-taking history.

The inclusion criteria were as follows:

(1). Primary hypertension: The diagnostic criteria were based on the revised edition of the guidelines for the management of essential hypertension in China (2014), which defined primary hypertension as systolic blood pressure (SBP) greater than or equal to 140 mmHg or diastolic blood pressure (DBP) greater than or equal to 90 mmHg measured several times on different days under resting conditions, and in the absence of hyperthyroidism, aortic stenosis, and other forms of secondary hypertension.
(2). Diabetes mellitus type 2: The diagnostic criteria were based on the World Health Organization's diagnostic criteria (1999): the presence of typical symptoms and a fasting blood glucose (FBG) level greater than or equal to 7.0 mmol/L. In cases with FBG levels greater than or equal to 7.0 mmol/L but no typical symptoms, the tests should be repeated again; if the aforementioned FBG values are noted again, a diagnosis of T2DM can be made.
(3). Overweight and obesity: The diagnostic criteria were based on China's Adult Overweight and Obesity Prevention Control Guide (2003): body mass index (BMI) greater than or equal to 24 kg/m^2 (overweight) or BMI greater than or equal to 27 kg/m^2 (obese).
(4). Age greater than or equal to 60 years.

The exclusion criteria were as follows: presence of acute complications, infectious disease; severe cardiac, hepatic, and nephrotic disease; hematological system disease; immune system disease; and malignant tumor.

Anthropometric and biochemical measurements

The physical examination data of the patients were collected, and the general demographic data were recorded. The anthropometric measurements included weight, height, waist circumference (WC), and blood pressure, including SBP and DBP; all these measurements were performed by the medical staff. The BMI was calculated as the weight (in kilograms) divided by the square of the height (in meters). WC was

measured to the nearest 0.1 cm at the level of the omphalion with a flexible inch tape while the patient was under conditions of minimal respiration.

Elbow vein peripheral blood was drawn in the morning (fasting 8 to 12 h) for all patients. All blood samples were stored in biochemical tubes. Some samples were used to detect biochemical indices, including FBG, cholesterol (CHOL), triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood routine, albumin (ALB), creatinine (Cr), and blood urea nitrogen (BUN) levels. Other samples were centrifuged (1000*g*, 20 min) after incubation for 2 h at room temperature; the upper layer, serum, was placed in an Eppendorf tube and stored at − 20 °C before the assessment of irisin levels.

FBG levels were measured using hexokinase, and blood lipid and liver function was determined using an enzymatic method (Hitachi Company's 7600 automatic biochemical analyzer); all examinations were performed by the medical staff.

Serum irisin measurement

Serum irisin concentrations were measured using am enzyme-linked immunosorbent assay kit (Sino Best Biological Technology Co., Ltd., Shanghai, China). The test proved to be highly sensitive to human irisin [3]. The sensitivity of the measurement was 1.0 ng/ml, and the linear regression of the standard product and the expected concentration correlation coefficient *R* value were greater than or equal to 0.9900. Variation between plates was less than 15%.

Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences version 19, and $p < 0.05$ was considered statistically significant. Descriptive statistical analysis was used to describe all data; tests included normality and homogeneity tests. Data showing normal distribution are presented as mean ± standard deviation, and those showing skewed distribution as median ± interquartile ranges. Analysis of variance with post hoc Bonferroni correction was performed for normally distributed data, and the Kruskal-Wallis test and post hoc Bonferroni correction were applied for non-normally distributed data. Spearman correlation analyses were performed to assess the correlation of irisin levels with different anthropometric and biochemical parameters. Multinomial logistic regression was performed to identify the risk of T2DM, hypertension, and overweight and obesity according to serum irisin levels. Receiver operating characteristic (ROC) curves were plotted to assess the sensitivity and specificity of serum irisin levels in differentiating between each condition.

Results

Table 1 shows that BMI and SBP, DBP, ALT, CHOL, TG, and FBG levels were significantly different between the groups. Post hoc analysis also demonstrated the significant difference between the control group and other groups. There were no

Table 1 Anthropometric characteristic and biochemical profiles of subjects

	Control	Hypertension	T2DM	Overweight and obesity	*p*
Age (year)	66.0 ± 4.8	69.7 ± 5.6	68.4 ± 6.4	66.0 ± 4.3	< 0.001
BMI (kg/m^2)	21.10 ± 2.08	22.17 ± 2.40	23.27 ± 3.87^{a}***	27.05 ± 2.07^{c}***,^{e}***,^{f}***	< 0.001
SBP (mmHg)	122 (112, 131)	148 (140, 165)^{b}***,^{d}*	142 (130, 158)^{a}***	130 (121, 134)^{c}*,^{e}***,^{f}***	< 0.001
DBP (mmHg)	73 (68, 79)	90 (80, 95)^{b}***,^{d}**	83 (75, 87)^{a}***	81 (76, 85)^{c}***,^{f}***	< 0.001
ALT (U/L)	18.9 (13.9, 24.6)	18.5 (16.0, 24.0)	19.6 (16.2, 25.5)	24.8 (18.3, 36.0)^{c}***,^{e}*,^{f}**	< 0.001
AST (U/L)	21.1 (18.6, 25.7)	21.1 (18.5, 23.1)	19.6 (17.5, 23.4)	21.5 (18.5, 26.7)	0.082
ALB (g/L)	45.6 (44.3, 47.5)	46.5 (44.8, 48.3)	46.6 (44.8, 47.9)	46.5 (45.0, 48.2)	0.281
Cr (umol/L)	66.4 (58.7, 75.9)	64.1 (55.3, 77.4)	60.7 (54.0, 79.5)	64.6 (54.0, 75.8)	0.433
BUN (mmol/L)	5.2 (4.2, 6.1)	5.1 (4.5, 5.9)	5.2 (4.2, 6.1)	5.3 (4.4, 6.2)	0.822
CHOL (mmol/L)	5.05 ± 0.56	5.56 ± 1.04^{b}**	5.64 ± 1.01^{a}***	5.39 ± 0.91	0.001
TG (mmol/L)	0.876 ± 0.463	1.330 ± 0.715^{b}**	1.660 ± 0.757^{a}***	1.520 ± 1.050^{c}***	< 0.001
FBG (mmol/L)	5.19 ± 0.41	5.30 ± 0.40^{d}***	7.84 ± 1.74^{a}***	5.26 ± 0.42^{e}***	< 0.001
Irisin (ng/ml)	777.13 (762.32, 801.01)	720.56 (647.68, 770.56)^{b}***,^{d}***	640.83 (574.51, 706.83)^{a}***	667.02 (604.37, 712.98)^{c}***,^{f}***	< 0.001

p is the result of comparison between four groups. [a] Comparison between control and T2DM, [b] comparison between control and hypertension, [c] comparison between control and overweight and obesity, [d] comparison between T2DM and hypertension, [e] comparison between T2DM and overweight and obesity, [f] comparison between hypertension and overweight and obesity

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 2 Correlation between irisin levels and anthropometric and biochemical index

		Variables										
		BMI	SBP	DBP	ALT	AST	ALB	Cr	BUN	CHOL	TG	FBG
Irisin	R	− 0.420	− 0.196	− 0.212	− 0.163	− 0.059	− 0.012	0.065	− 0.22	− 0.152	− 0.334	0.374
	p	0.001	0.001	0.001	0.006	0.325	0.839	0.276	0.717	0.010	0.001	0.001

significant differences in AST, ALB, Cr, and BUN levels between the groups. Serum irisin levels in the hypertension (720.56 ng/ml (647.68, 770.56)), T2DM (640.83 ng/ml (574.51, 706.83)), and overweight and obesity (667.02 ng/ml (604.37, 712.98)) groups were significantly lower than those in the control groups (777.13 ng/ml (762.32, 801.01)). A rank sum test was used for the comparison between the four groups, using a cutoff of $p < 0.001$, and the diversity was found to be statistically significant.

To determine which anthropometric and biochemical indices were correlated with serum irisin levels, rank correlation analysis was performed. Table 2 shows that serum irisin levels were negatively correlated with BMI and SBP, DBP, FBG, CHOL, TG, and ALT levels, but not with AST, ALB, Cr, and BUN levels.

Additionally, based on multinomial logistic regression analysis, we found that higher irisin levels were associated with a reduced risk of T2DM, hypertension, and overweight and obesity (Table 3).

Table 4 shows the sensitivity, specificity, positive predictive value, negative predictive value, and cutoff value of serum irisin levels for hypertension, T2DM, and overweight and obesity. ROC curve analysis was also performed to assess the diagnostic value of serum irisin levels and showed that the areas under the curve were 0.779 ($p < 0.001$) for hypertension (Fig. 1), 0.976 ($p < 0.001$) for T2DM (Fig. 2), and 0.957 ($p < 0.001$) for overweight and obesity (Fig. 3).

Discussion

Here, we conducted a nested case-control study on serum irisin levels in patients with hypertension, T2DM, and overweight and obesity, aged 60 years and above, in China. The study first found that the serum irisin level in patients with hypertension was significantly lower than that in healthy patients. Furthermore, the serum irisin levels in patients with hypertension were found to be negatively correlated with systolic and diastolic blood pressure, which indicates that the increase in irisin levels might decrease hypertension risk.

Previous studies have focused mostly on adults, and more on diabetes, obesity, and nonalcoholic fatty liver disease [9, 10]. Our results were consistent with these studies, but we found that the serum irisin levels in T2DM and overweight and obesity were significantly lower than those in the control group. The mechanism underlying these findings for irisin is unclear. However, some animal models suggest that reductions in serum irisin levels may be associated with the development of insulin resistance and T2DM [11].

Our study also revealed that serum irisin levels in patients with hypertension were significantly lower than those in the control group. This finding is similar to that from a clinical study from 2015 [12]. Animal model studies may explain the mechanism underlying the association between irisin and hypertension [13]. These studies provide evidence that corroborates our work, but it is important to understand that the

Table 3 Multinomial logistic regression analysis

		T2DM	Hypertension	Overweight and obesity
Model-1	*B* (SE)	− 0.033	− 0.024	− 0.031
	OR (CI)	0.968 (0.959, 0.976)	0.976 (0.968, 0.984)	0.969 (0.961, 0.978)
	p	< 0.001	< 0.001	< 0.001
Model-2	*B* (SE)	− 0.029	− 0.021	− 0.024
	OR (CI)	0.972 (0.963, 0.980)	0.979 (0.971, 0.987)	0.976 (0.967, 0.985)
	p	< 0.001	< 0.001	< 0.001
Model-3	*B* (SE)	− 0.03	− 0.018	− 0.019
	OR (CI)	0.971 (0.957, 0.985)	0.982 (0.971, 0.993)	0.981 (0.969, 0.993)
	p	< 0.001	0.002	0.001

Model-1: unadjusted

Model-2: adjusted for BMI

Model-3: adjusted for BMI, SBP, DBP, FBG

Table 4 Sensitivity and specificity

	Hypertension	T2DM	Overweight or obesity
Sensitivity	0.845	0.845	0.901
Specificity	0.662	0.972	0.831
Positive predictive value	71.43%	96.77%	84.21%
Negative predictive value	81.03%	86.25%	93.65%
Cutoff value (ng/ml)	754.77	754.77	739.88

association of irisin with high blood pressure and the related regulatory mechanisms still need to be further studied.

Serum irisin levels were found to be negatively correlated with SBP, DBP, FBG, CHOL, and TG levels when the correlation of serum irisin levels with other anthropometric and biochemical index was assessed, consistent with most previous studies. Serum irisin levels and BMI were found to be negatively correlated in our study, in contrast with reports from a study performed among Korean adolescents in 2017 [14]; this difference may be due to physiological and pathological differences among different populations [15, 16]. The high levels of irisin in obese patients may be the result of a form of irisin resistance (like insulin resistance), and irisin increases may be a compensatory mechanism aimed at improving insulin sensitivity [17]. However, there is no reasonable explanation for the decrease in irisin levels noted in obese people. Regarding the relationship of serum irisin levels with ALT and AST levels, a study from 2014 showed that irisin levels were not significantly associated with ALT and AST levels in patients with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) [18]; Rizk et al. showed that irisin levels in metabolic syndrome patients with elevated liver enzyme levels and fatty liver disease were significantly higher than those in normal controls [19]; in a study of patients with NAFLD performed by Shanaki et al. in 2017, ALT, AST, and irisin levels were found to be negatively correlated [10]. However, in our study, the serum irisin levels were negatively correlated with ALT levels, but there was no significant correlation with AST levels. These findings are slightly different to results from the published literatures [10, 18, 19] which might be due to the differences in the population.

In our study, after adjusting for BMI, SBP, DBP, and FBG levels, the logistic regression model showed that the rise in serum irisin levels has a protective effect against T2DM, hypertension, and overweight and obesity. In other words, the decrease in serum irisin levels was associated with an increased risk of T2DM, hypertension, and overweight and obesity.

The regression model had high sensitivity, specificity, positive predictive value, and negative predictive value to assess the relationship between irisin and hypertension, T2DM, and overweight and obesity. The ROC curve showed that irisin levels had a high diagnostic value for the three kinds of diseases. These results reveal that the irisin level could be used as a biomarker to assess the status of hypertension, T2DM, and overweight and obesity. In a previous study, the ROC curve showed that irisin was an effective parameter to distinguish

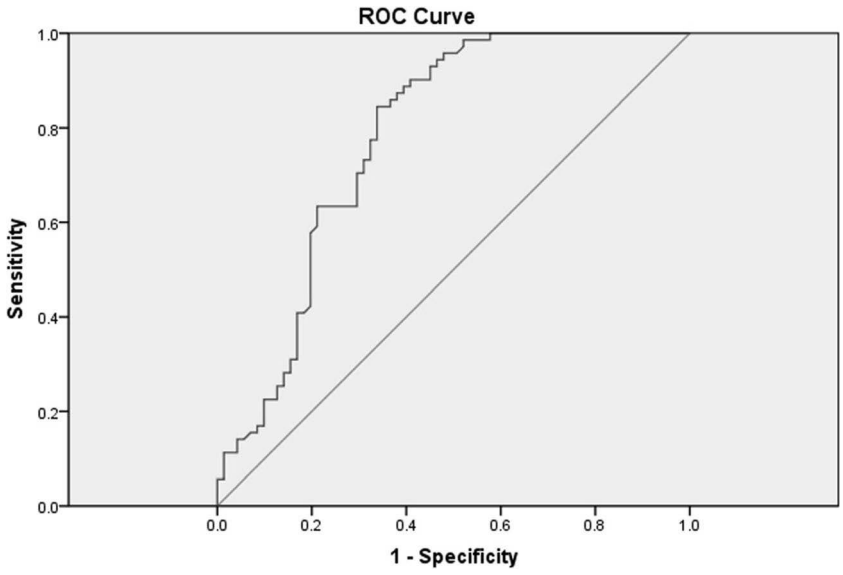

Fig. 1 ROC curve for diagnosis hypertension

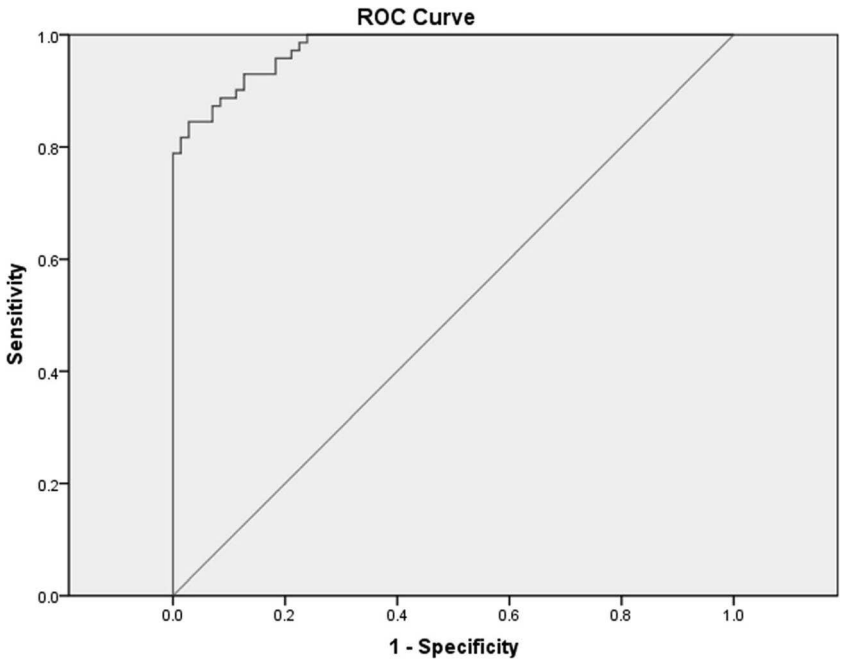

Fig. 2 ROC curve for diagnosis T2DM

patients with T2DM, NAFLD, or T2DM + NAFLD from those without these disorders [10]. Although the diseases examined in the two studies differ, both these results may be helpful in studying the diagnostic value of irisin. These results indicate that irisin plays a role in blood pressure control. Thus, we can promote the increase in irisin levels among middle-aged and elderly individuals by advocating moderate exercise and generating strategies aimed at the prevention, detection, and treatment of CVD factors. Ultimately, the burden of CVD can be reduced, thus improving health conditions. In our study, fasting lipid profile was of variable fasting of 8–12 h which is not standardized. This might affect our findings slightly. Although our study has some limitations, such as limited availability of information, lack of field investigation, a small sample size which might limit the validity of our study, and the fact that skeletal muscle mass or strength and exercise habits of the patients were not accounted for, all of which may affect the study's findings, our nested case-control study provides strong epidemiology evidence for irisin and verifies the results of previous studies on irisin. The mechanism by which this myokine affects the metabolic pathway is also not clear. Possibly, the mechanistic studies in the future will shed more

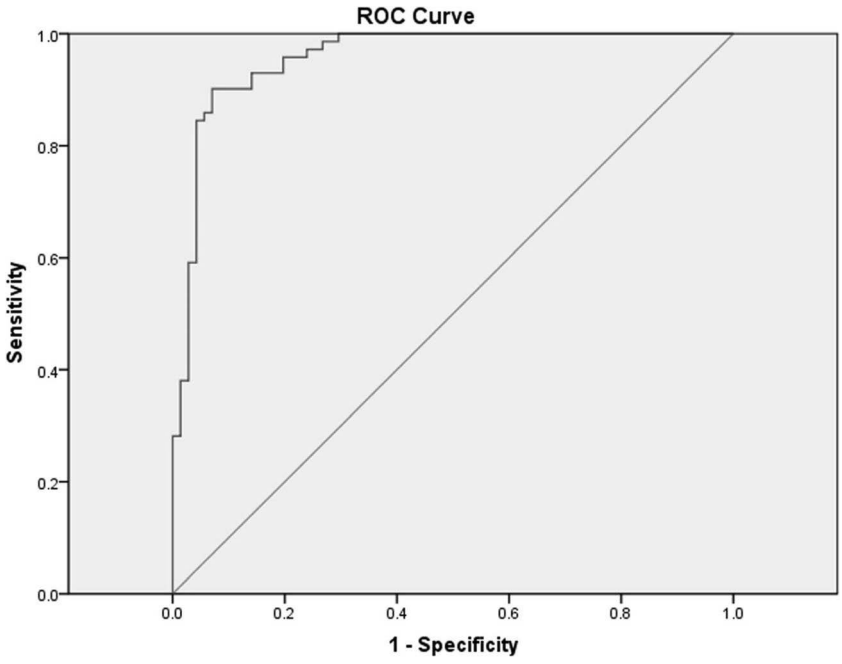

Fig. 3 ROC curve for diagnosis overweight and obesity

light into the pathophysiological mechanism by which irisin alters the metabolism in the human body. In the future, we will continue to set up and observe cohorts in order to understand the mechanism and principles of the effects of irisin.

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Compliance with ethical standards

The study were approved by the ethics committee of School of Public health, Xiamen University. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Competing interests The authors declare that they have no conflict of interest.

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

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ORIGINAL ARTICLE

Genetic polymorphism of *eNOS* (G894T) gene in insulin resistance in type 2 diabetes patients of Pakistani population

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Abstract

Background Insulin resistance associated with type 2 diabetes (T2DM) consequences in the development of metabolc syndrome. Due to the complex mechanism, various pathogenic factors like enviromental and genetic predispositon contribute to the disease pathogenicity. The reduced availability of *eNOS* has been determined as a mark of insulin resistance pathogenesis.

Objective The objctive of this study was to investigate genetic polymorphism of *eNOS* (rs1799983; G894T) with susceptibility of insulin resistance in type 2 diabetes (T2DM) patients from Pakistani population.

Methods Total of 322 (161 T2DM cases and 161 healthy controls) subjects were recruited for this study. Genomic DNA extraction was carried out by standard phenol-chloroform protocols. PCR amplification of the unique oligonucleotides of *eNOS* gene was done and restriction fragment analysis (RFLP) was performed by site-specific enzyme *BanII*.

Results The frequency of GG (wild genotype) was higher (77.6%) in cases than in controls (47.2%), heterozygous GT genotype higher in controls than patients (OR = 0.26, $p < 0.0005$ and after adjustment, OR = 0.34, $p = 0.091$). Different genetic models like dominant model of GT&TT was higher in controls than patients (OR 0.26, $p < 0.0005$; OR 0.29, $p = 0.038$ respectively) and log-additive model indicated the significant protective effect of the genotype before and after adjustment for the confounding factors.

Conclusion This study demonstrates that there is no association between *eNOS* rs1799983 polymorphism and insulin resistance in T2DM patients of Pakistani population.

Keywords Insulin resistance · T2DM · Genetic polymorphism · *eNOS* gene · Pakistan

Introduction

Type 2 diabetes (T2DM) is the most frequently metabolic disorder worldwide. Distal tissue's insulin resistance (IR) and inadequate insulin secretion from pancreas become the main two fundamental abnormalities involved in the pathogenesis of T2DM. IR along with other disorders like hypertension and dyslipidemia are considered as the main clustering risk factors of metabolic syndrome that are firmly related to development of T2DM and other cardiovascular illnesses [1, 2]. The genetic predisposition along with environmental factors has been considered to play an important role in the progression of diabetic complications, insulin resistance, and other metabolic syndrome constituents [3]. Many studies have been conducted to depict the susceptibility of genes in T2DM although very few data available in South Asian populations [4].

Nitric oxide (NO) is an important endogenous endothelial-derived relaxing factor synthesized by nitric oxide synthases family (NOS family). Three mammalian NOS isoforms are known to synthesis NO, most importantly eNOS. The eNOS form is to be involved in NO synthesis from molecular oxygen and L-arginine [5]. Endothelial nitric oxide synthase (eNOS) enzyme plays a vital function in normal insulin signaling via its role as an endothelial-derived relaxing factor [6]. Normal insulin signaling results in the modulating of two parallel

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pathways of which the PI3K-Akt pathway and another is Ras/Raf/MAP kinase system. The pathway of PI3 Kinase (PI3)-Akt regulates the vasodilation where Akt kinase performs phosphorylation at serine 1177 and activates the eNOS enzyme. The Glut4 transporter has been stimulated by Akt kinase and enhances the glucose uptake and vasodilation. In the presence of insulin resistance, there is a decreased effect of insulin due to a reduced sensitivity. The (PI3K)-Akt pathway is shown to be altered in the presence of insulin resistance, resulting in a diminished blood flow, while the Ras/Raf/MAP kinase pathway remains unaffected [7]. The inclusion of eNOS in enhancing uptake of glucose in skeletal muscle and evidence of decreased bioavailability of nitric oxide under diabetic states has led to the examining of *eNOS* gene and its relation with T2DM [8].

The *eNOS* gene is localized at chromosome 7q35-36 and its corresponding eNOS protein produces NO constitutively through the conversion of L-arginine to L-citrulline. There is a complex relationship between endothelial dysfunction (defect in NO production) and insulin resistance; insulin resistance is a known cause of endothelial dysfunction and conversely endothelial dysfunction also leads to insulin resistance perpetuating the problem [7].

Various studies have demonstrated the role of e*NOS* gene in different diseases in variable ethnic groups like Chinese [9], Japanese [10], Caucasians [11], Koreans [12], Italians [13], and North Indians [14]. Several studies have been carried out to study the effect of *eNOS* gene polymorphism on development of T2DM and its associated states, where *eNOS* polymorphisms have been revealed to be important diabetic risk factors [8, 15–20]. Nucleotide variation of *eNOS* occurs when the residue of guanine at position 894 in exon 7 is substituted by the residue of thymine (c.894G>T) at 298 codon. This polymorphism influences the response to vascular endothelial activity due to the oxidative stress and alterations in *eNOS* gene result in disturbances of metabolic activities involved in the development of T2DM and associated complications [15].

Aim of the study

The aim of present study was to detect the genetic association of *eNOS* rs1799983 (G894T; Glu298Asp) polymorphism with insulin resistance in T2DM patients of Pakistani population.

Subjects and methods

Before the start of the study, ethical approval was obtained from Advance Studies and Review Board (AS&RB) of University of Health Sciences, Lahore, Pakistan. Written informed consent was obtained from the human subjects according to Helsinki guidelines (2008) for sample obtaining.

Study population

Total of 322 participants including 161 cases diagnosed of T2DM, and 161 healthy controls were recruited for this study. Total of 5.0 ml of the venous blood sample was taken from each participant. Samples were collected in EDTA and gel vaccutainer tubes for DNA extraction and serum separation respectively.

Biochemical analyses

Various biochemical parameters were analyzed to establish the diagnosis of diabetes: Lipid profiles, glucose profile, and serum insulin levels were published in previous data [21]. Insulin resistance was documented by using HOMA-IR

Table 1 Comparison of basic study parameters among study subjects

Parameter	Over all	Controls	Cases	χ^2 (df)	*p* value
	n (%)	*n* (%)	*n* (%)		
Age					
< 50 years	181 (56.2)	70 (43.5)	111 (68.9)	21.209 (1)	*< 0.001**
> 50 years	141 (43.8)	91 (56.5)	50 (31.1)		
Body mass index					
Under weight	06 (1.9)	06 (3.7)	00 (0.0)	123.953 (3)	*< 0.001**
Normal weight	222 (68.9)	153 (95.0)	69 (42.9)		
Overweight	93 (28.9)	02 (1.2)	91 (56.5)		
Obese	01 (0.3)	00 (0.0)	01 (0.3)		
Gender					
Males	164 (50.9)	82 (50.9)	82 (50.9)	0.000 (1)	1.00
Females	158 (49.1)	79 (49.1)	79 (49.1)		

*p value < 0.05 considered statistically significant

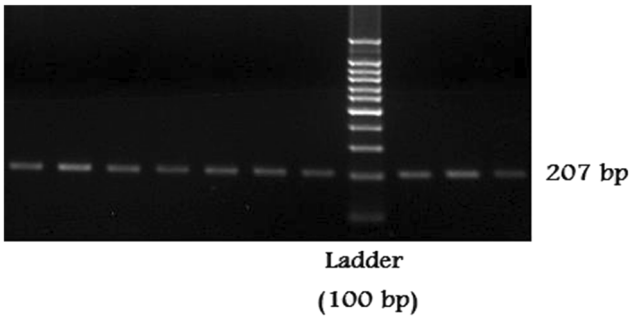

Fig. 1 PCR product size in genotyping for *eNOS* (rs1799983 G894T) polymorphism was 207 bp on 2.0% agarose gel with 100 bp DNA ladder

equation according to the International Diabetes Federation guidelines (Cut off value ≥ 1.6).

Genetic association studies of *eNOS* gene

The extraction of total genomic DNA was carried out by using standard phenol-chloroform method and for the small volume of sample, BioNeer Blood DNA extraction Kit (centrifugal column; Daejeon city, Korea) was used according to the manufacturer's specifications. Polymerase chain reaction of unique oligonucleotides (F′-5′-CATGAGGCTCAGCC CCAGAA-3′; R′-5′-AGTCAATCCCTTTGGTGCTCAC-3′) of *eNOS* gene was carried out and restriction fragment length polymorphism using *BanII* restriction enzyme was performed to genotype rs1799983 (G894T) polymorphism in T2DM patients and healthy controls. The digested products were resolved on 2.5% agarose gel to infer the genotype/alleles.

Statistical analysis

SPSS 22.0 was used for data analysis of the quantitative variables by performing Student's *t* test for continuous values and by the χ^2 test for nominal variables. The relationship between the *eNOS* G894T polymorphism and IR in T2DM was determined by calculating odds ratios and 95% of confidence interval (CI) through a univariate logistic regression analysis. Statistical significance was considered if p value ≤ 0.05.

Results

Population characteristics

The demographics parameters of participants are presented in Table 1. The range of age of the participants was 45 to 65 years. The cases or IR patients comprised 82 (50.9%) male patients and 79 (49.1%) female patients. An analysis of the body mass index in cases revealed 91 (56.5%) patients were overweight and 01 (0.3%) patient was obese (Table 1).

The clinical parameters including waist circumference (WC), HOMA-IR, and lipid profile were found to be significantly different in cases when compared with controls ($p < 0.001$) which explain the metabolic abnormalities among IR in T2DM patients [21].

Genetic studies

The amplification product of *eNOS* (rs1799983) gene polymorphism was 207 bp (Fig. 1). The RFLP digested products showed a single band (207 bp), double bands (125, 82 bp), and triple bands (207, 125, and 82 bp) for genotypes GG (wild), TT (homozygous), and GT (heterozygous) respectively as shown in Fig. 2.

Association between the *eNOS* (rs1799983 G>T) polymorphism and insulin resistance

To find the association between *eNOS* polymorphism and IR, the genotype frequency distributions of IR cases and controls are shown in Table 2. All three different genotypes were significantly associated with HOMA score in cases and controls ($p < 0.001$). The genotype frequency distributions of the *eNOS* polymorphism were 77.6% (GG), 17.4% (GT), and 5.0% (TT) in the cases and 47.2% (GG), 40.4% (GT), and 12.4% (TT) in the controls.

After adjustment for age, gender, BMI, HOMA, TC, TG, HDL, LDL, and VLDL, the relationship between the *eNOS* polymorphism and IR was analyzed. In co-dominant model, it was observed that minor TT genotype of *eNOS* polymorphism contributed only 5.0% occurrence of IR (OR = 0.24, 95%

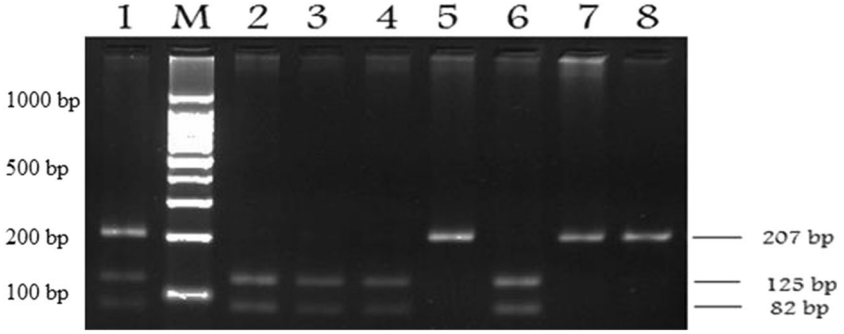

Fig. 2 Band fragments after restriction fragments length polymorphism in genotyping for *eNOS* polymorphism: lane 1 (digested product to three fragments: 207 bp, 125 bp, and 82 bp), lane M (DNA marker of 100 bp); lanes 2, 3, 4, and 6 (heterozygous; 125 bp and 82 bp); and lanes 5, 7, and 8 (wild-type single band of 207 bp)

Table 2 Comparison of HOMA score and different *eNOS* genotypes in T2DM and controls

Parameter	HOMA			Mann-Whitney *U*	*p* value
	Over all	Controls	Cases		
	Median (IQR)	Median (IQR)	Median (IQR)		
eNOS rs1799983SNP					
GG genotype	16.91 (19.5)	1.16 (0.53)	19.6 (6.33)	2970.0	< 0.001*
GT genotype	1.40 (14.1)	1.07 (0.54)	19.2 (6.24)	2355.0	< 0.001*
TT genotype	1.09 (10.9)	0.95 (0.50)	18.33 (9.18)	375.0	< 0.001*

IQR interquartile range, *p value < 0.05 considered statistically significant

CI = 0.10–0.58, $p < 0.001$) and after adjustment (OR = 0.13, 95% CI = 0.01–2.11, $p = 0.091$). In dominant model, we also found GT/TT genotypes contributed to 22.4% occurrence of IR (OR = 0.26, 95% CI = 0.16–0.42, p value < 0.001) and after adjustment (OR = 0.29, 95% CI = 0.08–1.01, p value 0.038) (Table 3).

Discussion

Insulin resistance is a characteristic feature of type 2 diabetes and can develop a broader clinical spectrum like glucose intolerance, obesity, diabetes, and metabolic syndrome [22]. Insulin resistance development lead to dyslipidemia (abnormal lipid metabolism) presented as low HDL-cholesterol and hypertriglyceridemia are common findings in T2DM patients which is considered a significant risk factor for premature atherosclerosis [2]. Complex diseases augmented due to the environmental factors and genetic predisposition. In T2DM, several genetic polymorphisms have been reported in different ethnic groups worldwide.

Nitric oxide (NO) is formed from L-arginine by nitric oxide enzymes family mainly eNOS and its reduced availability has been determined as a mark of insulin resistance pathogenesis [23]. The occurrence of SNP G894T in the gene encoding eNOS exon 7 results when guanine residue at position 894 is changed by thymine residue (Glutamate-GAG to Aspartate-GAT) at the codon 298. The occurrence of mutant phenotype GT/TT in some studies was found to be associated with T2DM [24, 25].

Variations in *eNOS* gene were assumed to result in the consequences of insulin resistance and hyperinsulinemia [19, 26]. In the present study, dominant model was appropriate to analyze results of *eNOS* G894T polymorphism association with IR-T2DM patients. This model showed that combined "GT&TT" genotypes were greater in controls (52.8%) than in cases (22.4%) with a frequency difference of 30.4% (OR 0.26, $p < 0.0005$; OR 0.29, $p = 0.038$). The frequency of IR risk factors combined (GT and TT) genotypes was low in cases as

Table 3 Genotype models for disease association of *eNOS* rs1799983 SNP genotype

Genotypes/ models	Controls	Cases	*p* value	OR crude [95% CI]	*p* value adjusted*	OR adjusted* [95% CI]
	n (%)	*n* (%)				
Dominant model						
GG	76 (47.2)	125 (77.6)	*< 0.001*	1.00	*0.038*	1.00
GT&TT	85 (52.8)	36 (22.4)		*0.26 (0.16–0.42)*		*0.29 (0.08–1.01)*
Recessive model						
GG&GT	141 (87.6)	153 (95.0)	*0.016*	1.00	0.16	1.00
TT	20 (12.4)	08 (5.0)		*0.37 (0.16–0.86)*		0.18 (0.01–2.71)
Over-dominant						
GG&TT	96 (59.6)	133 (82.6)	*< 0.001*	1.00	0.15	1.00
GT	65 (40.4)	28 (17.4)		*0.31 (0.19–0.52)*		0.41 (0.11–1.49)
Log-additive						
–	–	–	*< 0.001*	*0.37 (0.25–0.55)*	*0.029*	*0.35 (0.13–0.98)*

*Adjusted for age, gender, BMI, HOMA, TC, TG, HDL, LDL, and VLDL

*p value < 0.05 considered statistically significant

compared with controls, which indicated no association found between IR of T2DM and *eNOS* polymorphism. In this study, the genotype frequency of GG, GT, and TT was 77.6%, 17.4%, and 5.0% in the cases and 47.2%, 40.4%, and 12.4% in controls respectively. Similar to our results, previous studies documented the low frequency of TT genotype in Indian and Mexican ethnic groups [15, 27]. However, some other reports demonstrated the high frequencies of either TT genotype or T allele: South Indian (20% and 48%), North Indian (5% and 19%), Korean (0% and 4%), Caucasian (9% and 30%), Egyptian (normoalbuminuria TT 2% and T 20%; microalbuminuria TT 8% and T 39%; macroalbuminuria TT 4% and T 19%), Turkish (T2DM with diabetic foot 6% and 25%), Iranian (T2DM with diabetic foot 15% and 60%), and Asian Indian (10% and 36%) populations respectively [16–18, 27, 28].

Several studies have been conducted to identify the link between T2DM phenotype and *eNOS* polymorphism but no significant association was established [15, 20, 29, 30]. In previous studies, it has been determined an association between *eNOS* polymorphism and T2DM found to be significant, claiming a different genetic susceptibility factor for insulin resistance, hyperinsulinemia, and T2DM [18, 19, 31, 32].

According to the published data, this is the first ever report to explore the association of insulin resistance of T2DM and endothelial nitric oxide synthase (*eNOS*) G894T polymorphism in patients among Pakistani population.

Conclusion

It is concluded that no association between insulin resistance in T2DM patients and *eNOS* G894T polymorphism was established in Pakistani population. It is suggested that further study on larger scales to be performed to elaborate the precise association between the studied SNPs and insulin resistance in T2DM patients.

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Compliance with ethical standards

Ethical approval and consent to participate All participants provided written informed consent and all protocols were approved by the Ethics Committee of University of Health Sciences, Lahore. Additionally, the research and recruitment protocols were carried out according to the Ethical Principles for Medical Research Involving Human Subjects adopted in the Declaration of Helsinki by the World Medical Association. All samples used in the study followed standardized rules governing sample handling, and information obtained in the interview was recorded on standardized data collection forms.

Competing interests The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Association of SRB1 and PON1 gene polymorphisms with type 2 diabetes mellitus: a case control study

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Abstract

Objective Single-nucleotide polymorphism (SNP) in Paraoxonase 1 (PON1) and scavenger receptor class b member 1 (SRB1) gene has been associated with impairing high-density lipoprotein (HDL) functionality as an antioxidant and shown to diminish ability of PON1 in cholesterol homeostasis. Several studies found that SRB1 and PON1 polymorphism increases T2DM risk. Our study aimed to investigate the association and susceptibility of polymorphic variants in SRB1 rs9919713 and PON1 rs662 with type 2 diabetes mellitus.

Methods In the present case-control study, 250 type 2 diabetes mellitus patients (T2DM) and 250 healthy volunteer were recruited. The genotypes of PON1 and SRB1 were determined by using polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) technique, and biochemical analysis was done using standard protocol.

Results C and R alleles showed significant association with T2DM susceptibility with an odds ratio of 1.42 ($p < 0.005$) and 1.40 ($p < 0.007$), respectively. The frequency of CC and RR genotype was significantly higher in T2DM patients compared with healthy controls. Furthermore, CC and RR genotypes were significantly associated with higher LDL and low HDL levels. Additionally, no other significant association was observed.

Conclusions We conclude that the PON1 and SRB1 gene polymorphisms may probably surrogate biomarkers for T2DM susceptibility.

Keywords Paraoxonase 1 · Polymorphism · Type 2 diabetes mellitus · Single-nucleotide polymorphism

Introduction

International Diabetes Federation (IDF) estimates that the number of diabetes patients in India had increased doubled from 19 million in 1995 to 40.9 million in 2007 [1] and projected to increase up to 69.9 million by 2025. Diabetes mellitus also known as non-insulin dependent diabetes mellitus is a complex metabolic disorder in which pancreatic beta cells become dysfunctional and cause insulin resistance or decreased production of insulin [2]. Previous genetic studies show that > 25 mutants are associated with type 2 diabetes mellitus, and preponderance is from coding and non-coding region which modulates insulin secretion [3, 4].

PON1 gene clustered on chromosome 7q21.3–22.1 is HDL associated enzyme composed of 354 amino acids with molecular weight of 43 KDa secreted primarily in the liver and non-steroidogenic tissues [5]. PON family is known to inhibit LDL oxidative modification and prevents the buildup of oxidized LDL by elevating cholesterol efflux [6]. It has been suggested that PON1 utilizes VLDL as a passageway to get into HDL. Low activity may confer increased risk of type 2 diabetes by impairing the ability of HDL to inhibit LDL oxidation, and gene variations found to decrease the ability of HDL to compel cholesterol efflux from macrophages and reverse cholesterol transport from peripheral tissues [7, 8]. Previous genetic studies show PON1 association with diabetes [9]. Polymorphism in R192Q shows the protective effect of HDL against LDL oxidation. 192QQ homozygous is most valuable in inhibiting the accumulation of lipid peroxides on LDL [6, 10].

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The SRB1 gene has been located on chromosome 12 covering a region of 75 kb comprising 13 exons. The gene encodes a receptor protein of nearly 80 kDa, whose weight can differ based on its amount of glycosylation [11]. SRB1 mainly functions as a receptor of HDL and loss of hepatic SRB1 leads to compositional changes in HDL including increased sphingomyelin, which markedly reduces the ability of LCAT to bind HDL leading to accumulation of toxic-free cholesterol (FC) in HDL, resulting in reduced cholesterol efflux capacity and reverse cholesterol transport RCT.

The present study was designed to investigate the association and susceptibility of polymorphic variants in SRB1 rs9919713 and PON1 rs662 with type 2 diabetes mellitus. These polymorphisms have been studied previously in different geographical region, but since the impact of polymorphisms on disease risk is known to differ from population to population, the effects of these polymorphisms on T2DM risk remained poorly understood among the Indians. There were a few previous studies on the association of PON1 polymorphisms with T2DM risk in the Indian population, but these studies had small sample sizes and thus were underpowered. The present work was also the first study to investigate the association of SRB1 rs9919713 polymorphism and T2DM susceptibility among Indians.

Material and methods

Subject selection

This population-based case control study included a total of 500 subjects recruited randomly from May 2015 to June 2016 with matched on age and ethnicity from out-patient Department of Medicine, King George's Medical University. Overall 250 T2DM subjects and 250 control subjects were recruited. Survey related to demographic details and family history of diabetes was attained from each subject. Body mass index (BMI) was calculated as the weight in kilogram divided by meter square of height. The entire group of patients recruited was informed regarding aim of the study, and written consent was obtained from each subject. Blood samples were taken from both the groups which were used for genotyping and biochemical analysis. A case for the present study was defined as a diagnosed case of type 2 diabetes mellitus with no medical history of cardiovascular or cerebrovascular diseases, cancer, and chronic renal, liver, heart disease. The inclusion criteria for the control subjects were the following; no history of diabetes mellitus and fasting plasma glucose is less than 110 mg/dL and HbA1c levels ≤ 5.8%.

Diagnostic criteria for type 2 diabetes

T2DM subjects were diagnosed on the basis of fasting blood sugar (FBS ≥ 126) and glycated hemoglobin (HbA1C ≥ 6.5%) level. Diagnosis and classification of diabetes was based on the guidelines of American Diabetes Association (ADA) [12].

Ethical clearance

Ethical consent was approved by institutional ethics committee's KGMU, Lucknow (Ref. Code: 71 ECM II B Thesis/P 13).

Biochemical examination

Biochemical parameters analysis of very low-density lipoprotein (VLDL) was determined by enzymatic method. Low-density lipoprotein (LDL) cholesterol levels were calculated by using the Friedewald formula [13]. Serum total cholesterol (TC), serum triglyceride (TG), and high-density lipoprotein (HDL) levels were assessed by XL-300 Transasia fully auto analyzer. HbA1c was measured using a semi auto analyzer (Transasia).

Blood sample collection

About 5 mL of venous blood was withdrawn under aseptic precautions after fasting for 10 h and distributed as follows: 2 mL of whole blood was put into EDTA vials (BD Vacutainer® spray-coated) mixed up and down gently and then used to measure the HbA1c and for isolation of genomic DNA. About 1 mL of whole blood was put into Na fluoride serum test tubes and centrifuged at 1500 rpm for 10 min. The separated serum was used for the assay of fasting blood sugar. About 2 mL of blood was placed in a plain tube without anticoagulant, and the tubes were left till coagulation. After coagulation, samples were centrifuged at 1500 rpm for 15 min. The separated serum was used for the assay of lipid profile.

DNA extraction

Genomic DNA from whole blood was isolated using a standardized phenol/chloroform extraction method [14].The quantity and quality of DNA were checked by UV spectrophotometry on a NanoDrop spectrophotometer and 0.8% (*w*/*v*) agarose gel electrophoresis, respectively.

Analysis of polymorphism

SRB1

The SRB1 rs9919713 polymorphism was analyzed by PCR followed by *RFLP technique*. Genomic DNA was amplified using the following PCR conditions: 95 °C for 5 min followed by 30 cycles of 95 °C for 40s, 64 °C for 38 s, 72 °C for 45 s, and final extension at 72 °C for 7 min with specific SRB1 gene forward primer 5'-CCTTGTTTTTCCTCGACGC-3′

and reverse primer 5-'CACCACCCCAGCCACCAGC-3′. Amplification was performed with 25 μL PCR reaction mixture containing 1.2 μL template DNA, 10 pmol of each primer, and 2X PCR master mixes (Thermo Scientific). The amplified 218 PCR product was digested with 1 U of restriction enzyme AgsI after incubation at 63 °C for 3 h and separated on 2% agarose gel in 1X TBE (Tris/Borate/EDTA) buffer by ethidium bromide staining and visualized under UV light by BIORAD gel doc system (Bio-Rad Laboratories, Inc.). As marker, 1 Kb DNA ladder was used. Thus, results demonstrate that in the case of SRB1 rs9919713 polymorphism, TT genotype is wild homozygote for the absence of the site (218 bp), CT genotype is heterozygote for the presence and absence of the site (218 and 187, 31 bp), and CC genotype is variant homozygote for the presence of the site (187 and 31 bp) Fig. 1.

PON1

The PON1 rs662 polymorphism was analyzed by following PCR conditions: 93 °C for 5 min followed by 30 cycles of 93 °*C* for 40s, 56 °*C* for 38 s, 71 °*C* for 45 s, and final extension at 73 °*C* for 7 min with specific PON1 forward primer 5'-AAACCCAAATACATCTCCCAGAAT-3′ and reverse primer 5'-GCTCCATCCCACATCTTGATTTTA-3′. Amplification was performed with 25 μL PCR reaction mixture containing 1.2 μL template DNA, 10 pmol of each primer, and 2X PCR master mixes (Thermo Scientific). The amplified PCR product was digested with 1 U of restriction enzyme Hinf1 after incubation at 39 °*C* for overnight and separated on 2% agarose gel in 1X TBE buffer by ethidium bromide staining and visualized under UV light by Bio-Rad gel doc system (Bio-Rad Laboratories, Inc.). The results demonstrate that in the case of PON1 rs662 polymorphism, QQ genotype is wild homozygote for the absence of the site (214 bp), QR genotype is heterozygote for the presence and absence of the site (214 and 190, 24 bp), and RR genotype is variant homozygote for the presence of the site (190 and 24 bp) Fig. 2.

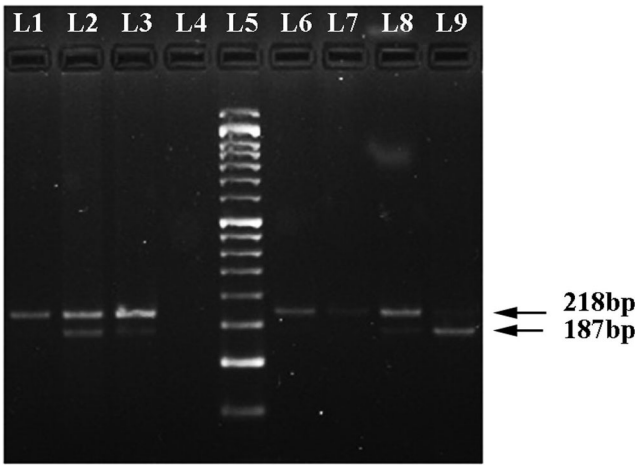

Fig. 1 Agarose gel electrophoresis for the rs9919713 polymorphism of scavenger receptor class B type 1 (SRB1) gene. The 218 bp bands correspond to wild homozygous TT genotype produced one fragment, while 187 bp and 218 bp corresponds to heterozygous CT that produced 3 fragments. The variant homozygous CC genotype produced 2 fragments of 187 bp and 31 bp. The 31 bp was invisible in the gel due to its fast migration speed. About 50 bp ladder marker (L1), TT genotype (L1, L6, L7), CT genotype (L2, L3, L8), and CC genotype (L9)

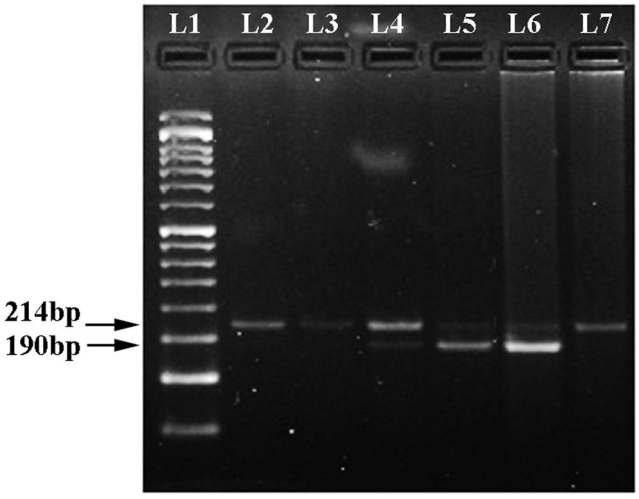

Fig. 2 Agarose gel electrophoresis for the rs662 polymorphism of paraoxonase 1 (PON1) gene. The 214 bp bands correspond to wild homozygous QQ genotype produced one fragment, while 190 bp, 214 bp, and 24 bp corresponds to heterozygous QR that produced 3 fragments. The 190 bp and 24 bp corresponds to variant homozygous RR genotype produced 2 fragments. The 24 bp was invisible in the gel due to its fast migration speed. About 50 bp ladder marker (L1), QQ genotype (L2, L3, L7), CT genotype (L4, L5), and RR genotype (L6)

Statistical analysis

Demographic and clinical data are reported as mean ± standard deviation (SD). Statistical comparisons between group means were analyzed using Mann-Whitney U test. Associations of genotypes with lipid parameters were analyzed by one-way ANOVA test. Association of PON1 and SRB1 genotypes with T2DM was done by odds ratio calculation. Allele and genotype frequencies were calculated by gene counting method. Observed genotype frequencies were compared with the expected frequency to confirm for Hardy-Weinberg equilibrium by Chi-square (χ^2) tests. $p \leq 0.05$ was considered significant. Statistical analysis was carried out using Statistical Program for Social Sciences (SPSS) version-19 (IBM SPSS Statistics, USA).

Results

Clinical and biochemical parameters as shown in Table 1 are compared to healthy control. No significant difference was observed in age, BMI between cases and controls. T2DM had significantly higher TC, TG, LDL, and VLDL, FBS,

Table 1 Clinical and biochemical parameters of controls and T2DM

Characteristics	Controls ($N = 250$)	T2DM ($N = 250$)	p value
Age (years)	47.5 ± 7.3	48.6 ± 9.7	0.11
BMI (kg/m^2)	24.8 ± 4.90	25.4 ± 5.10	0.14
HDL(mg/dl)	42.3 ± 8.5	36.5 ± 7.9	0.001*
LDL (mg/dl)	97.8 ± 30.7	139.2 ± 33.8	0.001*
VLDL(mg/dl)	30.8 ± 17.1	42.6 ± 10.6	0.001*
TG(mg/dl)	144.8 ± 79.4	206.1 ± 48.3	0.001*
FBS(mg/dl)	91.1 ± 11.7	149.1 ± 41.7	0.001*
HbA1c	5.6 ± 0.6	7.94 ± 0.99	0.001*

Data presented as mean ± SD; *Significant p value < 0.05

BMI body mass index, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very low-density lipoprotein, *TC* total cholesterol, *TG* triglycerides, *T2DM*, type 2 diabetes mellitus

Comparison between groups was performed with Mann-Whitney U test

and HbA1c. Furthermore, HDL levels were significantly reduced in T2DM patients when compared with controls.

Genotype and allele frequency of SRB1 and PON1

Both PON1 rs662 and SRB1 rs9919713 were consistent with Hardy-Weinberg equilibrium in all studied groups. In PON1 polymorphism, we observed R allele frequency was significantly associated with T2DM as compared to control with OR 1.40 ($\chi^2 = 7.09$, 95%CI = 1.09–1.80, $p = 0.007$). The frequency of RR genotype also showed significant increase in T2DM compared to controls with OR's 1.63 ($\chi^2 = 5.57$, 95% CI = 1.057–2.526, $p = 0.01$). R allele frequency was significantly increased in T2DM when compared to controls (46.4% vs. 38.6%, respectively), and it is associated with T2DM (Table 2). In SRB1 rs9919713 polymorphism, the frequency of CC ($\chi^2 = 7.47$, OR 2.0, 95%CI = 1.21–3.26, $p = 0.006$) genotypes was significantly increased in T2DM as compared to controls. The frequency of C allele was significantly associated with T2DM as compared to control with OR 1.42 ($\chi^2 = 7.74$, 95%CI = 1.11–1.82, $p = 0.005$) (Table 3).

Table 2 Comparison of genotypes and allele frequencies of PON1gene between healthy control and T2DM subjects

Genotype	Control $N = 250$	T2DM $N = 250$	OR (95% CI)	p value
QQ	118(47.2%)	93(37.2%)		
QR	75(30%)	81(32.4%)	1.37(0.904–2.076)	0.13 $\chi^2 = 2.21$
RR	57(22.8%)	76(30.4%)	1.63(1.057–2.526)	0.01* $\chi^2 = 5.57$
Allele				
Q	311(62.2%)	267(53.4%)		
R	193(38.6%)	233(46.4%)	1.40(1.09–1.80)	0.007* $\chi^2 = 7.09$

T2DM type 2 diabetes mellitus, *PON1* paraoxonase1

OR odds ratio, *CI* confidence interval; Comparison between group was performed with Chi-square test

*Significant p value < 0.05

Association of PON1 rs662 and SRB1 rs9919713 SNPs with lipid parameters

In PON1 polymorphism, QQ genotypes had higher HDL than the RR genotype. The levels of LDL were significantly increased in RR genotype as compared to QQ genotype (Table 4). Similarly, in SRB1 polymorphism, CC genotype had higher LDL level than TT genotypes. Further, HDL was significantly decreased in CC genotype (Table 5).

Discussion

The candidate gene method focuses on the association between genetic variations used in case control studies to find out the difference between allele and genotype frequency. SRB1 participates in the selective uptake of cholesterol ester [15] and binds a number of ligands with high affinity, including native HDL [16]. The selective uptake involves the transfer of cholesterol from the HDL particle and the release of the lipid-poor HDL particle into the plasma. In our previous findings, we observed significantly low level of HDL and PON1 protein level as the duration of diabetes increases. Numerous studies reported that PON1 and SRB1 play a significant role in worsening HDL's function and composition developing increased risk of T2DM.

Table 3 Comparison of genotypes and allele frequencies of SRB1 gene between healthy control and T2DM subjects

Genotype	Control $N = 250$	T2DM $N = 250$	OR (95% CI)	p value
TT	73(29.4%)	53(21%)	–	
CT	123(49%)	119(48%)	1.33(0.86–2.05)	0.19 $\chi^2 = 1.6$
CC	54(21.6%)	78(31%)	2 (1.21–3.26)	0.006* $\chi^2 = 7.47$
Allele				
T	269(53.8%)	225(45%)		
C	231(46.2%)	275(55%)	1.42 (1.11–1.82)	0.005* $\chi^2 = 7.74$

T2DM type 2 diabetes mellitus, *SRB1* scavenger receptor class B member I

OR odds ratio, *CI* confidence interval; Comparison between group was performed with Chi-square test

*Significant p value < 0.05

Table 4 Association of PON1 rs662 polymorphism with lipid parameters

Lipid parameter	QQ	QR	RR
Control	$N = 118$	$N = 75$	$N = 57$
LDL	79 ± 17.7	81.3 ± 17*	85.3 ± 18*
VLDL	34.5 ± 13.1	35.3 ± 13.2	33.2 ± 17.5
TC	137.4 ± 34.4	131.6 ± 33.4	135.3 ± 18.2
TG	179.1 ± 39.1	181.4 ± 33.5	184.1 ± 32
HDL	32.2 ± 11.7	34.2 ± 5.3	30.1 ± 7.5*
Cases	$N = 93$	$N = 81$	$N = 76$
LDL	81.3 ± 17	81.3 ± 17*	85.3 ± 18*
VLDL	43.4 ± 10.6	41.3 ± 10	43.7 ± 11.3
TC	178 ± 77.2	166.1 ± 67	176.5 ± 79.4
TG	215 ± 32.5	216.4 ± 31.5	220.4 ± 25.8
HDL	43.2 ± 9.7	41.1 ± 10.2	40.2 ± 10.8*

Data presented as mean ± SD; Genotypes were compared by one- way ANOVA

HDL high density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very low-density lipoprotein, *TC* total cholesterol, *TG* triglycerides, *PON*1 paraoxonase 1

*Significant P value < 0.05

Present study examined the association of R allele with type 2 diabetes mellitus. The association of PON1 variant and the risk of type 2 diabetes mellitus have been confirmed in several previous studies [17, 18]. On the other side, some contradictory results did not find association with PON1 polymorphism and T2DM [19, 20]. Several ethnic studies informed that significant association was observed with T2DM in Saudi and Egyptian individuals [21, 22]. However, in south Iranian population, no significant correlation was observed [23]. These outcomes might be the result of different racial, biological region, lifestyle, and socioeconomic factors. We also believe that the inconsistency between our study and these erstwhile studies was due to the sample size employed.

Table 5 Association of SRB1 rs9919713 polymorphism with lipid parameters

Lipid parameters Control	TT $N = 73$	CT $N = 123$	CC $N = 54$
LDL	75.4 ± 20	77.3 ± 19.1	82.5 ± 17.1*
VLDL	41 ± 9.4	39.5 ± 9.1	43.1 ± 10.6
TC	172.1 ± 39.7	177 ± 29.6	176.8 ± 33.8
TG	144.2 ± 42	142.6 ± 75	147.5 ± 26.1
HDL	42.8 ± 11.5	39.5 ± 9.7*	39.7 ± 10.2*
Cases	$N = 53$	$N = 119$	$N = 78$
LDL	62.1 ± 16.2	64.2 ± 16.6	71.5 ± 15.5*
VLDL	41.4 ± 10	43.5 ± 10.2	44 ± 12.5
TC	222 ± 27.9	228.3 ± 21.1	216 ± 26.1
TG	179.7 ± 73.9	182.5 ± 79.2	176.3 ± 82.3
HDL	35.1 ± 7.5	33.1 ± 12.5	30.3 ± 7.2*

Data presented as mean ± SD; Genotypes were compared by one- way ANOVA

HDL high-density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very low-density lipoprotein, *TC* total cholesterol, *TG* triglycerides, *SRB1* scavenger receptor class B member I

*Significant P value < 0.05

Additionally, Mackness et al. [20] reported that serum PON1 level was significantly lower in type 2 diabetes mellitus patients as compared to healthy individual, which is in steadiness with our earlier findings published by us [24]. The low levels of PON1 in T2DM might affect HDL functionality and decrease its ability to prevent LDL oxidation [19]. This implies that PON1 may reduce HDL activity in these individuals and also increase their susceptibility to T2DM. Recent studies demonstrated PON1 gene polymorphism and lipid parameters have also yielded conflicting results. Our findings also revealed that subjects carrying RR genotype had significantly higher levels of LDL. The results were in agreement with the outcomes of studies performed earlier [14]. But that is in contrast with the results of study that did not find association [19].

Moreover, In SRB1 gene polymorphism, we observed significant correlation of C allele with the risk of T2DM. Previously, findings proved that CC genotype had significant association with T2DM. We also observed lower frequencies of C allele in controls as compared to T2DM [23], which is in consistent with earlier findings that showed SRB1 rs9919713 gene polymorphism is associated with insulin resistance and T2DM [25]. Polymorphism may be implicated in the pathogenesis of insulin resistance by diminishing the concentration of PON1 and thus modulating the expression of GLUT-4. Earlier, human genetic studies confirmed that SRB1 carrying loss of function variant exhibit impaired cholesterol efflux to HDL. SRB1 polymorphism does not lead to change in amino acid sequence and cannot be linked at structure level. It is possible that some other variation in its region might effect, which is in linkage disequilibrium with SRB1 polymorphism and accountable for the observed association with T2DM.

Our results showed that increased HDL levels were associated with TT genotype in both the groups. We also found subjects carrying CC genotype had significantly increased LDL level. Our study supports previous findings that reported low HDL and high LDL levels in CC genotype [26, 27]. In a similar study of Tunisian population [28], T allele was found associated with higher HDL levels. However, some contradictory findings were reported in Chinese population [29]. Notwithstanding the clear functional evidence for an influence of PON1, SRB1 on HDL levels, the genetic epidemiological data is somewhat weak. Previous literature search identified polymorphic sites studied with HDL levels with varying results [30]. Large genetic epidemiological studies on this gene are required before a final conclusion can be drawn.

Conclusions

In conclusion, data suggested that the genetic variation in PON1 and SRB1 gene was independent influencing factors of diabetes mellitus and might be one of the candidate genes for conferring susceptibility to diabetes. The present study has certain limitations because it was conducted only on north Indian population with small sample size. Furthermore, we suggest study will be performed in larger sample size, in different ethnic groups in order to understand possible association of these loci alone or in linkage disequilibrium (LD) in development of diabetes which could possibly generate a more reliable result. However, efficient lifestyle modifications including implementation of a healthy dietary pattern like the Mediterranean diet, with physical activity, are vital in the prevention of type 2 diabetes. So, importance must be given to supporting a better lifestyle and finding solutions in order to increase devotion and compliance to the lifestyle modifications, especially for high-risk individuals.

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Compliance with ethical standards

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

Informed consent The study was approved by King George's Medical University Ethics Committee and informed written consent was taken from all the subjects.

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ORIGINAL ARTICLE

Association of adiponectin gene polymorphisms and their haplotypes with type 2 diabetes and related metabolic traits in an Iranian population

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Abstract

Introduction Adiponectin is an adipocyte-secreted protein that contributes to glucose homeostasis. Contradictory reports are available on single nucleotide polymorphisms (SNPs) in the adiponectin gene and the risk of type 2 diabetes (T2D). We investigate the association of adiponectin gene SNPs (+45T/G and +276G/T) with serum adiponectin, insulin resistance, lipid profile, and T2D risk in an Iranian population.

Method The +45T/G and +276G/T SNPs were genotyped in 211 non-familial T2D patients and 202 non-diabetic subjects by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and TaqMan probe, respectively.

Results T2D was associated with a decrease in serum adiponectin level. The G allele and the GG and TG genotypes of +45T/G SNP were more abundant than the T allele and the TT genotype in T2D patients compared with controls ($p < 0.001$). The risk of T2D in individuals with the GG and TG genotypes of +45T/G SNP was 4 and 2 times more than that with the TT genotype, respectively. There was no statistically significant difference in the frequencies of allele and genotype of +276G/T SNP between the control and T2D groups. The presence of +45G/+276G haplotype was associated with an increased risk of T2D (OR = 2.01, 95% CI = 1.34–3.03, $p = 0.04$).

Conclusion Therefore, our results showed that +45T/G SNP is associated with the risk of T2D higher than +276G/T SNP in the studied population.

Keywords Adiponectin · Type 2 diabetes mellitus · Gene polymorphism · Iran

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Introduction

The prevalence of type 2 diabetes (T2D), complications, and related deaths are rapidly rising around the world, accounting for the prevalence of 439 million people in 2030 [1]. In Iran, as one of the developing countries, T2D is one of the serious public health concerns, with a prevalence estimated at 11.4% (7.2–17.2) [2].

T2D is a polygenic disorder that develops through complex interactions between several genes and environmental and behavioral factors such as excessive stress, sedentary life, and inappropriate diets [3, 4]. Many studies [5, 6] have suggested that genetic factors are the main cause of differences in the risk of T2D. There are more than 50 loci associated with T2D [7].

The adiponectin gene with three exons located on chromosome 3q27 is one of the genes whose association with T2D has been considered in recent years [8]. The adiponectin is an adipocyte-secreted protein that contributes to regulating insulin sensitivity and glucose homeostasis [9]. Therefore, a decrease in the plasma adiponectin level is associated with all parameters of the metabolic syndrome, including T2D [8].

The association of single nucleotide polymorphisms (SNPs) in the adiponectin gene with low levels of plasma adiponectin, cardiovascular disease, obesity, insulin resistance, and an increased risk of T2D has been reported in various populations [10–14]. The substitution of T with G in exon 2 (+45T/G, rs2241766) and the substitution of G with T in intron 2 (+276G/T, rs1501299) are two of the most commonly studied polymorphisms in the adiponectin gene. However, the study of the association of these two polymorphisms with T2D in different populations has led to contradictory results [15, 16].

In Iran, as one of the Middle East countries, despite the geographic extent and existence of different ethnicities, only two studies in southern Iran have been carried out to examine the association of +45T/G SNP with T2D [17, 18]. The purpose of this study was to investigate the association of adiponectin gene SNPs (+45T/G and +276G/T) with serum adiponectin, insulin resistance, lipid profile, and T2D risk in the population of Semnan, a city located in north-central Iran.

Subjects and methods

Study population

Totally, 413 non-familial Iranian subjects (211 diabetic and 202 non-diabetic participants), age ≥ 40 years [19], were enrolled in the study, after clinical examination, related tests were performed in the Diabetes Research Center of Semnan University of Medical Sciences. Diagnosis of diabetes was based on the World Health Organization (WHO) criteria [9]. Diagnostic criteria for diabetes included fasting blood sugar of ≥ 126 mg/dl or blood glucose 2 h after eating 75 g glucose of ≥ 200 mg/dl. Inclusion criteria for the control group were fasting blood sugar of < 100 mg/dl or oral glucose tolerance test < 140 mg/dl and no history of T2D in the first- and second-degree relatives. Exclusion criteria were type 1 and other types of diabetes, history of liver, kidney, and heart disease and any type of illness, pregnancy, and history of insulin therapy, or treatment for hyperlipidemia and hypertension. All subjects enrolled in the study were Iranians with ancestry in the Semnan Province, Iran.

Body mass index (BMI) was calculated by dividing the body weight (kg) by the square of height (m^2) [9]. This study was approved by the Ethics Committee of Semnan University of Medical Sciences, and informed written consent was obtained from each participant in the project.

Biochemical analysis

Venous blood samples (10 ml) were taken after an overnight fasting. Glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by standard enzymatic methods using a Selectra auto-analyzer. Serum levels of insulin and adiponectin were determined by enzyme-linked immunosorbent assays (ELISAs) using kits (Monobind and BioVendor companies). Hemostasis model assessment-insulin resistance (HOMA-IR) was calculated by the equation of fasting plasma insulin (mU/l) × fasting glucose (mmol/l)/22.5 [20].

DNA extraction

The blood samples were collected in EDTA-containing tubes, and total DNA was extracted from the whole blood using a DNA extraction kit (GeNet Bio Co., South Korea). The genomic DNA was stored at − 20 °C for later testing.

The +45T/G genotyping

The +45T/G SNP was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The DNA fragment containing this polymorphism in the second exon of the adiponectin gene was amplified using the following primers [21]: F: 5′GAAGTAGACTCTGCTGAGATGG 3′ and R: 5′ TATCAGTGTAGGAGGTCTGTGATG 3′. All reactions occurred in a final volume of 20 μl containing 0.6 μl of each of the primers (10 mM), 1 μl of genomic DNA, 2 μl of 10x reaction buffer, 0.6 μl of $MgCl_2$ (50 mM), 0.6 μl of dNTP (10 mM), 0.2 μl of Taq DNA polymerase (5 U/ml), and 14.4 μl of H_2O. The PCR conditions were one cycle at 95 °C for 5 min, 45 cycles at 95 °C for 1 min, 45 cycles at 61 °C, and finally 1 min at 72 °C. Then, 3 μl of the PCR product was digested in the presence of 0.5 μl of SmaI restriction enzyme (10 μl/μl), 1.5 μl of 10X buffer, and 10 μl of water in a final volume of 15 μl at 30 °C for 16 h. The recognition site of the enzyme was CCC:GGG. The enzymatic digestion product was electrophoresed on 1.7% ethidium bromide–stained agarose gel (Fig. 1).

The +276G/T genotyping

The+276G/TSNP,theintron2intheadiponectingene,wasgenotyped using TaqMan 5′ nuclease method with MGB probe characteristics. The probe sequences labeled with two FAM and VIC fluorescence markers and corresponding primer pairs were designed by Applied

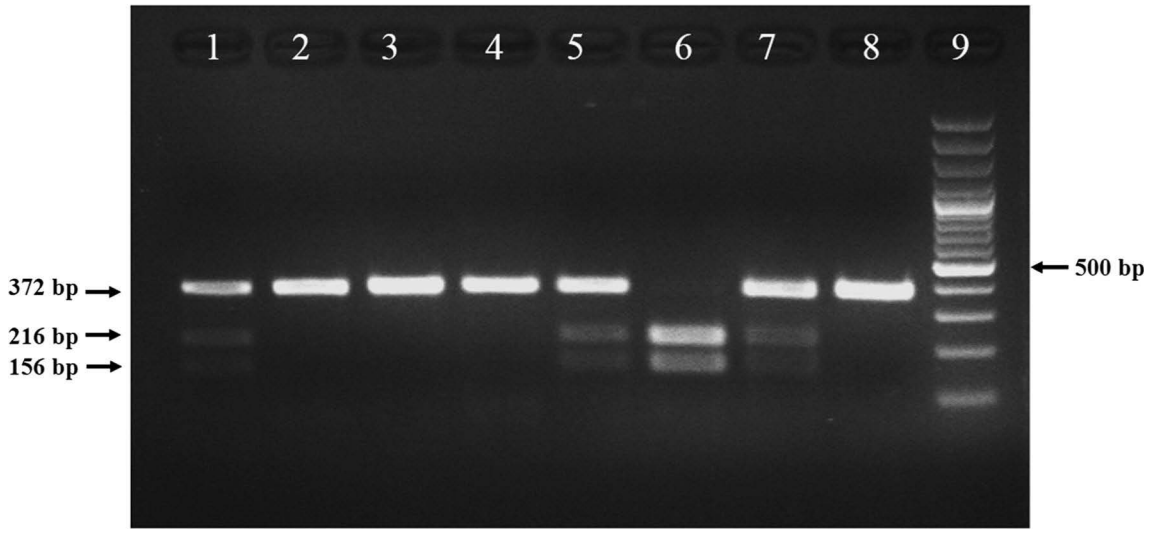

Fig. 1 Representative example of PCR-RFLP product for SNP +45T/G. lanes 1, 5 and 7, heterozygote TG genotype (372 bp, 216 bp and 156 bp); Lanes 2,3,4 and 8,homozygote TT genotype (372bp); Lane 6, homozygote GG genotype(216 bp and 156bp); Lane 9, 100 bp DNA ladder.(Agarose 1.7%, TBE buffer and staining with EB)

Biosystems (ABI) Co. The reactions, containing genomic DNA, probe, primer, deionized water, and TaqMan Universal PCR Master Mix buffer, were performed in a 96-well plate in accordance with the protocol of Applied Biosystems (ABI) Co. in a final volume of 10 μl. The PCR conditions were performed as follows: the first step consisting of primary denaturation at 95°C for 10 min and the second step comprising 40 cycles as follows: secondary denaturation at 92°C for 15 s, followed by annealing and extension for 1 min at 60°C. After completing the PCR, the fluorescence intensity of the two VIC and FAM dye was read using the ABI 7900HT-PCR device under SDS version 1.4 software.

Statistical analysis

Data were analyzed by SPSS 22 software. The mean biochemical parameters were compared between different genotypes using one-way ANOVA and Student's *t* test. The Hardy-Weinberg equilibrium (HWE) of both polymorphisms was analyzed by the chi-squared test. The frequency distribution of alleles and genotypes was compared between the diabetic and control groups using Pearson's chi-squared test. The distribution of haplotypes in the control and diabetic groups was estimated by the two-stage iterative method, called the expectation maximization algorithm, using the SNPStats software (http://www.bioinfo.iconcologia.net/SNPstats). The risk of diabetes in different genotypes of polymorphisms and haplotypes was determined using the logistic regression test; the most prevalent haplotype was considered a reference group. The continuous variables were reported as mean ± standard deviation. The significance level was considered being $p < 0.05$ in all tests.

Results

Clinical characteristics of studied subjects

The clinical and demographic information of all participants is shown in Table 1. The mean age, BMI, FBS, TG, TC/HDL-C, LDL-C/HDL-C, fasting insulin, and insulin resistance of the diabetic group exhibited a significant increase compared with those of the non-diabetic group ($p < 0.05$ or $p < 0.001$). The mean levels of HDL-C and adiponectin in the diabetic group were significantly lower than those in the non-diabetic group ($p < 0.001$).

Distribution of genotype and association of +45T/G SNP in the adiponectin gene with T2D

A 372-bp fragment in exon 2 with a +45T/G SNP site was amplified using specific primers. The fragments obtained from the enzymatic digestion (Fig. 1) were three 372-, 216-, and 156-bp fragments (a heterozygote TG), two 156- and 216-bp fragments (a homozygote GG), and a fragment 372 bp (a homozygote TT). The distribution of the genotypes observed for polymorphisms was fit with HWE (+45G/T, $x^2 = 2.52$, df = 2, $p > 0.05$).

The allele frequency and the genotype distribution of 45T/G SNP are reported in Table 2. Significant differences were observed in the distribution of +45T/G SNP genotypes and rare G allele between the two groups ($p < 0.001$, df = 2, $x^2 = 17.99$). The analysis of the risk factor of G allele in +45G/T SNP showed a significant association with the risk of diabetes ($p < 0.01$) so that the risk of diabetes was higher in individuals with GG genotype (OR = 2.061 (1.334–3.182), $p < 0.001$) and TG genotype (OR = 4.624 (1.667–12.825), $p < 0.001$) than

Table 1 General characteristics of study population

Variable	Control ($n = 202$)	Diabetic($n = 211$)
Age (year)	49.7 ± 8.7	51.2 ± 8.0*
Men/women	120/82	124/87
BMI(kg/m^2)	27.1 ± 2.9	27.8 ± 3.0*
SBP (mmHg)	129.6 ± 11.7	131.2 ± 11.0
DBP (mmHg)	76.3 ± 4.9	76.9 ± 4.8
FBS (mg/dl)	93 ± 11	163 ± 66**
TC (mg/dl)	189 ± 44	192 ± 36
TG (mg/dl)	176 ± 86	199 ± 95*
HDL-C (mg/dl)	49.3 ± 9.3	43.3 ± 8.0**
LDL-C (mg/dl)	108 ± 38	110 ± 33
TC/HDL	3.9 ± 1.1	4.4 ± 1.1**
LDL/HDL	2.3 ± 0.9	2.6 ± 0.9**
Fasting insulin (μIU/ml)	8.3 ± 6.81	11.2 ± 6.76**
HOMA-IR	1.9 ± 1.5	4.5 ± 3.4**
Adiponectin (μg/ml)	12.4 ± 3.6	8.3 ± 2.1**

Data present the mean ± SD

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol, *HOMA-IR* hemostasis model assessment-insulin resistance

*$p < 0.05$ and ** $p < 0.001$ compared with the control

TT genotype. No significant difference was observed in the clinical and demographic characteristics of different +45T/G SNP genotypes of the adiponectin gene in the control group (data not shown).

Distribution of genotype and association of +276G/T SNP in the adiponectin gene with T2D

The allele frequency and genotype distribution of +276G/T SNP (Table 2) were not significantly different between two groups ($p > 0.05$, df = 2, $x^2 = 1.34$). The distribution of genotypes observed for polymorphism was consistent with HWE (+276G/T, $x^2 = 5.84$, df = 2, $p > 0.05$). There was no significant association between the presence of T allele in +276G/T SNP and the risk of diabetes. No significant difference was observed in the clinical and demographic characteristics of the different +276G/T SNP genotypes in the adiponectin gene in the control group (data not shown).

Association of +45T/G and +276G/T SNPs of adiponectin gene with clinical and demographic characteristics of T2D patients

In T2D patients, individuals with GG genotype showed higher BMI, insulin level, and insulin resistance and lower adiponectin than other +45T/G SNP genotypes (Table 3). The individuals with TT genotype at the +276G/T SNP site had lower levels of total cholesterol, LDL-C, and LDL-C/HDL-C than other genotypes at this site ($p < 0.05$). The clinical and demographic characteristics of different genotypes of +45T/G and +276G/T SNPs in the adiponectin gene were not significantly different in the control group (data not shown).

Analyzing the haplotype of two adiponectin gene SNPs in association with T2D

The frequency of haplotypes of adiponectin gene SNPs was compared between the two diabetic and control groups(Table 4). Based on the four probable adiponectin gene haplotypes, the haplotype including +45SNP mutant alleles and +276SNP wild alleles in the diabetic group was more common than in the control group (OR = 2.01, 95% CI = 1.34–3.03, $p = 0.04$). The haplotypes containing mutant alleles of both SNPs (+45G/+276T) were uncommon and had the least frequency in both groups. Our study results showed that there is a significant association between one of the common haplotypes (+45G/+276G) and higher risk of T2D.

Discussion

In this study, for the first time, the association of the +45T/G and +276G/T polymorphisms of adiponectin gene with T2D and the frequency of their haplotypes in north-central Iran were investigated.

Results showed a significant difference between +45T/G SNP genotypes and rare G allele distribution between the two diabetic and control groups. T2D risk was two and four times higher in individuals with TG and GG genotypes after

Table 2 Comparison of genotype and allelic distribution of SNPs between diabetic (T2D) and control subjects

Polymorphisms	Genotypes (n (%))			p	Alleles (%)		p
+45T/G	T/T	T/G	G/G		T	G	
T2D	116 (55)	77 (36.5)	18 (8.5)	0.001	79	21	0.001
Control	149 (73.8)	48 (23.7)	5 (2.5)		85.64	14.36	
+276G/T	G/G	G/T	T/T		G	T	0.402
T2D	137 (64.9)	59 (28)	15 (7.1)	0.51	78.9	21.1	
Control	121 (60)	67 (33.1)	14 (6.9)		76.5	23.5	

Table 3 Characteristic of the type 2 diabetic group according to different genotypes of adiponectin polymorphisms

Variable	+45T/G genotype			*p* value	+276G/T genotype			*p* value
	T/T (*n* = 116)	T/G (*n* = 77)	G/G (*n* = 18)		G/G (*n* = 137)	G/T (*n* = 59)	T/T (*n* = 15)	
Age (year)	51 ± 8.3	51.6 ± 8.2	51.7 ± 5.3	NS	51.2 ± 8.1	51.1 ± 8.0	52.2 ± 8.6	NS
Men/women	67/49	45/32	12/6	NS	78/59	37/22	9/6	NS
BMI (kg/m^2)	27.2 ± 2.9	28.2 ± 2.2	29.3 ± 3.5	0.006	27.8 ± 3.2	27.6 ± 2.6	27.9 ± 2.5	NS
SBP (mmHg)	132 ± 11	130 ± 11	130 ± 10	NS	131 ± 11.5	129 ± 9.9	128 ± 9.2	NS
DBP (mmHg)	77 ± 5	76 ± 5	76 ± 4	NS	76.9 ± 4.8	76.2 ± 4.9	79.3 ± 4.4	NS
FBS (mg/dl)	166 ± 69	163 ± 63	151 ± 57	NS	159 ± 60	168 ± 70	182 ± 89	NS
TC (mg/dl)	192 ± 36	192 ± 37	197 ± 34	NS	196 ± 37	189 ± 34	169 ± 29	0.017
TG (mg/dl)	201 ± 94	197 ± 102	197 ± 80	NS	196 ± 93	212 ± 104	175 ± 79	NS
HDL-C (mg/dl)	43 ± 8	44 ± 9	43 ± 6	NS	44 ± 8	42 ± 7	47 ± 8	NS
LDL-C (mg/dl)	109 ± 33	111 ± 34	115 ± 30	NS	114 ± 33	106 ± 33	87 ± 28	0.005
TC/HDL	4.6 ± 1.2	4.5 ± 1.1	4.7 ± 1	NS	4.7 ± 1.1	4.6 ± 1.1	3.7 ± 0.8	NS
LDL-C/HDL-C	2.6 ± 0.9	2.6 ± 1	2.8 ± 0.9	NS	2.7 ± 0.9	2.6 ± 1	1.9 ± 0.7	0.004
Fasting insulin (μIU/ml)	9.5 ± 4.4	13 ± 8.1	13.7 ± 9.7	0.001	11 ± 6.6	11.5 ± 7.0	11 ± 7.7	NS
HOMA-IR	3.8 ± 2.4	5.4 ± 4.1	5.6 ± 4.5	0.001	4.3 ± 3	4.9 ± 3.4	5.3 ± 5.6	NS
Adiponectin (μg/ml)	8.7 ± 2.3	8 ± 1.6	6.4 ± 1.3	0.003	8.4 ± 2.1	8 ± 2.1	8.2 ± 1.7	NS

Data present the mean ± SD

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol, *HOMA-IR* -hemostasis model assessment-insulin resistance, *NS* not significant

adjustment for age and sex than those with TT genotype. Such a finding could help to better understand the mechanism and cause of the increased prevalence of T2D in the Iranian population. Our results are in line with the results of Mohammadzadeh and Zarghami [18] in a population in southwestern Iran. Such an association has not been observed in the study of Tabatabaei-Malazy et al. [17] in southeastern Iran; this contradiction can be due to the geographical extent, environmental factors, ethnic differences in Iran, and nonfunctionality of +45T/G SNP but is likely to be in linkage disequilibrium with other functional variants [22]. Our findings are consistent with the results of studying this polymorphism in other Asian countries, such as Iraq [23] and India [24]. The results of one meta-analysis study [12] also show the association of +45T/G SNP with increased risk of T2D in Asians though this association has not been reported in a meta-analysis of Han et al. in 2011 [25].

Table 4 The estimation of the association of haplotype frequencies and haplotypes with risk of type 2 diabetes (T2D)

Haplotype	Control	T2D	OR (95% CI)	*p* value[a]
Common haplotypes				
+45T/+276G	0.635	0.553	1.00	
+45T/+276T	0.222	0.178	0.94 (0.66–1.35)	0.74
+45G/+276G	0.130	0.235	2.01 (1.34–3.03)	0.04
Uncommon haplotype				
+45G/+ 276 T	0.0130	0.034	2.69 (0.65–11.10)	0.17

OR odds ratio, *CI* confidence interval

[a] Haplotype frequency determined by the maximum likelihood method

In accordance with some studies [8], the serum adiponectin level in diabetic subjects was significantly lower than that in the non-diabetic subjects in the present study. In addition, there was a significant reverse association between BMI and serum adiponectin levels. Our results, consistent with other studies [13, 15], showed that G allele in +45T/G SNP was associated with a significant decrease in serum adiponectin levels so that the serum level of adiponectin in diabetic patients with TT, TG, and GG genotypes showed a declining trend.

In the present study, the presence of G allele in the +45T/G SNP was associated with an increase in BMI and insulin resistance; these results are consistent with some studies [23]. On the other hand, some populations showed no association of +45T/G SNP with BMI, insulin resistance, and diabetes risk [26].

The association of the G allele of +45 SNP with decreased serum adiponectin levels, increased BMI, and T2D could be explained by several factors, which need to be explained. In any case, people with T2D have lower serum adiponectin levels than people without diabetes [8]. Also, increased BMI and obesity are associated with an increased risk of T2D [4, 10]. Thus, the role of the GG genotype in increasing the risk of T2D in some populations is consistent with its role in reducing

serum adiponectin and increasing BMI. This may be due to the effect of adiponectin on glucose/lipid metabolism [23]. Therefore, decreased adiponectin may contribute to the development of T2D.

The mechanism of the effect of different variants of the adiponectin gene on the serum adiponectin level and the incidence of T2D in different populations are still unknown. Although +45 SNP does not alter the amino acid in the adiponectin structure, silent +45 mutation alters the RNA splicing, thereby changing the adiponectin gene expression. There are also reports showing linkage disequilibrium between SNP+45 and other SNPs in the adiponectin gene or other adjacent genes, which may affect adiponectin structure such as − 11,377 and – 11391 in the 5′-promoter region [27].

In our study, there was no significant difference in serum levels of HDL-C, LDL-C, TG, and TC between different genotypes. We found a positive association between plasma adiponectin and HDL-C levels. The study of Mohammadzadeh et al. in an Iranian population showed that the serum adiponectin level in diabetic patients with cardiovascular disease was lower than that in diabetic patients without CAD. However, they did not investigate the association of serum HDL-C level with different +45T/G SNP genotypes [28].

In the present study, there was no significant difference in the allele frequency and genotype distribution of +276G/T SNP between the diabetic and control groups, though in the diabetic group, rare T allele was associated with a significant decrease in TC, LDL-C, and LDL-C/HDL-C ratio. Similar results have been reported in China [29] and Romania [15]. Contrary to our results, the association of +276G/T SNP with T2D has been observed in several populations such as Taiwan [22]. These contradictory results indicate that this polymorphism may have a link with other active SNPs.

In the present study, the haplotype analysis was used to determine the association between the two common SNPs of the adiponectin gene and the risk of T2D. Among the four haplotypes, the "double-mutant" (+45G/+276T) haplotype was lower in both groups than in other haplotypes; the presence of +45G/+276G haplotype was associated with an increased risk of T2D.

In this study, we concluded that there is significant association between the +45T/G SNP and T2D in our sample, whereas this association could not be replicated in the +276G/T SNP. Since the current research has been performed in a restricted area and due to different ethnic diversity in Iran, we suggest further studies to be performed on larger populations on different ethnic groups. Also, study of other common polymorphisms in adiponectin gene in Iranian population is recommended.

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Compliance with ethical standards

Human and animal rights The study has been approved by the appropriate local ethics committee at the Semnan University of Medical Sciences and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the Semnan University of Medical Sciences research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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

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ORIGINAL ARTICLE

MACF1 gene variant rs2296172 is associated with T2D susceptibility in Mizo population from Northeast India

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Abstract

Aim Microtubule actin cross-linking factor 1 (*MACF1*) has been identified as a type 2 diabetes (T2D) candidate gene, and variant rs2296172 of the gene was found associated with T2D in multiple populations. However, it has never been explored in Mizo population. The aim of the present study was to replicate the association of variant rs2296172 of *MACF1* gene with T2D in Mizo population of Northeast India.

Methodology The variation was genotyped using TaqMan allele discrimination assay in 755 individuals (425 cases and 330 healthy controls), belonging to the Mizo population.

Results The variant rs2296172 *MACF1* was found to be significantly associated with T2D (p value = 0.001) in Mizo population group with an observed odds ratio of 1.8 [1.3–2.8] at 95% CI after correction with age, gender and BMI.

Conclusion This study is the first replication report from Northeast India, showing variant rs2296172 of MACF1 gene associated with T2D in Mizo population. This independent study highlights *MACF1* as a candidate gene for T2D in Asian Indian populations, suggesting it is critical to evaluate the variant rs2296172 in other distinct endogamous Indian population cohorts.

Keywords Microtubule actin cross-linking factor 1 · Type 2 diabetes · Mizoram

Introduction

T2D is a heterogeneous disorder [1] with genetic predisposition, environmental exposure and epigenetic changes as key risk factors [2].With the development of high-throughput sequencing and SNP genotyping technologies, discoveries of genetic factors have paced up, and more than140 loci have been identified to be associated with T2D [3].However, these loci explain less than 20% heritability of T2D [3]. The most plausible reason for such less heritability could be ethnic disparity [4] and the fact that most of the discovery studies carried out were focused on European populations. Further, such data has been lacking in Asian populations, especially Indian populations, despite the fact that majority of diversity and effective population size to the global human population is contributed by the region. In India, the prevalence of T2D is estimated to escalate from 72.9 million in 2017 to 134.3 million in 2045 [5], and scanty genetic susceptibility data makes it pertinent to carry out gene discovery studies. Replication of already associated variants in different endogamous ethnic groups is also critical, keeping in mind the genetic heterogeneity and lack of such information for these groups of India.

The variation rs2296172 (A > G) [p.(met2290val)]of *MACF1*was discovered in an exome sequencing-based GWAS in European populations [6], and it has been replicated, showing association in an independent Indo-European

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linguistic group cohort from Punjab region of India by our group [7]. In the present study, we aimed to replicate the *MACF1* variant rs2296172 in distinct population group, the Mizo population, that primarily speaks language belonging to Tibeto-Burman linguistic group and is reported with very high prevalence of T2D [8]. Despite the fact that Mizo population is at high risk of T2D, the genetics studies for T2D susceptibility are almost absent, with only one such study report [9]. In this background, this is the first replication study highlighting the association of variant rs2296172 and potential role of *MACF1* with T2D susceptibility in Mizo population.

Materials and methods

Sampling

The study was approved by ethical committee of Mizoram University, Aizawl, and Institutional Ethical Review Board (IERB) of Shri Mata Vaishno Devi University. The clinical characteristics of the T2D cases and healthy controls are summarized in Supplementary Table 1. In this study, 2 ml of venous blood was collected, along with an informed written consent from 755 individuals (425 cases and 330 healthy controls), from Genesis Laboratory, Aizawl, Mizoram, India. T2D and control diagnosis were made in accordance with the World Health Organization criteria (WHO Expert Committee). Fasting and postprandial blood sugar levels were estimated by glucose oxidase-peroxidase (GOD-POD) method using Sys 200 biochemistry analyser. The genomic DNA was isolated from the blood samples in Mizoram University, Aizawl, by using the methodology [10]. Agarose gel electrophoresis was used to analyse the quality of the genomic DNA. Quantification was performed using bio-spectrophotometer (Eppendorf, Hamburg, Germany), and the working dilutions were made (5 ng/μl) for genotyping.

Genotyping

The genotyping of variant rs2296172 was performed using allele discrimination assay on real-time PCR make (Mx3005P Agilent USA) and has been adopted from our previous studies [7, 11]. UNG master mix (Applied Biosystem, USA) and TaqMan assay (Predesigned Primer and Probe, labelled with FAM and VIC, supplied by Applied Biosystem, USA) were used for genotyping at Human Genetics Research Group, Shri Mata Vaishno Devi University. As recommended by the manufacturer, dilution of the assay mix was made from 40X concentration to 20X with TE (Tris-EDTA) buffer. PCR reactions were carried out in 96-well plate format, with three negative controls (NTC), to check for extraneous nucleic acid contamination. The volume of the total PCR reaction mix in each well was 10 μl, contributed by 2.5 μl of TaqMan UNG master mix, 0.25 μl of 20X assay, 3 μl DNA (5 ng/μl) and 4.25 μl of water added to make up the final volume. The PCR conditions were as follows: hold for 10 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The post PCR detection system (Mx3005P Agilent USA) is used to measure allele-specific fluorescence, and alleles were called automatically. Eighty-three of the samples were picked and re-genotyped for cross-validation; genotyping calls were observed with 98% concordance.

Statistical analyses

After genotyping, statistical analysis of the data was done using SPSS software (version 23; Chicago, IL). Chi square ($\chi 2$) goodness fit analysis was performed, and genotypic frequencies were tested for the Hardy-Weinberg equilibrium (HWE). Logistic regression analysis was performed to estimate the corrected odds ratio (OR), confidence interval (CI) and level of significance (p value) for potential confounding factors like age, gender and body mass index (BMI).Population attributed risk (PAR) percentage was also calculated, with 95% CIs, by using adjusted OR. The power of the study was calculated by PS software version 3.1.2 [12].

Results

In the present replication study, we investigated association of variant rs2296172 of *MACF1* and T2D in the Mizo population of Northeast India. The allele frequency distribution has been summarized in Table 1, and it was observed to be following Hardy-Weinberg equilibrium ($p = 0.218$). In the population group, the frequency of risk allele (G) was found to be considerably higher in cases (0.13) as compared to that of controls (0.074). The variant was observed to be significantly associated with T2D in dominant mode of inheritance (GG + AG v/s AA) with $p = 0.001$ and OR = 1.87 (1.3–2.8) at 95% CI (Table 1) which appeared most appropriate. The PAR percentage observed was 5.82% (2.22–11.33%) at 95% CI.

Discussion

Interestingly, the present study followed the trend in association of this variant as in earlier reports [6, 7], and the G allele appeared as the risk allele; however, the frequency distribution of the G allele was different. Sample size included in this study was estimated assuming 80% to detect the association with OR of 1.7 (from our previous finding in Bania population group) having minor allele frequency of 0.20 [7]. The post hoc power analysis was performed, and the power of the study was estimated to be 74.4%. The study was under power; the potential reason could be the less frequency of minor allele in the

Table 1 Distribution of allele frequencies and risk associated with the *MACF1* variation in Mizoram population, India

Gene/ SNP	Cases ($n = 425$)	Controls ($n = 330$)	HWE			Allelic OR	p value	Dominant OR*	p value*
	A = 0.87	A = 0.926	Cases	Controls	Total population				
MACF1/rs2296172	G = 0.13	G = 0.074	0.272	0.98	0.218	1.87 [1.31–2.66]	0.002	1.8 [1.3–2.8]	0.001

*Corrected with age, gender and BMI

studied population. In European population [6], the G allele frequency in T2D patients was 0.23, whereas, in an earlier study from India carried out by us in Bania population from Punjab [7], it was 0.17.Further, in the present population, the G allele frequency was observed to be 0.13 in T2D cases. We hypothesise that this could be attributed to genetic heterogeneity that may be existing due to practices of endogamy in Indian population groups [11]. Also, it could be speculated that the presence of variant and gradient of risk allele frequency in Indian populations (from Indo-European linguistic group to Tibeto-Burman linguistic group) could be an outcome of ancient admixture, or higher frequency in European population could be an outcome of founder effect, or distribution of variant in Indian and European populations is a combined effect of both admixture and founder effects. However, the association of variant rs2296172 of *MACF1*, though with different risk allele frequencies in population of different linguistic background, indicates it as one of the common susceptibility factors in various endogamous Indian population groups. This perspective is important and justifies screening of the variant in other independent population cohorts in India to understand the extent of contribution of the variant in risk towards the disease.

With a strong background of potential role of *MACF1* either directly or through interaction with genes involved in insulin secretion pathway (supplementary data-Fig. 1), we evaluated further the variant rs2296172 of MACF1 gene in GTEx portal [13]. The variant showed eQTL for *RP11-69E11.8* in pancreatic tissues with p value of 5.5×10^{-5} [13]. The positive normalized effect size (NES) observed was 0.238 indicating change (increase) in expression of RP11-69E11.8 in pancreatic tissues due to allele G (risk allele). *RP11-69E11.8* is a non-coding antisense gene overlapped with Poly (A) Binding Protein Cytoplasmic 4 (*PABPC4*) located on chromosome 1 (www.ensembl.org) [14]. PABPs bind to poly A tail present at3' end of the eukaryotic mRNAs [15]. It is interesting to speculate that increased expression of RP11-69E11.8 in pancreatic tissues may have implications on PABPC4 expression which subsequently may affect insulin secretion. Further, an extensive literature support is building up highlighting role of PABPs in endocrine disorders [16] and metabolic syndromes [17, 18].These observations also warrant functional characterization of *RP11-69E11.8* and *PABPC4*, as well as their interaction with *MACF1*, to understand their biological role in etiology of T2D, and it is also possible that the variant might be serving as surrogate.

We also explored for the SNPs which are in linkage disequilibrium (LD) of variant rs2296172 using HaploReg v4.1 [19]. A total of 80 SNPs ($r^2 \geq 0.8$) were found to be in LD in European population (CEU) with the variant rs2296172 of MACF1 gene of which 4 were observed with potential functional annotations. rs16826069 and rs41270807 were reported to be exonic, whereas rs3768302 and rs17343193 were reported in 3'-UTR. For graphical representation and understanding potential functional implications of these SNPs, we used online single nucleotide polymorphism annotator (SNIPA) as described in supplementary data-Fig. 2 [20]. It could be concluded from the annotations that all the four variants are in the transcript region, and any alteration in this region may affect directly the role of MACF1 in regulation of filamentous actin(F-actin) and microtubule cytoskeletal dynamics [21, 22], resulting in impaired glucose homeostasis.

Our results and replication in an independent cohort indicate *MACF1* as an ideal T2D candidate gene for Indian populations. Thus, its variants are critical to be evaluated in different endogamous groups in India to validate if it can be attributed as a common biomarker of T2D in Indian populations. Further, as T2D is a complex multigenic disorder and as evident from small PAR associated with this variant (approx. 6%), it is emphasized that more genetic variants that are susceptible to T2D risk are required to be evaluated in the Mizo population group of Northeast India. These studies will facilitate us in better understanding of genetic predisposition in Mizo population and the genetic heterogeneity in Indian populations.

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Authors' contributions NSK and SWS primarily planned the study and provided guidance throughout the study. FL and VAS prepared the manuscript. FL collected samples and, INS, SUS, GUK, YU, HS performed experimental work and JZ and VAN primarily provided the samples. VAS and IS performed statistical analyses. ER, VIS, NSK, SWS critically evaluated and compiled the study.

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Compliance with ethical standards

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

Ethical approval Ethical committee of Mizoram University, Aizawl, and Institutional Ethical Review Board (IERB) of Shri Mata Vaishno Devi University approved the study. The work has been approved by ethical standards of the responsible committee on human experiments (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Statement of informed consent Informed consent was obtained from all the individuals for being included in the study.

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ORIGINAL ARTICLE

Liver iron concentration is an independent risk factor for the prediabetic state in β-thalassemia patients

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Abstract

Background Beta-thalassemia major (β-TM) comprises a group of inherited blood disorders characterized by the reduced synthesis of β-globin chains. Iron overload following blood transfusion can affect major tissues involved in glucose metabolism, leading to different glucose metabolic disorders in these patients. The aim of the present study was to compare glucose metabolism and iron overload indices in β-TM patients with prediabetic and normoglycemic states.

Methods This analytical study was performed on 49 patients with β-TM (age > 18 years), receiving regular blood transfusions. The fasting plasma glucose (FPG) and glucose tolerance tests indicated 32 normoglycemic and 17 prediabetic cases. The serum levels of C-peptide, fructosamine, fasting serum insulin, and serum ferritin were measured. In addition, T2*-weighted magnetic resonance imaging (MRI) of the heart and liver was carried out. Glycemic metabolism indices, including quantitative insulin sensitivity check index (QUICKI) and homeostatic model assessment for insulin resistance (HOMA-IR), were also calculated.

Results The HOMA-IR score was significantly higher, while the QUICKI score was significantly lower in prediabetic patients, compared to normoglycemic patients (median [IR], 2.59 [2.19] vs. 1.46 [1.03], $p = 0.007$; mean ± SD, 0.34 ± 0.03 vs. 0.37 ± 0.04, $p = 0.01$). On the other hand, β-cell function was not significantly different between the groups. The liver iron concentration (LIC) at a cutoff point of 5.82 mg/g dry weight showed 93% sensitivity and 70% specificity for differentiation of prediabetic and normoglycemic states (AUC, 0.81 [95% CI, 0.65–0.95]; $p = 0.002$).

Conclusion Based on the findings, HOMA-IR and QUICKI can be applied as useful glycemic metabolism indices for predicting prediabetic and normoglycemic states among β-TM patients. Also, LIC was an independent risk factor for the prediabetic state.

Keywords β-thalassemia major · Glucose metabolism · Glucose intolerance · Insulin resistance · Prediabetes · β-cell function

Introduction

Beta-thalassemia major (β-TM) comprises a group of inherited blood disorders, including hypochromic and hemolytic anemia, characterized by the reduced synthesis of β-globin chains [1]. Use of chronic and multiple blood transfusions in β-TM patients has increased the survival of these patients [2, 3]. However, blood transfusions can lead to iron overload, which frequently causes endocrine problems, such as abnormal glucose homeostasis (AGH), impaired fasting glucose (IFG) or prediabetes, and diabetes mellitus (DM) [4]. Also, iron overload can affect major tissues involved in glucose metabolism, including pancreatic β-cells, leading to different glucose metabolic disorders [5]. The incidence of impaired glucose tolerance (IGT) and DM in β-TM patients has been estimated at 4–30% [2, 4, 6] and up to 26% [2, 6], respectively.

The exact pathogenesis of AGH and the prediabetic state in β-TM patients has not been clarified yet. However, these conditions have been mainly attributed to insulin deficiency, caused by the toxic effects of iron deposits in the pancreas, as well as insulin resistance [7]. Although insulin deficiency secondary to pancreatic β-cell dysfunction after iron deposition has been assumed as a key factor in several studies [5, 8],

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reports of hyperinsulinemia with abnormal glucose tolerance may suggest insulin resistance [6, 9, 10].

An increase in insulin resistance has been described in TM patients, even without IGT or overt diabetes, suggesting the occurrence of insulin resistance before the onset of glucose intolerance or DM [2]. In this regard, Dandona et al. [11] performed a glucose tolerance test (GTT) among 15 patients (age, 6–36 years) with a history of blood transfusion associated with iron overload and severe hepatic injury and compared the results with those of 10 patients without a history of blood transfusion and normal iron levels [11]. Their findings were indicative of insulin resistance due to liver cell insult, as confirmed in other studies.

The aim of the present study was to compare glucose metabolism and iron overload indices in β-TM patients with prediabetic and normoglycemic states and to investigate the beneficial effects of these indices. Our findings can increase the objectivity and effectiveness of glucose metabolism and iron overload indices for differentiation of prediabetic and normoglycemic states in these patients.

Materials and methods

Study sample and design

This analytical study was performed at the Thalassemia Research Center (TRC) of Mazandaran University of Medical Sciences over 9 months from October 2015 to June 2016 after being approved by the Ethics Committee. Written informed consents were obtained prior to the study. The study sample included 49 patients with β-TM (age > 18 years), receiving regular blood transfusions. The inclusion criterion was diagnosis of transfusion-dependent β-TM, based on hemoglobin electrophoresis. On the other hand, diagnosis of severe diseases, infection, or DM and use of medications such as corticosteroids, leading to glucose intolerance, were considered as the exclusion criteria.

Glycemic status was assessed using FPG and GTT [12]. The patients were assigned to prediabetic and normoglycemic groups according to their glycemic status. The results of FPG and GTT indicated 32 normoglycemic and 17 prediabetic cases for the statistical analysis. Moreover, chemiluminescent immunoassay was used for measuring the serum level of ferritin (coefficient of variation [CV], 4.1 ng/mL; limit of detection [LOD], 0.5 ng/mL).

Fasting serum insulin (chemiluminescent immunoassay; CV, 4.3 mlU/L; LOD, 0.2 mlU/L), C-peptide (DiaSorin kit, Italy; CV, 5 ng/mL; LOD, 0.01 ng/mL), and fructosamine (Roche kit, Germany; CV, 2.8 μmol/L; LOD, 14 μmol/L) levels were measured in all patients. Age, gender, weight, body mass index (BMI), blood transfusion data, iron-chelating data, and history of splenectomy were also recorded for all patients.

Glycemic metabolism indices

The insulin resistance index based on the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) [13], β-cell function [13], and quantitative insulin sensitivity check index (QUICKI), as an insulin sensitivity index [14], were investigated according to the following formulae, respectively:

$$\text{Insulin resistance index} = [(\text{FPG, mmol/L}) \times (\text{fasting insulin, mIU/L})]/22.5$$

$$\beta\text{−cell function index} = [20\ (\text{fasting insulin, mIU/L})]/[(\text{FPG, mmol/L})-3.5]$$

$$\text{QUICKI} = 1/[\log(\text{fasting insulin, mIU/L}) + \log(\text{FPG, mg/dL})]$$

Diagnosis of the prediabetic state

The diagnosis of the prediabetic state was based on American Diabetes Association (ADA) criteria [15]: IFG: FPG > 100 mg/dL and < 126 mg/dL (5.6–6.9 mmol/L), and/or IGT: 2-h plasma glucose (2hPG) ≥ 140 mg/dL and < 200 mg/dL (7.8–11.0 mmol/L) (Fig. 1).

Magnetic resonance imaging (MRI)

Liver and cardiac MRI T2* was performed for all patients using a 1.5-Tesla MRI scanner (Symphony; Siemens, Germany) in Noor Medical Imaging Center, Tehran, Iran. In-house software was validated using the cardiovascular MRI tools of Brompton, and was used to calculate T2*-weighted as well as region of interest (ROI) elements [16]. Categorizations of quantitative MRI values to normal and abnormal were performed based on normal reference ranges of reported MRIs (Appendix Tables 6 and 7).

Statistical analysis

Data are presented as mean ± standard deviation (SD) for quantitative variables with a normal distribution, median and interquartile range (IQR) for quantitative variables without a normal distribution, and numbers (percentages) for qualitative variables. All quantitative data were checked for normal distribution using the Kolmogorov-Smirnov test. The baseline characteristics of the two groups were compared using Student's *t* test or Mann-Whitney *U* test for continuous variables and chi-square or Fisher's exact test for dichotomous variables.

We categorized quantitative variables, including HOMA-IR, T2*-weighted MRI, and serum ferritin, as nominal variables for chi-square test. The glycemic metabolism status was divided into two categories: (1) normal, HOMA-IR < 2.5, and (2) abnormal, and/or HOMA-IR ≥ 2.5 [17, 18]. The

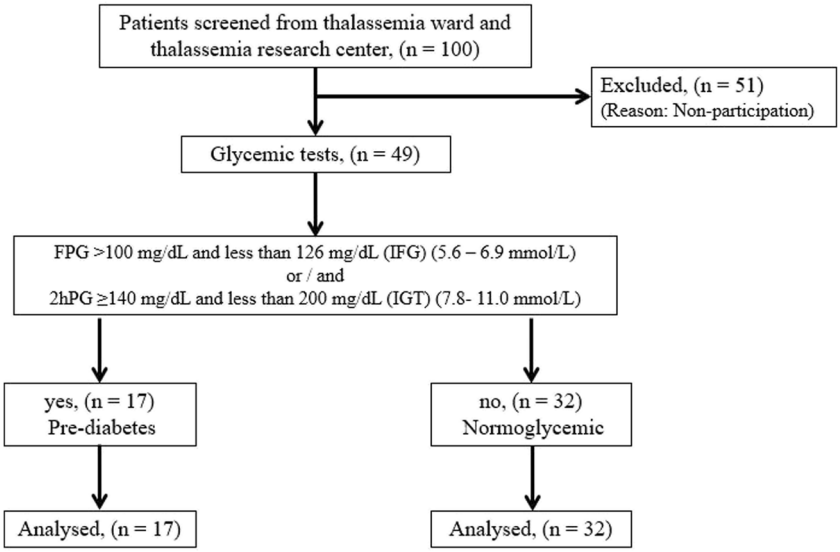

Fig. 1 Study flow chart. 2hPG, 2-h plasma glucose; FPG, fasting plasma glucose; IFG, impaired fasting glucose; IGT, impaired glucose tolerance

quantitative MRI findings were classified into normal and abnormal, based on the normal reference MRI ranges. The serum ferritin level was also classified into two groups according to the cutoff points of 500, 1000, and 1500 ng/mL.

A multivariate logistic regression model with an enter selection method to determine the predictors of prediabetes was used. Logistic regression analysis was also performed to confirm the effects of LIC and ferritin on the risk of the prediabetic state. First, variables with p values < 0.1 in the univariate analysis were entered in the logistic regression model after collinearity between quantitative variables was assessed. Moreover, after measuring the goodness of fit of the models, LIC and ferritin were considered as the independent variables in the multivariate logistic regression model with the constant term.

Finally, receiver operating characteristic (ROC) analysis was performed to determine the optimal cutoff point of LIC for diagnosis of the prediabetic state. Data were analyzed using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed, and p value < 0.05 was considered statistically significant.

Results

Forty-nine β-TM patients were recruited in this study (24 males, 49%; 25 females, 51%). Overall, 32 (65.3%) patients were normoglycemic, while 17 (34.7%) patients were prediabetic, including two cases of IGT and 15 cases of IFG. The mean ± SD values of FPG and 2hPG were 89.4 ± 7.2 and 88.2 ± 16.4 mg/dL in normoglycemic patients and 106.8 ± 9.3 and 104.2 ± 25.2 mg/dL in prediabetic patients, respectively (p < 0.0001 and 0.02, respectively). There was no significant difference in terms of age, gender, transfusion data, history of splenectomy, and type of iron chelators between the two groups (Table 1). All patients used standard iron chelators, including desferrioxamine (20–40 mg/kg/day), deferasirox (20–40 mg/kg/day), and deferiprone (50–100 mg/kg/day), with a consistent dose during the study.

The results showed significant differences in LIC, hepatic siderosis, and cardiac siderosis on T2*-weighted MRI between prediabetic and normoglycemic patients; also, ferritin level was significantly different between the groups (Tables 2 and 3). Moreover, the insulin resistance index and QUICKI were significantly different between the groups; however, β-cell function was not significantly different between the two groups (Table 3). The boxplots of the distribution of significant indices in normoglycemic and prediabetic patients are presented in Fig. 2.

Ferritin concentration above 500 ng/mL was considered to be abnormal in this study; accordingly, there was no significant difference between the prediabetic and normoglycemic groups (p = 0.53). However, a significant inter-group difference was observed by considering ferritin > 1000 ng/mL to be abnormal (Table 4). Furthermore, abnormal HOMA-IR, LIC, and cardiac T2*-weighted MRI (dichotomous variables) were significantly associated with the increased risk of the prediabetic state (Table 4). The results demonstrated no significant difference on abnormal hepatic T2*-weighted MRI between the two groups (p = 0.34).

The multivariate logistic regression analysis revealed a significant association between LIC and the prediabetic state. Increased LIC, as an independent risk factor, was related to the increased risk of prediabetes. The results of the multivariate logistic regression model are presented in Table 5. In order to find a significant cutoff point for LIC, the ROC of LIC for prediabetic and normoglycemic states was plotted, indicating higher sensitivity and specificity at a cutoff point of 5.82 mg/g dry weight (DW). Moreover, the Spearman correlation coefficient between the LIC and HOMA-IR was 0.42; p = 0.02.

Table 1 Basic and clinical characteristics of normoglycemic and prediabetic patients ($n = 49$)

Variables		Normoglycemic ($n = 32$)	Prediabetic ($n = 17$)	p value
Age, years		28 (6) [a]	27 (12.5) [a]	0.81 [b]
Gender, M/F		17/15	7/10	0.31 [c]
Weight, kg		57.87 ± 10.37 [d]	58.17 ± 9.88 [d]	0.84 [e]
BMI, kg/m^2		21.81 ± 3.13 [d]	22.01 ± 3.27 [d]	0.92 [e]
Duration of blood transfusion, years		24.7 (6.7) [a]	24.5 (10.5) [a]	0.72 [b]
Received iron following blood transfusion, mg		8500 (2062) [a]	7500 (2000) [a]	0.41 [b]
Splenectomy		16 (50)	9 (52.9)	0.83 [c]
Iron chelator	DFO	1 (3.1)	1 (5.9)	1 [c]
	DFO plus DFP	11 (34.3)	9 (52.9)	0.21 [c]
	Duration of DFO plus DFP	6.5 (6.5)	6 (6)	0.64 [b]
	DFX	11 (34.3)	5 (29.5)	0.72 [c]
	Duration of DFX	8 (7)	6 (6.5)	0.31 [b]
	DFO plus DFX	3 (9.3)	2 (11.8)	1 [c]
	Duration of DFO plus DFX	4 (7)	5 (6)	0.73 [b]
	DFP	1 (3.1)	–	
	DFX plus DFP	1 (3.1)	–	
	No treatment	4 (12.5)	–	

Qualitative variables presented as numbers (percentages)

M/F male to female ratio, *DFO* desferrioxamine, *DFX* deferasirox, *DFP* deferiprone, *BMI* body mass index

[a] Data presented as median (interquartile range; IQR)

[b] p value obtained by Mann-Whitney U test

[c] p value obtained by chi-square test

[d] Data presented as means ± standard deviations

[e] p value obtained by independent samples t test

Discussion

Understanding the pathophysiology of AGH and diabetes can improve blood glucose control and diabetes prevention in patients. The process of AGH progression is rather fast and leads to pancreatic β-cell dysfunction and overt diabetes [8, 19]. Involvement of the pancreas following iron siderosis results in reduced insulin secretion and circulating insulin in these patients, which is commonly an inevitable event [4, 20]. The mechanism of abnormal glucose homeostasis in β-TM patients is not completely clear yet. However, it has been mainly attributed to insulin deficiency because of pancreatic iron siderosis and insulin resistance, to some extent, due to iron deposition in both the liver and muscles [6].

Table 2 The comparison of cardiac and liver magnetic resonance imaging (MRI T2*) values between normoglycemic and prediabetic patients ($n = 49$)

Variables		Normoglycemic ($n = 32$)	Prediabetic ($n = 17$)	p value
Liver T2*, ms		4.72 (7.41) [a]	2 (6.29) [a]	0.06 [b]
LIC, mg/g/dw		2.77 (4.99) [a]	8.21 (2.42) [a]	*0.002* [b]
Liver siderosis	Normal	13 (40.6)	3 (17.7)	*0.02* [c]
	Mild	6 (18.7)	–	
	Moderate	11 (34.3)	11 (64.7)	
	Severe	–	–	
Cardiac T2*, ms		26.93 ± 7.97 [d]	22.25 ± 13.94 [d]	0.12 [e]
Cardiac siderosis	Normal	26 (81.2)	8 (47.1)	*0.01* [c]
	Mild	3 (9.3)	2 (11.8)	
	Moderate	1 (3.1)	–	
	Severe	–	4 (23.5)	

Qualitative variables are presented as number (percentage)

LIC liver iron concentration, *ms* milliseconds

[a] Data is presented as median (interquartile range; IQR)

[b] p value was obtained by Mann-Whitney U test

[c] p value was obtained by chi-square test

[d] Data is presented as mean ± standard deviation

[e] p value was obtained by independent sample t test

Table 3 The comparison of glucose metabolism indices and ferritin between normoglycemic and prediabetic patients ($n = 49$)

Variables	Normoglycemic ($n = 32$)	Prediabetic ($n = 17$)	p value
Ferritin, ng/mL	1370 (1638)	3383 (3263)	*0.01* [a]
Serum insulin level, mIU/L	6.60 (4.25)	9.75 (7.67)	0.14 [a]
C-peptide, ng/mL	2.07 ± 0.56	2.47 ± 1.02	0.25 [b]
Fructosamine, μmol/L	229.0 ± 15.65	226.0 ± 25.51	0.72 [b]
Beta-cell function	86.15 (77.41)	90.49 (89.57)	0.54 [a]
HOMA-IR	1.46 (1.03)	2.59 (2.19)	*0.007* [a]
QUICKI	0.37 ± 0.04	0.34 ± 0.03	*0.01* [b]

Data are presented as median (interquartile range; IQR) or mean ± standard deviation

HOMA-IR Homeostatic Model Assessment for Insulin Resistance, *QUICKI* Quantitative insulin sensitivity check index

[a] p value was obtained by Mann-Whitney U test

[b] p value was obtained by independent sample t test

Glucose metabolism indices can be beneficial in differentiating prediabetic and normoglycemic states in β-TM patients. Our results showed that IGT is associated with hepatic and cardiac siderosis. The higher HOMA-IR and lower QUICKI scores, as insulin sensitivity indices among AGH patients, indicate insulin resistance. The results also revealed that increased LIC was related to an increased risk of prediabetes.

The indices of insulin resistance and insulin sensitivity were significantly different between AGH and normoglycemic patients, unlike β-cell function. Therefore, it seems that insulin resistance first occurs, followed by pancreatic β-cell dysfunction and decreased insulin secretion. In a previous study, we showed that the main pathology of DM among β-TM patients involves iron siderosis in the pancreas [16]. In this regard, a clinical study showed that hyperinsulinemia compensates for insulin resistance, which occurs early, even before the onset of overt DM [10]. This case-control study aimed to assess glycometabolic function with respect to insulin secretion, glucose tolerance, β-cell function, and insulin resistance in transfusion-dependent β-TM patients and to identify its association with biochemical and clinical profiles.

In the mentioned case-control study, 30 homozygous TM children with regular blood transfusions (age range, 8–15 years) and 10 healthy children (sex- and age-matched),

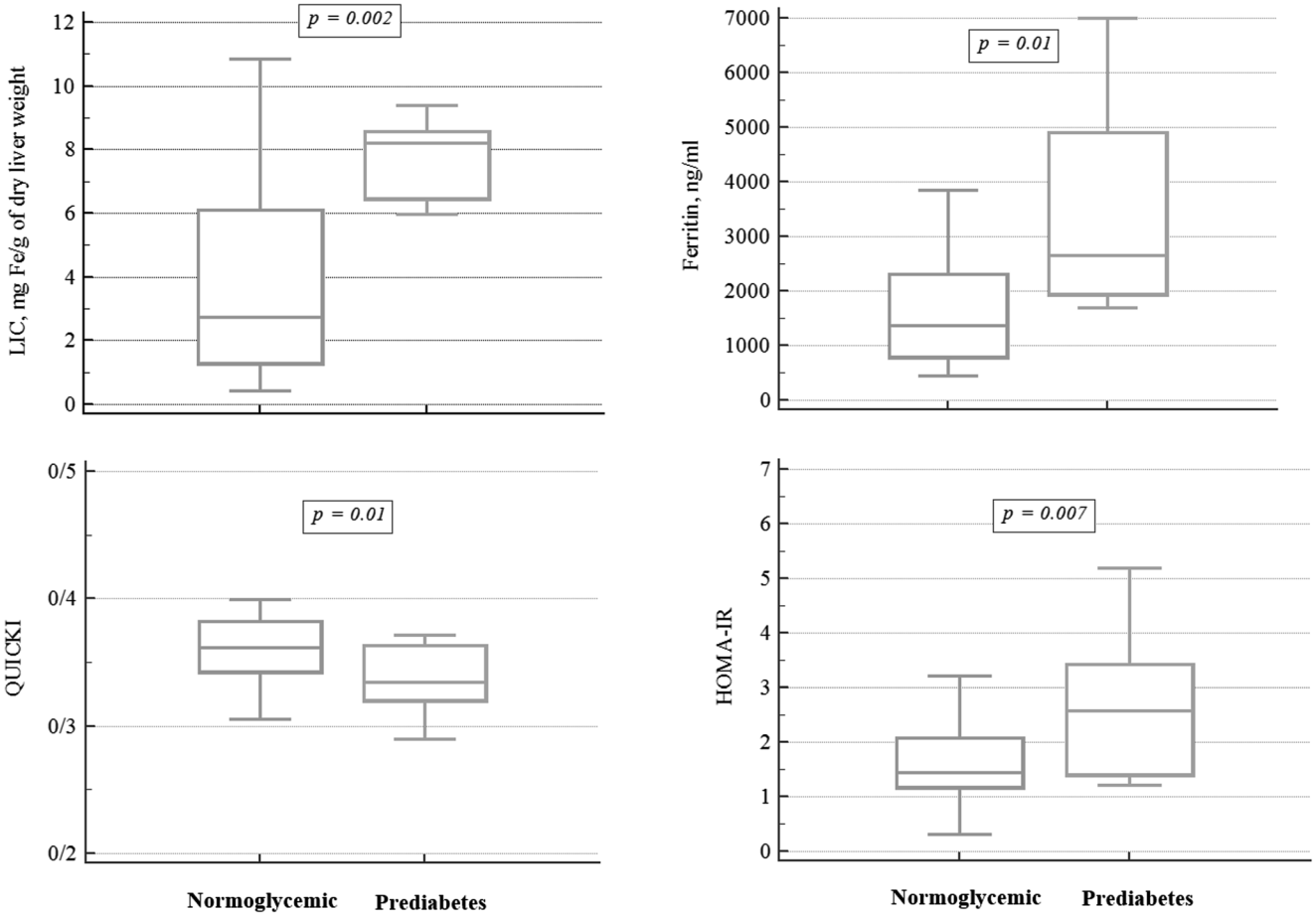

Fig. 2 Boxplots of the distribution of ferritin, liver iron concentration (LIC), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), and quantitative insulin sensitivity check index (QUICKI) based on the normoglycemic and prediabetic states in patients with β-thalassemia major ($n = 49$)

Table 4 Factors associated with the prediabetic state ($n = 49$)

Abnormal	n (%)		p value
	Normoglycemic ($n = 32$)	Prediabetic ($n = 17$)	
Ferritin, ng/mL [a]	13 (40.6)	10 (58.9)	*0.03*
Ferritin, ng/mL [b]	10 (31.2)	10 (58.9)	*0.004*
HOMA-IR	3 (9.3)	7 (41.2)	*0.01*
LIC, mg/g/dry weight	17 (53.1)	12 (70.6)	*0.03*
Cardiac T2*, ms	4 (12.5)	8 (47.1)	*0.01*

LIC ≥ 2 mg/g dry weight was considered as abnormal. HOMA-IR ≥ 2.5 as abnormal glycemic metabolism. Cardiac T2* < 20 ms was considered as abnormal

HOMA-IR Homeostatic Model Assessment for Insulin Resistance, *n (%)* number (percentage), *LIC* liver iron concentration

[a] Ferritin higher than 1000 ng/mL was considered abnormal

[b] Ferritin higher than 1500 ng/mL was considered abnormal

referred to a tertiary hospital, were exposed to GTT. Then, the insulin resistance index, fasting serum insulin, and β-cell function were measured. No cases of DM or IGT were reported in the case or control group. The case group showed a significantly higher serum insulin level, compared to the controls. The serum insulin level was well correlated with the total units of blood transfusion as an indicator of serum ferritin and iron overload ($r = 0.38$, $p = 0.038$ and $r = 0.41$, $p = 0.03$, respectively). Also, the case group showed higher insulin resistance in comparison with the controls ($p = 0.01$). Based on the findings, insulin resistance was correlated with the serum level of ferritin, fasting plasma insulin, and total blood transfusion units ($r = 0.4$, $p = 0.02$; $r = 0.95$, $p < 0.001$; and $r = 0.52$, $p = 0.005$, respectively). Moreover, the case group had a higher β-cell function, compared to the controls, although the difference was insignificant. Insulin resistance was found to be correlated with age, chelation therapy, iron overload, serum ferritin, and splenomegaly [10].

Progression towards diabetes can be divided into three phases [4]. The first phase is insulin resistance with normal insulin secretion and serum level. The second phase is characterized by increased insulin secretion, and the third phase involves insulin secretory defects and β-cell dysfunction following pancreatic iron siderosis. We observed the second phase in our prediabetic patients. Based on the findings, patients with a prediabetic state had higher serum insulin levels; however, the difference was not significant between prediabetic and non-prediabetic cases.

In addition, Messina et al. [13] followed three non-diabetic cases for 3 years to evaluate the evolution of glucose tolerance and insulin indices in β-TM patients (mean age, 26.2 ± 5.7 years; age range, 19–41 years). The mean FPG increased from 87 mg/dL at baseline to 94.4 mg/dL at the end of the study, while 2hPG increased from 150 to 191.4 mg/dL. Deterioration of 2hPG over time was observed, along with an increase in insulin resistance, with no concomitant change in insulin secretion. It was concluded that the glycometabolic state in TM adults is characterized by reduced 2hPG over time, particularly in patients with IGT, which may be indicative of an increase in insulin resistance.

In another study by Dandona et al. [11] on 15 patients with iron overload (ferritin, 1740–24,000), 47% of patients with oral glucose intake (1.75 g/kg) showed an increase in blood glucose level 120 min after GTT. Blood glucose level at 120 min was higher than blood glucose levels at 60 and 90 min in nearly 94% of patients. Moreover, the serum fasting immunoreactive insulin level increased concomitantly with blood glucose in patients with increased blood glucose at 120 min following glucose intake [11]. Importantly, in the study by Dandona et al., only diabetic patients with iron overload had

Table 5 The results of multivariate logistic regression analysis for the prediabetic state ($n = 49$)

Variable	B	SE	Wald	Adjusted OR (95% CI)	p value
LIC, mg/g/dry weight	0.51	0.25	4.19	1.66 (1.03 to 2.67)	*0.04*
Ferritin, ng/mL	0.001	0.001	1.15	1.01 (0.99 to 1.01)	0.27
Constant	− 4.69	1.78	7.02	0.01	*0.001*

The Spearman correlation coefficient between the LIC and ferritin was 0.54; $p = 0.03$. The logistic regression model was statistically significant, X^2 $(2, n = 49) = 11.09$, $p < 0.04$. The model explained 46.0% (Nagelkerke R^2) of the variance in the prediabetic state and correctly classified 82.15% of cases

OR odds ratio, *CI* confidence interval, *SE* standard error, *LIC* liver iron concentration

insulinopenia as the main pathogenic mechanism, which is probably attributable to iron overload in the pancreatic islet.

Epidemiologic observations in human studies and experimental studies on animal models have established a clear association between tissue iron storage and DM risk [4]. We determined an upper limit of hepatic iron for developing a prediabetic state at a cutoff point of 5.82 mg/g DW. Several studies have shown that serum ferritin level < 2500 ng/mL leads to the reduced incidence of diabetes [19, 21]. In our previous studies, we found a significant increase in the risk of the prediabetic state when the serum ferritin level was above 1000 ng/mL among β-TM patients.

We found LIC to be an independent risk factor for the prediabetic state. Therefore, monitoring and management of LIC as a reliable iron overload marker, along with adequate iron chelation therapy, can prevent glycemic complications, including prediabetes, and inhibit progression towards pancreatic β-cell dysfunction and overt diabetes. In this regard, Chern et al. [6] evaluated the prevalence and risk factors of IGT in 68 transfusion-dependent β-TM patients. The prevalence of IGT was reported to be 8.5%. The serum ferritin concentration was determined as a risk factor for IGT in these patients. This research, similar to our study, showed that increased serum ferritin level is associated with IGT [6].

There are several possible hypotheses in the literature about the role of LIC and ferritin in the etiopathogenesis of the prediabetic state in TM patients with iron overload. Insulin resistance can arise from iron deposition in both the liver and muscles. This may in fact disturb the insulin ability to suppress hepatic glucose production in the liver and decrease glucose uptake following muscle damage [6]. Transferrin-bound iron also interacts with hepatocyte TfR2 and may affect the insulin ability to ameliorate hepatic glucose synthesis, resulting in insulin resistance [6]. Excess iron accumulates in the event of insufficient hepcidin production. The subsequent trapping of iron in macrophages and hepatocytes can explain the presence of insulin resistance in patients with iron excess [4, 22].

Hepcidin is a negative-feedback regulatory protein, which controls iron absorption and its distribution among tissues [23]. A severe decline in hepcidin mRNA has been observed in patients with higher insulin resistance, compared to the controls [24]. Recently, use of hepcidin agonists for increasing the serum hepcidin level has been proposed in order to manage patients with high iron burdens, besides impaired glucose homeostasis [23]. Insulin signal loss may be explained by the reduced hepcidin and prohepcidin levels in diabetic patients with excessive iron storage (hepcidin-dependent mechanisms) [25–27].

The first step of iron siderosis may happen in reticuloendothelial systems (bone marrow, spleen, and liver) and subsequently in the hepatocytes, the myocytes, and the endocrine glands [16]. So cardiac iron siderosis may occur before or concurrently with emerging AGH in β-TM patients. We previously determined a moderate positive correlation between pancreatic and cardiac T2*-weighted MRI ($r = 0.4$; $p < 0.001$) [16]. Moreover, cardiac T2*-weighted MRI was significantly lower in β-TM patients affected by AGH compared to normoglycemic cases ($p < 0.02$). The severity of cardiac iron siderosis was also meaningfully higher in patients with AGH ($p < 0.04$).

Considering the role of iron overload in the pathogenesis of IGT, a more aggressive and extensive iron chelation treatment is recommended [4]. It is also suggested that physicians consider the risk of prediabetes and diabetes in TM patients. Moreover, regular assessment of LIC should be considered in the prediction of the prediabetic state among β-TM patients. Further research is needed to develop guidelines for identifying patients who would benefit from iron homoeostatic balance.

It should be mentioned that the numbers of participants of the two groups were not equal in our study. Therefore, further studies with a larger sample size are needed to confirm our results. Also, further research, incorporating subgroup analysis for glycemic metabolism indices, is essential in IFG and IGT patients. We also suggest measuring muscle iron deposition as an important part of insulin resistance and hepcidin levels for studies which will be carried out in the future.

Conclusion

Based on the findings, HOMA-IR and QUICKI could be applied as useful glycemic metabolism indices for predicting prediabetic and normoglycemic states in β-TM patients. In addition, LIC was found to be an independent risk factor for the prediabetic state in these patients.

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Compliance with ethical standards

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

Statement of human rights The study conformed to the Declaration of Helsinki principles.

Informed consent All subjects gave informed consent and were informed of their ability to discontinue participation at any time.

Appendix. Interpretation and categorization of cardiac and liver MRI

Table 6 Cardiac MRI

Myocardial loading	Myocardial T2* (ms)
Normal	≥ 20
Mild	19.99–14
Moderate	13.99–10
Severe	< 10

ms milliseconds

Table 7 Liver MRI

Hepatic loading	Liver T2* (ms)	LIC (mg/g/dw)
Normal	> 6.3	< 2
Mild	6.3–2.80	2–4.99
Moderate	2.79–1.4	5–9.99
Severe	< 1.4	> 10

LIC liver iron concentration, *ms* milliseconds

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ORIGINAL ARTICLE

Hematocrit levels and arterial stiffness: the Cardiometabolic Risk in Chinese (CRC) Study

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Abstract

Objective We aimed to investigate the association of haematocrit (HCT) level with central and peripheral arterial stiffness in adults in China. We particularly focussed on the association between HCT and other cardiometabolic risk factors.

Methods Adults aged ≥ 20 years were included in the study. Carotid radial pulse wave velocity (crPWV), carotid femoral pulse wave velocity (cfPWV), carotid dorsalis pedis pulse wave velocity (cdPWV), and HCT were measured.

Results Overall, 3225 individuals were included in the study. After adjustment for age, sex, and body mass index, HCT level was found to increase significantly with increases in cfPWV, crPWV, and cdPWV ($p = 0.001$). Following adjustment for heart rate, blood pressure, and blood lipids, the association of HCT level with crPWV and cdPWV was not significant ($p = 0.090$ and 0.053, respectively); however, the association between HCT level and cfPWV remained significant ($p = 0.007$). We found significant interactions of HCT level with hypertension and metabolic syndrome in the effect on cfPWV ($p = 0.0419$ and 0.026, respectively).

Conclusions In adults in China, HCT level was associated with elevated central arterial stiffness, independent of conventional cardiovascular risk factors. As a serological marker, HCT can predict the degree of central arterial stiffness. HCT combined with other traditional cardiovascular risk factors can better assess vascular heart disease.

Keywords Hematocrit · Pulse wave velocity · Hypertension

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Abbreviations

HCT	hematocrit
crPWV	carotid-radial pulse wave velocity
cfPWV	carotid-femoral pulse wave velocity
cdPWV	Carotid dorsalis pedis pulse wave velocity
MetS	metabolic syndrome
CRC	Cardiometabolic Risk in Chinese
BMI	bodymass index
BP	blood pressure
HR	heart rate
SUA	serum uric acid
FBG	fasting blood glucose;
TC	total cholesterol
TG	triglycerides
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein cholesterol

Introduction

Hematocrit (HCT), the proportion of blood volume occupied by red blood cells, is a routinely measured hematological

parameter. Recent studies have shown an association between high HCT level and the development of hyperviscosity, hypertension, and insulin resistance [1–3]. Furthermore, recent prospective studies have shown that HCT is associated with metabolic syndrome (MetS) [4, 5].

Aortic stiffness is known to be associated with cardiovascular outcomes [6]. As a gold standard for assessing arterial stiffness, pulse wave velocity (PWV) is widely used as an early predictor of atherosclerosis [7, 8]. Studies have shown that many factors, including age, body mass index (BMI), blood pressure (BP), and blood lipids, can influence PWV [9]. Several studies have investigated the relationship between HCT level and PWV [10–12]; however, the relationship of HCT with hypertension and PWV remains unclear.

In this study, we examined the associations between HCT level and PWV in adults in China. We particularly focussed on the interactions between HCT and metabolic factors, including age, BMI, BP, and blood lipids.

Methods

Study population

As a part of the Cardiometabolic Risk in Chinese (CRC) Study, individuals living in the city of Xuzhou were examined in the Central Hospital of Xuzhou from January 2015 to December 2015. The age range was 21–87 years. The study was reviewed and approved by the ethics committee of the Central Hospital of Xuzhou, the affiliated hospital of the Medical School of Southeast University, China. All participants provided written informed consent.

Adults aged ≥ 20 years were included in this study. PWV, BP, BMI, heart rate (HR), serum uric acid (SUA) level, and other metabolic markers were recorded. Individuals with a history of vascular disease, diabetes, chronic obstructive pulmonary disease, hemopathy, or renal failure (glomerular filtration rate reduced to 10–20% and serum creatinine level increased to 451–707 μmol/L) were excluded [13]. In addition, we excluded individuals who did not undergo both PWV determination and blood sampling. In total, 3225 men and women were included in the final analyses.

Assessment of PWV

Before PWV, we measured the BP of the participants. Physicians measured BP using a mercury manometer after the subject had rested for ≥ 5 min. The mean arterial pressure was calculated as 2/3 diastolic blood pressure (DBP) + 1/3 systolic blood pressure (SBP). The mean of the three measurements, spaced 60 s apart, was used for the analysis. We used an automatic waveform analyser (Complior System, Artech-Medical Corp.) to measure the PWV. All participants were in the supine position to set up the baroreceptor, which was placed in the most obvious part of the carotid and femoral pulsations. We measured the distance between the two points and input it into the computer. The automatic waveform analyser continuously recorded 16 pulse wave velocity values in the subject. After removing the three highest and three lowest values, the average of the other 10 measured values was considered for the final analyses [14].

Assessment of biomarkers and covariates

Blood samples were collected from the participants in the morning after they had fasted overnight. Blood was drawn from the antecubital vein and collected in tubes containing EDTA. Immediately after this, the clotted serum samples were centrifuged for 15 min at 3000 rpm. Fasting blood glucose (FBG), HCT, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, SUA, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDLC), and low-density lipoprotein cholesterol (LDLC) were determined by an auto-analyser (Type 7600; Hitachi Ltd.).

Height was measured to the nearest 0.5 cm, and body weight was measured to the nearest 100 g (none of the participants were wearing shoes during measurement). BMI was calculated as weight (in kilogrammes) divided by the square of height (in metres). Information regarding education level, income, smoking status, and alcohol consumption was collected using a questionnaire.

MetS

In the study, MetS was defined as the presence of three or more of the following four medical conditions: (1) overweight or obesity, i.e., BMI ≥ 25.0 kg/m^2; (2) hypertension, i.e., SBP ≥ 140 mmHg, DBP ≥ 90 mmHg, or previously diagnosed; (3) dyslipidemia, i.e., fasting TG ≥ 1.7 mmol/L or fasting HDL < 0.9 mmol/L; and (4) hyperglycemia, i.e., FBG ≥ 6.1 mmol/L or 2 h post-meal glucose (PG) ≥ 7.8 mmol/L or previously diagnosed.

Statistical analysis

The relationship between HCT level (divided in quartiles as follows: Q1: female HCT ≤ 37.5%, male HCT ≤ 43.2%; Q2: female 37.5% < HCT ≤ 39.3%, male 43.2% < HCT ≤ 44.9%; Q3: female 39.3% < HCT ≤ 41.0%, male 44.9% < HCT ≤ 46.8%; and Q4: female HCT > 41.0%, male HCT > 46.8%), and PWV was examined using general linear regression models adjusted for covariates, including age, sex, BMI, HR, FBG, blood lipids, and BP. SUA, TG, and HCT levels were log-transformed to make them normally distributed before analyses. The interactions between HCT and other

cardiometabolic risk factors were assessed by introducing a cross-product term into the regression model. All reported *p*-values were two-tailed. Variables with p values < 0.05 were considered statistically significant. SAS statistical software (version 9.1; SAS Institute, Inc.) was used for data management and statistical analyses.

Results

Characteristics of the participants based on HCT level

In total, 3225 participants were included in the analyses. Table 1 shows the characteristics of the study participants based on HCT level (in quartiles). Weight, BMI, neck circumference, waist circumference, body fat rate, oral glucose tolerance, insulin, TC, TG, LDL-C, SBP, DBP, overweight, hypertension, dyslipidemia, and SUA values showed significant

Table 2 The association between hematocrit and pulse wave velocity

	cfPWV		crPWV		cdPWV	
	Beta	*p*	Beta	*p*	Beta	*p*
Model 1	0.118	< 0.001	0.095	< 0.001	0.103	< 0.001
Model 2	0.114	< 0.001	0.063	0.006	0.067	< 0.001
Model 3	0.076	0.001	0.062	0.007	0.066	< 0.001
		27				

Model 1 was adjusted for sex, age, and BMI. Model 2 was adjusted for sex, age, BMI, SBP, and DBP. Model 3 was adjusted for sex, age, BMI, SBP, DBP, and FBG. Model 4 was adjusted for sex, age, BMI, SBP, DBP, FBG, TG, TC, LDL-C, HDL-C, SUA, HR, smoking status, drinking, and physical exercise

BMI, body mass index; *FBG*, fasting blood-glucose; *TC*, total cholesterol; *TG*, triglycerides; *HDL-C*, high-density lipoprotein cholesterol; *LDL-C*, low-density lipoprotein cholesterol; *SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *SUA*, serum uric acid; *HR*, heart rate

Table 1 Characteristics of participants by hematocrit levels (in quartiles)

Variables	Q1 (F, HCT < 37.5, M, HCT < 43.2)	Q2 (F, 39.3 > HCT ≥ 37.5, M, 44.9 > HCT ≥ 43.2	Q3 (F, 41.0 > HCT ≥ 39.3, M, 46.8 > HCT ≥ 44.9)	Q4 (F, HCT ≥ 41.0, HCT ≥ 46.8)	*p*
N	834	820	800	771	
Age	42.85 ± 10.59	44.63 ± 12.68	44.59 ± 11.04	43.09 ± 11.05	0.718
Sex (Male %)	539 (64.6%)	519 (63.3%)	510 (63.8%)	497 (64.5%)	0.938
Weight (kg)	67.73 ± 11.51	68.74 ± 11.69	68.77 ± 11.79	70.27 ± 11.87	< 0.001
BMI (kg/m^2)	24.10 ± 3.05	24.37 ± 3.02	24.36 ± 3.15	24.84 ± 3.11	< 0.001
NC (cm)	35.77 ± 3.37	35.83 ± 3.44	35.86 ± 3.51	36.38 ± 3.71	0.003
WC (cm)	84.71 ± 9.78	85.17 ± 9.83	85.19 ± 9.69	86.79 ± 9.75	< 0.001
HC (cm)	96.95 ± 6.25	96.98 ± 6.00	97.12 ± 6.27	97.67 ± 6.44	0.095
BFR%	26.20 ± 5.05	26.55 ± 5.05	26.96 ± 5.13	27.84 ± 5.01	< 0.001
FBG (mmol/l)	5.18 ± 1.24	5.18 ± 1.04	5.18 ± 0.97	5.25 ± 1.27	0.479
OGTT (mmol/l)	7.59 ± 3.44	7.18 ± 2.70	7.10 ± 2.37	7.28 ± 2.70	0.011
FINS (μU/ml)	8.05 ± 6.56	8.64 ± 6.04	9.18 ± 5.01	10.12 ± 5.51	< 0.001
TC (mmol/l)	4.88 ± 0.89	4.94 ± 0.86	5.07 ± 0.85	5.21 ± 0.92	< 0.001
TG (mmol/l)	1.45 ± 1.66	1.54 ± 1.42	1.69 ± 1.56	1.88 ± 1.63	< 0.001
HDL (mmol/l)	1.25 ± 0.30	1.24 ± 0.30	1.27 ± 0.31	1.25 ± 0.32	0.401
LDL (mmol/l)	2.87 ± 0.76	2.90 ± 0.76	3.02 ± 0.77	3.12 ± 0.78	< 0.001
SBP (mmHg)	121.39 ± 15.84	121.98 ± 15.69	123.86 ± 15.41	124.79 ± 16.14	< 0.001
DBP (mmHg)	77.31 ± 11.08	78.90 ± 11.13	79.93 ± 10.80	81.31 ± 11.33	< 0.001
SUA (umol/l)	286.55 ± 83.56	292.86 ± 77.25	301.47 ± 79.40	300.14 ± 77.37	< 0.001
Overweight	299 (35.9%)	334 (40.7%)	330 (41.2%)	366 (47.5%)	< 0.001
Hypertension	194 (23.3%)	194 (23.7%)	205 (25.6%)	212 (27.5%)	0.025
Dyslipidemia	171 (20.5%)	189 (23.1%)	205 (25.6%)	238 (30.8%)	< 0.001
Hyperglycemia	178 (21.3%)	189 (23.1%)	193 (24.1%)	191 (24.7%)	0.383

BMI, body mass index; *NC*, neck circumference; *WC*, waist circumference; *HC,* hip circumference; *BFR%,* body fat percentage; *FBG*, fasting blood-glucose; *OGTT*, oral glucose tolerance test;*FINS*, fasting insulin; *TC*, total cholesterol; *TG*, triglycerides; *HDL-C,* high density lipoprotein cholesterol; *LDL-C*, low-density lipoprotein cholesterol; *SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *SUA*, serum uric acid

differences across the HCT groups; in contrast, sex, age, and hyperglycemia were not significantly different.

Association between HCT level and central and peripheral arterial stiffness

Table 2 shows the associations between PWVs and HCT. In the models adjusted for age, sex, and BMI, HCT was positively associated with carotid femoral pulse wave velocity (cfPWV), carotid radial pulse wave velocity (crPWV), and carotid dorsalis pulse wave velocity (cdPWV) ($p < 0.001$). For each 1 SD increase in HCT, the cfPWV, crPWV, and cdPWV increased by 0.118 m/s, 0.095 m/s, and 0.103 m/s, respectively. Further adjustment for FBG and BP did not significantly change the association ($p < 0.01$). However, after adjusting for TG, TC, LDL, HDL, SUA, and HR, the association of HCT level with crPWV and cdPWV was no longer significant ($p = 0.090$ and $p = 0.053$, respectively). Meanwhile, the association between HCT level and cfPWV remained significant ($p = 0.007$). For each 1 SD increase in HCT, cfPWV increased by 0.065 m/s.

Association stratified by cardiometabolic risk factors

We examined the association between HCT and cfPWV in different cardiometabolic risk factor groups (Table 3). Considering the study power, we grouped the stratification factors (age, BMI, FBG, and TC) into three categories (tertiles). We found a significant BMI-dependence of the association between the HCT level and cfPWV ($p = 0.003$): The association was significant in the group with a BMI < 22.53 kg/m^2 ($p < 0.001$), but not significant in the groups with a BMI of 22.53–25.63 kg/m^2 ($p = 0.358$) and > 25.63 kg/m^2 ($p = 0.996$). HCT showed no significant interaction with sex, age, FBG level, or TC level in its effect on cfPWV.

After adjustment for age, sex, BMI, FBG level, blood lipid level, and HR, we found a significant interaction between hypertension status and the HCT level in the effect on cfPWV ($p = 0.0419$): The association between HCT level and cfPWV was significant among those with normal BP but not among those with hypertension (Fig. 1).

We also examined the association between HCT and cfPWV according to the number of MetS components. These two markers showed an additive effect on cfPWV (Fig. 2). The cfPWV was nearly 2.5 m/s higher in those with the highest number of MetS components and the highest HCT than in those with the lowest number of MetS components and the lowest HCT.

Discussion

In the present study, we found a significant association between HCT and cfPWV, a measure of central arterial stiffness, in adults in China. These associations were

Table 3 Associations between hematocrit and carotid-to-femoral pulse wave velocity, stratified by sex, age, and biomark

Variables		Q1 (F, HCT < 37.5, M, HCT < 43.2)	Q2 (F, 39.3 > HCT ≥ 37.5, M, 44.9 > HCT ≥ 43.2	Q3 (F, 41.0 > HCT ≥ 39.3, M, 46.8 > HCT ≥ 44.9)	Q4 (F, HCT ≥ 41.0, M, HCT ≥ 46.8)	*p* for trend	*p* for interaction
Sex	Female	9.57 ± 1.69	9.62 ± 1.34	9.92 ± 1.60	10.18 ± 1.57	0.014	0.081
	Male	10.84 ± 1.85	10.83 ± 1.78	10.87 ± 1.67	10.91 ± 1.61	0.045	
Age (years)	> 60	12.94 ± 2.53	12.66 ± 2.44	12.30 ± 2.44	12.68 ± 3.04	0.385	0.094
	< 60	10.06 ± 1.51	10.21 ± 1.53	10.39 ± 1.56	10.53 ± 1.43	0.002	
BMI (kg/m^2)	< 22.53	10.08 ± 1.94	9.92 ± 1.56	10.24 ± 1.79	10.55 ± 1.86	< 0.001	0.003
	22.53~25.63	10.36 ± 1.86	10.45 ± 1.81	10.53 ± 1.72	10.64 ± 1.59	0.358	
	> 25.63	10.87 ± 1.81	10.84 ± 1.72	10.79 ± 1.55	10.75 ± 1.53	0.996	
FBG (mmol/l)	< 4.77	10.08 ± 1.92	10.08 ± 1.57	10.27 ± 1.66	10.16 ± 1.40	0.712	0.912
	4.77~5.20	10.17 ± 1.60	10.18 ± 1.47	10.38 ± 1.49	10.63 ± 1.52	0.054	
	> 5.20	10.97 ± 2.02	10.94 ± 1.99	10.90 ± 1.87	11.13 ± 1.79	0.014	
TC (mmol/l)	< 4.58	10.14 ± 1.69	10.29 ± 1.72	10.31 ± 1.76	10.46 ± 1.41	0.062	0.326
	4.58~5.32	10.33 ± 1.90	10.24 ± 1.67	10.46 ± 1.59	10.59 ± 1.79	0.011	
	> 5.32	10.77 ± 2.04	10.68 ± 1.76	10.79 ± 1.75	10.80 ± 1.61	0.343	

Analyses were adjusted for age, BMI, sex, smoking status, drinking, and physical exercise and biomarkers (when they were not the strata variables). *BMI*, body mass index; *FBG*, fasting blood glucose; *TC*, total cholesterol

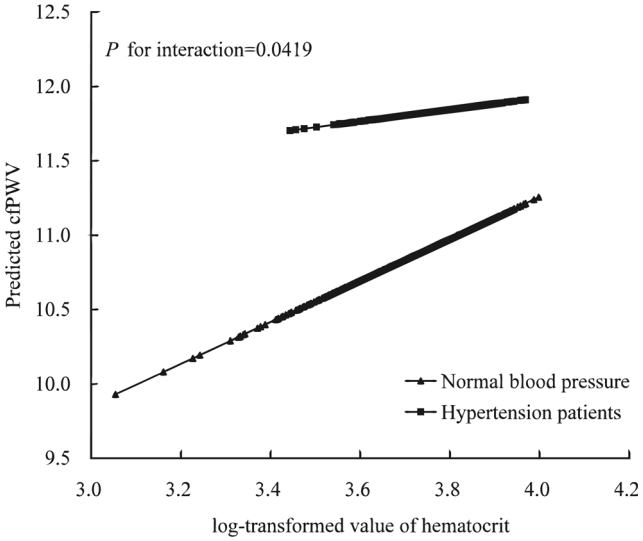

Fig. 1 The association between HCT and cfPWV in different blood pressure status. (cfPWV carotid femoral pulse wave velocity; HCT, hematocrit)

Log transformation was used for hematocrit which value is abnormal distribution. Figure 1 shows different blood pressure status of participant change the association between HCT and cfPWV. Compare the hypertension patients, cfPWV of normal blood pressure participants increase with HCT more notably. There is an interaction between HCT and blood pressure to cfPWV.($p = 0.0419$).

independent of conventional risk factors, including sex, BMI, BP, blood lipids, and glucose metabolism. HCT level was not associated with peripheral arterial stiffness measured by crPWV and cdPWV. In addition, we found significant interactions of HCT with age, hypertension, and MetS in the effect on cfPWV.

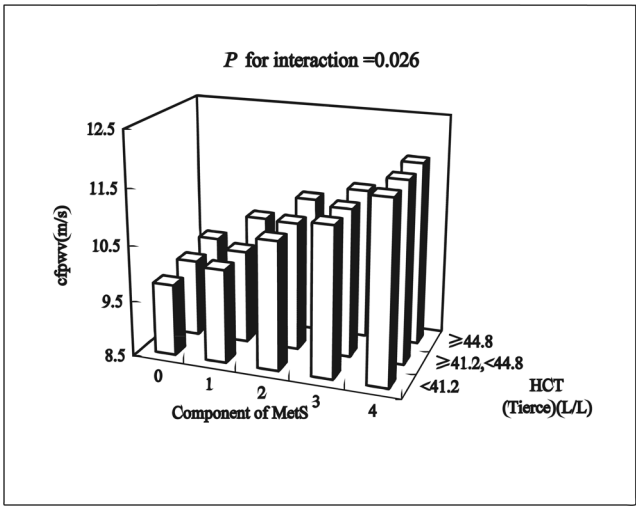

Fig. 2 The association between HCT and cfPWV in different number of MetS components. (cfPWV, carotid femoral pulse wave velocity; HCT, hematocrit). These two markers showed an additive effect on cfPWV. The cfPWV was nearly 2.5 m/s higher in those with the highest number of MetS components and highest HCT than that in those with the lowest number of MetS components and lowest HCT

Several previous studies have shown that high HCT levels are associated with cardiovascular diseases. In a study conducted in Bulgaria, HCT correlated with intima-media thickness in carotid atherosclerosis and with other major risk factors for carotid atherosclerosis, including hypertension and coronary heart disease [15]. In a cross-sectional study, it was demonstrated that the HCT level was elevated in patients with

[29] 16]. Our results are consistent with the findings of these studies. Our data indicate that HCT was positively correlated with PWV, an early indicator of atherosclerosis. After adjustment for age, sex, BMI, FBG level, blood lipid level, and HR, the association of HCT with crPWV or cdPWV, signifying peripheral arterial stiffness, was not significant. However, the association between HCT and cfPWV, signifying central arterial stiffness, remained significant. This suggests that HCT is an independent risk factor for central arterial stiffness in adults in China.

In this study, we found that age modified the deleterious effects of HCT on central arterial stiffness; this association was stronger in older subjects. In a retrospective, observational study, cfPWV showed a positive correlation with early carotid atherosclerosis in elderly patients [17]. Increased HCT levels may be beneficial due to the related increase in blood viscosity with ageing [18]. Carallo et al. reported a marked increase in blood viscosity with ageing, which suggests a role of factors directly affecting red blood cell membranes [19]. High oxidative stress decreases antioxidant defences, which in turn decreases erythrocyte membrane fluidity in elderly adults [20, 21]. Ageing causes an increase in blood viscosity, probably through a direct effect on erythrocyte membranes; this may contribute to the risk of atherosclerosis in advanced age [19]. The results of our study suggest that older individuals may be more sensitive to the atherosclerosis-promoting effect of elevated HCT level.

Interestingly, we found that the adverse effects of HCT level on central arterial stiffness appeared to be more evident in participants with normal BP than in those with hypertension. Notably, on an average, the cfPWV was much lower and its variation was larger in individuals with normal BP than in those with hypertension (Fig. 1). Given the low variation, it is not surprising that no association was observed between HCT level and cfPWV in participants with hypertension. A recent study reported that higher HCT level, even within the normal range, was associated with the incidence of hypertension irrespective of other cardiovascular risk factors in men [22]. Arterial stiffness is strongly associated with hypertension [23]. PWV reflects functional and structural changes in the vascular wall structure, a potential cause of future BP elevation [24]. The present findings suggest that a high HCT level may play a more important role in arterial stiffness before the development of hypertension. This interesting finding may have an important clinical implication in reducing the risk

and thereby preventing adverse cardiovascular events in people with early atherosclerosis. Further investigations are required to explore the potential mechanisms.

MetS is a cluster of risk factors including elevated TG level, decreased HDL-C level, hyperinsulinemia, and hypertension [25]. MetS is an increasingly prevalent risk factor for cardiovascular diseases. MetS is reportedly associated with alterations in the arterial system [26, 27] and inflammation [28]. MetS also accelerates arterial ageing [29]. A previous study showed that elevated TG level, BP and FBG level, and abdominal obesity were associated with a twofold higher odds ratio of presenting with extremely stiff arteries [30]. In our study, we found that HCT and components of MetS exerted an additive effect on central arterial stiffness. Kawamoto et al. found that HCT level was positively associated with insulin resistance and the prevalence of MetS in community-dwelling individuals in Japan [31]. In a cross-sectional study of 1339 patients, Lohsoonthorn et al. reported that HCT concentration increased with an increasing number of MetS components [32]. A high HCT level has been correlated with various metabolic changes. We assume that certain changes may interact with MetS components in promoting and accelerating the development of atherosclerosis. Further investigation is warranted to explore the potential mechanisms.

While the sample size of this study was large, the study had several limitations. First, since the study was cross-sectional, a causal relationship between HCT level and central arterial stiffness could not be established. Second, potential confounding factors that may exist as risk factors, such as cigarette smoking, alcohol intake, and lifestyle factors, as well as intima-media thickness and the ankle-brachial index, are complementary parameters for evaluating the severity of early atherosclerosis that were not measured in our study. Finally, the study included only participants from China. To further confirm our findings, additional studies must be conducted with individuals of different races and ethnicities.

Conclusions

In summary, in the present study, we showed that HCT level was associated with elevated central arterial stiffness, independent of conventional cardiovascular risk factors in adults in China. In addition, we found that age, hypertension, and MetS amplified the impact of HCT on central arterial stiffness.

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Availability of data and materials All data generated or analysed during this study are included in this manuscript.

Authors' contributions CY Z and XK L were the performer of this study and written this manuscript; QQ Q performed the recruitment of patients; J L and HF G were the guarantor of integrity of the entire study and responsible for the study concepts, study design, and approval of the final version of the manuscript; L Q was responsible for the analysis the data onto this study.

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Compliance with ethical standards

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

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was reviewed and approved by the ethics committee of the Central Hospital of Xuzhou, Affiliated Hospital of Medical School of Southeast University, China. (No. 2015.428).

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

Consent for publication None application.

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ORIGINAL ARTICLE

Phenotypic characterization of patients with type 2 diabetes mellitus

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Abstract

Introduction Type 2 diabetes mellitus harbors heterogeneity. This study describes the phenotypic subgroups based on anthropometric and biochemical characteristics.

Methods This was a cross-sectional study involving 96 patients with early type 2 diabetes conducted from July 2017 to December 2018. Clinical history was recorded and anthropometric measurements were done. Beta cell function was estimated using oral disposition index (DIo). Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the fasting plasma glucose and fasting insulin levels. The cut off to define insulin resistance was 1.93.

Results The median age was 53.5 years. Fifty-five (57.3%) patients were males. Thirty-two percent of females and 33% of males had a BMI of less than 23 kg/m^2. The average DIo was 0.54. Insulin resistance was present in 59 (61.5%) of the total patients. Three subgroups of patients were discernible. One, predominantly insulin resistant (HOMA-IR > 1.93)]: 61.45%; second, predominant beta cell dysfunction (HOMA-IR 1–1.93)]: 26%; and third, severely reduced beta cell function (HOMA-IR < 1)]: 12.5%. Insulin-resistant men could be identified with 87.5% specificity if BMI of more than 25.56 and waist circumference (WC) more than 91.8 cm. Insulin-resistant women could be identified with 100% specificity if their WC was more than 97 cm.

Conclusion Three phenotypic subgroups were discernible with 40% of patients having predominant insulin deficiency. It is important to identify patients belonging to these subgroups so as to tailor their treatment accordingly.

Keywords Type 2 diabetes mellitus · Phenotype · Subgroups · Beta cell dysfunction

Introduction

Type 2 diabetes mellitus (DM) constitutes about 85 to 95% of all DM. Majority of patients with type 2 DM have obesity in the Western population [1]. The clinical phenotypes in India are different when compared to them, with around 30% of the patients being lean [2]. The typical description of a patient with type 2 DM in India is that of an Asian Indian phenotype with characteristics of visceral obesity and insulin resistance even though the BMI is lower than what is seen in the West [3]. Type 2 DM can occur due to predominant insulin resistance or predominant beta cell dysfunction. Though the insulin resistance is highlighted in the "Asian Indian phenotype," predominant beta cell dysfunction has been emphasized in recent studies [4]. In these studies, beta cell dysfunction has been demonstrated in patients with any degree of insulin resistance. This role of early beta cell dysfunction in the pathophysiology of diabetes may be especially important in populations or subgroups where the prevalence of type 2 DM is high at a lower body mass index, a phenomenon that is quite common in populations of emerging economies. Life style interventions as a preventive or treatment modality may have a blunted effect in these patients [5]. Based on these heterogeneous findings, it has been suggested to subclassify type 2 DM as one with predominant insulin resistance and the other with predominant reduced insulin secretion [5]. Similar heterogeneity has been noted in other countries. In a recent, data-driven cluster analysis of newly diagnosed Swedish diabetes cohorts, Ahlqvist et al. had found five subgroups [6]. They ranged from extreme insulin deficiency to extreme insulin resistance. With these new developments, it is imperative to investigate the clinical and biochemical characteristics of patients with type 2 DM [7]. A better understanding of the pathophysiology may help in better tailoring of the available treatment modalities [8].

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Methodology

This was a cross-sectional observational study conducted in the Medicine outpatient department and Diabetes Clinic from July 2017 to December 2018 after obtaining clearance from the Institute Ethics Committee. Patients with early type 2 DM were included. A sample size (96 patients) was calculated in order to obtain proportions of subgroups with 10% level of precision and a confidence interval of 95%. Patients were excluded if there was a history of diabetic ketoacidosis, if they were receiving corticosteroids, if there was a history of pancreatitis or presence of pancreatic calcification on an abdominal X-ray, if patients were receiving sulphonylureas or insulin, and if they had any microvascular or macrovascular complication. Informed written consent was obtained from all patients. The demographic details, disease-related history, and family history were collected as per proforma. A review of hospital records if available was done. Anthropometric measurements such as height, weight, BMI, waist circumference, hip circumference and skinfold thickness over triceps, subscapular area, suprailiac region, and abdomen were measured. For body fat percentage, body density was first calculated by the Jackson-Pollock skinfold method and later body fat percentage was calculated using the Siri formula [9]. Five millilitres of venous blood were collected from the median cubital vein in a fasting state for estimation of fasting plasma glucose levels, fasting insulin levels, HbA1c, serum adiponectin levels, and highly sensitive C-reactive protein. Then patients were given 75 g of oral glucose, and 3 ml of venous blood was collected after 30 min. This sample was subjected for estimation of plasma glucose and plasma insulin levels by ELISA.

Beta cell function was estimated using oral disposition index (DIo). It was calculated using the formula: $DIo = ([\Delta I_{0–30}/\Delta G_{0–30}] \times [1/\text{fasting insulin}])$. Homeostatic model assessment for insulin resistance (HOMA-IR), a measure of insulin resistance was calculated from the computer model (HOMA 2) available from University of Oxford (https://www.dtu.ox.ac.uk/ToolsSoftware) using the fasting plasma glucose and fasting insulin levels. The HOMA-IR cut off to define insulin resistance was 1.93. This had been obtained in an epidemiological study done in Chennai, wherein data had been collected for 1072 individuals. The 75th percentile HOMA-IR value (derived by the first-generation formula) was 1.93 [10].

The data was tabulated using Microsoft Excel software and analyzed using Statsdirect software, version 2.7.9. Descriptive statistics were used to summarize the results. The continuous variables were expressed in mean and standard deviation (SD) if the distribution was normal. It was expressed as median and inter-quartile range (IQR) if the data was skewed. The categorical variables were expressed in the form of proportions. The Pearson correlation coefficient was used to measure the strength of association between continuous variables. Multiple linear regression analysis was performed to examine the relationship between HOMA-IR and variables that found to have a significant relationship during univariate analysis. The Kruskal-Wallis test was used to compare continuous variables among the subgroups of diabetes patients. Analysis of the receiver-operating-characteristic (ROC) curve was used to assess the diagnostic accuracy and to derive best cut off values of BMI and WC in predicting HOMA-IR of more than 1.93. It was considered to have no discriminatory value if the area under the ROC curve (AUC) was 0.5 or less. All statistical analysis was carried out at 5% level of significance, and a p value < 0.05 was considered as significant.

Results

A total of 241 patients with type 2 DM were screened and 96 patients were recruited into the study during the period between July 2017 and December 2018.

Clinical characteristics (Table 1)

The median age of the patients was 53.5 years. Fifty-five (57.3%) patients were males. Type 2 DM had been newly diagnosed in 18 (18.8%) patients. The duration of diabetes was less than 1 year and 1 to 2 years in 32 and 46 patients respectively. Forty (41.7%) patients had a family history of DM. Thirty patients (31.3%) were not on any treatment. Sixty-six patients were on metformin. Twenty-three (24%) patients had hypertension and two patients had bronchial asthma. The mean FBS was 181.3 (± 85.4) mg/dl. The mean HbA1C was 8.7 (± 2.1) %.

Table 1 Clinical characteristics of the patients ($N = 96$)

Characteristic	Number of patients (n (%) or median (IQR) or mean (SD))
Median age in years	53.5 (48–60)
Males	55 (57.3%)
Females	41 (42.7%)
Place: Tamil Nadu	63 (66%)
Pondicherry	33 (34%)
Duration of diabetes	
Newly detected	18 (18.8%)
Less than 1 year	32 (33.3%)
1 year to 2 years	46 (47.9%)
Mean FBS (mg/dl)	181.3 (± 85.4)
Mean HbA1c (%)	8.7 (± 2.1)
Treatment naive	30 (31.3%)
Metformin dose less than 1 g	22 (22.9%)
Metformin dose 1 g to 2 g	44 (45.8%)
Comorbidities	
Hypertension	23 (23.9%)
Bronchial asthma	2

Anthropometric characteristics and adiposity (Fig. 1)

The mean BMI in males was 24.3 kg/m^2 (± 2.9), and it was 24.9 kg/m^2 (± 3.2) in females. Thirty-two percent of females and 33% of males had a BMI of less than 23 kg/m^2. The body fat percentage was higher in females (29.8%) when compared with males (26.5%). The average waist circumference was higher in women (93.3 cm) when compared to men (89.9 cm). The waist to height ratio a reflection of this was higher in women. The males had a higher mean waist-hip ratio (0.94) than the females (0.92).

Insulin resistance and beta cell function

The average oral disposition index among the study population was 0.54 with males having an average DIo of 0.40 compared to females with an average DIo of 0.72. DIo did not correlate with age ($r = 0.1$), BMI ($r = 0.12$), or the duration of diabetes in months ($r = 0.09$). The mean fasting insulin level in the study population was 17.3 (± 12.4) μU/ml. Patients with HOMA-IR of more than 1.93 had a higher fasting insulin level of 23.39 (± 12.1) μU/ml. The rest also had low DIo but the fasting insulin levels were low (mean 3.6 μU/ml in patients with HOMA-IR < 1) representing insulin deficiency.

Average HOMA-IR was 3.45 in males and 2.98 in females. Insulin resistance (HOMA-IR > 1.93) was present in 59 (61.5%) of the total patients in whom the average HOMA-IR was 4.5 (± 4.3). Having a HOMA-IR of less than 1.93 were 46.3% of females and 32.7% males. Twelve (12.5%) of the patients had a HOMA-IR of less than one.

Presence of acanthosis nigricans was 76% sensitive and 47% specific for insulin resistance (HOMA-IR > 1.93). Proportion of patients with BMI of > 23 kg/m^2 having insulin resistance was 78.4% for men and 57% for women. HOMA-IR showed positive correlation in males with BMI ($r = 0.44$, $p = 0.000$) and WC ($r = 0.41$, $p = 0.001$), but there was no such correlation in females. Multiple linear regression analysis using HOMA-IR as dependent variable did not show BMI ($r = 0.19$, $p = 0.15$) and WC ($r = 0.09$, $p = 0.51$) as significant independent predictors in males.

The average serum adiponectin value among the patients with type 2 DM was 3.06 mcg/ml. Females had a higher

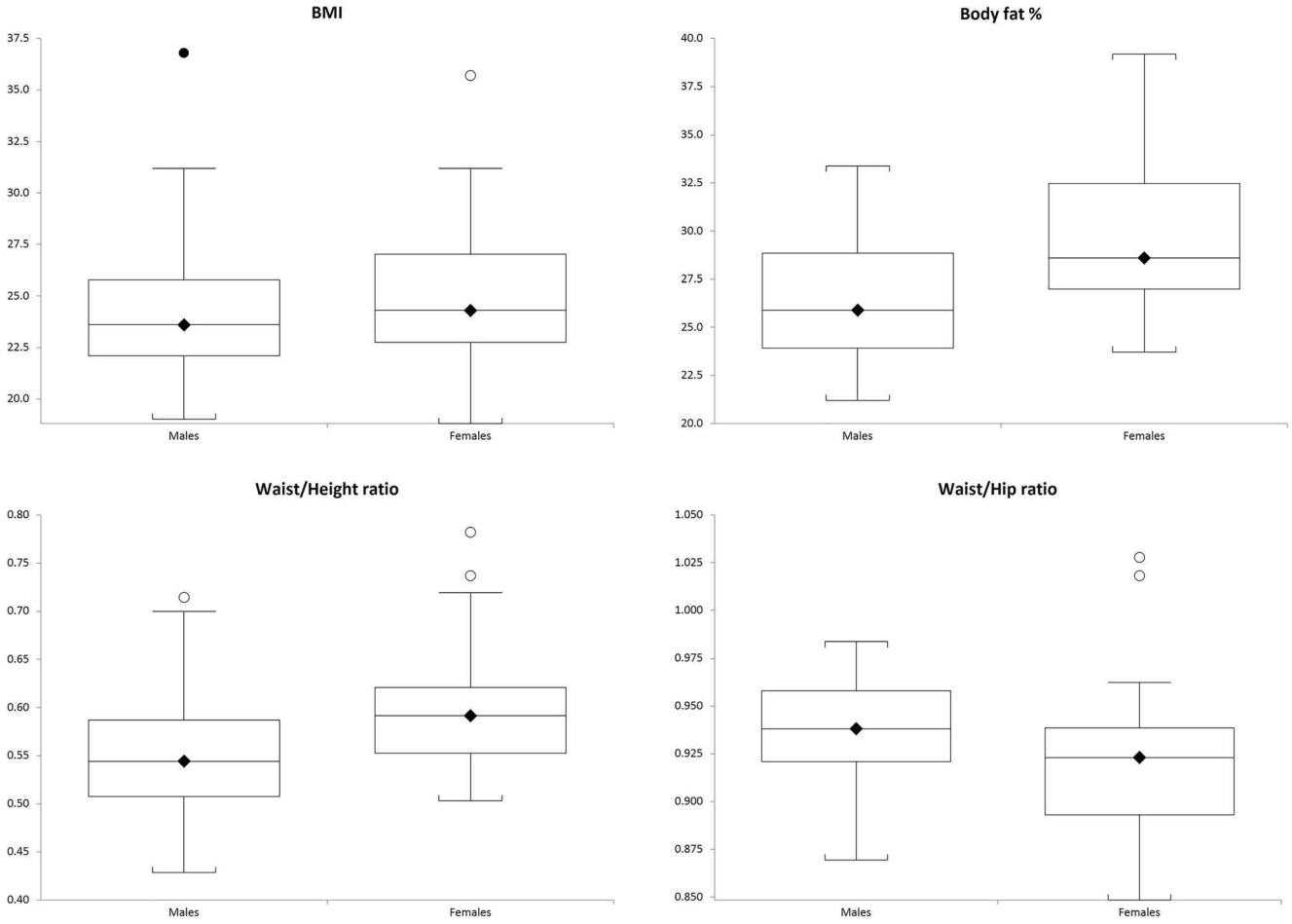

Fig. 1 Anthropometric characteristics of patients presented gender-wise

average serum adiponectin of 4.1 mcg/ml compared to males whose average serum adiponectin was 2.3 mcg/ml. The average hsCRP value among the study population was 1.6 mg/dl with males and females having an average hsCRP value of 1.4 mg/dl and 1.7 mg/dl respectively. Serum adiponectin and hsCRP levels did not correlate with HOMA-IR. hsCRP levels also did not correlate with FBS.

Phenotypic subgroups

The following subgroups were obtained based on HOMA-IR values:

Group 1. [Predominantly insulin resistant (HOMA-IR > 1.93)]: 61.45% (CI 51.5–70.6%)
Group 2. [Predominant beta cell dysfunction (HOMA-IR 1–1.93)]: 26% (CI 18–35%)
Group 3. [Severely reduced beta cell function (HOMA-IR < 1)]: 12.5% (CI 7.3–20.6%)

The characteristics of patients belonging to the subgroups have been provided in Tables 2 and 3 gender-wise. BMI was different in the three subgroups in males, but not in females. WC was significantly different in both males and females. Acanthosis nigricans was not discriminatory in females. Serum adiponectin and hsCRP levels were more in the subgroup with insulin deficiency in males, but the opposite was true in females.

Clinical differentiators of the subgroup with insulin resistance (HOMA-IR > 1.93)

Considering two subgroups, one with HOMA-IR more than 1.93 (with insulin resistance) and other with HOMA-IR less than 1.93, and considering males and females separately, the following results were obtained. BMI, WC, and acanthosis nigricans were considered for differentiating these two subgroups. In males, using ROC analysis, a BMI cut off of 25.56 provided a 83% specificity (AUC = 0.72) in identifying insulin-resistant men. Similarly, WC cut off of 91.8 cm provided 84% specificity (AUC 0.73) (Fig. 2).

In women, difference in BMI was not statistically significant between insulin-resistant women and those without insulin resistance. A WC cut off of 97 cm provided a 100% specificity (AUC = 0.76) in identifying insulin-resistant women. The odds ratio for having insulin resistance in women, when acanthosis nigricans was present, was 4.56.

With our data set, insulin-resistant men could be identified with 87.5% specificity if they had a BMI of more than 25.56 and WC of more than 91.8 cm. Insulin-resistant women could be identified with 100% specificity if their waist circumference was more than 97 cm.

Discussion

In this study, we have described the anthropometric characteristics, insulin resistance pattern, and beta cell function in 96 adult patients (mean age 54 years) with an early type 2 DM. Males had constituted 57% of the study population.

Oral disposition index was severely reduced in all the patients and this was similar to a study done in Chennai by Staimez et al. [4]. Using a cut off for HOMA-IR of 1.93, 60% of the patients had insulin resistance. So, 40% of diabetes patients did not have significant insulin resistance, and this proportion was larger among females than males (46% vs. 33%). These patients had predominantly insulin deficiency. We have not investigated for the presence of anti-glutamic acid decarboxylase antibodies as one of the causes for insulin deficiency. Primary beta cell dysfunction may be the cause for diabetes in these patients. This has been demonstrated in previous studies. In a study from Chennai, investigating beta cell function and insulin resistance in a spectrum of individuals having normoglycemia to diabetes, it has been shown that beta cell function was reduced in any form of dysglycemia, and it

Table 2 Characteristics of male patients according to the HOMA-IR-based subgroups

Characteristic	HOMA-IR < 1 ($n = 4$)	HOMA-IR 1–1.93 ($n = 14$)	HOMA-IR > 1.93 ($n = 37$)	p value
Average age (years)	51.5	52.21	53.1	$p = 0.95$
Average BMI	22.6	23.1	25	$p = 0.02$
Average WC (cm)	84	86	92	$p = 0.02$
Waist/height ratio	0.52	0.53	0.56	$p = 0.08$
Waist/hip ratio	0.91	0.93	0.95	$p = 0.01$
Average HbA1C (%)	8.9	9.24	8.57	$p = 0.85$
Acanthosis nigricans present	0%	28%	43%	$p = 0.18$
Average adiponectin (μg/ml)	3.32	2.89	1.97	$p = 0.006$
hsCRP (mg/L)	3.04	1.88	1.1	$p = 0.02$

Table 3 Characteristics of female patients according to the HOMA-IR-based subgroups

Characteristic	HOMA-IR < 1 ($n = 8$)	HOMA-IR 1–1.93 ($n = 11$)	HOMA-IR > 1.93 ($n = 22$)	p value
Average age (years)	58.25	54.3	54.81	$p = 0.45$
Average BMI	24	23.8	25.8	$p = 0.27$
Average WC (cm)	87	87	97	$p = 0.01$
Waist/height ratio	0.56	0.58	0.62	$p = 0.05$
Waist/hip ratio	0.9	0.9	0.93	$p = 0.09$
Average HbA1C (%)	8.02	9.32	8.83	$p = 0.29$
Acanthosis nigricans present	25%	27%	54%	$p = 0.18$
Average adiponectin (μg/ml)	3	4.04	4.52	$p = 0.31$
hsCRP (mg/L)	1.14	1.25	2.22	$p = 0.83$

appeared to contribute more than the insulin resistance to the dysglycemic state [4]. The predominant-insulin secretory defect has been found across Southeast Asia but with ethnic differences. Among Asian men, it has been found that Malays have the lowest beta cell function when compared to Chinese and Asian Indians [11]. Similar results have been found in Danish patients but not in Japanese patients [4].

We have chosen to subclassify the patients based on the degree of insulin resistance as this is an important determinant of therapy. This yielded three subgroups, but primarily two or one groups with insulin resistance (60%) and other with predominant insulin deficiency (40%). It is already known that these subgroups exist [12], and our study provides their relative proportions at our setting. Other Indian studies providing information on this are not yet available. In a recent data-driven cluster analysis of newly diagnosed Swedish diabetes cohorts, Ahlqvist et al. have found five subgroups [6]. This is based on the age of onset, HbA1c, insulin sensitivity, β-cell function, and body mass index. Two subgroups were insulin-deficient and the other three had varying degrees of insulin resistance. The total proportion of insulin-deficient patients was 24% which is lower than found in our study population (40%).

It is important to differentiate the subgroup of patients who are insulin-deficient from those who are predominantly insulin-resistant. It can be noted that, in the Swedish study, the cluster 2 which comprised of insulin-deficient patients received metformin in higher proportion than compared to cluster 3 which comprised of insulin-resistant patients [6]. At present, fasting C-peptide, plasma glucose, and GADA levels have been recommended to allow proper classification of newly diagnosed patients with diabetes [13]. Following this may not be possible in resource limited settings [14]. With our data set, we have found that insulin-resistant men can be identified with 87.5% specificity if they have a BMI of more than 25.56 and WC of more than 91.8 cm. Insulin-resistant women can be identified with 100% specificity if their waist circumference is more than 97 cm.

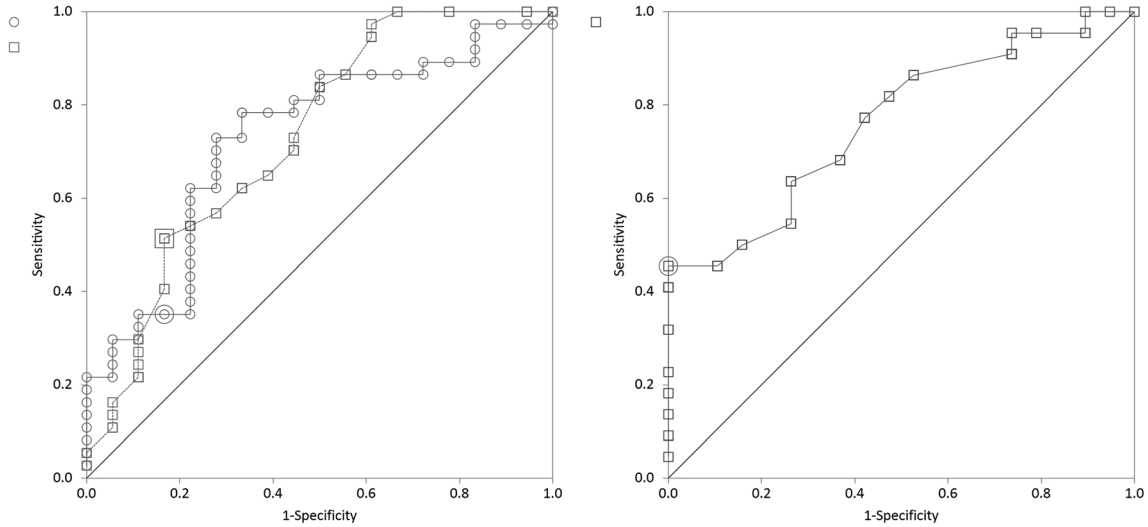

Fig. 2 Receiver-operating-characteristic (ROC) curves. Depicted for the body mass index (BMI) (AUC = 0.72) and waist circumference (WC) (AUC = 0.73) in males and WC alone (AUC = 0.76) in females for the presence of insulin resistance (HOMA-IR > 1.93)

There are limitations to the study. First, with a better sample size, we would have been able to provide the proportion of patients with insulin deficiency with a higher precision. Similarly, a sample size of 48 (current 41) of female patients would have provided a reliable waist circumference cut off value to predict insulin resistance. Second, since 70% of the patients were receiving metformin, it may have affected insulin sensitivity [15]. But, recently, the target of metformin has been found to be a mitochondrial enzyme [16]. Its selective inhibition leads specifically to the inhibition of gluconeogenesis. This should now be considered as the main mechanism of action [16]. It also has been demonstrated that metformin does not affect insulin sensitivity at the muscle and adipose tissue [17]. Third, a subset of patients with insulin resistance but with a BMI of less than 23 kg/m^2 has not been explored further here. Fourth, the applicability of the study should be considered limited to this region until studies are done in other parts of India.

In conclusion, three phenotypic subgroups were discernible with 40% of patients having predominant insulin deficiency. It is important to identify patients belonging to these subgroups so as to tailor their treatment accordingly.

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Compliance with ethical standards

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

Ethics approval and consent to participate The study was approved by Institute Ethics Committee JIP/IEC/2016?1138 and written informed consent was obtained from all the participants.

Ethics statement All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments.

Consent for publication Not applicable as this contains only de-identified information.

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ORIGINAL ARTICLE

Changes in SFRP5, WNT5A, HbA1c, BMI, PBF, and insulin resistance in men with type 2 diabetes after 12 weeks of combined exercise (HIIT and resistance)

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Abstract

Background Secreted frizzled-related protein 5 (SFRP5) and WNT family member 5A (WNT5A) have been found to associate with glucose metabolism and insulin sensitivity through noncanonical WNT signaling. The purpose of this study was to investigate changes in SFRP5, WNT5A, glycated hemoglobin (HbA1c), body mass index (BMI), percentage body fat (PBF), and insulin resistance in men with type 2 diabetes after 12 weeks of combined exercise (high-intensity interval training (HIIT) and resistance) and examine the relationship between these variables in men with type 2 diabetes.

Methods Nineteen men with type 2 diabetes (mean age of 58.3) were divided into two groups: experimental group (10 individuals) and control group (9 individuals). The experimental group performed 12 weeks of combined exercise (HIIT and resistance).

Results Twelve weeks of combined exercise (HIIT and resistance) increased SFRP5 serum level and decreased WNT5A serum level, HbA1c, insulin resistance, BMI, and PBF in the intervention group compared with the control group. SFRP5 had a significant negative relationship with WNT5A, insulin resistance, BMI, and PBF. In addition, BMI had a significant positive relationship with WNT5A, insulin resistance, and PBF.

Conclusion The improvement of type 2 diabetic men's health can be a result of increased anti-inflammatory factor SFRP5 and decreased inflammatory factor WNT5A through reduction of BMI and PBF.

Trial registration IRCT20151026024717N3

Keywords SFRP5 · WNT5A · HbA1c · Exercise · Insulin resistance

Introduction

Type 2 diabetes is a chronic disease resulting in debilitating complications in the body. This disease includes a group of hyperglycemic disorders caused by defects in insulin secretion or action. Lifestyle and genetic factors lead to the development of this disease. It spreads among all age groups throughout the world. In 2010, nearly 220 million people with type 2 diabetes were reported, a 50% increase over 2001, and it is estimated that this number will exceed 366 million by 2030 [1]. It has been clearly evidenced that increasing prevalence values of obesity and sedentary lifestyle are among the most important factors in the incidence of type 2 diabetes. Studies have shown that various proteins are involved in glucose homeostasis and inflammatory processes of adipose tissue associated with diabetes. Several adipokines have been identified so far are associated with glucose metabolism and insulin sensitivity. Among these adipokines, some improve insulin sensitivity and others play a part in the development of insulin resistance [2]. SFRP5 and WNT5A are among these adipokines. SFRP5 is a member of the secreted frizzled-related protein (SFRP) family that increases the expression of pro-inflammatory cytokines [3]. SFRP family has different roles. The function of SFRP1-5 in modifying the WNT pathway has been revealed in studies. Direct evidence for the direct interaction of SFRPs with WNT ligand has also been provided in studies [4]. The molecular mechanism of WNT

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signaling is divided into two groups of beta-catenin dependent (canonical) and beta-catenin independent (noncanonical) [5]. WNT5A is a glycoprotein that increases inflammatory responses by activating the noncanonical WNT signaling pathway. SFRP5 connects to the frizzled receptors competing with WNT5A and inhibits the noncanonical WNT signaling. Therefore, increasing the expression of WNT5A along with decreasing the expression of SFRP5 leads to an increase in inflammatory symptoms and insulin resistance. It has been argued that the balance between WNT5A and SFRP5 can control inflammation and insulin sensitivity [6]. Therefore, creating balance between these two factors is important for people with type 2 diabetes.

Combined exercise, which involves performing both aerobic and resistance exercise in the form of a workout protocol, is recognized as the most complete type of exercise for health and therapeutic purposes by the Diabetes Association. So far, there has been limited information about the role of combined exercise in anti-inflammatory adipokines such as SFRP5 and WNT5A inflammatory factor and their effects on inflammation and their mechanism of action. In this regard, Fayaz et al. (2019) investigated the effect of 12 weeks of high-intensity aerobic exercise with or without cinnamon extract on the noncanonical WNT pathway in ovariectomized rats and presented these findings: reduction in LDL indices, insulin resistance, and TNF-α and increase in serum SFRP5 level in the exercise group, the supplement group, and the exercise with supplement group. Also, serum WNT5A decreased only in the exercise with supplement group [7]. In general, due to the limitations of conducted research studies and lack of definite results on the effect of combined exercises (HIIT and resistance) on levels of these inflammatory and anti-inflammatory markers, researchers of this study decided to investigate the effect of 12 weeks of combined exercise (HIIT and resistance) on SFRP5, WNT5A, HbA1c, glucose and insulin serum levels, insulin resistance, and anthropometric indices in men with type 2 diabetes.

Materials and methods

This research was conducted following an intervention study design and was approved by the Regional Scientific Ethics Committee, Ferdowsi University of Mashhad, in accordance with the ethical standards of the 1983 Helsinki declaration. The study was performed from February to May 2019 in Behnood Rehabilitation Center, Sabzevar, Iran. The inclusion criteria of the study were as follows: having type 2 diabetes for more than 1 year, being treated only with oral hypoglycemic drugs, and sedentary lifestyle. The exclusion criteria of the study were these conditions: having retinopathy, nephropathy, and severe neuropathy, history of serious cardiovascular diseases, stroke, and having musculoskeletal problems limiting physical activity. Before conducting the research, subjects were asked to complete medical history and exercise preparation questionnaires (PAR-Q) and were referred to a physician for health checkup. The sample size was measured 8.36 for each group according to the study by Kadam and Bhalerao [8]. Sample size requirements were as follows: $p < 0.05$, power of study = 80%, and effect size = 1.37. To ensure that the number of subjects in each group would not reach less than 9 considering probable sample loss, 22 subjects were selected after obtaining approval of the physician and were divided into two groups of control (11 subjects) and experimental (11 subjects) by restricted randomization (random allocation rule) using the SAS software. During the study, 3 subjects (one in the experimental group and two in the control group) were excluded from the study due to their report on changes in their medication regimen.

Training protocol

The combined exercise program was designed based on the previous literature and existing prescribed guidelines for patients with type 2 diabetes [9–11], which lasted for 12 weeks and three sessions per week. Each exercise session included two exercise protocols. The first protocol was to perform HIIT exercise on a treadmill, which included three 4-min courses with an intensity of 70–90% HR_{max} done with a 3-min rest with an intensity of 50–70% HR_{max} after each course. The second protocol was to perform resistance exercise in the range of 55–80% of one repetition maximum with details shown in Table 1. Resistance exercises in each session consisted of 6 exercises: leg press, chest press, front thigh, lat pull-down, back thigh, and shoulder press which included the main muscles of the upper and lower extremities. Heart rate was monitored throughout the session using a digital heart rate meter, wearing the chest belt and a wrist-strapped heart rate monitor (Beurer® PM60, Germany). If the heart rate exceeded 90% HR_{max} or reached below 70% HR_{max}, exercise intensity was decreased or increased, respectively. Subjects' systolic and diastolic blood pressures were measured using standard clinical methods to ensure the health of the subjects before and after each exercise session in a rest state with the ALPK2 V-500 pressure gauge. There was a nurse and a personal trainer at all training sessions. There were no injuries or collapses during the entire training program. Control group subjects were advised to continue their sedentary lifestyle until the end of the project, and they were asked to inform researchers about any changes in their medications. All participants were under treatment with different dosages and types of oral anti-diabetic agents (metformin, glibenclamide, pioglitazone, and repaglinide) without any changes in their medication regimens for the past month and continued their medications throughout the study.

Table 1 Resistance exercise

Mesocycles	Number of sets	Number of repetitions per set	Intensity (a percentage of one repetition maximum)	Rest time (s/second)	
				Between sets	Between exercises
Weeks 1–3	2	15–18	55–65	60–90	90–120
Weeks 4–6	3	12–15	60–70	60–90	90–120
Weeks 7–9	3	10–12	65–75	90–120	120–150
Weeks 10–12	3	8–10	70–80	90–120	120–150

Anthropometric test

In order to evaluate the composition of the body, subjects' heights were measured with SECA height gauge (made in Germany) with an accuracy of 5 mm, and subjects' weights and PBFs were measured with a body composition analyzer (InBody-720 model, South Korea) with an accuracy of 100 g using bioelectrical resistance method. BMIs were measured in kilograms per square meter by dividing the body weight by the square of height in meters. All measurements were taken after the subjects had stopped eating and taking liquids for 4 h and their bladder, stomach, and intestine had been relatively empty.

Blood collection and preparation

In two steps, blood samples were collected from the left-hand vein of each subject in sitting and resting position 48 h before the start of exercises and 48 h after the last session of exercises. All samples were obtained at 6 to 7 am in the laboratory after subjects had been fasting for 10 to 12 h. The samples were immediately centrifuged at 4 °C and serum was separated. Serums were kept in a freezer at − 80 °C until measurements. ELISA Kit (ZellBio GmbH, Germany) was used to measure serum SFRP5 with CV of 9% and WNT5A with CV of 8%. HbA1c was measured using the immunoturbidimetric assay. Glucose oxidase method and Beckman analyzer (USA) were used to measure fasting glucose with sensitivity 5 ml/dl and CV of 2.5%. Radioimmunoassay and Stillwater kit (USA) were used to measure insulin with sensitivity 0.75 μIU/ml and CV of 6.3%. Also, the insulin resistance index was obtained through the HOMA-IR formula [12].

Statistical analysis

All data were analyzed using SPSS-23 software. At first, all data were investigated using the Shapiro-Wilk test to determine the normality (data normality). Levene's test was then used to check the homogeneity of data. After confirming the normality and homogeneity of data, the dependent *t* test was used to examine intra-group changes and the independent *t* test was used to examine inter-group changes. To investigate the relationship between variables, the Pearson correlation test was also used. The significance level of the tests was considered less than 0.05.

Results

Baseline characteristics of the participants are shown in Table 2. The average ages of the control and experimental groups were 57.7 and 58.9 years, and the BMIs were 28.21 and 28.77 kg/m^2, respectively. The average systolic blood pressures of the control and experimental groups were 115.3 and 116.7 mmHg, and the diastolic blood pressures were 77.6 and 76.5 mmHg, respectively.

Changes in physical and biochemical indices of the control and experimental groups are presented in Table 3. PBF decreased from 31.87 to 30.74% ($p < 0.001$), BMI decreased from 28.77 to 27.51 kg/m^2 ($p < 0.000$), glucose decreased from 162 to 142 mg/dl ($p < 0.001$), insulin decreased from 7.67 to 6.64 IU/ml ($p < 0.004$), insulin resistance index decreased from 3.12 to 2.33 ($p < 0.001$), SFRP5 concentration increased from 0.62 to 1.26 ng/ml ($p < 0.002$), WNT5A decreased from 0.20 to 0.07 ng/ml ($p < 0.005$), and HbA1c decreased from 7.49 to 6.97%. Changes in all the above factors were statistically significant in the intervention group, but they were not statistically significant in the control group. Also, the mean changes in all the above variables were statistically significant ($p < 0.05$) between the control and intervention

Table 2 General characteristics of study participants

Variables	Groups	
	Control ($n = 9$)	Experimental ($n = 10$)
Age (in years)	57.7 ± 4.57	58.9 ± 3.54
Height (cm)	171.3 ± 5	172.1 ± 6
Weight (kg)	85.3 ± 6.09	85.03 ± 8.35
BMI (kg/m^2)	28.21 ± 2.42	28.77 ± 3.12
Systolic blood pressure	115.3 ± 9.6	116.7 ± 7.2
Diastolic blood pressure	77.6 ± 5.3	76.5 ± 4.7

Data shown as mean ± SD

Table 3 Changes in physical and biochemical indices

Variables	Groups	Blood samples		Changes	
		Pre-test	Post-test	Inter-group Significance level	Intra-group Significance level
SFRP5 (ng/ml (	Experimental	0.62 ± 0.44	1.26 ± 0.51	*0.002	0.001*
	Control	1.07 ± 0.73	0.93 ± 0.59	0.405	
WNT5A (ng/ml)	Experimental	0.20 ± 0.14	0.07 ± 0.08	*0.005	0.013*
	Control	0.13 ± 0.14	0.23 ± 0.17	0.221	
HbA1c (%)	Experimental	7.49 ± 0.86	6.97 ± 0.89	*0.027	0.002*
	Control	7.13 ± 0.68	7.54 ± 0.70	0.125	
FBS (mg/dl)	Experimental	162.2 ± 32.54	142.2 ± 31/27	*0.001	0.004*
	Control	162.56 ± 4.52	166 ± 44.33	0.580	
Insulin (IU/ml)	Experimental	7.67 ± 1.71	6.64 ± 1.47	*0.004	0.021*
	Control	6.73 ± 2.20	7.30 ± 2.37	0.218	
HOMA-IR	Experimental	3.12 ± 1.06	2.33 ± 0.69	*0.001	0.012*
	Control	2.65 ± 1.07	3 ± 1.35	0.161	
BMI (kg/m^2)	Experimental	28.77 ± 3.12	27.51 ± 2.66	*0.000	0.001*
	Control	28.21 ± 2.42	28.23 ± 2.46	0.489	
PBF (%)	Experimental	31.87 ± 3.04	30.74 ± 3.10	*0.001	0.002*
	Control	31.03 ± 2.16	31.33 ± 2.52	0.385	

Data shown as mean ± SD

SFRP5 secreted frizzled-related protein 5, *WNT5A* WNT family member 5A, *HbA1c* glycated hemoglobin, *FBS* fasting blood sugar, *HOMA-IR* homeostasis model assessment-insulin resistance, *BMI* body mass index, *PBF* percentage body fat

*Significance level $p < 0.05$

groups. In addition, the Pearson correlation between changes in variables in pre-test and post-test in the experimental group indicated that SFRP5 had a significant negative relationship with WNT5A, insulin resistance index, BMI, and PBF. Also, BMI had a significant positive relationship with WNT5A, insulin resistance, and PBF ($p < 0.05$) (Table 4).

Discussion

The present study is aimed at investigating changes in SFRP5, WNT5A, HbA1c, BMI, PBF, and insulin resistance in men with type 2 diabetes after 12 weeks of combined exercise (HIIT and resistance) as well as the relationship between these variables in men with type 2 diabetes. This study showed that 12 weeks of combined exercise in men with type 2 diabetes significantly reduced serum levels of WNT5A, HbA1c and glucose, insulin resistance index, BMI, and PBF and significantly increased serum SFRP5 levels. Also, SFRP5 had a significant negative relationship with WNT5A, insulin resistance index, BMI, and PBF, and BMI had a significant positive relationship with WNT5A, insulin resistance, and PBF. This finding is consistent with the results of Fayaz et al. (2019) [7] and Teliewubai et al. (2018) [13]. Fayaz et al. (2019) investigated the effect of 12 weeks of high-intensity aerobic exercise with or without cinnamon extract supplement on the noncanonical WNT pathway in ovariectomized rats and reported these findings: reduction in LDL indices, insulin resistance, and TNF-α and increase in serum SFRP5 level in the exercise group, the supplement group, and the exercise with group supplemented. Also, serum WNT5A level decreased

Table 4 Pearson correlation coefficient values among the indices in the exercise group

Index *i*	Index *j*	Correlation of changes	
		r	*p*
SFRP5	WNT5A	− 0.877	*0.001
	HbA1c	− 0.246	0.494
	HOMA-IR	− 0.688	*0.028
	BMI	− 0.821	*0.004
	PBF	− 0.773	*0.009
WNT5A	HbA1c	0.154	0.672
	HOMA-IR	0.630	0.051
	BMI	0.776	*0.008
	PBF	0.472	0.168
HbA1c	HOMA-IR	0.165	0.649
	BMI	0.373	0.289
	PBF	0.360	0.307
HOMA-IR	BMI	0.734	*0.016
	PBF	0.562	0.091
BMI	PBF	0.774	*0.009

*Significance level $p < 0.05$

only in the exercise with supplement group [7]. Teliewubai et al.'s (2018) studies showed that plasma levels of SFRP5 are negatively associated with BMI, WHR, and fasting blood glucose. Also, men had a lower SFRP5 level than women and SFRP5 level was lower in subjects with diabetes compared with those without diabetes [13]. Increased SFRP5 level in response to combined exercise (HIIT and resistance) in subjects with diabetes is probably the result of improving body composition such as fat loss and decreasing BMI and thereby reducing inflammation. However, the results of our research were not consistent with the results of Canivell et al. (2015) [14]. Canivell et al. (2015) showed that SFRP5 levels are higher in patients with type II diabetes compared with subjects with pre-diabetes and control subjects [14]. The lack of studies on the effect of training exercises on the levels of SFRP5 and WNT5A makes it harder to justify findings. In research studies not associated with exercises, some studies showed that high levels of WNT5A in obese subjects were reduced after gastric bypass. The expression of WNT5A mRNA in visceral adipose tissue was increased in obese subjects, and gene expression levels of SFRP5 were decreased. Also, expression of WNT5A mRNA was significantly increased by induction of lipopolysaccharide and TNF-α, but no effect was observed at gene expression levels of SFRP5. Additionally, the induction of exogenous WNT5A increased IL-6, IL-1β, MMP2, MMP9, and the expression of SSP1 mRNA in adipose cells [15]. At the cellular level, recombinant SFRP5 treatment reduced IL-6 release to 49% in adipose cells induced by TNF-α in the laboratory. Also, SFRP5 had no effect on JNK activation but reduced the activation of NFκB [16]. In obese mice, SFRP5 reduced the levels of TNF-α, IL-6, and MCP-1 proinflammatory proteins and macrophage contents in adipose tissue [6]. In other studies, there was a positive relationship between plasma SFRP5 and HDL-C and a negative relationship between SFRP5 and body mass index, waist-hip circumference ratio, PBF, blood pressure, fasting glucose, hemoglobin, insulin resistance index, triglyceride, and free fatty acid. Also, SFRP5 plasma level was significantly lower in men than women and in obese and overweight people was less than normal people. Fasting plasma concentration of SFRP5 was significantly lower in subjects with type 2 diabetes and glucose intolerance compared with normal glucose tolerance. In addition, a strong positive relationship has been found between plasma SFRP5 and circulating concentrations of adiponectin, and women with insulin resistance had a lower level of SFRP5 and adiponectin than healthy women. During blood glucose increase with the oral glucose tolerance test, the concentration of SFRP5 decreased significantly and rapidly, but the concentration of adiponectin increased slightly. Also, the level of SFRP5 in obese and overweight subjects was lower than that in lean subjects [17]. However, researchers did not find any differences in SFRP5 levels between obese and lean subjects [18]. These differences appear to be due to the drugs used by obese patients. It is reported that SFRP5-deficient mice were resistant to nutrition-stimulated obesity, and their mitochondrial metabolism had increased [19]. According to other studies consistent with these research studies, in obese mice, the expression of SFRP5 mRNA was increased in white adipose tissue, and nutrition-induced obesity was also increased [20]. But in another study, SFRP5 mRNA level was low in white adipose tissue and did not change with obesity and also was not effectively secreted from white adipose tissue [21]. Several studies have shown that SFRP5 serum level was inversely associated with hsCRP, leptin, TNF-α, and IL-1β and was directly associated with adiponectin [22–25]. The activity of the noncanonical WNT pathway through upregulation of WNT5A and downregulation of SFRP5 may create a pro-inflammatory state in visceral adipose tissue and thus plays a role in the progression of obesity-related diseases. A positive relationship between WNT5A and serum leptin concentration has also been found indicating the role of WNT5A in obesity [15]. WNT5A is recognized as a potent inhibitor of adipogenicity in human fat cells [26]. It has also been observed that adipose tissue macrophages in obese individuals with type 2 diabetes express WNT5A, and WNT5A secreted from macrophages leads to the inhibition of pre-adipocyte differentiation [27]. These findings suggest that WNT5A can act as an important proinflammatory factor in mild inflammation of adipose tissue of obese or overweight people. TNF-α increases the secretion of WNT5A from adipocytes and can disrupt the ratio of SFRP5 to WNT5A [15, 28]. WNT5A also reduces the storage capacity of the inflamed adipose tissue by reducing adipogenicity in adipose tissue. In fact, WNT signaling increases fat storage in tissues other than adipose tissue, such as the liver and muscles, thereby increasing insulin resistance [29] by activating the JNK pathway and serine phosphorylation of insulin receptor substrate [6]. The activation of the JNK pathway and serine phosphorylation of insulin receptor substrate cause metabolic disturbances with increasing proinflammatory setting [15, 30]. Regarding the effect of weight loss, a study found that SFRP5 had less sensitivity to weight and PBF changes than WNT5A [15]. On the other hand, it has been shown that SFRP5 was associated with weight gain, and expression of SFRP5 had a strong relationship with fat increase and fat extent [20].

Resistance exercises can modulate inflammatory cytokines and increase insulin sensitivity. Evidence also suggests that lifestyle changes, including weight loss produced in response to aerobic exercises, can also reduce inflammatory factors [31]. Combined exercise, which involves performing both aerobic and resistance exercise in the form of a workout protocol, is recognized as the most complete type of exercise for health and therapeutic purposes by the Diabetes Association. Increasing the expression as well as the activity of PGC-1α and mitochondrial biogenesis,

glycolytic oxidative muscle function and phenotype switch, increasing the oxidative capacity of muscles in removing and metabolizing fats, reducing the systematic chronic inflammation, and improving insulin resistance [32, 33] are all the results of activating pathways such as AMPK and p38MAPK and increasing calcium which generally performed through the aerobic part of this exercise. Aerobic physical activity with the higher-than-average intensity seems to play a more important role in activating these factors. Resistance exercises also cause hypertrophy and muscle volume development through the activation of the PI3K-Akt/PKB-mTOR pathway and the inactivation of the myostatin-smad3 pathway which ultimately leads to an increase in insulin sensitivity and a decrease in PBF [34]. Based on this, it seems that combined physical activity in which both resistance and aerobic exercises have simultaneously been utilized can partly take advantage of both resistance and aerobic exercises, and its effects will be greater than those of resistance or aerobic exercises alone. It has been shown that those exercises accompanied by reducing PBF decrease HbA1c levels by up to 66%, which is a desirable reduction for improving glycemic control, and this improvement can be more effective in patients with inadequate blood glucose control. Combined exercise activity increases GLUT4 levels in the practiced muscles which improve insulin action on glucose metabolism and can significantly reduce HbA1c and fasting blood glucose levels in men with type 2 diabetes. Such reduction has been found to be due to exercise volume and subsequently decreasing body mass index. A meta-analysis study has also revealed that resistance exercises reduce blood HbA1c levels. Several studies have also reported that combined (aerobic-resistance) exercises can be more effective in reducing HbA1c and improving insulin sensitivity [35]. In the present study, level of glycosylated hemoglobin in the exercise group was significantly reduced, although it was not significantly associated with other variables of the study.

Conclusion

Improvements in health-related factors of men with type 2 diabetes, such as decreased HbA1c, blood glucose, and insulin resistance, after combined exercise (HIIT and resistance) can occur through changes in the noncanonical WNT pathway, including increased anti-inflammatory factor SFRP5 and decreased inflammatory factor WNT5A. This mechanism appears to be linked to changes in BMI and PBF.

Compliance with ethical standards

This research was conducted following an intervention study design and was approved by the Regional Scientific Ethics Committee, Ferdowsi University of Mashhad, in accordance with the ethical standards of the 1983 Helsinki declaration. The subjects of this study were 19 men with type 2 diabetes aged 50–60 years, BMI 25–30 kg/m^2, who voluntarily participated in the study after obtaining a written consent.

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

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ORIGINAL ARTICLE

Mild hyperamylasemia in type 1 diabetic children without diabetic ketoacidosis is associated with C-peptide

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Abstract

Background The aim of this study is to investigate the relationship between plasma amylase concentration and C-peptide level in type 1 diabetic and healthy children.

Methods The cross-sectional study involved 31 Chinese type 1 diabetic children without complications and 107 healthy control subjects. Clinical examination and laboratory examinations were assessed for all participants.

Results Significantly higher amylase concentrations were measured in type 1 diabetic children than in controls (102.8 U/l vs. 70.2 U/l, respectively; $p < 0.001$), but plasma lipase was significantly lower in type 1 diabetic children. The mean concentrations of pancreatic amylase were also lower in type 1 diabetic children than controls, but this difference did not reach statistical significance. Plasma amylase was significantly negatively correlated with lipase and C-peptide concentrations and was significantly positively correlated with both blood glucose and glycemic control. There were also negative correlations among blood glucose, glycemic control, and plasma lipase and an interaction between plasma lipase and C-peptide concentrations. Pancreatic amylase concentration was significantly correlated with the dose of insulin administered to the type 1 diabetic children. A total of 22.1% of the type 1 diabetic children were temporarily positive for urine protein but did not have diabetic nephropathy.

Conclusions It is recommended that clinicians perform amylase assays to assess the extreme low level of C-peptide which is difficult to detect in children with T1DM.

Keywords Amylase · C-peptide · Pancreatic amylase · Lipase · Type 1 diabetes · β cell

What is already known on this topic?
- Severe pancreatitis may cause secondary diabetes.
- A reason for the failure of islet cell transplantation in diabetes is pancreatitis.
- There may be high concentrations of plasma amylase in diabetic ketoacidosis.

What this study adds?
- Mild hyperamylasemia in type 1 diabetes may imply the presence of persistent low-grade pancreatic injury.
- The amylase concentration in children with type 1 diabetes is not reduced by decreasing HbA1c, but C-peptide concentration is associated.
- Mild hyperamylasemia may be associated with diabetic complications.

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Introduction

The prevalence of type 1 diabetes mellitus (T1DM) in pediatric populations (aged < 18 years) has been increasing globally over the last decade [1], but no permanent cure is available. Islet transplantation may be effective, but its use is limited by the complication of pancreatitis, in which pancreatic amylases digest islet cells. Amylases that can be routinely detected in clinical analyses include amylase (AMY) and pancreatic amylases (PAMY), as well as lipases (LPS), which are secreted by the pancreas, as biomarkers of pancreatic inflammation, are routinely measured to diagnose pancreatitis [2].

AMY is a calcium-containing enzyme that belongs to family 13 of the glycoside hydrolase group. It is composed of three different domains and has a common calcium-binding site stabilizing the interface between the central A domain and the variable B domain [3]. The activities of key enzymes, such as pancreatic amylases, are associated with postprandial changes in blood glucose concentrations, control of which is an important goal in the treatment of T1DM. Pancreatic lipase catalyzes the hydrolysis of lipid substrates at specific positions on the glycerol backbone of lipid substrates [4] and plays an essential role in the digestion and transportation of dietary lipids in the small intestine. It is believed that the inhibition of lipase activity not only reduces the absorption of lipids, but also protects the pancreas, allowing β cells to produce normal levels of insulin [5].

Plasma amylase concentrations sometimes rise in patients with diabetic ketoacidosis (DKA) that do not have acute pancreatitis. Hyperamylasemia in DKA can occur for a variety of reasons, including because of a reduction in amylase elimination from the kidneys, pancreatic cell necrosis, or an increase in cell membrane permeability. In addition, high circulating amylase concentrations, derived from pancreatic gland vesicles, can occur in metabolic disorders [6]. Although great attention is paid to blood glucose concentrations in children with T1DM, amylase concentrations are often ignored. Similarly, amylase concentrations in T1DM patients without DKA are seldom reported, and no normal range for plasma amylases in normal children has been documented. Therefore, the aim of this study is to investigate the relationship between plasma amylase concentration and C-peptide level in type 1 diabetic and healthy children.

Materials and methods

This study was cross-sectional. We performed a power analysis to determine the sample size using AMY as the primary outcome. With an alpha of 0.05 (two-sided test) and 90% power, a sample size of 18 in type 1 diabetes group and 36 in control group were needed. Assuming a dropout rate of 10%, we determined that 31 cases in type 1 diabetes group and 107 in healthy control group would be needed to show a significant difference between the groups. The han nationality children with T1DM who regularly attended Shandong Provincial Hospital and the Second hospital of Shandong University (Jinan, China) Diabetes clinic between December 2017 and May 2018 was conducted. In addition, healthy controls with similar age, sex, race, and body mass index (BMI), attending Shandong Provincial Hospital and the Second hospital of Shandong University Child Care clinic for routine health examinations, were also recruited. Inclusion criteria for the T1DM group were age 3–14 years, meeting the diagnostic criteria for T1DM [7]: (1) symptoms of diabetes and a casual plasma glucose ≥ 200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss. OR (2) Fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/l). (3) Two-hour plasma glucose 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test. The test should be performed as described by the World Health Organization, using a glucose load of 75 g anhydrous glucose dissolved in water or using a glucose load of 1.75 g/kg body weight if weight is <40 pounds (18 kg). (4) Fasting basal plasma insulin ≤ 5 μIU/ml and at the same time fasting plasma glucose ≥ 126 mg/dl; absence of diabetic complications, especially DKA, absence of acute disease for at least 3 months, and not in honey-moon status, and use of a basal-bolus routine involving basal insulin treatment or continuous subcutaneous insulin therapy. All the participating control children were required to be free of chronic and acute disease, especially acute gastrointestinal disease, for at least 3 months, and not to have taken any medication for at least 3 months, and they were performed OGTT and were normal glucose tolerance.

Fasting blood samples were obtained from all participants, after an overnight fast, for laboratory measurements. Fasting plasma glucose (FPG), carbon dioxide combining power (CO2CP; normal range 20–30 mmol/l), blood urea nitrogen, serum creatinine, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and serum triglyceride were determined using an automated biochemical analyzer (AU5400, Beckman Coulter, Tokyo, Japan). Routine urinalysis was conducted using a HT-2000 Urine Chemistry Analyzer. Glycated hemoglobin (HbA1c) was analyzed using a spectrophotometric method (TOSOH, G8, Japan). The non-diabetic range of HbA1c was accepted to be 4.0–6.0%. Fasting basal plasma C-peptide and fasting basal plasma insulin (INS) were evaluated to define the degree of residual beta-cell function and measured using a Cobas 602 fully automatic electrochemiluminescence immunoassay analysis system. The reference range used for fasting C-peptide was 1.1–4.4 ng/ml. The intra-assay and inter-assay coefficients of variation were both < 7.0% in these assays. Serum insulin-like

growth factor-1 (IGF-1) levels were measured by chemiluminescence immunoassay (Immulite 2000, Siemens, Eschborn, Germany). Plasma lipase (pancreas-specific; normal range 5.6–51.3 U/l), amylase (α-amylase; normal range 35–135 U/l), and pancreatic amylase (normal range 0–53 U/l) were measured using an automated biochemical analyzer (Beckman coulter AU680, USA). Better glycemic control of T1DM was defined by HbA1c of < 7.0% and worse glycemic control group by HbA1c of ≥ 7.0%, according to ADA criteria. [8]. Insulin dose was defined as the total insulin dose/kg body mass (u/kg).

Statistical analyses

Normally distributed variables are summarized as the mean ± standard deviation, and the median and range are used for variables which were not normally distributed. Independent sample *t* tests were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Pearson's correlation was used to analyze correlations between normally distributed data, but the Kolmogorov–Smirnov test indicated that AMY was not normally distributed; therefore, Spearman's correlation was used to evaluate its associations. The relationships between AMY, C-peptide, and clinical and laboratory variables were analyzed by logistic and linear regression analysis. Statistical significance was accepted when $p < 0.05$ for all clinical and laboratory variables.

Results

A total of 14 boys and 17 girls with T1DM and a mean age of 7.33 years and 58 girls and 49 boys who were healthy and had a mean age of 9.09 years were recruited. None of the T1DM children had DKA for at least 3 months. The mean insulin dose in the T1DM group was 0.83 ± 0.29 u/kg. Renal function (urea nitrogen, creatinine) was normal. Their general characteristics are shown in Table 1. CO2CP levels were normal in T1DM group, so DKA was excluded. Other baseline clinical data were not significantly different between the two groups. The T1DM children had a mean HbA1c of 7.2% and 22.1% were positive for proteinuria at baseline. Urine culture was performed to exclude urinary tract infection, and urine protein quantification was performed in this group of children. Urine protein in these children was < 0.5 g/24 h. Diabetic kidney disease is defined by characteristic structural and functional changes. Microalbuminuria defined as urinary albumin excretion between 30 and 300 mg/day or between 30 and 300 mg/g creatinine on a random urine sample. Macroalbuminuria means nephropathy [9]. We followed up this group of children for 6 months, during which the proteinuria largely disappeared; therefore, they did not meet the diagnostic criteria for diabetic nephropathy. Microalbuminuria was temporary.

Table 1 General characteristics of healthy and type 1 diabetic children

	Control group ($n = 107$)	T1DM ($n = 31$)
Age (years)	9.09 ± 3.1	7.33 ± 2.9
Boy (*n*)	49	14
Girl (*n*)	58	17
BMI	17.19 ± 3.6	16.2 ± 2.9
Course of T1DM (years)		2.9 ± 0.43
CO2CP (mmol/l)	25.3 ± 1.4	25.1 ± 2.2
AST (U/l)	27 ± 3.2	29 ± 2.4
ALT (U/l)	14 ± 2.1	16 ± 1.9
Urea nitrogen (μmol/l)	4.7 ± 0.25	5.1 ± 0.16
Creatinine (μmol/l)	35.9 ± 5.6	37.4 ± 4.2
Triglyceride(mmol/l)	0.39 ± 0.1	0.46 ± 0.2

Normal ranges were calculated for the children (as 95% confidence intervals) to be 65.56–74.81 U/l for AMY, 18.47–20.73 U/l for PAMY, and 19.16–21.37 U/l for LPS. The T1DM group also had a significantly lower concentration of plasma C-peptide than the control group (Fig. 1a), as well as a significantly lower plasma LPS concentration (13.7 U/l vs. 20.3 U/l; $p < 0.001$). Significantly higher AMY was present in the T1DM group than the control group (102.8 U/l vs. 70.2 U/l, respectively; $p < 0.001$). Mean plasma PAMY concentration was lower in children with T1DM than in controls, but the difference was not significant (Fig. 1b).

The mean plasma LPS concentration in the healthy boys was significantly lower than that of the girls, but there were no differences in AMY and PAMY concentrations (Fig. 2a). There were no differences in AMY, LPS, and PAMY concentrations between boys and girls with T1DM (Fig. 2b). There were no differences in the concentrations of AMY, PAMY, or LPS between groups of diabetic children with better or worse glycemic control (comparison of number 13 vs. 18) (Fig. 3) and no differences in the other measurements (FPG, plasma C-peptide, plasma insulin, and insulin dosage). The concentrations of PAMY and LPS were significantly higher in the control group than in the diabetic children with better glycemic control, but AMY was similar (Fig. 4). However, the control children were significantly older than the diabetic children with worse glycemic control (9.1 years vs. 6.0 years).

In all recruited children, AMY was significantly negatively correlated with both LPS and C-peptide (Spearman's correlation: $\rho = -0.21$, $p = 0.03$ and $\rho = -0.25$, $p = 0.01$, respectively) and was also significantly positively correlated with both FPG and HbA1c (Spearman's correlation: $\rho = 0.59$, $p = 0.00$ and $\rho = 0.42$, $p = 0.00$, respectively). As expected, the plasma concentration of PAMY was also associated with LPS (Pearson's correlation: $r = 0.61$, $p = 0.00$). A significant negative correlation between FPG and LPS was also identified (Pearson's correlation: $r = -0.29$, $p = 0.03$), as well as a

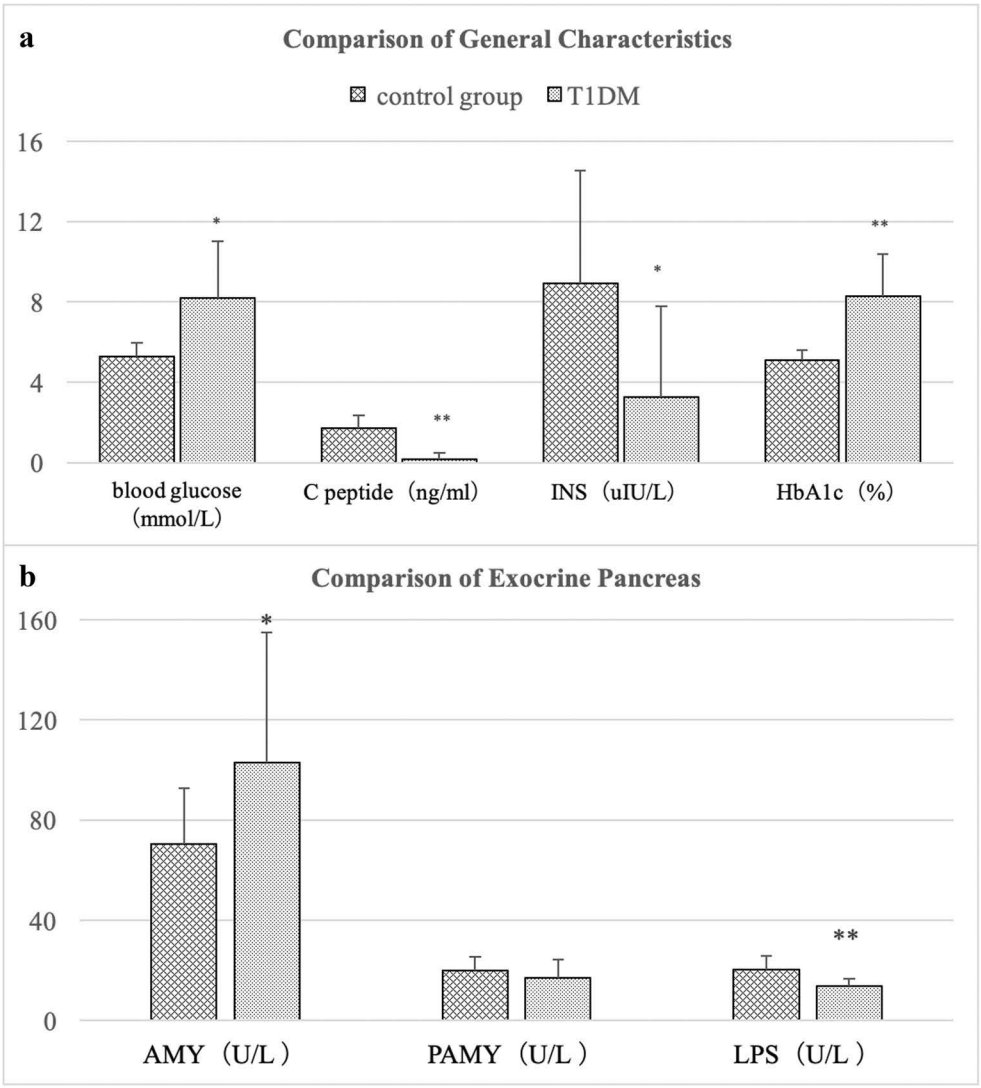

Fig. 1 Comparison of the control and type 1 diabetic groups. **a** General characteristics of the control and type 1 diabetic groups. "INS" represents the fasting basal plasma insulin concentration and "C-peptide" represents the fasting plasma C-peptide concentration. **b** Exocrine pancreatic function of the control and type 1 diabetic groups

positive interaction between LPS and C-peptide (Pearson's correlation: $r = 0.21$, $p = 0.03$). In addition, higher concentrations of LPS were present in children with lower FPG and HbA1c (Pearson's correlation: $r = -0.29$, $p = 0.03$ and $r = -0.24$, $p = 0.02$, respectively). In the control group, AMY was significantly positively correlated with LPS and PAMY concentrations (Spearman's correlation: $\rho = 0.24$, $p = 0.02$ and $\rho = 0.35$, $p = 0.00$, respectively), and PAMY was significantly positively correlated with LPS (Pearson's correlation: $r = 0.63$, $p = 0.00$). In the T1DM group, PAMY was significantly positively correlated with insulin dose (Pearson's correlation: $r = 0.64$, $p = 0.03$).

Logistic regression analysis was used to identify factors associated with AMY. The median with 25 and 75 percentile values of AMY was 66.50 (53.0, 84.25). C peptide decreased significantly, especially AMY above 53.0. Participants in the median of AMY showed a higher risk of β cell failure (OR 0.13; 95% CI 0.016–1.072 p for trend 0.058).

Discussion

This study was designed to evaluate the plasma concentrations of amylases in children with T1DM. Hyperamylasemia has been reported in some recent studies [10, 11], but to the best of our knowledge, this is the first study to report amylase data from children with T1DM uncomplicated by DKA. Our study shows that significantly higher plasma amylase concentrations within the normal range are present in T1DM children without DKA than control, but plasma amylase was more high when DKA is present [10]. DKA develops alongside various degrees of acute pancreatic injury. The pancreas has both an exocrine and an endocrine component, which reciprocally interact in a system that is important for digestion, absorption, and homeostasis of nutrients. Thus, it is not surprising that disorders of the exocrine pancreas also affect the endocrine system, and vice versa. On the basis of the mechanisms whereby hyperamylasemia develops in DKA, the slightly

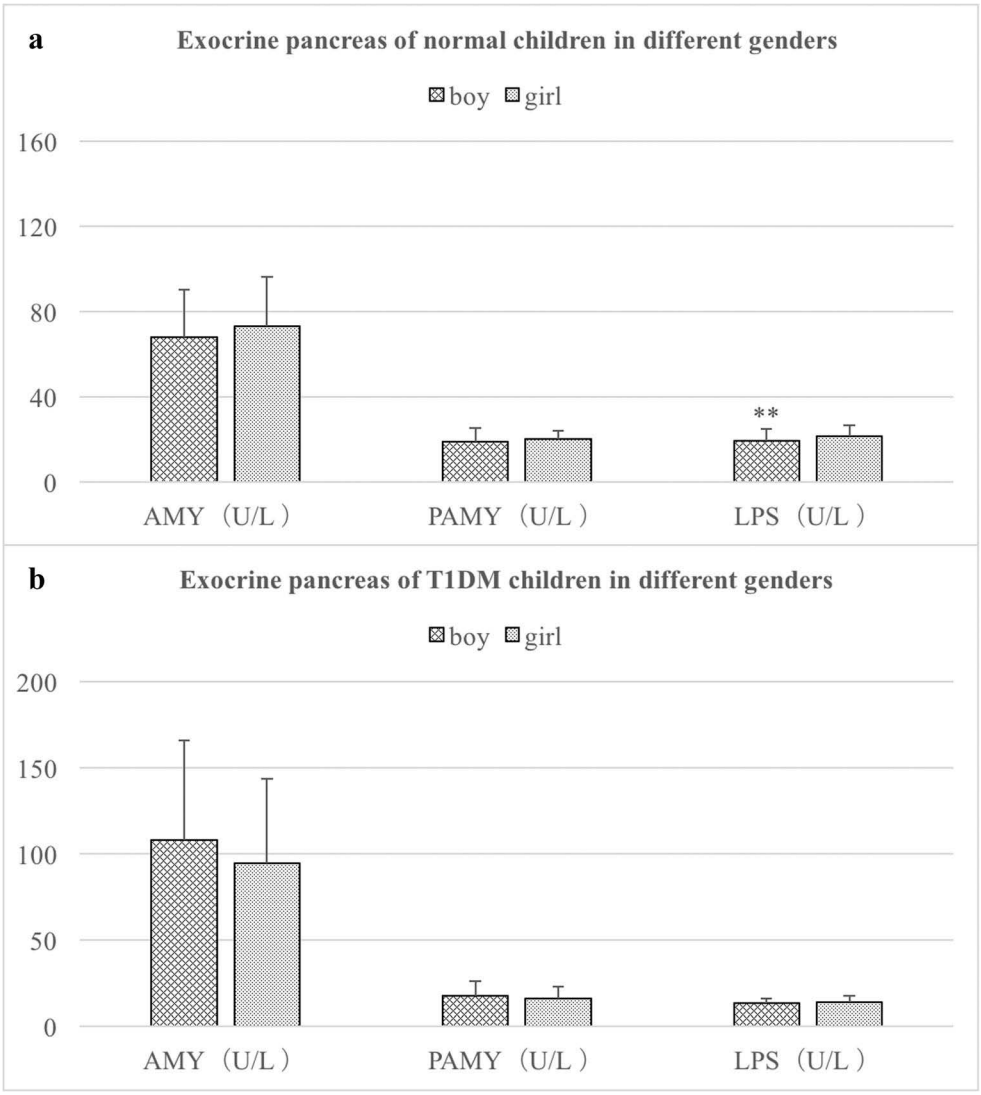

Fig. 2 Comparison in different genders. **a** Exocrine pancreas of healthy children of each sex. **b** Exocrine pancreas of type 1 diabetic children of each sex

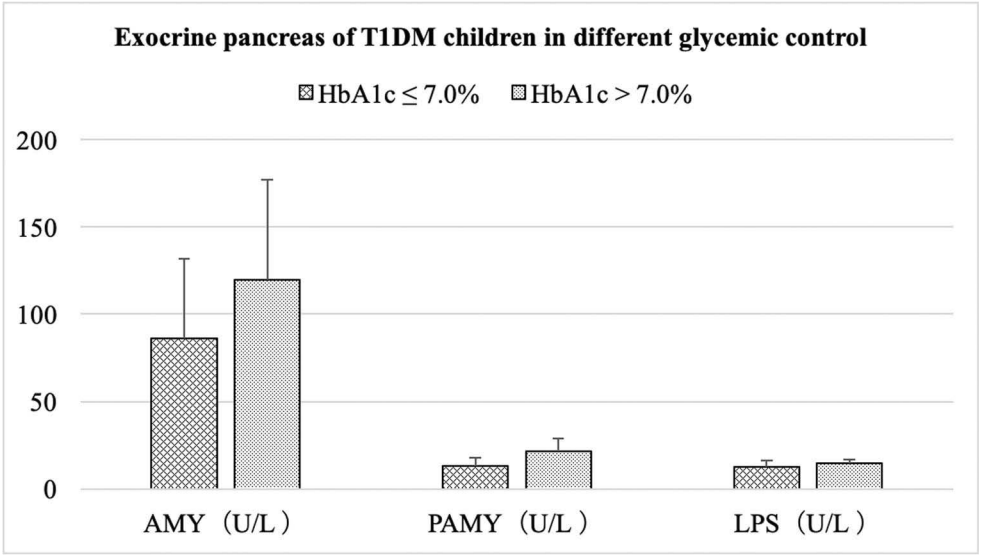

Fig. 3 Comparison of the exocrine pancreases of type 1 diabetic children with various degrees of glycemic control

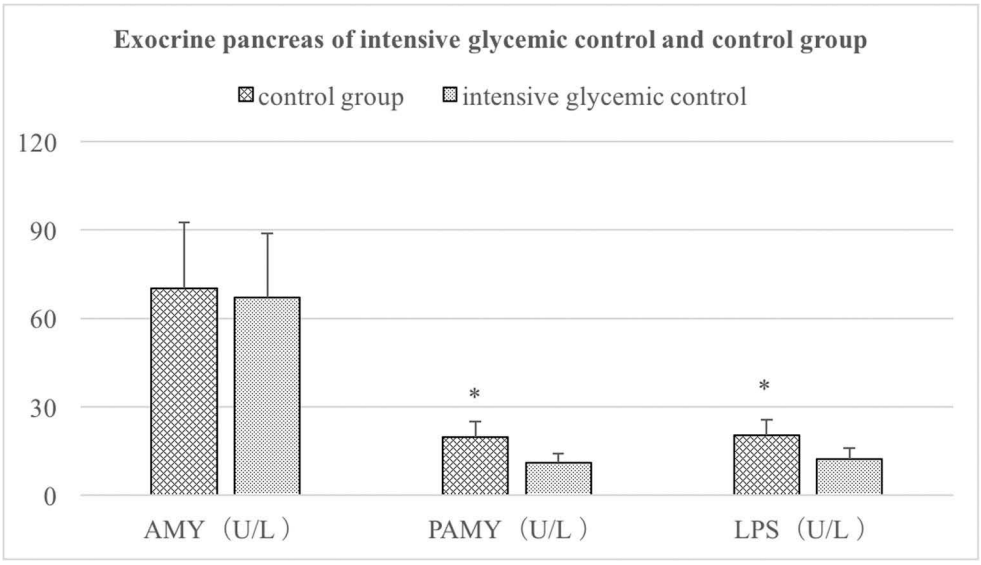

Fig. 4 Comparison of the exocrine pancreases of type 1 diabetic children with better glycemic control and the control group. Values are mean ± SD. Statistically significant differences are shown by $*p < 0.05$ and $**p < 0.01$ vs. the corresponding control values

higher AMY concentration present in T1DM without DKA may arise for the following reasons: (1) inadequate insulin secretion, leading to greater fat mobilization, higher concentrations of free fatty acid and lecithin, and the activation of pancreatic enzymes, causing chemical damage to pancreatic gland tissue; (2) dysfunctional arachidonic acid metabolism and atherosclerosis in capillaries, resulting from chronic hyperglycemia, and causing microcirculatory defects and pancreatic necrosis; or (3) cellular dehydration, resulting in sticky, thick pancreatic juice, which can cause an obstruction; (4) an involvement of the exocrine tissue in the autoimmune destruction of islet cells [10, 12]. These processes may lead to failure of pancreatic β cells. Slightly high amylase concentrations in the absence of symptoms may be associated with abnormalities on pancreatic imaging and pancreatic steatosis. Pancreatic steatosis is associated with β cell dysfunction and exocrine insufficiency. Greater acinar activity can induce cellular growth and increase both plasma enzyme concentrations and pancreatic volume [11, 13–15], and indeed, abnormalities in the histological and imaging features of the pancreas of diabetic patients have been reported, which are associated with pancreatic exocrine insufficiency [16, 17]. Therefore, it is necessary to evaluate pancreatic volume when hyperamylasemia is detected in T1DM. In a previous study, patients with diabetes are also more likely to develop periodontal disease than non-diabetic individuals [18], with changes of salivary amylase [19]. Therefore, it is also necessary to routinely evaluate plasma amylase concentrations as an indicator of a higher risk of periodontal disease. A finding of mild hyperamylasemia in children may suggest the presence of T1DM or a higher risk of developing T1DM in the future. In addition, persistent low-grade inflammation of the pancreas in T1DM may induce hyperamylasemia. The reason for pancreatic injury in type 1 diabetic patients needs further research.

In our study, AMY was shown to be significantly positively correlated with FPG and HbA1c in all the children studied. In the T1DM group, there were no differences in amylase concentrations between children with HbA1c > 7.0% and those with HbA1c ≤ 7.0%. Therefore, it seems that the concentration of AMY in children with T1DM cannot be reduced by lowering HbA1c. AMY was also shown to be significantly negatively correlated with C-peptide across all the children studied. AMY reflects the control of blood glucose and the level of C-peptide. As reported, animal studies have shown that pancreatic amylase expression is strictly insulin-dependent [20], and indeed, insulin influences pancreatic growth and exocrine function [21]. However, this correlation was not significant in T1DM group alone, which may be due to extremely low C-peptide concentrations in this group. These findings imply that changes in HbA1c concentration do not influence AMY, although it is affected by worsening function of β cells. In summary, the major contributing factor to AMY concentration is the concentration of C-peptide, while the effect of HbA1c is only indirect. The concentrations of PAMY and LPS in children with better glycemic control were lower than those in the control group. Additionally, data from the present analysis suggest that worsening glycemia is associated with baseline lipase levels [22]. A total of 22.1% of diabetic children identified to be positive for proteinuria in this study did not meet the diagnostic criteria for diabetic nephropathy. Similarly, it has been shown previously that older age, lower BMI, and renal impairment are associated with slightly high baseline amylase/lipase concentrations in patients with type 2 diabetes [23].

Type 1 diabetes, which is linked to a primary autoimmune process and is characterized by early occurrence, severe insulin deficiency, and long standing disease, with a high rate of neural and vascular complications, seems to be more

frequently associated with pancreatic injury than type 2. Glutamic acid decarboxylase antibody (GAD antibody) was typical marker of immunological disorder. GAD antibody was related to the state of T1DM while some T1DM has no GAD antibody at the point of the diagnosis. The antibodies were detected at the time of diagnosis. The level of amylase in this study was not the level at the time of diagnosis, and therefore, antibodies were not included in the statistics. More research about this will be proceeded in the future. This study included a larger number of children in the control group, principally to ensure that the mean values of these parameters were within the normal range.

In conclusion, we recommend performing amylase assays in children with T1DM. This inexpensive and rapid process helps to assess the extreme low level of C-peptide which is difficult to detect in children with T1DM.

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Data availability statement The data used to support the findings of this study are included within the article.

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from both parents of each child. The study was approved by the ethics committee of Shandong Provincial Hospital and the Second hospital of Shandong University and was carried out according to the Helsinki declaration.

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ORIGINAL ARTICLE

Prevalence and predictors of osteopenia and osteoporosis in patients with type 2 diabetes mellitus: a cross-sectional study from a tertiary care institute in North India

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Abstract

Background Patients with type 2 diabetes mellitus (T2DM) have an increased risk of hip and vertebral fractures**.** The increased fracture risk has largely been attributed to poor bone quality and microarchitecture. The contribution of bone quantity, measured as areal bone mineral density (BMD), to the risk of fracture is variable with most studies showing an increase in BMD in T2DM. The present study was undertaken to find out the prevalence of osteoporosis and osteopenia (based on BMD) in a cohort of patients with T2DM and delineate the possible modifiable and non-modifiable risk factors.

Methods In this cross-sectional observational study, 252 otherwise ostensibly healthy patients with T2DM underwent dual energy X-ray absorptiometry (DEXA) scan. Osteoporosis and osteopenia were defined based on *T*-scores. The effect of modifiable and non-modifiable risk factors on BMD and osteoporosis were assessed.

Results The mean age of the cohort was 59.9 years with a M:F ratio 2.9:3.4. The mean BMD at the lumbar spine and hip were 0.892 g/cm^2 and 0.715 g/cm^2, respectively. Males had significantly higher BMD at both the sites compared to females. The prevalence of osteoporosis and osteopenia was 33% and 40%, respectively. Female gender, increasing age, normal body mass index (BMI), low serum 25-hydroxyvitamin D, and use of pioglitazone were significantly associated with the risk of osteoporosis.

Conclusion The prevalence of osteoporosis and osteopenia in patients with T2DM is high. Female gender, increasing age, normal BMI, low serum 25-hydroxyvitamin D, and pioglitazone use further increase the risk of osteoporosis.

Keywords Bone mineral density · Body mass index · Osteoporosis · Type 2 diabetes mellitus · Vitamin D · Pioglitazone

Introduction

The prevalence of diabetes mellitus (DM), specifically type 2 diabetes mellitus (T2DM), is on the increase. The World Health Organization (WHO) had estimated that 8.5% of adults over 18 years of age were affected by diabetes mellitus, a figure amounting to 422 million people worldwide. The number is expected to rise to 642 million by 2040 [1]. The rise has been more noticeable in low- and middle-income countries. Apart from contributing to micro- and macro-vascular disease, DM exerts a detrimental effect on bone health. Patients with T2DM have a moderately increased risk of hip and vertebral fractures [2, 3]. Hip and vertebral fractures not only contribute to significant morbidity but have also been shown to independently predict mortality [4]. The presence of diabetes further worsens mortality [5]. The increased fracture risk in T2DM is multifactorial; bone quality and microarchitecture are adversely affected by hyperglycemia and advanced glycemic end products (AGEs) [6]. Data on bone quantity, as measured by bone mineral density (BMD), is controversial with many studies showing increased BMD in T2DM [7, 8] while others showing reduced BMD [9, 10]. Among a cohort of 243

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patients with T2DM, the prevalence of osteoporosis (as defined by WHO on the basis of BMD *T*-scores) was 21.9% in women and 13% in men [10].

India is home to about 69.1 million patients with DM [1] with an overall prevalence of 7.3% [11]. Data on the prevalence of osteoporosis and osteopenia in Indian patients with T2DM is limited [12–15]. Hence, the present study was undertaken to find out the prevalence of osteoporosis and osteopenia (based on BMD) in a cohort of patients with T2DM and delineate the possible modifiable and non-modifiable risk factors.

Material and methods

This was a cross-sectional analytical study wherein patients of T2DM attending the diabetes clinic of the Post Graduate Institute of Medical Education and Research, Chandigarh, a tertiary care institute in North India underwent dual-energy X-ray absorptiometry (DEXA) scan for evaluation of BMD at the lumbar spine and non-dominant hip. Inclusion criteria included patients aged 50 years and above with T2DM for more than 5 years. In addition, they must have had been regularly attending the diabetes clinic and have had their glycated hemoglobin (HbA1c) evaluated at least thrice over the past 1 year. Patients with prior history of fractures, renal dysfunction (eGFR < 60 ml/min/1.73 m^2), chronic smokers and alcohol consumers, primary hyperparathyroidism, thyroid disorders, celiac disease, multiple myeloma, active malignancy, lactose intolerance, obvious bone and mineral disorders, chronic pancreatitis, rheumatological and systemic inflammatory disorders, malabsorption syndromes or those receiving corticosteroids, immunosuppressants, anticonvulsants, diuretics, calcium, vitamin D supplements, and anti-resorptive/anabolic therapy were excluded from the study.

Detailed history of each patient was obtained regarding age, duration of T2DM, anti-diabetic drug treatment over the past 1 year, symptoms of neuropathy, retinopathy, and nephropathy. Glycemic control of the participants was assessed by enquiring about HbA1c values available over the past 1 year. Height of the participant was measured three times by a standard stadiometer to the nearest centimeter and the mean of the three readings was taken as the final height. Similarly, weight was measured three times using a digital weighing machine to the nearest of 0.1 kg and the mean of the three readings was considered as the final weight. The accuracy of the weighing machine was checked every day using an ISI standardized weight of 5 kg. Finally, body mass index (BMI) was calculated using formula: weight (in kg)/height (in meter)^2. Neuropathy was assessed using vibration perception threshold (Vibrometer—VPT®, Diabetic Foot Care, Madras Engineering Service, India). A threshold of ≥ 15 V was chosen to define neuropathy. A dilated fundus examination was performed by a single, dedicated ophthalmologist to look for retinopathy. Albuminuria was defined as urinary albumin-to-creatinine ratio ≥ 30 mg/g creatinine on two separate occasions.

Six milliliters of blood sample was collected from each participant after 8 h of overnight fasting for biochemical investigations. Serum calcium (reference range (RR) 8.8–10.2 mg/dl), inorganic phosphate (RR 2.7–4.5 mg/dl), albumin (RR 3.5–4.5 g/dl), alkaline phosphatase (RR 40–129 IU/L), and creatinine (RR 0.8–1.2 mg/dl) were measured by autoanalyzer (Roche Diagnostics, Modular P 800). Calcium values were corrected for respective serum albumin levels. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI equation [16]. HbA1c, serum intact parathyroid hormone (iPTH, RR 10–65 pg/ml), and 25-hydroxyvitamin D (RR 11.7–42.2 ng/ml) [17] were measured by electrochemiluminescence assay using commercially available kits (Elecsys 2010 system, Roche diagnostic, Germany). Additionally, thyroid function test, serum protein electrophoresis, and tissue transglutaminase (tTG) antibody (IgA) were measured to rule out underlying thyroid disorders, multiple myeloma, and celiac disease, respectively. For the purpose of final analysis, the mean of the four HbA1c values (three available over the past 1 year and one performed at the time of study inclusion) was considered.

A total of 518 patients of T2DM attending the diabetes clinic of Post Graduate Institute of Medical Education and Research, Chandigarh over a period of 6 months were initially screened, out of which 252 met the inclusion criteria and were enrolled in the study. Each patient satisfying inclusion criteria underwent dual energy X-ray absorptiometry (DEXA) scan of lumbar spine and non-dominant hip. DEXA scan was performed using the HOLOGIC Discovery A machine (HOLOGIC Viewer 6). A single, dedicated, International Society for Clinical Densitometry–certified technician performed all the scans. *T*-score and *Z*-scores were measured using Hologic reference population. Osteoporosis was diagnosed as per the WHO definition of *T*-score ≤ − 2.5 at the lumbar spine (L1-L4) or total hip. *T*-score of − 1 to − 2.4 was defined as osteopenia and a *T*-score ≥ − 1 was defined as normal.

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) 23.0 software program (SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to check normality of data. Normally distributed variables were expressed as mean ± SD while non-parametric data were expressed in terms of median (interquartile range, IQR). Correlations between BMI, diabetes duration, HbA1c, serum calcium, serum 25-hydroxyvitamin D, and BMD were calculated using Pearson/Spearman correlation. Based on BMI, the cohort was divided into 4 groups: underweight (BMI < 18.5), normal (BMI 18.5–22.9), overweight (BMI 23–24.9), and obese (BMI ≥ 25). Patients were

divided into poor and good glycemic control groups based on HbA1c cutoff of 7.5%. Similarly, patients were divided into vitamin D sufficient, insufficient, and deficient groups according to vitamin D levels of ≥ 30 ng/ml, 20–29 ng/ml, and < 20 ng/ml, respectively. Between-group comparisons in the prevalence of osteopenia and osteoporosis were made using independent samples *t* test/one-way ANOVA. Binary logistic regression/multinomial logistic regression was used to calculate odds ratio. A p value of < 0.05 was considered significant.

Results

Baseline characteristics

The enrolled group of 252 participants included 116 male (46%) and 136 female (54%) with a mean age of 59.9 ± 6.9 years and a BMI of 25.9 ± 4.7 kg/m^2. There was no statistically significant difference in age or BMI between males and females. The median duration of diabetes was 7 years. The mean HbA1c of the group was 7.6 ± 1.8%. The baseline characteristics are summarized in Table 1.

The prevalence of osteoporosis, osteopenia, and normal BMD in the entire group was 83 (33%), 101 (40%), and 68 (27%), respectively. The mean lumbar spine and hip BMD were 0.892 ± 0.184 g/cm^2 and 0.715 ± 0.202 g/cm^2, respectively. BMD at the lumbar spine was significantly higher in the 50–59-year age group compared with the 60–69-year age group (p = 0.014). Males had significantly higher BMD at both the lumbar spine (p < 0.001) and the hip (p < 0.001) compared with females (Fig. 1a, b). BMI had a positive correlation with both lumbar spine (r = 0.138, p = 0.028) and hip BMD (r = 0.189, p = 0.003) (Fig. 2a, b). Similarly, serum 25-hydroxyvitamin D levels had a positive correlation with lumbar spine (r_s = 0.216, p = 0.001) and hip BMD (r_s = 0.241, p < 0.001). Duration of diabetes, HbA1c, and serum calcium had no significant correlation with BMD at either site.

Risk factors for osteoporosis

Age

The prevalence of osteoporosis in the 50–59-year, 60–69-year, 70–79-year, and 80–89-year age groups was 29%, 36%, 39%, and 33%, respectively. Similarly, the prevalence of osteopenia in the 50–59-year, 60–69-year, and 70–79-year age groups was 36%, 44%, and 44%, respectively. Subjects belonging to the 60–69-year age group had an OR of 2.2 (95% CI; 1.1–4.3, p = 0.023) for osteoporosis and a similar OR of 2.2 (95% CI; 1.1–4.2, p = 0.018) for osteopenia compared with individuals who were a decade younger (50–59-year age group).

Table 1 Table showing baseline characteristics of the study population (N = 252)

Characteristic	Data
M:F	2.9:3.4
Mean age ± SD (years)	
-Total	59.9 ± 6.9
-Male	60.3 ± 7.4
-Female	59.4 ± 6.4
Mean BMI ± SD (kg/m^2)	
-Total	25.9 ± 4.7
-Male	25.8 ± 4.2
-Female	26.0 ± 5.1
Median duration of DM (IQR) (years)	7.0 (5.0–10.0)
Retinopathy	70 (27.8%)
Neuropathy	118 (46.8%)
Nephropathy (proteinuria)	62 (24.6%)
Anti-diabetic drug use	
-Metformin	245 (97.2%)
-Sulfonylureas	183 (72.6%)
-Thiazolidinediones	53 (21%)
-α-glucosidase inhibitors	28 (11.1%)
-DPP4 inhibitors	26 (10.3%)
-GLP1 receptor agonist	3 (1.2%)
-SGLT2 inhibitors	12 (4.7%)
-Insulin	47 (18.7%)
Mean HbA1c ± SD (%)	7.6 ± 1.8
Glycemic control	
-Good (HbA1c < 7.5%)	150 (59.5%)
-Poor (HbA1c ≥ 7.5%)	102 (40.5%)
Mean creatinine ± SD (mg/dl)	0.8 ± 0.2
Mean eGFR ± SD (ml/min/1.73 m^2)	81.2 ± 13.4
Mean calcium ± SD (mg/dl)	8.9 ± 0.8
Median ALP (IQR) (IU/L)	122.5 (94.0–147.0)
Median 25-hydroxy vitamin D (IQR) (ng/ml)	26.1 (14.8–37.1)
Vitamin D status	
-Deficient	83 (33%)
-Insufficient	60 (24%)
-Sufficient	109 (43%)
Median iPTH (IQR) (pg/ml)	45.9 (36.1–56.8)
Mean lumbar spine (L1-L4) BMD ± SD (gm/cm^2)	
-Total	0.892 ± 0.184
-Male	0.970 ± 0.148
-Female	0.826 ± 0.186
Mean hip BMD ± SD (gm/cm^2)	
-Total	0.715 ± 0.202
-Male	0.766 ± 0.144
-Female	0.672 ± 0.232

SD, standard deviation; *DM*, diabetes mellitus; *IQR*, interquartile range; *HbA1c*, glycated hemoglobin; *eGFR*, estimated glomerular filtration rate; *ALP*, alkaline phosphatase; *iPTH*, intact parathyroid hormone; *BMD*, bone mineral density

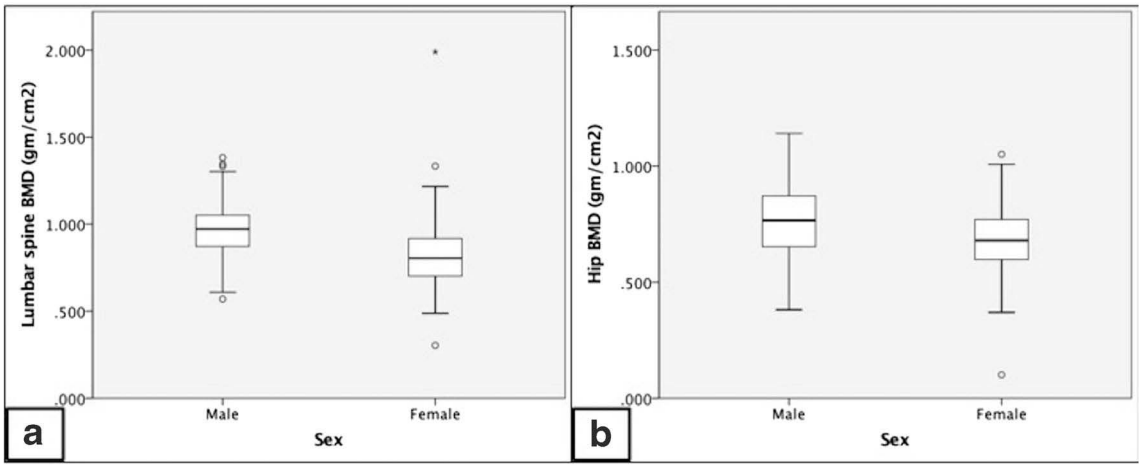

Fig. 1 Box and whisker plots showing lumbar spine (**a**) and hip (**b**) bone mineral density (BMD) in males and females (*N*=252)

Sex

The prevalence of osteoporosis, osteopenia, and normal BMD in males was 15%, 50%, and 35%, respectively; that in females were 48%, 32%, and 20%, respectively (Fig. 3). Osteoporosis was more prevalent in females than in males ($p < 0.001$) across both decades (50–59 years and 60–69 years). Binary logistic regression showed that females had an odds ratio (OR) of 5.9 (95% CI; 2.8–12.1, $p < 0.001$) for having osteoporosis compared with males.

Modifiable risk factors

BMI, duration of T2DM, presence of nephropathy, neuropathy, retinopathy, vitamin D status and use of specific antidiabetic drugs, and the risk for developing osteoporosis/osteopenia were analyzed by multinomial logistic regression. Patients with a normal BMI (18.5–22.9 kg/m^2) had an OR of 3.9 (95% CI; 1.6–9.4, $p = 0.002$) for having osteoporosis compared with obese subjects (BMI ≥ 25.0 kg/m^2). Subjects who were vitamin D deficient were more likely to have osteopenia (OR 7.8 (95% CI; 3.1–19.5, $p < 0.001$)) and osteoporosis (OR 7.3 (95% CI; 2.8–18.8, $p < 0.001$)) as opposed to those who were vitamin D sufficient. Amongst the antidiabetic drugs, only use of pioglitazone was significantly associated with osteoporosis with an OR of 10.6 (95% CI; 3.8–29.3, $p < 0.001$).

The modifiable and non-modifiable risk factors are summarized in Table 2.

Discussion

The present study shows that the prevalence of osteoporosis and osteopenia in a cohort of 252 otherwise ostensibly healthy patients with T2DM was 33% and 40%, respectively. Women were more likely to have osteoporosis than men. Similarly, increasing age, normal BMI, thiazolidinedione use, and vitamin D deficiency were associated with an increased risk of having osteoporosis. Duration of diabetes or the degree of glycemic control over the past 1 year did not have any significant effect on BMD or prevalence of osteoporosis.

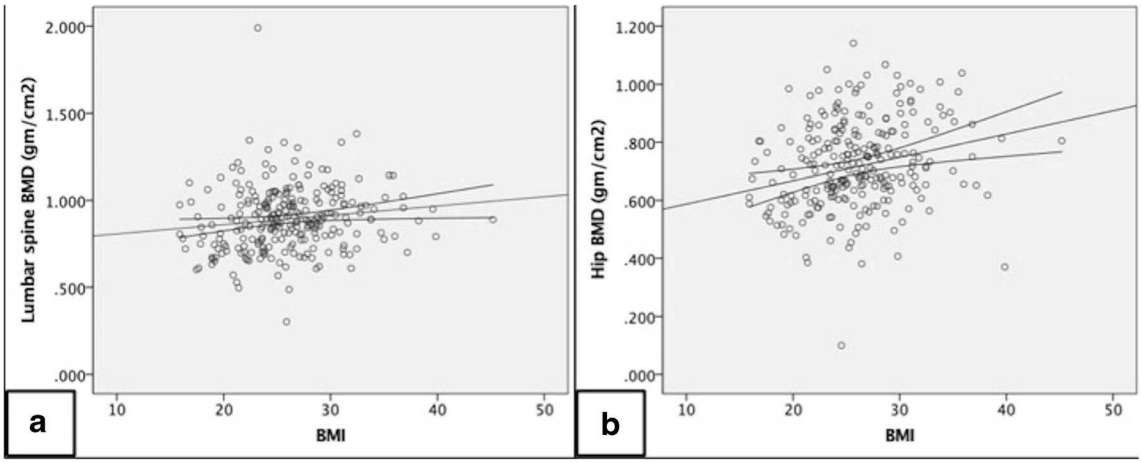

Fig. 2 Scatter diagrams showing correlation of lumbar spine (**a**) and hip BMD (**b**) with body mass index (*N*=252)

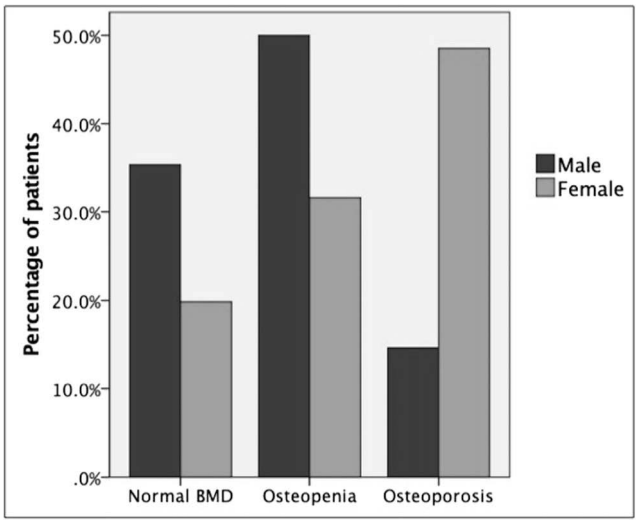

Fig. 3 Bar diagram showing prevalence (in %) of osteoporosis, osteopenia, and normal bone mineral density (BMD) in males and females ($N = 252$)

Diabetes is unequivocally associated with an increased risk of fractures. The risk is higher in patients with T1DM compared with patients with T2DM. In the prospective Nurses' Health Study, the risk of hip fracture in patients with T1DM was 6-folds higher than the study population and 2.5-folds higher than T2DM patients [18]. Such a high risk of fracture in patients with T1DM has largely been attributed to decreased BMD, generally up to the range of 22–37% [19]. Absolute insulin deficiency, low levels of insulin-like growth factor-1 (IGF-1), and lack of amylin all contribute to low peak bone mass and low BMD in T1DM [6, 20]. Although T2DM is also associated with an increased risk of hip and vertebral fractures [2, 3, 19], the relation between BMD and fracture risk in T2DM is not straightforward. Many studies have found that BMD in T2DM is increased, in the range of 5–10% above an age-matched non-diabetic population [7, 8]. This increase in BMD is predominantly a feature of the weight-bearing skeleton (femur, hip, spine) rather than the non-weight-bearing bones (forearm). The increase in BMD has traditionally been attributed to high BMI, hyperleptinemia, hyperinsulinemia, and frequent use of certain medications like thiazides and statins in T2DM [8]. However, in spite of an increase in areal BMD, patients with T2DM have low trabecular bone score at the lumbar spine implying that underlying bone microarchitecture, an entity not reflected in BMD, is compromised [21]. Factors that lead to deterioration of bone quality in T2DM include direct toxic effect of hyperglycemia on osteoblasts, AGE-mediated cross-linking of bone collagen, elevated circulating levels of sclerostin, and pro-inflammatory cytokines mediated increased bone resorption [6].

Table 2 Table showing risk factors for osteoporosis and corresponding odds ratios

Risk factor	Odds ratio (95% confidence interval)
Female	5.9 (2.8–12.1)
Increasing age	2.2 (1.1–4.2)
Normal body mass index	3.9 (1.6–9.4)
Vitamin D deficiency	7.8 (3.1–19.5)
Use of pioglitazone	10.6 (3.8–29.3)

That BMD elevated in patients with T2DM is not universal and contradictory data exist [9, 10]. There is limited data from India; however, all available data support the fact that BMD is low and osteoporosis is more prevalent in patients with T2DM than an age-matched non-diabetic population [12–15]. Similarly, our study shows that the prevalence of osteoporosis and osteopenia is as high as 33% and 40%, respectively, in a group of otherwise healthy patients with T2DM. Our data is in line with Sharma et al. who had shown a prevalence of osteoporosis of 35.5% in a cohort of 200 patients with T2DM aged > 50 years [12]. However, the data needs to be interpreted with caution. A cross-sectional study conducted by Marwaha et al. in 1600 apparently healthy North Indian men and women aged > 50 years found that the prevalence of osteoporosis and osteopenia was 35.1% and 49.5%, respectively [22]. The mean age of the aforementioned study population was 57.67 years while that of our index study was 59.9 years. Moreover, our study was also conducted in the northern part of India, thereby ruling out any major ethnic variations. Though we do not have a control group to make a direct comparison, we would like to conservatively conclude that patients with T2DM probably have the same risk of developing osteoporosis (based on BMD) as non-diabetic healthy subjects.

As per as the risk factors were concerned, female sex and increasing age were associated with the risk of having osteoporosis. Females had an odds ratio of 5.9 for having osteoporosis compared to males while subjects belonging to the 60–69-year age group had an OR of 2.2 compared to subjects who were a decade younger. Osteoporosis has a predilection for females and increasing age is well-known. Among the modifiable risk factors, normal BMI, vitamin D deficiency, and use of pioglitazone were more likely to be associated with osteoporosis. BMI has consistently been inversely associated with osteoporosis and risk of fractures with higher BMI being osteo-protective, possibly by exerting a mechanical effect on the bones [23]. Weight is known to affect bone loss from spine and hip. Finkelstein et al. [24] had reported 35–55% slower rate of bone loss from spine and hip in women belonging to the top tertile compared with those in the lowest weight tertile[25]. Sharma et al. [12] and Dutta et al. [15] have also demonstrated a positive effect of BMI on BMD in patients with T2DM.

Observational studies have shown a positive relationship between serum 25-hydroxyvitamin D levels and hip BMD [26]. Our study too showed a positive correlation between

serum 25-hydroxyvitamin D levels and BMD at the lumbar spine and hip, with vitamin D–deficient subjects having an OR of 7.3 for developing osteoporosis compared with those who were vitamin D sufficient. This is in stark contrast to most of the studies that show no correlation between BMD and serum 25-hydroxyvitamin D levels in patients with T2DM [12, 14]. Such a disparity is difficult to explain. Nevertheless, the relation of vitamin D and fracture risk in T2DM is currently uncertain [27] and administering vitamin D might not be beneficial as a recent meta-analysis strongly refutes the fact that vitamin D supplementation reduces the risk of fractures and falls [28].

Lastly, use of pioglitazone for at least 1 year was associated with an increased risk of osteoporosis with an OR of 10.6. Use of thiazolidinediones (TZD) has consistently been associated with bone loss and fractures in patients with T2DM [29]. Data from the Health, Aging and Body Composition cohort showed that postmenopausal TZD users experience bone loss at the rate of − 0.61% per year compared with non-users [29]. Even short-term use of pioglitazone for only 16 weeks in patients with polycystic ovarian syndrome leads to a significant decline in BMD of the lumbar spine (− 1.1%) and femoral neck (− 1.4%) [30]. The mechanism leading to bone loss is speculative; activation of PPARγ by TZD is believed to promote osteoclast recruitment and differentiation from hematopoietic progenitors leading to increased bone resorption and bone loss [32]. The risk of osteoporosis and incident fractures is higher in elderly women and those with a prior history of TZD-unrelated fracture; hence, it is best to be avoided in this group of diabetic patients.

Our study did not find any correlation between duration of diabetes and glycemic control with lumbar spine or hip BMD. Jang et al. [31] had shown that T2DM patients with diabetes duration of > 5 years had lower hip and femoral neck BMD compared with those with a diabetes duration ≤ 5 years [33]. Most of the available data point towards an increased incidence of fractures in patients with a diabetes duration > 10 years [33]. The median duration of diabetes of our cohort was only 7 years; moreover, fracture incidence was not assessed. Data on the effect of glycemic control is highly variable with poor glycemic control implicated in both high [6] and low BMD [6, 33]. The mean HbA1c of our cohort was 7.6% with 60% of the patients having HbA1c < 7.5%, perhaps nullifying the effect of glycaemia on BMD.

Our study did have certain limitations. The absence of an age- and BMI-matched control group makes comparison with non-diabetic population impossible. The study was a cross-sectional one with no follow-up and fracture incidence data. Patients with T2DM are at a high risk of fractures even at a higher BMD, thereby making incident fracture a better end point for assessment. We did not measure the serum estrogen levels of the female participants as estrogen levels have a direct bearing on the BMD. Lastly, we did not assess bone quality and neither we perform bone histomorphometry.

In conclusion, we have demonstrated a high prevalence of osteoporosis and osteopenia in a cohort of patients with T2DM. Lack of a control group makes it impossible for us to make a comparative statement. However, the prevalence of osteoporosis (as assessed solely by BMD) is probably not increased when compared with the general non-diabetic population. This reinforces the fact that BMD may be increased (or remain normal) and BMD alone may be poor predictor of fracture risk in patients with T2DM. Nevertheless, we recommend that all patients with T2DM aged 50 years and above, especially females, should be screened for osteopenia and osteoporosis; in addition, avoidance of thiazolidinediones and regular supplementation of vitamin D is advisable in this patient population.

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Compliance with ethical standards

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

Ethical approval The study was approved by the Institute Ethical Committee, Post Graduate Institute of Medical Education and Research, Chandigarh, India (Reference number NK1739/MD 11259-60).

Informed consent Informed consent was obtained from all the participants prior to enrollment.

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ORIGINAL ARTICLE

High-fat diet with alcohol elevate oxidative stress which cause hyperlipidemia by inducing mutation in the ANGPTL3 locus

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Abstract

Background Angiopoietin-like 3 (ANGPTL3) is a secreted protein which predominantly expressed in the liver, and involves in upregulation of lipid metabolism. Mutation in ANGPTL3 leads to decrease in the concentration of triglycerides (TGs), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), which makes it a promising candidate for hyperlipidemia.

Objectives The present study aims to identify the effect of alcohol and high-fat diet, on the ANGPTL3 locus, in the development of hyperlipidemia.

Methods 24 male Wister rats divided into four groups viz. control group (regular diet), HFD group (high-fat diet), ALC group (alcohol consuming), and HFD + ALC group (high-fat diet + alcohol consuming). All the groups were followed up for 12 weeks. Malondialdehyde (MDA), total body weight, TG, LDL, and HDL level were measured at the endpoint of study, and gene mutation analysis was also done for ANGPTL3.

Results MDA level found to be significantly increases in HFD + ALC group, HFD group, and ALC group compared to the control group. RFLP analysis revealed a mutation in the ANGPTL3 gene which occurred only in HFD + ALC group. Moreover, body weight, TG, LDL, and HDL found to increase significantly in HFD + ALC, HFD, and ALC group as compared to the control group.

Conclusions The present finding indicates is that high-fat diet with alcohol increases the oxidative stress in the liver, which causes a gain of function mutation in ANGPTL3 gene, resulting in hyperlipidemia.

Keywords Lipid · Fat diet · Metabolism · Gene mutation · Alcohol · Obesity

Introduction

With the continuous influence of technology, life has considerably become comfortable nowadays. However, on the bright side, where the machinery reduces much of the physical work and saves a lot of time and energy; on the dark side, it causes severe damage to public health over long terms. Every technological advancement reduces the physical work, which ultimately leads to decrease in the energy demand of the body. By this law, to balance energy demands of body, one should reduce calories intake, but what happens is exactly opposite. On one side, individuals are continuously adapting to new technologies to reduce physical work; on the other side, individuals are taking more calories than required by eating the foods with high caloric values like cold drinks, chips, chocolates, and cakes, etc. But this is not the end of the story; another grooming problem is of alcohol consumption, which is nowadays known as a status symbol in modern cultures. The deadly combination of above-cited factors is causing the metabolic abnormalities including the impaired glucose tolerance and blood pressure, dyslipidemia (increase in triglycerides and high-density lipoprotein cholesterol), cardiovascular alterations, liver problems, obesity [1], and pain [2]. High-fat diet and alcohol cause obesity, which is considered a prominent lifestyle disorder. Various treatment regimens available to treat the obesity including the Ayurvedic herbal drugs [3, 4,

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5–7] or modern medicines, which generally fails to give promising results without side effects. Therefore, prevention of disease by the adoption of a healthy lifestyle to the daily routine, by imparting physical exercise, yoga, gym, or swimming, remains the first choice [18–22]. However, no matter what the approach is, every solution requires the support of scientific data about the deep existing molecular mechanism to design effective prevention as well as treatment strategies.

The angiopoietin-like protein-3 (ANGPTL3) is a secreted glycoprotein composed of N-terminal coiled-coil domain and a fibrinogen-like C-terminal domain. ANGPTL3, which is linked to lipid metabolism as inhibit the enzyme endothelial lipase and lipoprotein lipase (LPL), thereby reduces plasma triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) [8]. Variants of ANGPTL3 gene locus have associated with its function and loss of function mutations in these variants cause lower levels of TG, LDL, and HDL level [9]. The present study aims towards finding the role of high-fat diet and alcohol in inducing oxidative stress in liver tissue, which causes severe damage to hepatic cells; and its relation to other factors including plasma lipid profile and body weight. Moreover, the current study also deciphers the role of ANGPTL3 locus in the development of hyperlipidemia.

Material and methods

Chemical and reagents All the reagents were procured from standard suppliers, including Himedia, Sigma, and Thermo Scientific.

Animals Healthy Wister rats weighing around 150 g were procured from the College of Veterinary Science, Khanapara, Guwahati, Assam (26.1206° N, 91.8225° E). Animals were housed in 4 groups (6 in each cage) in a room under controlled environment with temperature (22 ± 1 °C), humidity (60%), and 12 h light–12 h dark cycle. Food and water were provided ad libitum, bedding, and cages of the animals were changed twice a week throughout the experiment duration. The animals were acclimatized for 2 weeks prior to commencement of the experiment.

Experiment design For this, 24 male Wister rats divided into four groups (6 animals in each group) viz. control group (regular diet), HFD group (high-fat diet), ALC group (alcohol consuming), and HFD + ALC group (high-fat diet + alcohol consuming). Animals were sacrificed via cervical dislocation on reaching the endpoint of the study, i.e., after 12 weeks, for obtaining the liver tissue sample.

Diet feeding High-fat diet in HFD and HFD + ALC group animals were administrated orally daily for 12 weeks. High-fat diet (ingredients gram/kg of HFD) were prepared by mixing the powdered NPD (365 g), lard (310 g), casein 250 g, cholesterol (10 g), vitamin and mineral mix (60 g), DL-methionine (3 g), yeast powder (1 g), and sodium chloride (1 g).

Alcohol consumption Ethanol solution (3–15% ethanol + 0.2% saccharin) were administrated orally to ALC and HFD + ALC group animals with the water bottle for 12 weeks orally at increasing concentration of ethanol gradients (starting from 3% with gradual increase of 3% per week up to 5th week and then continued until 12th week) with subsequent weeks.

Lipid peroxidation (MDA analysis) Malondialdehyde (MDA) is produced during polyunsaturated fatty acid peroxidation and increase in free radicals leads to overproduction of MDA, commonly known as a marker of oxidative stress. Lipid peroxidation was done by assessment of MDA level in the liver tissue sample. Homogenized sample was centrifuged at 700×*g* for 10 min, and the supernatant was subjected to protocol. Briefly, 200 μl supernatant and 200 μl of SDS (8.1%) were mixed followed by addition of 1.5 ml acetic acid (20%) of pH 3.4 and 1.5 ml thiobarbituric acid (0.8%). The volume was made up to 4 ml, and the mixture was kept in a water bath at 95 °C for 60 min. After cooling tubes under running tap water, the mixture was centrifuged at 10,000 rpm for 10 min. Finally, the supernatant from the mixture was collected, and absorbance was measured at 532 nm. The results were obtained as MDA (in micromoles) per gram of tissue weight.

DNA extraction DNA was extracted by using Hipure blood genomic DNA minipreparation kit (HiMedia). To 200 μl fresh blood, 200 μl of lysis solution was added. The blood pellet was thawed by continuous pipetting. To the above solution, 20 μl of proteinase-k was added. The solution was allowed to vortex for 10–15 s. This solution was incubated at 55 °C for 10 min. 200 μl of ethanol (96–100%) was added to lysate obtained from the above step. The lysate obtained from the above step was transferred into the spin column. These spin columns with lysate were centrifuged at 14,000 rpm for 1 min. The flow-through liquid was discarded, and the column was placed in a new 2-ml collection tube. Five hundred microliters of prewash solution was added to the spin columns and were centrifuged at 12,000 rpm for 1 min. Flow through was discarded, and the column was placed in the same collecting tube. Five hundred microliters of wash solution was added to the above spin columns, and they were centrifuged at 12,000 rpm for 3 min. Flow-through liquid was discarded, and columns were placed in same collecting tube, and these empty columns were centrifuged at 12,000 rpm for 3 min. One hundred microliters of elution buffer was added to the above columns; then, they were centrifuged at 14,000 rpm for 1 min.

Again, the same step was repeated. The spin columns were discarded, and the DNA collected in elution buffer was stored at − 20°c.

Agarose gel electrophoresis Agarose (0.4 g) was added to 50 ml 1× TAE buffer in a glass beaker or flask. The mixture was heated on a microwave or hot plate or burner, swirling the glass beaker/flask occasionally, until agarose dissolves completely. The solution was allowed to cool down to about 55–60 °C. Added 0.5 μl ethidium bromide mixed thoroughly and poured the gel solution into the gel tray. Allowed the gel to solidify for about 30 min at room temperature. Two microliters of 6× gel loading dye was added to 10 μl of DNA sample and loaded onto the agarose gel well. Electrophorese run at 100–120 V and 90 mA until dye markers have migrated to an appropriate distance, depending on the size of DNA, and finally visualized under gel dock.

Mutagenic PCR DNA was amplified by a mutagenic PCR assay. A mismatched upstream primer for ANGPTL3 gene amplification was used. The primer was designed by doing BLAST in PubMed. The forward primer was 5′-AGCA CACAGACCTGATGTTTTCTAC-3′, and the reverse primer was 5′-CCACCTGAGTAACTTTCTGGACAGT-3′. Final PCR volume of 20 μl was made in PCR tube by adding PCR master mix (10 μl), forward primer (1 μl), reverse primer (1 μl), sample DNA (1 μl), and nuclease-free water (7 μl). The tubes were placed on thermal cycler to 95 °C for 5 min followed by 35 cycles of amplification as follows: for ANGPTL3 denaturation at 95 °C for the 30s, annealing at 58 °C for the 30 s, and extension at 72 °C for 45 s. The final extension step was performed at 72 °C for 10 min. After amplification, the 5 μl aliquot was analyzed by electrophoresis on a 2% (*w*/*v*) agarose gel.

RFLP analysis The expected size of the PCR product was 372 bp. Each sample (20 μl) was then digested overnight at 60 °C with five units of restriction endonuclease *DraI*. The standardization of *DraI* was done at a different temperature, concentration, and time. The products were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide. *DraI* digestion of wild-type ANGPTL3 allele yields two bands at 293 and 79 bp, while the mutant type remains intact at (372 bp).

Statistical analysis The statistical analysis was carried out using GraphPad Prism software version 8.02. The mean and standard deviation were calculated. Dunnett's multiple comparison test was used to compare the means and p value < 0.005 was considered as significant.

Language, grammar, and plagiarism The references were inserted using EndNote software version X9 (Thomson Reuters, Toronto, Canada), language and grammar were checked by Grammarly software version 6.6 (Grammarly, Inc., San Francisco, CA, USA), plagiarism was checked with the help of Turnitin plagiarism detection service (Webster St., CA), proofreading, and editing by doc navigator©, Chandigarh.

Results

Oxidative stress Oxidative stress was analyzed by estimating the MDA level in the liver tissue (Fig. 1). HFD, ALC, and HFD + ALC group show higher oxidative stress (MDA) compared to the control group. Oxidative stress found to be highest in the HFD + ALC group.

Gene mutation analysis Restriction endonuclease DraI, was used in the present study, which cuts the wild-type ANGPTL3 gene into two fragments (293 bp and 79 bp), but the mutated group does not show any cuts and gives a single band at 372 bp. RFLP analysis shows the wild-type bands in the control, HFD, and ALC groups but shows mutated band in HFD + ALC group (Fig. 2).

Lipid profile Lipid profile was measured from the liver tissue sample (Tables 1). TG, LDL, and HDL concentration were found to be increased in HFD, ALC, and HFD + ALC group compared to the control. Highest lipid profile was observed in the case of HFD + ALC group.

Body weight Total body weight of animals was measured at 5-time points (Table 2). Body weight of animals found to be higher in HFD, ALC, and HFD + ALC group compared to the control group. Highest body weight observed in HFD + ALC group after the 12th week.

Discussion

High-fat diet [10, 11] and alcohol [12] found to increase total body weight in many previous studies, and similar results were observed in the present study when rats were made to feed on high-fat diet and alcohol. Increase in body weight is evident because calories intake is high, but energy demands of the body remain the same, resulting conversion of extra calories into the fat with the parallel increase in total body weight. Although specific pathways from crosstalk which leads to increase body weight are well understood in previous studies, in the present study, an attempt was made to fill the remaining gaps in the underlying mechanism behind the weight gain by revealing the role of oxidative stress and ANGPTL3 gene mutation in hyperlipidemia.

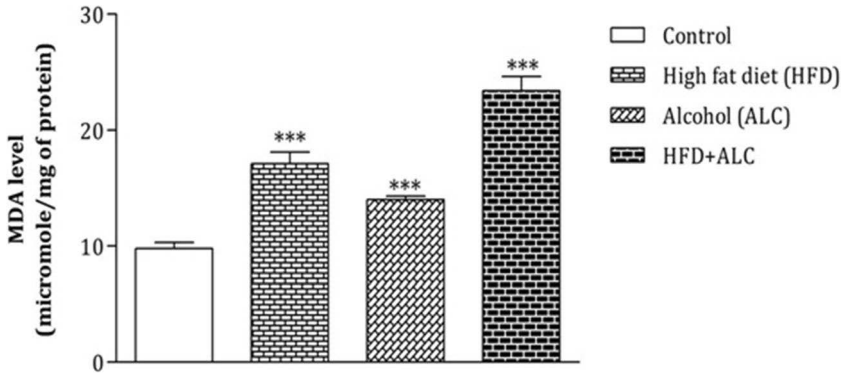

Fig. 1 All the three groups, including HFD, ALC, and HFD + ALC, show higher oxidative stress (MDA) compared to the control group (***$p > 0.001$ as compared to normal control group)

Reactive oxygen species (ROS) are the agents to increase oxidative stress in the tissue, which ultimately leads to cellular damage causing a severe problem in the liver, including alcoholic liver disease and non-alcoholic steatohepatitis [13]. High-fat diet reported to increases the oxidative stress in the liver, by decreasing expression of antioxidant enzymes like GSH-Px-1, Cu/Zn-SOD, and paraoxonase [13]. Alcohol is also found to increase oxidative stress by two pathways: dehydrogenase system and microsomal ethanol-oxidizing system. Dehydrogenase pathway initiated by alcohol dehydrogenase which oxidizes alcohol to acetaldehyde which enters into the mitochondria where it is oxidized to acetate by aldehyde dehydrogenases. The microsomal ethanol-oxidizing system involves an NADPH-requiring enzyme, the cytochrome P450 enzyme CYP2E1. Also, ethanol can also be oxidized by catalase in peroxisomes [14]. Both pathways lead to increase of reactive oxygen species which is also found in the present study indicated by increased malondialdehyde (MDA) level, which is a standard marker of oxidative stress in liver produced during polyunsaturated fatty acids peroxidation and free radical formation.

Elevated oxidative stress is associated with the gene mutation in liver cells [15]. Numerous research have been reported ANGPTL family as secreted glycoproteins which play major roles in angiogenesis, inflammation, and lipid metabolism [16]. ANGTPL3, ANGPTL4, and ANGPTL8 are primely found involved in lipoprotein metabolism. Among these, ANGPTL3 predominantly expressed in the liver and involved in upregulation of lipid metabolism and mutation in it leads to decrease in the concentration of triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), and making it as a promising candidate for hyperlipidemia [9]. The present study revealed that gain of function mutation caused by oxidative stress in the ANGPTL3 locus leads to the development of hyperlipidemia, which is thought to be associated with obesity and weight gain.

Sanger sequencing of ANGPTL3 exon carried out by Musunuru et al. [17] confirmed the two nonsense mutations in ANGPTL3. First was a single-nucleotide variant (GAA → TAA, at nucleotide 62,836,210 on chromosome 1) that introduces a nonsense mutation at position 129, resulting in the amino acid mutation E129X. The second mutation was a double nucleotide variant (TCC → TGA, at nucleotides 62,835,875 to 62,835,876 on chromosome 1) that introduces a nonsense mutation at position 17, resulting in the amino acid mutation S17X. The X in both amino acid mutations denotes a

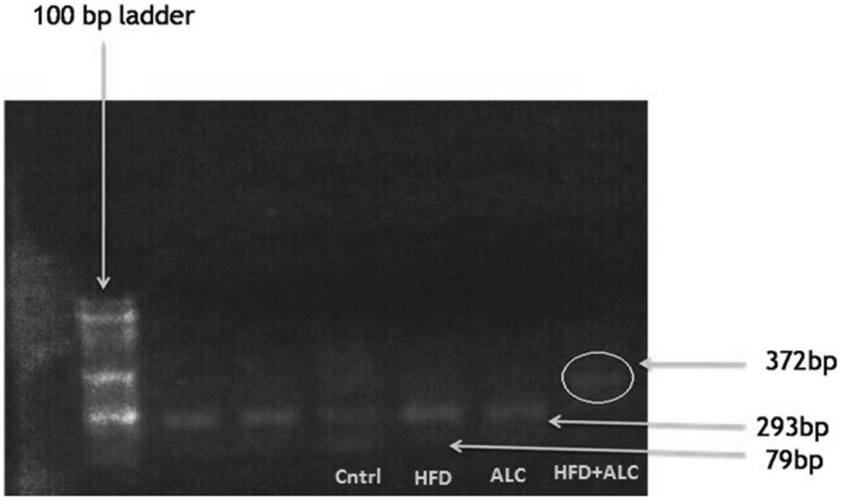

Fig. 2 RFLP analysis shows the wild type bands (293 bp and 79 bp) in the control, HFD, and ALC groups but shows mutated band (372 bp) in HFD + ALC group

Table 1 All the three groups, including HFD, ALC, and HFD + ALC, show higher lipids concentration compared to the control group (**$p > 0.01$ as compared to normal control group; ***$p > 0.001$ as compared to normal control group)

S. no.	Parameters	Control	HFD	ALC	HFD + ALC
1	TG (mg%)	279.74 ± 32.70	485.98 ± 27.03***	437.78 ± 20.81**	587.17 ± 20.65***
2	LDL (mg%)	73.36 ± 3.92	152.76 ± 5.76**	186.12 ± 8.48***	250.29 ± 14.54***
3	HDL (mg%)	60.44 ± 9.07	175.93 ± 5.18***	172.87 ± 8.84***	202.76 ± 18.51***

Table 2 All the three groups, including HFD, ALC, and HFD + ALC, show higher body weight compared to the control group (**$p > 0.01$ as compared to normal control group; ***$p > 0.001$ as compared to normal control group)

S. no.	Time point	Control	HFD	ALC	HFD + ALC
1	Day 1	178.38 ± 5.32	174.30 ± 8.38	179.30 ± 5.39	170.30 ± 5.39
2	3 weeks	209.31 ± 3.29	258.38 ± 7.83**	200.38 ± 7.48	238.40 ± 6.0
3	6 weeks	250.51 ± 10.48	302.81 ± 13.29**	248.49 ± 7.44	312.11 ± 9.9***
4	9 weeks	270.34 ± 14.22	350.54 ± 9.38***	300.41 ± 10.39	395.87 ± 11.38***
5	12 weeks	275.18 ± 6.11	309.49 ± 8.44***	333.69 ± 9.48***	352.49 ± 8.47***

stop codon. Individuals heterozygous for just one of the two nonsense mutations are found to have significantly lower plasma levels of LDL cholesterol and triglycerides compared to individuals with neither mutation. Individuals who are compound heterozygotes are found to have even lower plasma LDL cholesterol and triglyceride levels. Thus, the nonsense mutations in ANGPTL3 affected phenotypes in a manner dependent on the gene dosage, which is the number of alleles present in a given person [17].

As a strength, the present study revealed the putative underlying mechanism (Fig. 3), which shows that a high-fat diet with alcohol causes the formation of ROS in the liver. This ROS leads to an elevation in oxidative stress, which causes a gain of function mutation in ANGPTL3 gene. Wild-type ANGPTL3 which forms two fragments (293 bp and 79 bp) upon restriction endonuclease DraI digestion gene formed a single mutated fragment of 372 bp. This gain of function mutation leads to enhance expression of ANGPTL3 gene and results in the elevated level of TG, LDL, and HDL. Hyperlipidemia finally causes weight gain, which indicates obesity.

Although the current study explained the possible mechanism for oxidative stress-induced hyperlipidemia, it has still several limitations. Gene mutation found to occur only in animals which were made to feed on a high-fat diet with alcohol, but no mutation was detected in animals feeding on high-fat diet only or alcohol only, even if these groups show elevated oxidative stress and lipid profile. One possible explanation for this is that the magnitude of oxidative stress is not sufficient enough to cause a mutation in the gene. The present study is a primary study; therefore, it lacks sufficient convincing data about oxidative stress directly responsible for the putative "mutation" in ANGPTL3. Further, in-depth follow-up studies are required to give a well-founded shape to the putative

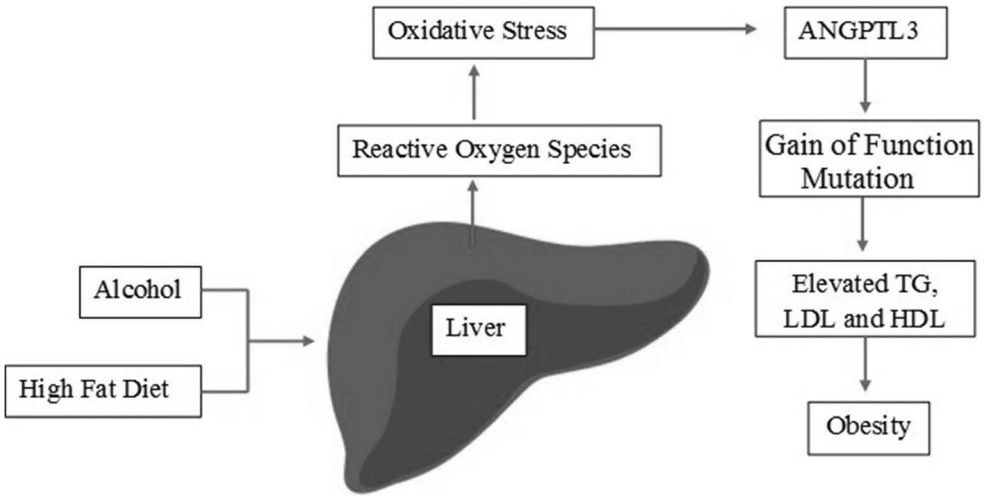

Fig. 3 Putative underlying mechanism of hyperlipidemia caused by alcohol and high-fat diet

mechanism. Also, in the present study, we directly checked the mutation in ANGPTL3, being ANGPTL3 most common studied gene for hyperlipidemia, it may also be possible that reactive oxygen species which are generated by the HFD and ALC may cause the random non-specific mutation, which still needs to be deciphered.

Conclusion

A high-fat diet, which is very common these days among an especially young generation of the country, is alone enough to cause metabolic disorders, and when it combines with alcohol, the situation goes from bad to worst. The present study demonstrates that intake of high-fat diet and alcohol elevates the oxidative stress in the liver, which induces gain of function mutation in the ANGTPL3 locus leading to hyperlipidemia. Therefore, it is necessary to have awareness programs for a healthy lifestyle on general public level, and more research should be done at a scientific level to understand the deep existing molecular mechanism to design new prevention and treatment tools.

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Author contributions Jyoti Saini and Ashok K Jangra designed the concept, conducted the study, and generated the raw data. Atul K Goyal compiled the raw data, did statistical analysis, and drafted the manuscript.

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Compliance with ethical standards

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

Ethical statement The study was approved by the Institutional Animal Ethics Committee (IAEC), Guwahati Medical College & Hospital (CPSCEA Registration No. 351;3/1/2001) under Approval No. MC/32/2013/36.

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ORIGINAL ARTICLE

Association of advanced glycation end products (AGEs) with endothelial dysfunction, oxidative stress in gestational diabetes mellitus (GDM)

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Abstract

Background Advanced glycation end products (AGEs) are major risk factors for vascular complications in diabetes. Its role in gestational diabetes mellitus (GDM) and vascular complications in GDM is not known.

Objective The present study was aimed to study the association of AGEs with GDM and vascular inflammation.

Methods Plasma samples from normal pregnant and GDM women ($n = 50$ each) were obtained from two tertiary referral centers in Tamil Nadu, India. Quantification of AGEs, methylglyoxal (MGO), ICAM-1, and malondialdehyde (MDA) were performed by commercially available ELISA kits.

Results and conclusions The third trimester fasting blood sugar (101.35 ± 26.15 vs. 81.63 ± 6.14, $p < 0.002$) and postprandial blood sugar (150.69 ± 23.07 vs 105.79 ± 11.99, $p < 0.0001$) were significantly high in GDM women compared to normal pregnant women. The concentrations of AGEs (13.18 ± 8.74, $p < 0.001$), MGO (15.7 ± 13.54, $p < 0.02$), and ICAM-1 (217.8 ± 86.92, $p = 0.005$) were significantly higher in GDM women compared to AGEs (2.68 ± 0.89), MGO (9.26 ± 5.38), and ICAM-1 (142.3 ± 38.21) in normal pregnant women. Further, elevated levels of MDA concentration (0.64 ± 0.08, $p < 0.002$) and low GSH levels (0.19 ± 0.1, $p < 0.0001$) in the GDM women were indicative of oxidative stress. AGE levels significantly correlated with MDA concentration which indicates AGEs may be responsible for oxidative stress in GDM women. Further, elevated level of ICAM-1 in GDM women suggests endothelial activation which may impact endothelial function. Thus, AGEs may be used as a biomarker during pregnancy to predict vascular complications due to GDM.

Keywords AGEs · GDM · Hyperglycemia · Vascular dysfunction

Sundar Krishnasamy and Barathi Rajaraman contributed equally to this work.

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Introduction

GDM (gestational diabetes mellitus) is a state of glucose intolerance where a woman develops diabetes for the first time during pregnancy. There is an increased prevalence of GDM globally. It affects nearly 16–17 % of pregnant women in India [1]. In addition to increased pregnancy risks to mother and fetus, 30–50 % of these women go on to have GDM in the next pregnancy and within 10 years develop type 2 diabetes mellitus (T2DM) [2]. A major pathological consequence of chronic hyperglycemia is the formation of advanced glycation end products (AGEs) which are formed due to non-enzymatic glycation of proteins, lipids, and nucleic acids [3–5]. Several studies have also shown that AGEs and its precursors promote oxidative stress and inflammation by binding to its receptors and thereby causing vascular damage [6–8].

AGEs elicit its function upon binding to receptor for advanced glycation end products (RAGEs). AGEs are reported to activate certain signaling cascades that damage endothelium and strongly suggested to play a role in the development of diabetic vascular complications [9, 10]. Further, AGEs are also reported to deactivate the eNOS enzyme which leads to reduction in nitric oxide and poses a greater risk to vascular endothelium that could damage the vascular permeability [11–13]. In addition, AGEs induce inflammation which is evident by the production of pro-inflammatory cytokines and inflammatory molecules such as TNF-α and IL-6 [14]. Among the prominently studied AGEs, the most stable and relatively inert is Nε-carboxy-methyl-lysine (CML), and of all the AGEs precursors studied, methylglyoxal (MGO) and its derivatives are found to be more potent dicarbonyl compounds. MGO is found to be harmful since it binds to arginine residues in the proteins forming hydroimidazolone adducts (MG-H1). These adducts are targeted for protein degradation and thus reducing the lifespan of proteins. Since arginine is found predominantly in the active sites of many enzymes, MGO inactivates or alters the function of many enzymes [15].

Exposure of the fetus to maternal diabetes is associated with the high risk of abnormal glucose homeostasis in the offspring. Abnormal endothelial function is a common feature in GDM; however, cellular mechanisms underlying altered umbilical endothelial function in gestational diabetes are still not understood completely. It is evident from the above literature that AGEs play a crucial role in the development of vascular complications. However, to date, no studies have demonstrated the role of AGEs in GDM pathophysiology. Hence, the objective of the present study is to understand the association of AGEs with oxidative stress and vascular inflammation in GDM. This study may serve as a prelude to study the mechanisms underlying the endothelial inflammation in GDM.

Materials and methods

We recruited the third trimester pregnant women visiting the K.A.P. Viswanathan Government Medical College and Hospital, Trichy, Tamil Nadu, India and Kovai Medical Center and Hospital, Coimbatore, India (normal = 50 and GDM women = 50). Women with singleton pregnancy were considered for this study. The sample size was calculated using a power analysis software [16]. The estimated required sample size to give a power of 90 % with $\alpha = 0.05$ significance for AGEs is 32, 58 samples for ICAM-1, MGO, and MDA, 28 samples for GSH and safely we considered $n = 100$ (normal = 50 and GDM women = 50) to negate any inter-individual confounding results [16]. The template for the patient consent information is provided as supplementary (Figure S1). GDM was diagnosed using the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria if one or more values equaled or exceeded the following thresholds: fasting plasma glucose of 5.1 mmol/L (92 mg/dL), 1-h plasma glucose level of 10.0 mmol/L (180 mg/dL), and 2-h plasma glucose level of 8.5 mmol/L (153 mg/dL) [17]. Age and BMI matched pregnant women were included in the study (Table 1). In this study, 63 % of first parity, 33 % of second parity, and 3 % of third parity of GDM women were included.

However, those with history of chronic illness including chronic kidney, liver, or heart disease, pregnancy complications such as hypertension, pre-eclampsia, pre-pregnancy diabetes, preexisting maternal diseases (thyroid, renal), and smoking were excluded in the study based on their clinical history. Blood was collected at 28–32 weeks of gestation for biochemical analysis.

Anthropometric measurements and biochemical parameters

We followed the standard procedures for measuring blood pressure, height, and weight as given in the earlier study [18]. Blood pressure was recorded from the right arm in a sitting position to the nearest 2 mmHg with a mercury sphygmomanometer (Diamond Deluxe BP apparatus, Pune, India). Two readings were taken 5 min apart and the mean of the two was taken as the blood pressure.

Height was measured to the nearest centimeter. Weight was measured by using the balance placed on a horizontal surface [18]. The fasting and postprandial blood glucose were measured by the GOD-POD method. Glucose measurements were analyzed by the Hitachi-912 autoanalyzer (Hitachi, Mannheim, Germany). The postprandial plasma was used for AGEs, methylglyoxal, ICAM-1, GSH, and MDA detection.

ELISA for MGO and AGEs Quantitative determination of human endogenous MGO and AGEs were determined by ELISA using commercially available kit (Cusa Bio kit, USA) according to manufacturer's protocol. Briefly, 0.1 ml of plasma was

Table 1 Clinical characteristics and anthropometric measures of the individuals participating in the present study

Status of the women	Normal pregnant women ($n = 50$) (mean ± SD)	GDM ($n = 50$) (mean ± SD)	p value
Age (years)	25.00 ± 4.09	27.00 ± 4.41	0.07
First trimester (weight (kg))	59.31 ± 9.95	60.14 ± 7.76	0.74
Weight (28–32 weeks pregnancy) (kg)	60.24 ± 9.78	63.18 ± 12.16	0.15
Baby birth weight (kg)	2.99 ± 0.40	3.16 ± 0.48	0.19
Height (cm)	156.6 ± 6.84	155.1 ± 6.20	0.26
BMI (kg/m^2)	24.22 ± 4.67	25.72 ± 5.48	0.25
Systolic BP (mmHg)	100.0 ± 9.36	108.1 ± 9.17	0.28
Diastolic BP (mmHg)	72.76 ± 7.91	73.74 ± 6.69	0.47
FBS (mg/dL)	81.63 ± 6.14	101.35 ± 26.15	*0.002**
PPBS (mg/dL)	105.79 ± 11.99	150.69 ± 23.07	*0.0001**
TSH (mIU/L)	2.9 ± 0.39	2.09 ± 0.22	0.140
Creatinine (mg/dL)	0.55 ± 0.02	0.55 ± 0.22	0.145
MDA (nmol)	0.55 ± 0.06	0.64 ± 0.08	*0.002**
ICAM-1 (ng/mL)	142.3 ± 38.21	217.8 ± 86.92	*0.005**
GSH (pmol/mL)	0.31 ± 0.12	0.19 ± 0.10	*0.0001**
MGO (ng/mL)	9.26 ± 5.38	15.7 ± 13.54	*0.02**
AGEs (μg/mL)	2.68 ± 0.89	13.18 ± 8.74	*0.001**

To distinguish between the significant and non-significant in the parameters, significant values are italicized

pipetted out to each 96-well plate one specific for MGO and AGEs identification. Each plate has wells pre-coated with antibody specific for MGO and AGEs respectively. After 2 h of incubation at 37 °C, wells were washed, and biotin-conjugated antibodies were added. After incubation, the wells were washed and avidin-conjugated horseradish peroxidases were added to the wells followed by a substrate solution. The amount of color developed was directly proportional to the amount MGO and AGEs bound in the initial step, and the color developed was read at 450 nm. Multi-mode microplate reader (Bio-Tek Synergy H1) from Bio-Tek USA was used to measure the optical density. Inter- and intra-assay co-efficient of variation was calculated to be 9.4 and 1.2 respectively for MGO and 12.5 and 8.2 respectively for AGEs.

ELISA for ICAM-1 ICAM-1 was measured by ELISA, Bio Vision, USA according to the manufacturer's protocol. Briefly, 100 μL of plasma is added to the 96-well plate and incubated for 90 min. ICAM-1 present in the plasma sample binds to monoclonal antibody from mouse specific for ICAM-1 which was pre-coated in the 96-well plate. A biotinylated antibody specific for ICAM-1 was subsequently added. After washing, an avidin-biotin-peroxidase complex was added to the wells. Finally, the TMB substrate was used to visualize the enzyme reaction which was read at 450 nm. The inter- and intra assay co-efficient of variation was calculated to be 12 and 2 respectively for ICAM-1.

ELISA for MDA levels MDA levels were measured by ELISA, Sigma-Aldrich, USA by following the instructions in the kit. Before initiating formation of MDA-TBA adduct, the plasma samples were prepared for the study. Briefly, 10 μL of plasma samples were mixed with 500 μL of 42 mM sulfuric acid and 125 μL phospho-tungstic acid, and centrifuged for 3 min at 13000 *g*. The pellet was suspended in 100 μL of butylated hydroxyl toluene (BHT), and it was made up to 200 μL with water. MDA standards were prepared in the range of 0–20 nmol. To form MDA-TBA adduct, 600 μL of thio-barbituric acid (TBA) was added to samples and standards and incubated at 95 °C for 60 min. After incubation, 200 μL of the reaction mixture was read at 532 nm. The inter- and intra assay co-efficient of variation was calculated to be 15 and 2.88 respectively for MDA.

ELISA for glutathione kinetic assay Glutathione kinetic assay was performed using commercially available Sigma-Aldrich ELISA kit. Briefly, 200 μL of plasma samples were initially deproteinized by 5 % 5-sulfosalicylic acid and then used for the glutathione kinetic assay. Glutathione standards were prepared by diluting an aliquot of standard glutathione (10 mM) stock solution 200 folds to get 50 μM of concentration with 5 % 5-sulfosalicylic acid. It was then diluted serially to get a concentration range of 0–50 μM. Ten microliters of each of samples and standards were pipetted out into a 96-well plate. One hundred fifty microliters of working mixture containing buffer, glutathione reductase, and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution were added and incubated for 5 min at room temperature. Fifty microliters of NADPH solution were added after incubation, and optical density at 412 nm was measured with a kinetic read for 5 min at a 1-min interval.

Statistical analyses PS program software version 3.1.2 was used to calculate the power and sample size for the present study. Student's non-parametric *T* test was used to detect significant differences among mean values of each parameter studied in healthy and GDM women; a significance level was taken as $p < 0.05$. Pearson correlation was performed to test the association of AGEs concentration with other risk variables and multiple regression analysis was done to test the influence of risk variables on the disease outcome. All analyses were performed using the SPSS Statistics version 20.

Results

The biochemical and anthropometric measures of participants with and without GDM are shown in Table 1. Age, height, weight (first and third trimester), birth weight of babies, and BMI of normal and GDM women were not significantly different. Systolic and diastolic BP of normal pregnant women were 100 ± 9.36 and 72.76 ± 7.91 mmHg respectively which were not significantly different from GDM women 108.1 ± 9.17 and 73.74 ± 6.69, $p < 0.28$ and 0.47 respectively.

FBS (101.35 ± 26.15 mg/dL, $p < 0.002$) and PPBS (150.69 ± 23.07 mg/dL, $p < 0.0001$) levels were higher in GDM women compared to normal healthy pregnant women (81.63 ± 6.14 mg/dL and 105.79 ± 11.99 mg/dL) respectively. Table 1 also shows higher levels of MDA (0.64 ± 0.08 nmol, $p < 0.002$) and ICAM-1 (217.8 ± 86.92 ng/mL, $p < 0.005$) in GDM women compared to normal pregnant women (0.55 ± 0.06 nmol and 142.3 ± 38.21 ng/mL) respectively. GSH concentration was found to be significantly lower ($p < 0.0001$) in GDM women (0.19 ± 0.1 pmol/mL) compared to normal pregnant women (0.31 ± 0.12 pmol/mL). Furthermore, MGO and AGEs concentration in GDM women found to be high (15.7 ± 13.54 ng/mL, $p < 0.02$ and 13.18 ± 8.74 (μg/mL), $p < 0.001$ respectively) when compared with normal pregnant women (9.26 ± 5.38 ng/mL and 2.68 ± 0.89 μg/mL respectively).

Pearson correlation analysis showed a significant positive correlation of AGEs concentration with PPBS levels ($r = 0.330$, $p < 0.05$), MGO concentration ($r = 0.454$, $p < 0.001$) (Table 2), MDA concentration ($r = 0.630$, $p < 0.001$) (Table 2), and ICAM-1 concentration ($r = 0.490$, $p < 0.05$) (Table 2).

Multiple logistic regression analysis revealed that AGEs was significantly associated with GDM with odds ratio (OR) 1.184, 95 % confidence interval (CI) 1.055–1.327, $p = 0.004$*. Association of AGEs with GDM was significant even after adjusting for body weight [OR 1.174, 95 % CI 1.037–1.329, $p = 0.011$*] and ICAM-1 with OR 1.148, 95 % CI 1.014–1.300, $p = 0.029$*. However, the significant association was lost after adjusting with MDA with OR 1.156, 95 % CI 0.966–1.384, $p = 0.098$ (Table 3).

Table 2 Pearson's correlation analysis of AGEs concentration with other risk variables

Parameters	*R*	Significance (*p* value)
Age	− 0.062	0.681
BMI	0.145	0.338
FBS	0.144	0.277
PPBS	0.330	*< 0.05**
MGO	0.454	*< 0.001**
MDA	0.630	*< 0.001**
ICAM-1	0.49	*< 0.05**
GSH	0.015	0.912

To distinguish between the significant and non-significant in the parameters, significant values are italicized

Discussion

GDM is a heterogeneous disorder which involves various factors acting in concert leading to hyperglycemia and terminating into vascular complications in mother as well as the offspring. This may be due to various factors such as reduction in insulin sensitivity and impaired insulin secretion, pancreatic beta cell dysfunction, and increased inflammation and many others [19]. The primary cause for the maternal hyperglycemia that induces changes in the intrauterine milieu is found to be oxidative stress [20, 21]. However, there is no evidence on how ROS signaling is mediated in the GDM pathophysiology. AGEs play a pivotal role in the induction of oxidative stress in diabetes. The present study, as per our knowledge for the first time demonstrates the association of AGEs with GDM in south Indian mothers. Further, we also show that elevated AGEs have a strong correlation to lipid peroxidation. Recently, a study published from our group has clearly established role of AGE in causing vascular inflammation in GDM through activation of early growth response-1. In the present study, it had shown the association of AGEs with GDM and its correlation with inflammation and oxidative stress. This study may be the starting point of understanding the role of AGEs in pathophysiology of GDM and the impact on the feto-placental vasculature which may have a long-term influence on the off-spring and the mother [22].

Earlier studies have demonstrated an increased systemic level of AGEs, markers of pro-inflammation and oxidative stress in women with pre-diabetes, and metabolic syndrome and in patients with type 2 diabetes mellitus [23–28]. In addition to the endogenous AGEs that are formed due to chronic hyperglycemia, the dietary AGEs too contribute to the pool of AGEs in vivo. Evidences suggest that higher dietary AGEs added to the pool of endogenous AGEs cause beta cell damage and insulin resistance. Mericq et al. [29] had reported food derived AGEs when consumed during pregnancy is transmitted to

Table 3 Multiple Logistic Regression with GDM as dependent variable

Model No.	Variable	Odds Ratio	95% Confidence Interval (CI)	*p-value*
Model 1	Unadjusted AGEs	1.184	1.055-1.327	*0.004**
Model 2	Model 1 with Gestational weight	1.174	1.037-1.329	*0.011**
Model 3	Model 2 with ICAM-1	1.148	1.014-1.300	*0.029**
Model 4	Model 3 with MDA	1.156	0.966-1.384	0.098

To distinguish between the significant and non-significant in the parameters, significant values are italicized

the developing fetus. They have demonstrated that serum CML and MGO present during pregnancy positively correlated with the newborn CML and MGO concentration.

In the present study, a significant rise in the MGO levels correlate with the AGE levels. Further, the elevated MGO and AGEs may contribute to oxidative stress and inflammation in the feto-placental vasculature. To date, MGO is the best characterized AGEs precursor. Earlier studies in animals have shown that MGO and AGEs mediate endothelial dysfunction, in part, by RAGE driven mechanisms [30]. MGO is considered a major risk factor as it is one of the predominant AGE precursors formed early during the progression of diabetic vascular complications.

AGE-RAGE interaction induces oxidative stress in vascular endothelial cells [31]. AGEs induce ROS and RNS generation by activating the NADP(H) oxidase pathway [32, 33]. In the present study, the MDA which is formed due to lipid peroxidation is found to be significantly high in GDM women compared to normal pregnant women indicative of oxidative stress.

This is further supported by the reduced GSH levels in the GDM women compared to healthy controls. The reduced GSH activity demonstrates the reduced antioxidant capacity in GDM women compared to normal pregnant women. AGEs are reported to inactivate GSH-related enzymes and thus affect GSH concentration [34–36]. Thus, the increased AGEs may be responsible for the reduced GSH activity and in turn contribute to the diminished antioxidant status as observed in case of GDM women.

The activation of AGE-RAGE signaling cascade involves the upregulation of transcription factor NF-kB which triggers the secretion of pro-inflammatory cytokines such as IL-6, IL-1α, ICAM-1, and tumor necrosis factor-α [37, 38] and damages the endothelial cells.

Elevated level of cellular adhesion molecules is seen in GDM women [39] which suggests the persistent damage to endothelium due to inflammation. In the current study, elevated levels of ICAM-1 in GDM women also indicate enhanced inflammatory response. Increased ICAM-1 induces pro-inflammatory effects and transmigration of leukocytes across vascular endothelium and thus affecting the endothelial integrity. Increased circulatory ICAM-1 also causes vascular activation and vascular dysfunction in endothelial cells. The results were consistent with the early study by Poniedziałek-Czajkowska et al. [40]. These data confirm that elevation in AGEs levels accompanied by increased expression of ICAM-1 in GDM women may result in the damage to endothelium. Regression analysis revealed that AGEs are significantly associated with GDM, suggesting vascular complication. Further adjusting with weight and subsequently with ICAM-1, vascular inflammation marker, the significance was not lost, suggesting significant oxidative stress in GDM women. However, the significance was lost when adjusted with MDA. The results suggest AGEs cause oxidative stress in GDM women. Longitudinal studies with serial measurements of AGEs need to be done at different stages of pregnancy to further clarify the predictive role of AGEs/glucose levels for vascular complications.

Beyond genetic considerations, maternal transmission of disease risk has recently been raised as a serious contributing factor to diabetes, which increasingly affects younger adults and children. Researchers have shown evidence linking poor diet to risk of diabetes and other age-related diseases in later stages of life. High levels of AGEs have been observed in the offsprings born to GDM mothers. In summary, our study demonstrates association of AGEs with GDM. We have shown that AGE induced oxidative stress in GDM might contribute to vascular dysfunction. This is a clinical association study and future work focusing on the cause and effect of AGEs in causing feto-placental vascular dysfunction in GDM may be interesting. Nevertheless, this study highlights the significance of pursuing AGE as a potential biomarker for predicting complications in GDM.

Conclusions

The study for the first time has shown a clinical association of AGEs with GDM in southern Indian mothers. Elevated AGE levels accompanied by increased oxidative stress and inflammation might contribute to the vascular complication in GDM. AGEs may be used as an important biomarker in predicting GDM. AGEs may be an important biomarker to predict alteration in vasculature of endothelium during GDM, the influence of which may be studied separately as a prospective cohort.

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical committee approval (EC/AP/423/12/2015 & MCRC.IEC#001, 2015) and written informed consent were obtained from all study participants.

Abbreviations GDM, gestational diabetes mellitus; AGEs, advanced glycation end products; MGO, methylglyoxal; MDA, malondialdehyde; ICAM-1, intercellular adhesion molecule-1; GSH, glutathione reduced; BMI, body mass index; FBS, fasting blood sugar; PPBS, postprandial blood sugar

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ORIGINAL ARTICLE

Blockade of receptor for advanced glycation end products improved essential response of inflammation in diabetic wound healing

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Abstract

Background Inflammation in impaired diabetic wound healing has been described as caught in a persistent inflammatory state. Accumulation of advanced glycation end products (AGEs) is closely relevant to impaired diabetic wound. Recent studies identified that blockade of AGEs-RAGE increased neovascularization and granulation tissue formation to improving diabetic wound healing.

Objective This study aimed to evaluate the correlation among the pathogenic effects of AGEs-RAGE, persistent abnormal inflammatory inflammation stage, and impaired wound healing on diabetes.

Methods Authors examined the levels of inflammatory factor secretion and inflammatory leukocytes infiltration and the connection with AGEs-RAGE interaction on diabetic mice.

Results Blockade of AGEs-RAGE improved essential secretion of inflammatory factors and infiltration of inflammatory leukocytes in the early inflammatory stage. Furthermore, it also improved the clean of neutrophils by macrophages.

Conclusions In summary, these findings suggest that rather than a hyper-inflammatory state, diabetic wounds are lack of an essential inflammatory response in the early phase, thus may induce a paradoxical and persistent inflammation state, and AGEs-RAGE play a vital role in these pathogenic progresses.

Keywords Diabetes mellitus · Inflammation · Wound healing

Introduction

Impaired wound healing is one of the hallmarks of diabetes, which results in a significant financial and healthcare burden. Diabetes is characterized by persistent hyperglycemia, which causes a number of physiological and biochemical changes. As a result of the glycosylation of various proteins, advanced glycation end products (AGEs) accumulated [1]. In addition, the interaction between AGEs and the receptor for AGEs (RAGE) have been found to be closely related to impaired diabetic wound healing [2]. Our previous study also had shown an acceleration of wound healing after blocking AGEs-RAGE [3].

Normal skin wound healing is composed of three sequential but overlapping phases: (1) the inflammatory phase; (2) the proliferative phase; and (3) the maturation and remodeling phase [4–6]. As the initiative progress of wound healing, inflammation in the diabetic wound has been described as caught in a persistent inflammatory state, characterized by the accumulation of pro-inflammatory cytokines and proteases [5, 7]. Therefore, the inflammatory response remains in an unresolved state, which is recognized as a major culprit contributing to impaired diabetic wound healing [8, 9].

Given the importance of a well-balanced inflammatory response in wound healing, as well as the potential role of AGEs-RAGE signaling in the pathology associated with the impaired healing of diabetic wounds, we aimed to study the inflammatory responses during the early inflammatory phase in STZ-induced diabetic mice. Our prior work has shown the confirmation of the diabetic state and expression localization of AGEs and RAGE in the animal models, and topical application of anti-RAGE antibody accelerated diabetic wound

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healing [3]. In this study, we examined the levels of inflammatory factor secretion and inflammatory leukocytes infiltration and the connection with AGEs-RAGE interaction on diabetic mice. Our results suggest that, during the early phase of wound healing, rather than existing in hyper-inflammatory state, there is an insufficient essential inflammatory response. The deficiency may induce a paradoxical and persistent inflammatory state, which had close connection with AGEs-RAGE.

Materials and methods

Induction of diabetes mellitus

Male C57BL/6 mice (8–10-week-old; 20–25 g) were purchased from the Experimental Animal Center of Ruijin Hospital, Shanghai, China, and maintained in a conventional facility with a 12-h light/dark cycle with access to standard laboratory food and water. All experimental procedures were in compliance with the institutional laboratory guidelines and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Diabetes was induced by multiple intraperitoneal injections of streptozocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 50 mg/kg body weight (dissolved in citrate buffer, pH 4.5) for five consecutive days. For the preparation of citrate buffer, we put citric acid (2.1 g) into distilled water (100 mL) to prepare liquid A, while citrate sodium (2.94 g) was out into distilled water (100 mL) to prepare liquid B. When used, liquids A and B are mixed in a ratio of 1:1. The pH value is determined by a pH meter, and the pH value is adjusted to be 4.2–4.5. Before injection, STZ was dissolved in citrate buffer at 1% concentration. Blood glucose measurements were performed for 8 weeks after the injection. The occurrence of polyuria, polydipsia, polyphagia, weight loss, and elevated blood glucose (16.7 mmol/L) confirmed the desired effects.

Creation of full-thickness skin wounds

Mice were anesthetized using intraperitoneal injections of sodium pentobarbital (60 mg/kg body weight). After shaving their backs, we applied a depilatory agent onto the skin to remove any remaining hair, and prepped the surgical area of the skin with normal saline and benzalkonium bromide. A sterilized punch (9 mm diameter) was used to remove the skin and panniculus carnosus. Throughout the entire experimental period, the mice were housed individually and a semipermeable transparent dressing (Tegaderm; 3 M Health Care, St. Paul, MN) was used to cover the wounds; the dressing was replaced every 2 days until day 7. The mice were randomly assigned to three groups: In group R (n = 24), anti-RAGE antibodies (20 μg/dose; Abcam, UK) were topically administered to the wounds on postoperative days 0, 2, 4, and 6. The other control groups were administered normal saline (group C, n = 24) or a rabbit IgG isotype (Bioss, Beijing, China) (group I, n = 24), respectively.

Analysis of wound healing

On days 0, 1, 3, 7, and 14 after wounding, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital. The edge of the wound was traced onto a transparent plastic membrane, which was then scanned for analysis using ImageJ 1.49v software.

Preparation of the wound tissue

The wound and the surrounding tissues (approximately 5 mm beyond the wound) were excised on days 1, 3, 7, and 14 after wound formation. The excised tissues included the subcutaneous fat underneath the wound. The tissue samples were fixed in 10% neutral buffered formalin and stored at − 80 °C until further analysis.

Hematoxylin and eosin staining

The prepared wound sections were embedded in paraffin and sections were generated (6–7 μm thick). The samples were then deparaffinized in xylene, rehydrated through a graded series of alcohol to phosphate buffered saline (PBS), and stained with hematoxylin and eosin (H&E) for subsequent histological and morphometric evaluation. For the quantitative analysis of neutrophils, six tissue section slices from each group were randomly chosen, and five fields per slice at × 200 magnification were captured using a light microscope (Zeiss, Axioskop 2 Plus, Germany).

Immunohistochemistry

After deparaffinizing, rehydrating, and washing, the tissue sections underwent immunohistochemical staining for CD68 using pancreatin for antigen retrieval. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. To block nonspecific reactions, the sections were incubated with PBS containing 10% fetal bovine serum. The sections were then incubated with mouse monoclonal anti-CD68 (1:400; Abcam, UK) at 4 °C overnight. Following washing, HRP-labeled secondary antibody (Dako, Denmark) was applied at room temperature for 1 h, followed by staining with diaminobenzidine (DAB), and counterstaining with hematoxylin. Stained cells on the wound edge were manually counted on a Zeiss microscope at × 200 magnification. The images were captured and processed with SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI).

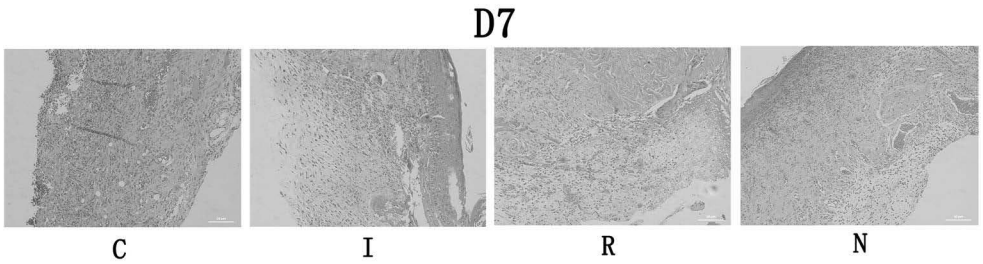

Fig. 1 The hematoxylin and eosin (H&E) staining showed the anti-RAGE antibody–treated diabetic wounds exhibited an increased number of granulation tissue forming, angiogenesis occurring, dermal papilla, and well-constructed epidermal compared with diabetic control mice (× 200 magnification) C: group C; I: group I; R: group R; N: group N

Enzyme-linked immunosorbent assay

Frozen skin tissue samples were ground into a homogenate. Following centrifugation, the expression of in inflammatory factors (TNF-α, IFN-γ, IL-1α, IL-6, and MPO) in the supernatants was measured using commercial ELISA kits (Senxiong Biotech, Shanghai, China) according to the manufacturer's instructions.

Transmission electron microscopy

Tissues obtained on days 1 and 3 after wound formation were fixed, dehydrated, and embedded in Araldite CY212. Ultrathin sections were stained with uranyl acetate and lead citrate, and visualized via TEM (CM-120 BioTwio, Philips).

Statistical analysis

All data were represented as the means ± SD and analyzed with SPSS for Mac 21.0 (SPSS, Chicago, IL, USA). An analysis of variance (ANOVA) and Student's *t* test were applied to determine the statistical significance of differences between the groups. Statistical significance was defined as $p < 0.05$.

Results

Topical application of anti-RAGE antibodies promotes granulation tissue formation during wound healing in diabetic mice

H&E staining of 7-day-old wounds revealed that the anti-RAGE antibody–treated wounds exhibited an increased level

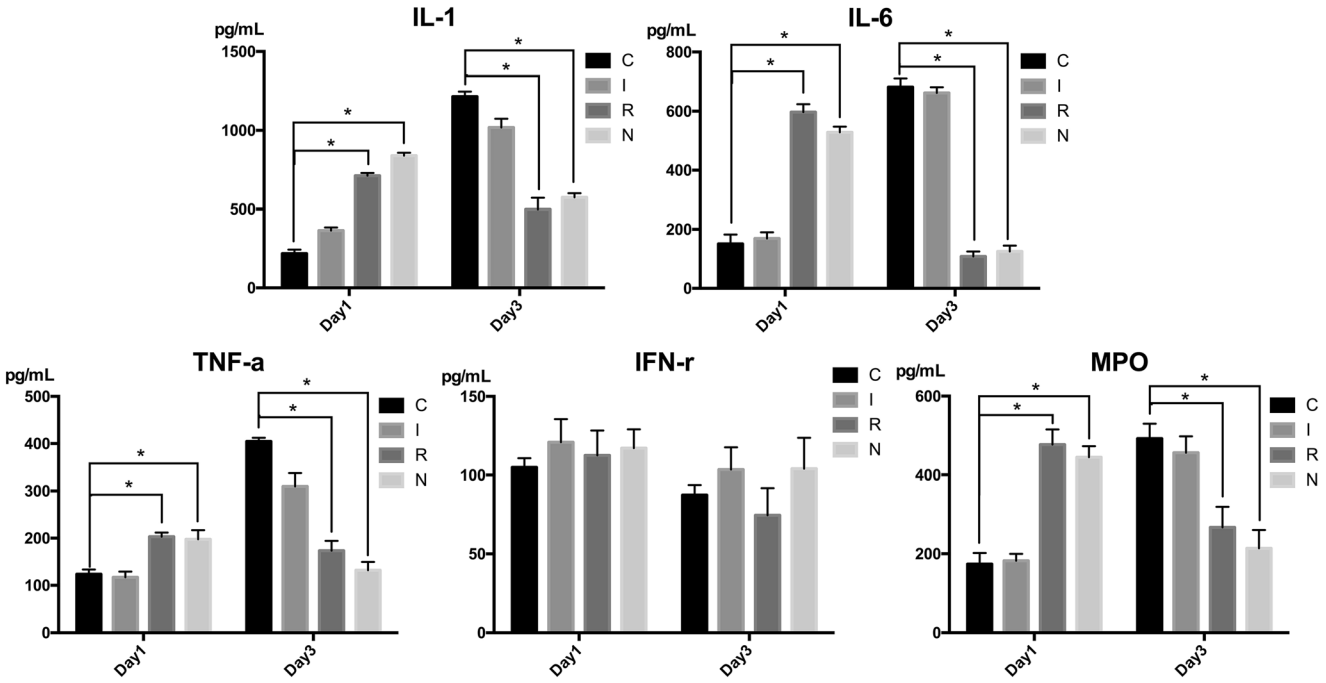

Fig. 2 ELISA showing the secretion levels for TNF-α, IFN-γ, IL-1α, IL-6, and MPO of each experimental group on days 1 and 3 ($n = 6$, $^*p < 0.05$). On day 1, the secretion levels of TNF-α, IL-1α, IL-6, and MPO in group R are much higher than those in group C, which were the opposite on day 3 C: group C; I: group I; R: group R; N: group N

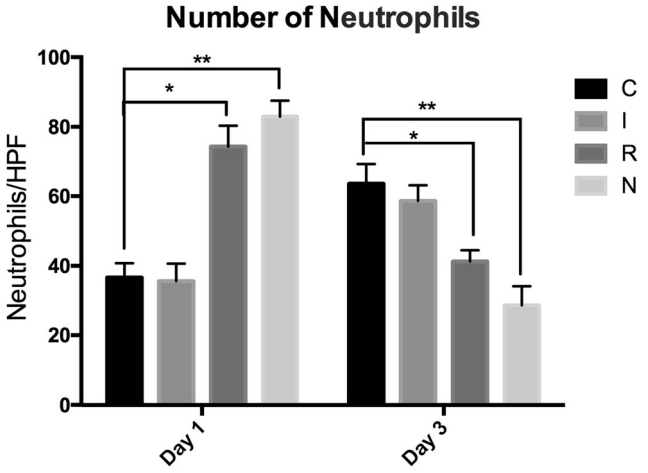

Fig. 3 The number of neutrophils on days 1 and 3 after wounding for each experimental group (× 200 magnification; $n = 6$; *$p < 0.05$, **$p < 0.01$). On day 1, the number of neutrophils in group R is much higher than that in group C, while lower than that in group C on day 3 C: group C; I: group I; R: group R; N: group N

of granulation tissue formation, angiogenesis, dermal papilla, and well-constructed epidermis, which confirmed that a greater extent of healing was achieved when the AGEs-RAGE pathway was inhibited in diabetic mice (Fig. 1).

Blockade of AGEs-RAGE signaling improved the secretion of essential inflammatory factors.

Considering that diabetic wound healing exists in a state of persistent hyper-inflammation characterized by harmful increased levels of inflammatory factors, we examined whether the interaction of AGEs-RAGE contributed to the secretion of abnormal levels of inflammatory factors. We performed an ELISA to assess the level of TNF-α, IFN-γ, IL-1α, and IL-6 secretion. The results showed that the level of inflammatory factor secretion increased rapidly after wounding on day 1 then declined on day 3 in the normal control mice (group N) and anti-RAGE-treated diabetic mice (group R). In contrast, in the diabetic mice (groups C and I), the levels of inflammatory factors were higher on day 3 compared with those on day 1, (Fig. 2). On day 1, the level of TNF-α, IL-1α, and IL-6 on group R is much higher than that on group C ($t = 10.275$,

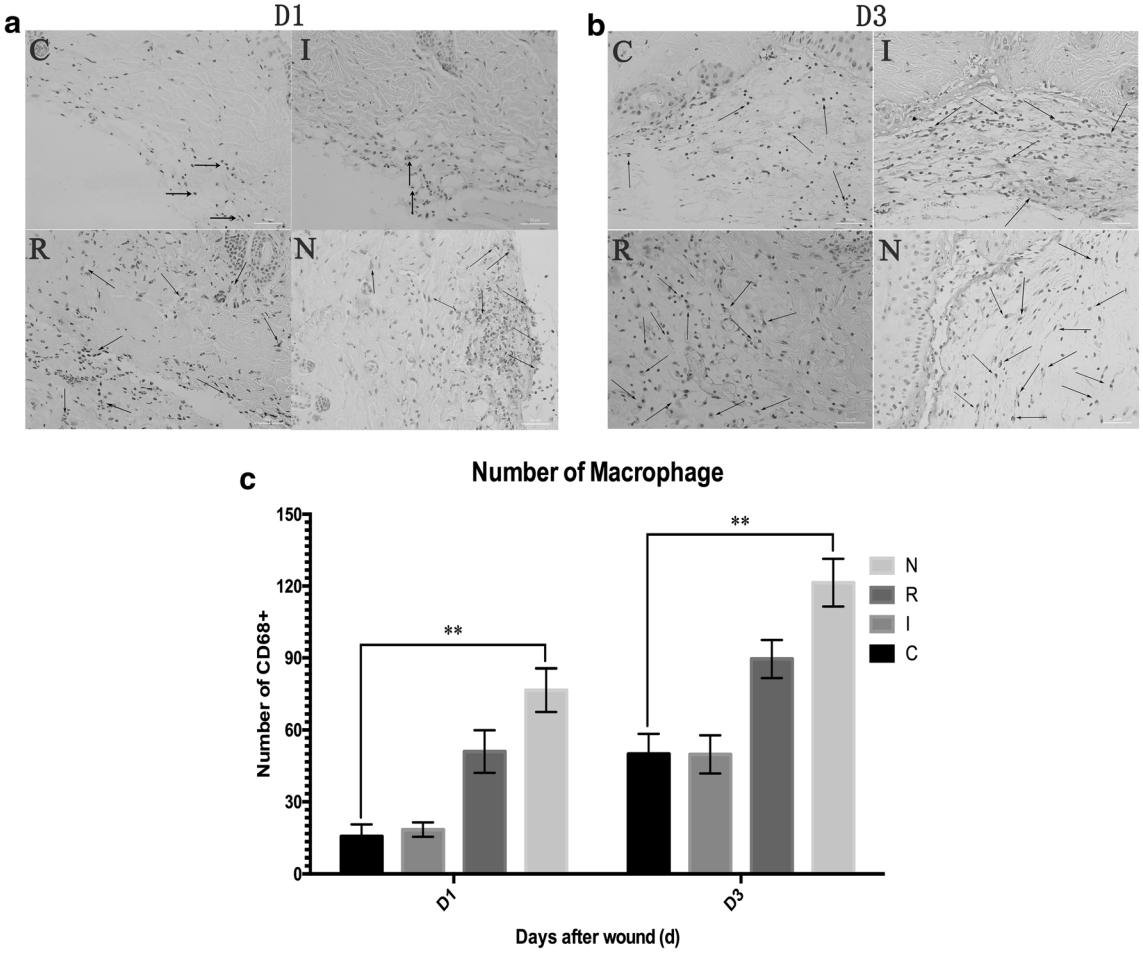

Fig. 4 **a**, **b** Representative immunohistochemical sections showing the number of $CD68^+$ cells in all experimental groups on days 1 and 3 of wound healing (× 200 magnification). **c** The number of $CD68^+$ cells increased slowly in the diabetic group. This was partly rescued by inhibition of AGEs-RAGE signaling ($n = 6$, *$p < 0.05$, **$p < 0.01$) C: group C; I: group I; R: group R; N: group N

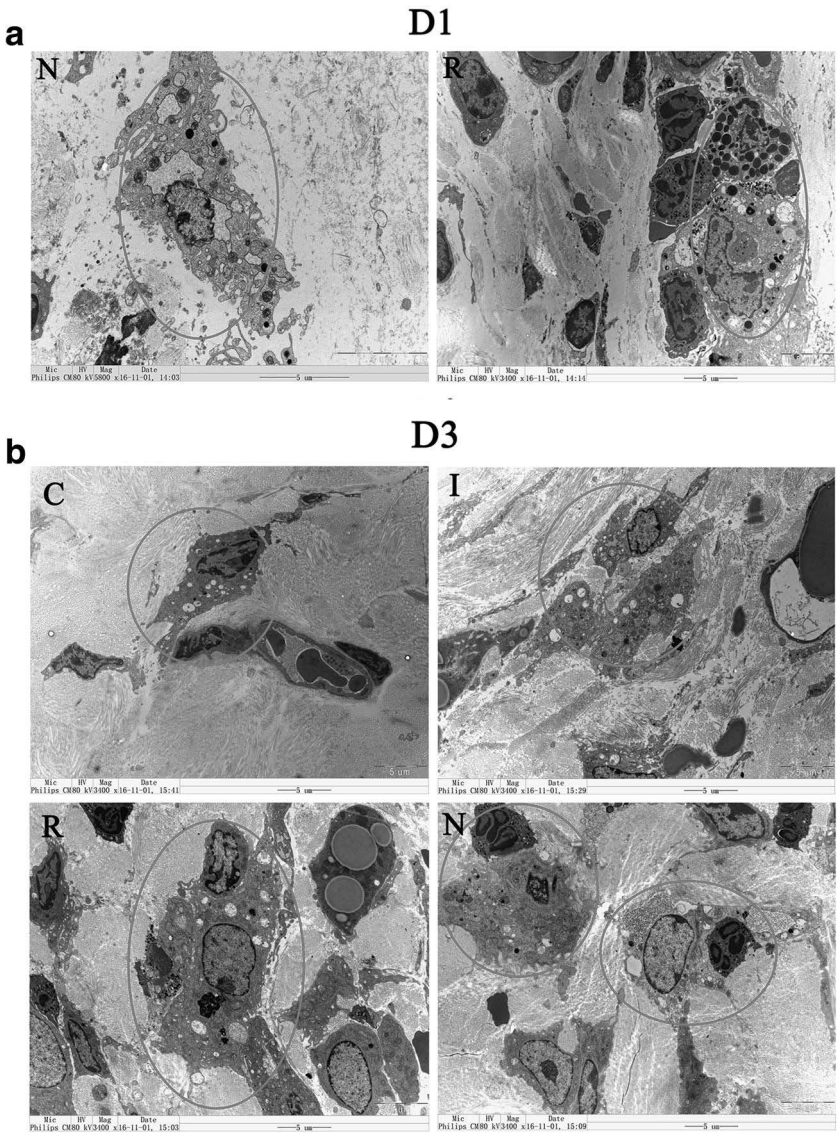

Fig. 5 **a** TEM images of wound tissue from anti-RAGE antibody–treated diabetic groups (R) and normal mice group (N) on D1 of wound healing (× 3400 magnification). **b** Uptake of neutrophils by macrophages in groups N and R on day 3 of wound healing, visualized by TEM (× 3400 magnification) C: group C; I: group I; R: group R; N: group N

27.295, 18.591; $p < 0.05$). On day 3, the level of TNF-α , IL-1α, and IL-6 on group R is lower than that on group C (t = 17.819, 15.584, 29.360; $p < 0.05$).

Blockade of AGEs-RAGE signaling improved the neutrophil response in vivo

Given the abnormal level of inflammatory factor secretion, we next evaluated the infiltration of inflammatory leukocytes, including neutrophils and macrophages. We quantified the number of neutrophils on days 1 and 3. After wounding, neutrophils rapidly arrived and aggregated at the edge of the wound (day 1) in the normal control mice (group N) and anti-RAGE antibody–treated diabetic mice (group R), while the diabetic control mice (groups C and I) did not. At the key time point of neutrophil resolution (day 3), a large number of neutrophils persisted at the wound edges of the diabetic control mice compared with the normal control mice and anti-RAGE antibody–treated diabetic mice (Fig. 3).

In the diabetic control mice, the level of MPO (Fig. 2) examined by ELISA also reduced on day 1, albeit to a higher degree on day 3 compared with the normal control mice and anti-RAGE antibody–treated diabetic mice. Both the results of the number of neutrophils and MPO levels indicated a delayed neutrophil response in diabetic wounds.

AGEs-RAGE signaling blockade improves macrophage infiltration and neutrophil removal

When it comes to macrophage infiltration, immunohistochemical staining for CD68 revealed that on day 1, the normal mice (group N) and anti-RAGE-treated diabetic mice (group R) already exhibited a high quantity of $CD68^+$ cell infiltration, while the control diabetic mice (groups C and I) was

significantly lower. On day 3, the macrophage infiltration was increased in all the groups; however, the diabetic control groups remained lower than that of the normal and anti-RAGE antibody–treated mouse groups (Fig. 4). These findings suggested a delayed infiltration of macrophages in the diabetic mice due to the interaction of AGEs-RAGE.

We evaluated the level of phagocytosis based on transmission electron microscopy [10]. Samples collected on days 1 and 3 after wounding were examined for the presence of macrophages by TEM. One day after wounding, the normal mice (group N) and anti-RAGE antibody–treated mice (group R) displayed macrophage infiltration at the wound edge, while the diabetic control mice (groups C and I) did not (Fig. 5a). On day 3, groups N and R exhibited the typical uptake of neutrophils by macrophages at the wound edge (the average numbers of macrophages phagocytosing neutrophils in groups N and R are 1 and 2 under the × 3400 magnification field of view, $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$), while the macrophages in groups C and I did not show any signs of phagocytosis (Fig. 5b).

Discussion

Diabetic wounds are characterized by delayed healing, due primarily to disorders of the inflammatory and proliferative phases of wound healing [1]. Recently, many studies have suggested that diabetic wound healing is characterized by heightened levels of reactive oxygen species and harmful levels of inflammatory factors in response to wound healing, which induce continuous damage during the early inflammatory stage [5, 11–13]. The findings of the present study imply that compared with a hyper-inflammatory state, diabetic wounds lack an essential inflammatory response during the early healing phase. Therefore, a paradoxical and persistent inflammation state appears to be induced, in which AGEs-RAGE may play a vital role in these pathogenic progresses.

Prior studies have shown that the topical application of anti-RAGE antibodies improves diabetic wound healing by blocking AGEs-RAGE signaling [2]. In the present study, H&E staining revealed improved formation of granulation tissue on day 7 firstly, implying an ideal transition between the inflammatory phase to the healing progress following the blockade of RAGE. We next examined the level of secreted inflammatory factors. A rapid increase in TNF-α, IFN-γ, IL-1α, and IL-6 was released following the blockade of AGEs-RAGE signaling, which more closely resembled the wound healing progress observed in normal mice. These findings suggest a slow inflammatory response in diabetic wounds, which is associated with AGEs-RAGE signaling.

MPO is a heme-containing enzyme that is abundant in neutrophils, and macrophages to a much lesser extent, and functions as a key mediator of the phagocytic oxidative burst and inflammatory process. MPO levels and activity are often used to estimate the number and aggregation of neutrophils [14–16]. The MPO levels and quantitative neutrophil analysis in the present study suggest a delayed infiltration of neutrophils during the early inflammatory phase of wound healing (day 1). Furthermore, the removal of neutrophils by macrophages was also impaired on day 3.

Neutrophils undergo apoptosis soon after infiltrating a wound, and the release of cytokines during this process is an important component involved in macrophage recruitment [17, 18]. Moreover, macrophages have been observed to extensively infiltrate a wound 2 days post-injury and induce intense phagocytic activity [8, 19]. In this study, the quantitative analysis of macrophage infiltration by immunohistochemical staining and macrophage phagocytosis assay via TEM demonstrated that both macrophage infiltration and phagocytic activity were impaired by AGEs-RAGE interaction during the early stages of wound healing.

In contrast to the prevailing view of the hyper-inflammatory state of diabetic wound healing, our data indicate a lack of an essential inflammatory response during the early stage of wound healing, due in part to the interaction of AGEs-RAGE. Furthermore, other studies have discussed the deleterious impact of the prolonged use of anti-inflammatory agents on various aspects of wound healing (e.g., anti-proliferative effects), reduced wound contraction, impaired angiogenesis, and delayed re-epithelialization [20–22]. The results of the present study indicate that the blockade of AGE-RAGE interaction restored the essential inflammatory response during the early phase of wound healing in diabetic mice. Therefore, these findings may provide a theoretical basis for the prevention of impaired diabetic wound healing.

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Compliance with ethical standards

All experimental procedures were in compliance with the institutional laboratory guidelines and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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

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ORIGINAL ARTICLE

Combination of negative pressure wound therapy using vacuum-assisted closure and ozone water flushing for treatment of diabetic foot ulcers

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Abstract

Objective To investigate the efficacy of negative pressure wound therapy (NPWT) using vacuum-assisted closure (VAC) and ozone water flushing for the treatment of diabetic foot ulcers.

Methods The present study included 136 consecutive diabetic foot ulcers (DFUs) patients who went to our hospital during April 2016 to August 2017. The patients were randomly divided into two groups: the combined group in which patients received both VAC and ozone water flushing, and the VAC group in which patients received only VAC. Clinical outcomes were recorded and the change of the wound surface area was calculated. The pain condition was evaluated using the visual analogue scale (VAS) scores.

Results The duration of the treatment was significantly shorter and the dressing change times and peak VAS scores were both dramatically lower in the combined group than the VAC group. The reduction of the wound surface area was significantly larger after 1-week, 2-week, and 3-week treatment in the combined group; however, no significant difference was found after 1-month treatment. The bacterial clearance rate was significantly higher after 2-week treatment in the combined group compared with the VAC group; however, no significant difference was found after the whole treatment. No significant difference was observed in the recurrence and amputation rates.

Conclusion The combination use could enhance the recovery of DFUs, shorten the treatment duration, and reduce the pain during treatment.

Keywords Negative pressure wound therapy · Vacuum-assisted closure · Ozone water flushing · Diabetic foot ulcers

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Introduction

Diabetic foot ulcers are one of the most common diabetic complications, which are defined as ulceration of the foot (distally from the ankle and including the ankle) associated with neuropathy and different grades of ischemia and infection by World Health Organization (WHO) [1–3]. DFUs represent the first cause of hospitalization in diabetic patients and bring more than 20–40% of health care resources related to diabetes-related foot care [4–6]. Moreover, DFUs account for more than 85% foot amputations in developing countries [7, 8]. The diabetic ulcers can often remain unhealed for months, even years, and the healing of a DFUs needs the well-established processes of inflammation, granulation tissue formation, and epithelialization [9, 10]. Thus, it is still a clinical challenge for the treatment of DFUs.

Among the treatment methods for DFUs, negative pressure wound therapy (NPWT) using vacuum-assisted closure (VAC) is now a widely applied method in the treatment of DFUs, which uses a noninvasive system to create a localized controlled

negative pressure environment [11, 12]. The negative pressure can cause contraction of the dressing and deformation of the cells in the wound bed, and further stimulate neo-angiogenesis and granulation tissue formation, as well as remove wound exudate, reduce edema, and promote perfusion [13, 14]. Many studies show the NPWT treatment using VAC is a more effective method than the traditional moist wound therapy [15].

Ozone treatment is widely applied in the treatment of many diseases, such as nerve injury [16], wounds [17], and oral lichen planus [18]. Studies also showed ozone treatment can be used in treatment of DFUs [19]. However, to the best of our knowledge, few studies reported the combination use of ozone water flushing and negative pressure wound therapy using VAC. In the present research, we demonstrated that the combined use of ozone water flushing and negative pressure wound therapy using VAC could promote the recovery of DFUs and reduce the pain during treatment. This study might give more clinical evidence for ozone treatment in DFUs.

Methods and materials

Patients

This is a prospective study which enrolled 136 consecutive DFUs patients who went to our hospital during April 2016 to August 2017. All patients who were diagnosed as DFUs during the study period and met the inclusion criteria were included. The inclusion criteria included the following: (1) the patients were diagnosed as DFUs according to WHO criteria [19]; (2) patients did not have other vascular lesions or dermatosis; (3) the patients were Wagner's stage 2 or 3; (4) patients agreed to participate in the study. Among all patients, 74 patients were diagnosed as Wagner's stage 2 and 62 patients were diagnosed as Wagner's stage 3. The areas of foot ulcers for all patients ranged from 2.5 to 76 cm^2 on calcaneal, dorsal, or plantar after debridement. The exclusion criteria were as follows: (1) patients with other vascular lesions or dermatosis; (2) patients with severe infection or severe immune system diseases; (3) patients with severe liver or renal diseases and cancers. The patients were randomly divided into two groups: the combined group in which patients received both VAC and ozone water flushing, and the VAC group in which patients received only VAC. Informed consent was obtained from all patients. The present study was approved by the Ethics Committee of the Shanghai Tenth People's Hospital.

Treatment strategy

The surgical debridement and negative pressure wound therapy using VAC were conducted as described previously [20]. Briefly, glucose control and surgical debridement were conducted to remove necrotic tissues. Then, the wound surface was washed by 1.5% hydrogen peroxide solution and normal saline for 10 times following by diluted Anerdian III skin disinfectant and 0.9% saline. The KCI negative pressure system (KCI, USA) was used for negative pressure wound therapy using VAC, with continuous negative pressure ranging from 80~125 mmHg. The wound surface was washed by 0.9% saline for 3–5 days after debridement and the condition of the wound was monitored every day. The treatment lasted until ulcer closure.

For the ozone water flushing, the 10 μg/mL O_3 water was prepared using an ozone generator (Herrmann Apparatebau GmbH, German). The prepared O_3 water was then introduced into the VAC system and the treatment lasted for 60 min (Fig. 1). The ozone water flushing treatment was conducted twice every day and lasted until ulcer closure.

For both groups, postoperative nursing management was performed, such as mental intervention, monitoring the patients' blood circulation, skin color, limb sensation, and dressing condition.

Data collection and measurement

Demographic characteristics such as age and gender were collected. Clinical outcomes including DFU stage, course of disease, and duration of the treatment were recorded. The pictures of the wound surface were taken by a digital camera and analyzed by Image J software (Rasband; NIH, USA). The change of the wound surface area was measured and calculated at 1 week, 2 weeks, 3 weeks, and 1 month during the treatment. Dressing changing times were recorded during the whole treatment period. The pain condition was evaluated using the visual analogue scale (VAS) scores. For measurement of bacterial clearance rate, the secretion of wound surface was collected before the treatment, as well as at 2-week treatment and after the treatment. Then, bacterial culture was conducted and the number of bacterial species was recorded. The bacterial clearance rate was calculated as: (bacterial number before treatment − bacterial number after treatment)/bacterial number before treatment × 100%. Recurrence rate and amputation rate were also collected and analyzed. All patients were followed up for 1 year after admission.

Statistical analysis

The measurement data was expressed by mean ± SD. Comparison between two groups was performed using the Student *t* test. Categorical variables were analyzed using chi-square test or Fisher's exact test. *p* value less than 0.05 was considered as statistically significant. All calculations were made using SPSS 20.0 (SPSS Inc., Chicago, USA).

Fig. 1 Management for DFUs patients using negative pressure wound therapy using vacuum-assisted closure and ozone water flushing

Results

Basic clinical information for all patients

Among all patients, 72 patients were male and 64 patients were female. The mean age of the patients was 55.0 ± 9.6 and the mean area of wound surface was 38.4 ± 22.1. No significant difference was found in age, gender, and mean area of the wound surface, DFU stage, and course of disease between the two groups of patients (Table 1).

Comparison for clinical outcomes of different groups of patients

To study the efficacy of O_3 water flushing in treatment of DFUs, duration of the treatment, wound surface area change, dressing changing times, pain conditions, and bacterial clearance rate were measured for both of the groups. Results showed the duration of the treatment in the combined group was significantly shorter than the VAC group ($p < 0.05$, Table 2). Meanwhile, the reduction of the wound

Table 1 Basic clinical characteristics of all patients

Variables	VAC group ($n = 68$)	Combined group ($n = 68$)
Mean age (years)	56.4 ± 10.4	53.5 ± 8.6
Gender (male:female)	37:31	35:33
Course of diabetes mellitus (year)	13.5 ± 6.1	13.3 ± 6.9
Course of DFU (day)	94.5 ± 21.5	91.5 ± 25.3
DFU stage (n (%))		
Wagner's stage 2	35 (51.5)	39 (57.4)
Wagner's stage 3	33 (48.5)	29 (42.6)
Postoperative wound surface area (cm^2)	39.3 ± 22.8	37.5 ± 21.6

Table 2 Clinical outcomes of different groups of patients

Variables	VAC group ($n = 68$)	Combined group ($n = 68$)	p value
Duration of the treatment (day)	25.8 ± 4.3	12.6 ± 4.2	< 0.001
Dressing changing times	26.1 ± 5.0	7.4 ± 2.1	< 0.001
Peak value of VAS score	7.4 ± 1.8	3.4 ± 1.3	< 0.001
Bacterial clearance rate (%)			
2-week treatment	77.3 ± 3.2	89.0 ± 2.9	< 0.001
After the whole treatment	94.5 ± 2.0	94.6 ± 2.3	0.704

surface area was significantly larger after 1-week, 2-week, and 3-week treatment in the combined group (Fig. 2, $p < 0.05$); however, no significant difference was found after 1-month treatment between the 2 groups (Fig. 3). The dressing change times and peak VAS scores were both dramatically lower in the combined group ($p < 0.05$). The bacterial clearance rate was significantly higher after 2-week treatment in the combined group compared with the VAC group ($p < 0.05$); however, no significant difference was found after the whole treatment for both the two groups. All these results showed the treatment of O_3 water flushing could facilitate the recovery of the DFUs and reducing the pain during the treatment.

Comparison for recurrence and amputation rates for different groups of patients

At last, we compared the recurrence and amputation rates of the two groups. As shown in Table 3, the recurrence rate was 11.8% in the combined group and was 8.8% in the VAC group. The amputation rate was 5.9% in the combined group and was 4.4% in the VAC group.

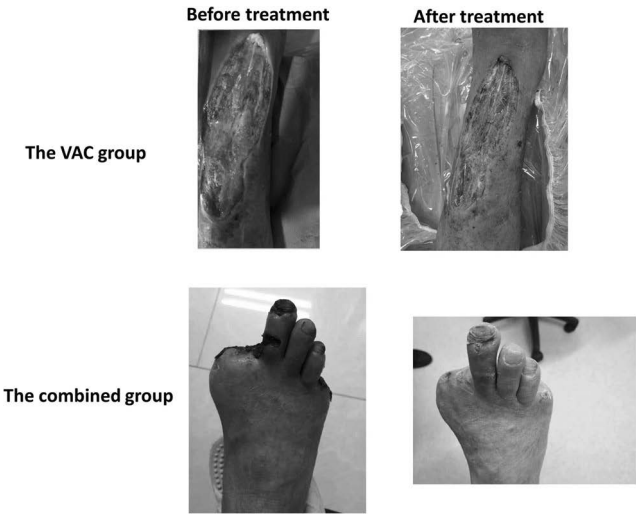

Fig. 2 Wound surface of DFUs patients in VAC group (**a**) and combined group (**b**) before and after treatment

Discussion

Diabetic foot ulcers are a common diabetic complication affecting millions of patients all over the world. Many studies have reported different new treatment methods for DFUs. It was reported that growth factors could be used in the treatment of DFUs [21], such as transforming growth factor-beta [22] and intralesional epidermal growth factor [23]. Other treatment methods include skin substitutes [24] and electric stimulation [25]. Among the treatment strategies, the negative pressure wound therapy using vacuum-assisted closure is now widely used and is proven to be effective in DFUs. However, the diabetic ulcers can often remain unhealed for months, even years; it is still a complex challenge for treatment of DFUs. Thus, new treatment methods for DFUs are still needed. In the present research, we reported a novel treatment method, the combination use of VAC negative pressure wound therapy and ozone water flushing. Results showed the combination use of VAC negative pressure wound therapy and ozone water flushing could enhance the recovery of DFUs, shorten the treatment duration, and reduce the pain during treatment.

The VAC negative pressure wound therapy has been reported in the treatment of DFUs in many studies. Muhammad et al. compared the efficacy and safety of VAC negative pressure wound therapy and advanced moist wound therapy in DFUs treatment and found NPWT using VAC was more effective than the traditional moist wound therapy [15]. In a systematic review, Guffanti et al. also showed NPWT systems were more effective than standard moist wound therapy with regard to proportion of healed wounds and rate of

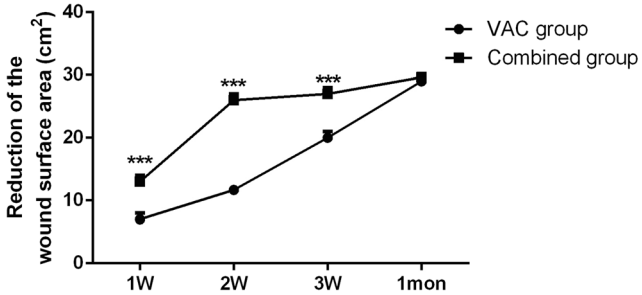

Fig. 3 Wound surface change at treatment time for 1 week, 2 weeks, 3 weeks, and 1 month

Table 3 Comparison for recurrence and amputation rates for different groups of patients

Variables	VAC group ($n = 68$)	Combined group ($n = 68$)	p value
Recurrence (n (%))	8 (11.8)	6 (8.8)	0.485
Amputation (n (%))	4 (5.9)	3 (4.4)	0.631

wound closure [9]. In a recent study, Borys et al. found NPWT was also effective in chronic neuropathic non-infected DFUs [26]. In this research, we also found that the NPWT using VAC was effective in treatment of DFUs.

The clinical use of ozone has been reported in many researches. Ozone is considered to be effective in the treatment of wounds [17]. Studies also showed treatment with ozone could benefit the ulcers. Studies reported that ozone-oxygen therapy, as well as intralesional ozone injection, could be used in treatment of DFUs [19, 27]. It was also demonstrated that ozone could prevent fungal infection in DFUs [28]. In our study, we demonstrated for the first time that the ozone water flushing was an effective way in treatment of DFUs, and the combination use of ozone water flushing and VAC treatment could enhance the recovery of DFUs. The manuscript also has some limitations. First, the study sample size is small. Secondly, all cases are from a single center. All these need more studies to confirm.

In conclusion, we conducted a prospective study to investigate the efficacy of the combination use of VAC negative pressure wound therapy and ozone water flushing. Results showed the combination use could enhance the recovery of DFUs, shorten the treatment duration, and reduce the pain during treatment. This study might give more clinical evidences for application of ozone water flushing in DFUs treatment.

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Compliance with ethical standards Informed consent was obtained from all patients. The present study was approved by the Ethics Committee of the Shanghai Tenth People's Hospital.

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

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ORIGINAL ARTICLE

Quality of metabolic control, serum potassium, and aging are the major determinants of severity of musculoskeletal disorders in hospitalized diabetic patients

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Abstract

Background Diabetes is strongly associated with numerous musculoskeletal disorders (MSD) and may be considered as another group of diabetic complications.

Objective We explored the association of diabetes type, duration, quality of metabolic control, applied therapy, and serum electrolytes, with prevalence and extent of musculoskeletal disorders as well as radiographic and EMG changes.

Methods The case-control clinical study involved 80 patients with type 1 and 2 diabetes hospitalized at General Hospital Berane and Clinical Center Podgorica, Montenegro, in 2013. More than half of the diabetics had MSD. Peripheral nerve disorders were diagnosed by EMG. The musculoskeletal disorders were evaluated using the translated Short Musculoskeletal Function Assessment (SMFA) questionnaire. The statistical methodology involved *T*-testing, $\chi 2$ test, Spearman's multiple correlation, and ordinal logistic regression.

Results The incidence and the severity of MSD were significantly related to patients age, duration of diabetes, quality of metabolic control, serum potassium, radiographic, and EMG findings. However, in the regression analysis, only aging, mean blood glucose levels, and serum potassium were associated with the significant risk for development of MSD.

Conclusion Advanced age, metabolic control, and hypokalemia are the main determinants of both prevalence and extent of musculoskeletal disorders in diabetics. Along with the strict glycemic control, an additional effort should be taken to avoid potassium depletion associated with diabetic ketoacidosis and to maintain optimal potassium homeostasis in diabetic patients receiving insulin, diuretics, or suffer gastrointestinal motility disorders.

Keywords Diabetes mellitus · Long-term diabetic complications · Musculoskeletal disorders · Glycemic control · Aging · Potassium

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Background

Diabetes mellitus may be associated with various types of musculoskeletal and neuromuscular disorders. While the degree of association of these disorders with diabetes may vary [1], the reported prevalence of over 80 % [2] permits the inclusion of musculoskeletal derangements in the list of chronic diabetes complications, even though the precise mechanisms interconnecting those two categories of the illness are not quite clear. Some authors argue that reducing glycemic levels and better metabolic control might lead to reduction in musculoskeletal complications [3], comparable to diabetic microangiopathy. Musculoskeletal diseases are thought to be dependent on duration of diabetes and can be harbinger or marker of underlying microvascular complications [4].

Although the exact pathophysiology of most of these musculoskeletal disorders remains obscure, the connective tissue disorders, neuropathy and vasculopathy, may underlie the increased incidence of musculoskeletal disorders in DM: it has been difficult to show a direct correlation with the metabolic control of DM [5]. However, it seems that nonenzymatic glycosylation of protein and advanced glycation products formation, abnormal collagen deposition in the periarticular connective tissues, autonomic nervous system neuropathy, vascular damage, hyperuricemia, reduced bone density, low-grade chronic inflammation, and abnormal levels of insulin and insulin-like growth hormone may be responsible for development of MSD in long term [6].

The recognized forms of musculoskeletal disorders related to diabetes [7] are diabetic hand syndrome, diabetic cherioarthropathy, Dupuytren's contracture, flexor tenosynovitis, carpal tunnel syndrome, shoulder-hand syndrome ("frozen shoulder syndrome"), Charcot joint, ankylosing spondylitis, osteoarthritis, osteopenia, diabetic osteolysis, muscle infarction, and diffuse idiopathic skeletal hyperostosis (DISH). The most frequent musculoskeletal disorders in diabetics hospitalized and treated in medical institutions in Berane and Podgorica were previously referred at 32nd Balkan medical week, September 21–23, 2012, Nis, Serbia (Artromuscular disorders in diabetes mellitus, https://umbalk.org/32rd-balkan-medical-week/).

Methods

The case-control clinical study involved 80 patients consecutively admitted and hospitalized at General Hospital Berane and Clinical Center Podgorica, Montenegro, from March to November 2013. The survey was conducted respecting ethical principles for human clinical trials by the Declaration of Helsinki of 1983 [8] The study was approved by Ethical Committee, Faculty of Medicine, University of Pristina/K.Mitrovica.

The power analysis confirmed that the statistical power of the study was over 0.95 for p value of 0.05 and the sample size (80 patients) was adequate regarding performed statistical procedures.

All patients involved in the trial had diagnostically confirmed diabetes mellitus type 1 and 2, detected by one of the following procedures:

- Fasting plasma glucose ≥ 7 mmol/L in at least two consecutive measurements
- Plasma glucose ≥ 11.1 mmol/L during a 2-h OGTT using a 75-g oral glucose load
- Clinical diagnosis and positive history of diabetes therapy use ranging from diabetic diet to intensified insulin therapy, according to the indications

More than half of the diabetics (46, e.g., 57.5 %) had clinical and radiological features associated with diagnosis of one of the several musculoskeletal disorders listed in Table 3. Clinical parameters scrutinized during the follow-up were patients' age, gender, diabetes type, diabetes duration, diabetes therapy course, and the existence of diabetic micro- and macrovascular complications (including the diagnosis of concomitant cardiovascular diseases). Sensory and motor peripheral nerve disorders, including diabetic polyneuropathy, were diagnosed by EMG. Blood glucose was measured by a glucose-automated enzymatic method (along with serum cholesterol, HDL cholesterol, and triglyceride concentrations) throughout the hospitalization period and the mean values were taken into statistical analysis; glycosylated hemoglobin HbA1c was determined by affinity chromatography shortly after the admittance to the hospital. Serum electrolytes were measured spectrophotometrically.

The musculoskeletal disorder in patients was clinically evaluated using the translated Short Musculoskeletal Function Assessment (SMFA) questionnaire, developed by Swiontkowski MF, Department of Orthopedic Surgery, Medical School, University of Minnesota. The rationale for this kind of evaluation was focusing not only on prevalence but rather on extent and severity of musculoskeletal changes, symptoms and signs, and their relation with diabetes. The SMFA is freely available on the web (https://www.ortho.umn.edu/research/mfa-smfa-resources). Permission for use can be found at https://www.ortho.umn.edu/sites/ortho.umn.edu/files/smfa-statement.pdf. The questionnaire can be used to assess the impact of a musculoskeletal condition on self-reported patient functioning and on the impact of the condition in impeding everyday life [9]. The questionnaire determines functional impairment in 5 categories (Daily Activities, Emotional Status, Arm and Hand Function Category, Mobility and Bother), providing the scoring system with the least impairment being close to 0. It is a 46-item questionnaire—all of which contribute to an overall Bother

Index (SMFA-BI). The survey uses a 5-point Likert-type response system that is further transformed into a score ranging from 0 to 100 [10]. The patients' history evaluation, physical examination, and the SMFA assessment were diagnostically supported by conventional musculoskeletal radiography.

The exclusion criteria comprised degenerative rheumatic diseases (coxarthrosis, gonarthrosis), inflammatory rheumatic disorders (ankylosing spondylitis, systemic lupus erythematosus, rheumatoid arthritis, fibromyalgia, systemic sclerosis, psoriatic arthritis), discus hernias, and gout.

The statistical methodology involved *t*-testing for small independent samples, $\chi 2$ test for non-parametric data, Spearman's multiple correlation analysis, and ordinal logistic regression analysis (consistent with non-parametric data distribution and unequal distances of the variables involved in the calculation). The variables entering the multiple regression analysis were diabetes type and duration, patients' gender and age, the presence of cardiovascular diseases and/or arterial hypertension, positive EMG findings, blood glucose, HbA1c, serum potassium, and type and duration of diabetic treatment; these variables showed significant correlation with dependent variable(s) and achieved at least $p \geq 0.2$ in univariate regression, thereby fulfilling criteria for entering the multivariate regression analysis.

Results

The main baseline features of the patients involved in the study are shown in Tables 1 and 2. The mean age was 54.71, and the gender distribution and the prevalence of patients with type and type 2 diabetes were even. Musculoskeletal disorders were diagnosed by physical examination and history in more than half of the diabetics, while the slightly lesser percentage of patients (52.5 %) had radiologically detectable pathological changes.

Diabetic microangiopathy was present in the majority of patients with diabetes and almost one-third of them had some form of cardiovascular manifestations considered to be a consequence of diabetic macroangiopathy—most often coronary artery disease and atherosclerotic/ishaemic cardiomyopathy. In addition, almost one-third of the diabetic patients had manifest arterial hypertension, with no significant difference between the groups (Table 2). Similar results and were obtained for statistical analysis of BMI.

Table 1 The main clinical features of diabetic patients

Age	54.71 ± 11.0
Gender	Male 41 (51.25 %
	Female 39 (49.75 %)
Diabetes type	Type 1 40 (50 %)
	Type 2 40 (50 %)
Diabetes duration	7.64 ± 5.59 years
Musculoskeletal disorders	46 (57.5 %)
Musculoskeletal pathology—radiographically verified	42 (52.5 %)
Diabetic microangiopathy	49 (61.25 %)
Diabetic macroangiopathy	27 (33.75 %)

Parameters of glycemic control were clearly distinguishable between the patients with and those without MSD ($p < 0.0001$). While the lipids and lipoprotein testing results reflect a highly prevalent diabetic dyslipidemia among patients in both groups, the obtained means for serum cholesterol and triglycerides, HDL, LDL, and VLDL cholesterol were not significantly different among the group of patients with and the one without MSD; consequently, lipid aberrancies did not correlate significantly either with clinical or with radiologic manifestations of diabetic MSD in our study, so they did not enter the subsequent regression analyses.

The distribution of diabetic patients according to their respective musculoskeletal pathology is shown in Table 3. None of our patients suffered Charcot joint, diabetic osteolysis, muscle infarction, or DISH. Maximum SMFA score in individual patient was 67. The most prevalent was neuropathy, carpal tunnel syndrome, and osteoarthritides, followed by few cases of ankylosing spondylitis, Dupuytren/s contracture, diabetic cherioangiopathy, and frozen shoulder syndrome.

Comparing to patients without such complications (Table 4), patients who suffered musculoskeletal disorders had significantly more advanced age, diabetes duration, and history of antidiabetic medications (including insulin) application, worse metabolic control—higher mean blood glucose and HbA1c levels, as well as lower serum potassium level. Serum calcium, phosphate, and natrium did not differ significantly. Of course, EMG and radiographic changes were far more prevalent in patients with musculoskeletal disorders.

The severity of MSD signs and symptoms, as evaluated by SMFA questionnaire were significantly related to patients age; duration of diabetes; radiographic changes; EMG findings; quality of metabolic control—mean serum glucose and HbA1c; and serum potassium (Table 5). Radiographic diagnostic findings in patients with MSD were related to same parameters and, additionally, with prevalence of type 1 diabetes and history of insulin application: the latter might indicate at least weak relation of type 1 diabetes immunologic mechanisms and the radiologically apparent changes.

Although the correlations the severity of MSD and radiographic changes had with different clinical and laboratory findings (Table 4) were highly significant, not all of these correlations showed a causality; for example, correlation between the history of insulin usage and radiographic findings is simply the consequence of the more prevalent radiographically detectable musculoskeletal disorders in patients with diabetes type 1 and does not mean that insulin might have caused musculoskeletal disease.

Table 2 The baseline parameters in two groups of patients

	Diabetics with MSD	Diabetics without MSD	*p*
Mean age (years)	61.38 ± 8.32	48.05 ± 9.22	$T = 6.79$, $p < 0.0001$
Gender			
Female	45 %	52.50 %	$\chi 2 = 0.909$
Male	55 %	47.50 %	$p > 0.05$
Diabetes type			
1	50%	50%	$\chi 2 = 1$
2	50%	50%	$p > 0.05$
Diabetes duration (years)	10.65 ± 5.63	5.62 ± 3.59	$T = 4.76$, $p < 0.0001$
BMI (kg/m^2)	27.5 ± 8.17	24.59 ± 11.33	$T = 1.31$
			$p > 0.05$
Arterial hypertension	32.5 %	27.4 %	$\chi 2 = 0.6$
			$p > 0.05$

In order to discover which of the variables are more directly related with one another, we performed multiple ordinal logistic regression (Table 6); The results (Table 6) showed that the significant risk for generating and/or aggravating MSD in diabetics was derived only from patients' age, mean serum glucose, hypokalemia, and positive EMG findings. Additionally, age, hypokalemia, and hyperglycemia were closely related to occurrence of radiologically detectable musculoskeletal changes. Furthermore, a rise in mean serum glucose of 1 mmol/L increased SMFA score by 3.3 points (confidence interval 0.5–6.2, $p < 0.02$) and risk and lowering of serum potassium for 1 mmol/L increased SMFA score for 19.3 points (CI 33.0–5.5). Not surprisingly, SMFA scores and EMG changes were closely related. The results of multiple regression using radiologically detectable MSD in diabetic patients as a dependent variable were similar to those for SMFA scoring system.

Discussion

Although musculoskeletal disorders commonly occur in patients with diabetes, they are often overlooked. Sometimes, it is difficult to show a direct correlation of metabolic control in DM with musculoskeletal pathology [5] and its role in the pathogenesis remains somewhat controversial. The results of Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) focused on a specific complication (diabetic cherioarthropathy) in type 1 diabetes confirmed its association with other types of microangiopathic complications and high levels of blood glucose and HbA1c [11]. Other study dealing with type 2 diabetes musculoskeletal complications find that female gender, age over 60 years and long duration of diabetes were associated with hand abnormalities [12]. On the other hand, Cagliero et al. suggest that glycemic control, evaluated by HbA1c levels does not play a major role in the pathogenesis of hand and shoulder abnormalities in diabetic patients [13]; also, type of diabetes in this study was not associated with hand and shoulder syndromes, while there was significant and strong association of musculoskeletal and microangiopathic complications [14].

Our results revealed a strong and significant correlation between metabolic control parameters (both mean glycemic level and HbA1c) and the prevalence and severity of musculoskeletal disorders in diabetic patients. It should be

Table 3 Musculoskeletal disorders observed in patients with diabetes

Disorder	Number of patients	%
Osteopenia	4	8.69 %
Ankylosing spondylitis	5	10.87 %
Carpal tunnel syndrome	11	23.91 %
Dupuytren's contracture and diabetic cherioarthropathy	2	4.34 %
Frozen shoulder	2	4.34 %
Neuropathy	38	82.6 %
Osteoarthritis	9	19.56 %
Small joints	6	13.04 %
Large joints	3	6.52 %

Total no. of patients with MSD is 46. Some of the patients have more than one disorder

Table 4 The main clinical, biochemical and radiographic findings in two groups of diabetic patients and the results of statistical differences and distribution testing

	Diabetics with MSD	Diabetics without MSD	*p*
Diabetes duration (years)	10.65 ± 5.63	5.62 ± 3.59	$T = 4.76$, $p < 0.0001$
Mean serum glucose (mmol/L)	10.62 ± 2.80	7.24 ± 1.34	$T = 6.88$ $p < 0.0001$
HbA1c (%)	8.50 ± 1.78	6.18 ± 0.90	$T = 7.35$, $p < 0.0001$
Serum cholesterol (mmol/L)	7.52 ± 2.74	6.6 ± 2.58	$T = 1.54$ $p > 0.05$
Serum triglycerides (mmol/L)	1.94 ± 2.2	2.44 ± 2.28	$T = 0.99$ $p > 0.05$
LDL cholesterol (mmol/L)	3.93 ± 1.2	3.52 ± 1.66	$T = 1.26$ $p > 0.05$
HDL cholesterol (mmol/L)	1.11 ± 0.19	1.07 ± − 0.23	$T = 0.85$ $p > 0.05$
VLDL cholesterol (mmol/L)	0.87 ± 0.27	0.95 ± 0.32	$T = 1.2$ $p > 0.05$
Serum potassium (mmol/L)	3.92 ± 0.49	4.67 ± 0.41	$T = 7.42$, $p < 0.0001$
Serum sodium (mmol/L)	141.02 ± 7.15	141.91 ± 7.41	$T = 0.54$, $p > 0.05$
Serum calcium (mmol/L)	2.33 ± 0.49	2.38 ± 0.62	$T = 0.40$, $p > 0.05$
Serum phosphate	1.29 ± 0.66	1.18 ± 0.48	$T = 0.85$, $p > 0.05$
Antidiabetic therapy history (years)	6.62 ± 5.51	4.32 ± 3.55	$T = 2.2$, $p < 0.02$
Insulin therapy utilization	67.50 %	52.50 %	$\chi 2 = 4.103$, $p < 0.05$
Peripheral nerve pathology (EMG changes)	82.6 %	22 %	$\chi 2 = 99.7$, $p < 0.0001$
Diabetic macroangiopathy/cardiovascular diseases	20 %	15 %	$\chi 2 = 0.62$, $p < 0.05$
Radiographic changes	91.3 %	0 %	NA—one of the frequencies equals 0

emphasized, though, that hemoglobin A1c represents a relatively short-lived and reversible Amadori compound [15], formed during the nonenzymatic glycation of proteins and not the advanced glycation end-product responsible for the

Table 5 The results of multiple correlation analysis of the clinical, biochemical and laboratory parameters in diabetic patients

	Spearman's correlation coefficient (ρ) and the significance of correlation (p)	Musculoskeletal disorders SMFA score	Radiographic findings
Diabetes type	ρ	n.s.	− 0.5
	p	n.s.	$p < 0.0001$
Age	ρ	0.58	0.56
	p	p < 0.0001	$p < 0.0001$
Radiographic findings	ρ	0.95	/
	p	$p < 0.0001$	/
EMG changes	ρ	0.74	0.73
	p	$p < 0.0001$	$p < 0.0001$
Serum glucose	ρ	0.59	0.67
	p	$p < 0.0001$	$p < 0.0001$
HbA1c	ρ	0.56	0.69
	p	p < 0.0001	$p < 0.0001$
Serum potassium	ρ	− 0.57	− 0.69
	p	p < 0.0001	p < 0.0001
Diabetes duration	ρ	0.46	0.62
	p	$p < 0.001$	$p < 0.0001$
Insulin utilization	ρ	n.s.	0.44
	p	n.s.	$p < 0.0003$

n.s., nonsignificant

Table 6 Results of the ordinal logistic regression of the main factors influencing musculoskeletal SMFA score and the radiological changes

	Regression coefficient	St. error	Wald	*p*	95 % CI	
					Lower bound	Upper bound
Musculoskeletal disorders SMFA score						
Age	1.2	0.3	13.37	0.0003	0.6	1.8
Mean serum glucose	3.3	1.5	5.19	0.02	0.5	6.2
Serum potassium	− 19.3	7.0	7.51	0.006	− 33.0	− 5.5
EMG findings	33.2	11.4	8.51	0.004	10.9	55.5
Radiological changes						
Age	0.17	0.07	4.97	0.03	0.02	0.31
Mean serum glucose	1.97	0.79	6.29	0.01	0.43	3.51
Serum potassium	− 3.49	1.60	4.75	0.03	− 6.63	− 0.35

development of late skin, joint, tendons, muscular, or bone complications. The accumulation of collagen and other AGE proteins are involved [16] in the occurrence of diabetic musculoskeletal complications.

Aging was one of the main determinants of both MSD prevalence and severity. The effect of advanced age on promotion of degenerative processes involving musculoskeletal structures, [17, 18] is well known. Majjad et al. [19] found age over 50 and dyslipidemia associated with one or more musculoskeletal disorder.

By the results of our study, it has been demonstrated that hypokalemia has strong influence on incidence and extent of MSD in diabetic patients. Electrolytes are essential to normal skeletal muscle contraction. Excessively low levels of these ions in the serum are associated with symptoms such as muscle weakness or cramping [20]. Interstitial potassium concentration, [K +], is linked to muscle activity due to a number of different mechanisms [21]. The role of hypokalemia on neuromuscular function is well documented. Although a hereditary in nature, hypokalemic periodic paralysis is known to cause episodes of extreme muscle weakness. Additionally, there is a heterogeneous group of muscle diseases known as periodic paralyzes (PP) is characterized by episodes of flaccid muscle weakness occurring at irregular intervals [22]. It should be emphasized, however, that serum potassium levels are typically affected by acute conditions; therefore, it is unlikely to have long-term effect on most of the MSDs discussed here. However, undertaking a future study focused on relation of muscular abnormalities with frequency and severity of diabetic ketoacidosis and/or hyperosmolar nonketotic states would be worthwhile. On the other hand, this finding may reflect frequent and more complex electrolyte disturbances which can be registered in diabetic patients, including calcium, magnesium and vitamin D disturbances, renal hyper and hypofiltration, treatment with loop diuretics, hyperphosphatemia, and hypoalbuminemia. Ultimately, our study draws attention on electrolyte disbalances as a possible cause of musculoskeletal problems; it is often assumed that the mechanism of MSD development may not differ much from those involved in diabetic microangiopathy (such as collagen glycation); undoubtedly, loss of glycemic control contributes significantly to the development of MSD, but the exact mechanism may not be the direct one.

Furthermore, this case-control study was performed at hospitalized patients during the limited period which did not exceed 14 days. The visualization and laboratory methods at our disposal were not as ample as in larger medical centers. Therefore, a future prospective study that would include diabetic patients under more permanent and broader follow-up might provide a better insight into etiology of musculoskeletal complications.

Finally, in our study, we demonstrated a positive and strong correlation between the findings obtained by SIMFA questionnaire and other, less subjective assessment methods such as radiography and EMG.

Conclusion

- Advanced age, metabolic control, and hypokalemia are the main determinants of incidence and severity of musculoskeletal disorders in diabetics.
- SMFA questionnaire was accurate in the diagnosis of the extent of musculoskeletal disorders and was closely related to EMG and radiographic findings.
- To avoid the development of musculoskeletal complications, patients should retain strict glycemic control and avoid potassium depletion states such as diabetic ketoacidosis.
- Additional effort should be taken to maintain optimal potassium homeostasis in diabetics receiving insulin, diuretics, or suffer gastrointestinal motility disorders; a future study focused on relation of muscular abnormalities

with frequency and severity of diabetic ketoacidosis and/or hyperosmolar nonketotic states would be worthwhile.

Author contributions ANJ and SMJ composed the manuscript and wrote it including the tables.

JM and AJ identified the patients, took the medical history data, and filled in the forms.

LJS and BSJ performed the rheumatologic and radiologic diagnostic.

TS and VA and performed the endocrinologic diagnostic.

RDR and NM did the statistical tests and interpretations.

Data availability The research data and materials available at: https://1drv.ms/x/s!AnToNOskCFMggehCKd16jy4nmNTj6g

Compliance with ethical standards

Ethical approval The survey was conducted respecting ethical principles for human clinical trials by the Declaration of Helsinki of 1983 [8] The study was approved by Ethical Committee, Faculty of Medicine, University of Pristina/K.Mitrovica.

Informed consent All patients gave their written consent to use data from the medical documentation gathered during their hospitalization period for the research purposes.

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

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ORIGINAL ARTICLE

The status of frailty in poor older adults with type 2 diabetes mellitus or hypertension: the case of Mexico

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Abstract

Background There are limited studies on type 2 diabetes mellitus (DM2) and hypertension (HTN) related to frailty, functionality, and depression-anxiety. Such data are important for planning public health policies for older adults who are users of Family Medicine clinics, located in the most popular urban area of Guadalajara, Mexico.

Aims To establish the frequency of frailty, functionality, and depression-anxiety in older persons with DM2 and HTN and establish differences statistically between the variables which are being tested.

Design Observational, cross-sectional study

Materials and methods Four hundred older adults aged over 60, users of 30 office clinics in Family Medicine Clinic no. 3, of the Mexican Institute of Social Security (IMSS). From these subjects, we obtained a random-quota sample, with 95% confidence and 80% study power, which had, as units of analysis, two groups (DM2 and HTN), each group comprising 200 persons, divided proportionally by gender and clinical office. We applied Fried's clinical criteria and the following scales: Physical Activity Scale for the Elderly (PASE), Katz, Lawton-Brody, and Calderon Narvaez

Results In individuals with DM2 prevalence, there was frailty of 17%, 56% reported no physical activity, 20 % had not basic occupations, 3% has deficiency in instrumental activities, and 31% showed signs of depression-anxiety. In persons with HTN, 8% has frailty, 52%had not physical activity, 15%had not developed basic activities, 3% had a deficiency in instrumental activities, and 40% showed signs of depression-anxiety.

Conclusions By sexes we observed significant frequencies of frailty and pre-frailty in males with DM" and in women with HTN. In the Katz scale, women with DM2 have high percentages of disability in basic activities of daily living. Associating DM2 and HTN with frailty, dysfunction and depression reveals a lack of prevention.

Keywords Type 2 diabetes mellitus · Hypertension · Frailty · Physical and instrumental activity · Depression-anxiety

Introduction

The increase in life expectancy and the Western lifestyle have paradoxically favored an increase in morbidity and mortality for degenerative diseases principally in people aged over 50 years, such as hypertension (HTN) and type 2 diabetes mellitus (DM2), which, in Mexico, demonstrate higher prevalence, 40% and 15%, respectively, in this age group [1].

In Mexico, little study has been devoted to the relationship between DM2 and HTN related with conditions such as frailty, functionality, and depression, to which are credited an increase in the risk of morbidity, hospitalization, and mortality in older adults, either independently or associated with these [2, 3].

This risk situation is complex since the illnesses and conditions previously mentioned may demonstrate, during or after, stressful situations, which reveal the efficiency of the organic systems involved.

Thus, frailty is characterized by the expression of physical dysfunction, including muscular weakness, exhaustion, low weight, slow walking, and a low level of physical activity as

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originally reported by Fried et al. [4], as a phenotype syndrome, communicating a prevalence of 6.4%. These comprise the criteria under which, in Latin America [5], according to the type of group, there are frequencies that have been reported as ranging from 7.7–42.6%.

In reference to age, patients with DM2 and HTN are related with the following: damage in physical performance (such as slow walking); a more rapid functional decline, and that, together with frailty, give rise to vicious negative cycle, which implies greater risk of adverse effects on health, both pathologies reporting that frailty is associated with HTN in between 55 and 84% and with DM2 in between 12 and 22% [5–9].

For example, there are studies indicating that among older adults with HTN and DM2, 45.7% presented severe frailty in magnitude, which involves the need for effective actions of prevention and control [10].

With regard to depression, in Mexico, a general prevalence of 9% was reported among the elderly [11] in 2005. Another prevalence of 25% was reported in 2013 in Ecuador, where Molina et al. [12] found a prevalence of moderate and severe depression in older adults with DM2 and HTN of 81.4%.

Therefore, our objective was to determine the prevalence of the frailty syndrome under the Fried criteria, in addition to functionality and level of depression in older adults with HTN or DM2 who were users of a Family Medicine Clinic of the Mexican Institute of Social Security (IMSS), located in the area of a poor economic stratum of the Mexican city of Guadalajara, Jalisco, and establish statistical differences between the variables which are being tested under a hypothesis of equality.

Materials and methods

We applied an observationally designed, descriptive, and cross-sectional study in Family Medicine Unit no. 3 in Guadalajara, Mexico, where 28,766 eligible persons aged over 60 years who were users of outpatient services at the clinic were seen.

The study sample, calculated with 95% confidence and 80% study power, was estimated at 200 individuals, but we selected 400, proportionally grouped by gender for each of the two branches of analysis, patients with DM2 and patients with HTN.

For the purpose of consideration, for each of the 30 room clinics of UMF #3, a sub-sample with quota of 6 or 7 adults over the age of 60 years, who were consecutive outpatient-services users at the clinic, met the following inclusion criteria: accepted to participate in the study, with ages of 60 or more years, both genders, with a medical diagnosis of HTN or DM2, and a clinical record.

Exclusion criteria included patients with a diagnosis of dementia, cognitive disability, and physical disability of the lower limbs, fractures, or recent surgery, an individual who was alone and unable to answer questions, or to be subjected to medical or surgical procedures that may affect their nutrition or their weight within the last 6 months, or whose clinical records were not accessible.

The researchers piloted the study prior to its commencement to improve the cultural adaptation of instruments.

Next, in a separate office, interviews were conducted with patients, recording their socio-demographic data. Frailty syndrome-related data [13] and data related to the scales included the following: PASE [14] (Physical Activity Scale for the Elderly) with 12 questionnaire items on the individual's degree of physical activity (professional, home, and leisure) during the past week; Katz index [15] (six basic activities for life (BADL)); Lawton and Brody [16] (eight instrumental activities of daily living (IADL); and the Calderon-Narvaez questionnaire [17] (which measures anxiety and depression by means of 20 reagents, concerning the most common symptoms in our sociocultural environment). The scales have more than 80% of validity and reliability [13–17].

Average questionnaire application time was 45 ± 10 min.

The socio-demographic characteristics gathered included age, gender, level of schooling, income in minimum wage, civil status, and body mass index in the groups of only DM2 or HTN patients, excluding another morbidity, for medical diagnosis.

Frailty is defined according to the criteria of Fried as follows: 3 or more positive components = Frailty, 1 or 2 positive components = Pre-frailty, and 0 positive criteria = NOT frailty.

To contrast frailty, the questionnaires of the PASE, Katz, Lawton-Brody, and Calderón-Narváez scales, according to their specifications, were applied by the researchers and were filled in with the responses that the patients gave.

Each answered questionnaire was recorded in a spreadsheet, of the SPSS ver. 15 software program to create a database and to be validated.

The descriptive and inferential statistical analysis was carried out according to the scale of the studied variables, for example, categorical variables, percentages, and the chi-square test (χ^2)[1] or Fisher's exact test (FET), and interval variables, average, standard deviation (SD), and the Student t test[1].

Ethical aspects

This study was conducted according to the General Law of Health of Mexico in relation to the research for health, in which the latter is typified as "minimal risk research," since the procedures include the application of a questionnaire and testing physical activity (walking and grip). Ethical principles

[1] These were considered significant differences in terms of those with $p \leq 0.05$.

are safeguarded for the following: confidentiality, autonomy, justice, beneficence, and non-maleficence, respecting at all times the confidentiality of information obtained, and each patient was asked for verbal and written informed consent to participate in the study. The National Ethics Committee of IMSS approved the protocol with the admission number FIS/IMSS/PROT/PRIO/13/022 R-2012-785-099 2013-TE.

Results

We compiled information from a total of 400 patients, which is presented in the following tables. In order to determine the characteristics of the sample and to determine the prevalence of the frailty syndrome under the Fried criteria, in addition to the functionality and level of depression in older adults with HTN or DM2 and show statistical differences between the variables which are being tested under a hypothesis of equality.

Table 1 shows the occurred similitudes or different inter-groups (DM2 versus HTN) in their general characteristics.

In the group with DM2 versus the group with HTN, there are significant differences only in two variables: in persons with low schooling (that include null and elementary education, less education in HTN) and individuals with high body mass index (BMI ≥ 25, more obesity in HTN) but not in age, 69.5 years old in DM2 group and 70.3 years old in HTN group; feminine gender, 53% in DM2 group and 64% HTN group; income with < 3 minimum wage (approximately < 4 US dollars per day), 92% in DM2 group and 93% in HTN group; and civil status (for married and common law), 53% in DM2 group and 57% in HTN group.

Table 2 shows inside the groups DM2 and HTN, divided for gender, differences and similarities for the scales of Frailty, PASE, Katz, Lawton-Brody, and Calderon-Narvaez.

We observed significant differences in frequencies of frailty and pre-frailty in masculine gender with DM2 and frailty and pre-frailty in the feminine gender of the group with HTN.

In the DM2 and HTN groups, masculine and feminine patients have similar proportions of physical activity according to the PASE scale.

In the Katz scale, only women with DM2 have higher percentages of disability in basic activities of daily living (BADL) ($p < 0.05$), while in the HTN group, there were no differences in disability by gender.

Table 1 General characteristics of the studied groups

Concept		DM2, $N = 200$		HTN, $N = 200$		Comparison groups, variables, significance
Age, mean ± standard deviation (SD)		69.5 ± 9.6		70.3 ± 7.5		T = 0.92 $p = 0.35$
		#	%	#	%	
Gender	Feminine	105	53	128	64	χ^2=2.49
	Masculine	95	47	72	36	$p = 0.11$
Schooling	Null	10	5	24	12	χ^2=4.55
	Elementary	85	43	102	51	$p = 0.04$
	High school	58	29	42	21	
	Preparatory	28	14	14	7	
	Professional	19	9	18	9	
Income in minimum wage	< 1	67	33	51	25	FET = 0.07
	1–2	117	59	135	68	$p = 0.78$
	3–5	14	7	12	6	
	6–9	0	0	2	1	
	10 or +	2	1	0	0	
Civil status	Single	13	6	19	10	FET=0.32
	Widowed	62	31	53	26	$p = 0.56$
	Separated	10	5	15	7	
	Divorced	10	5	0	0	
	Married	99	50	113	57	
	Common law	6	3	0		
BMI (Body Mass Index)	< 18 under-weight	2	1	2	1	χ^2=12.85
	18–24 normal	116	58	66	33	$p = 0.001$
	25–29.9 overweight	65	32	78	39	
	> 30 obesity	17	9	54	27	

Table 2 Frailty, functionality, and gender

CONCEPT	DM 2					HTN			
	Frailty scale					Frailty scale			
	Frailty	Pre	Normal	Significance between sexes		Frailty	Pre	Normal	Significance between sexes
Feminine (n = 105) (53%)	10 (9%)	44 (42%)	51 (49%)	χ^2 = 10.84	Feminine (n = 128) (64%)	14 (11%)	56 (44%)	58 (45%)	FET = 30.13
Masculine (n = 95) (47%)	24 (25%)	41 (43%)	30 (32%)	p = 0.0044	Masculine (n = 72) (36%)	2 (3%)	14 (19%)	56 (78%)	p = 0.0000
Total (n = 200) (100%)	34 (17%)	85 (42%)	81 (41%)		Total (n = 200) (100%)	16 (8%)	70 (35%)	114 (57%)	
Significance between totals of boot groups. χ^2 = 0.0012; p = 0.0011									
Specifications	*Pre* pre-frailty								
	PASE scale					PASE scale			
	Physical activity		No activity	Significance between sexes		Physical activity		No activity	Significance between sexes
Feminine (n = 105) (53%)	53 (50%)		52 (50%)	χ^2=3.46	Feminine (n = 128) (64%)	57 (45%)		71 (55%)	χ^2=1.25
Masculine (n = 95) (47%)	35 (37%)		60 (63%)	p = 0.0524	Masculine (n = 72) (36%)	38 (53%)		34 (47%)	p = 0.2623
Total (n = 200) (100%)	88 (44%)		112 (56%)		Total (n = 200) (100%)	95 (48%)		105 (52%)	
Significance between totals of boot groups. χ^2 = 0.4936; p = 0.4823									
Specifications									
	Katz scale					Katz scale			
	Ind	Light Dis	Mod Dis	Sev Dis	Significance between sexes	Ind	Light Dis	Mod Dis	Sev Dis	Significance between sexes
Feminine (n = 105) (53%)	76 (72%)	14 (13%)	11 (11%)	4 (4%)	χ^2=9.37	Feminine (n = 128) (64%)	107 (84%)	12 (9%)	6 (5%)	3 (2%)	FET = 2.31
Masculine (n = 95) (47%)	84 (88%)	6 (6%)	5 (6%)	0	p = 00247	Masculine (n = 72) (36%)	64 (89%)	6 (8%)	2 (3%)	0	p = 0.5098
Total (n = 200) (100%)	160 (80%)	20 (10%)	16 (8%)	4 (2%)		Total (n = 200) (100%)	171 (85%)	18 (9%)	8 (4%)	3 (2%)	
Significance between totals of boot groups. FET = 6.0977; p = 0.1070											
Specifications	*Ind* independence, *Slight Dis* slight disability, *Mod Dis* moderate disability, *Sev Dis* severe disability, *BADL* basic activities of daily living										
	Lawton-Brody scale						Lawton-Brody scale					
	4	5	6	7	8	Significance between sexes		4	5	6	7	8	Significance between sexes
Feminine (n = 105) (53%)	2 (2%)	2 (2%)	7 (7%)	18 (17%)	76 (72%)	χ^2=6.33	Feminine (n = 128) (64%)	3 (2%)	2 (2%)	8 (6%)	10 (8%)	105 (82%)	χ^2=6.21
Masculine (n = 95) (47%)	0	1 (1%)	2 (2%)	25 (26%)	67 (71%)	p = 0.175	Masculine (n = 72) (36%)	0	0	8 (11%)	2 (3%)	62 (86%)	p = 0.183
Total (n = 200) (100%)	2 (1%)	3 (2%)	9 (4%)	43 (22%)	143 (71%)		Total (n = 200) (100%)	3 (2%)	2 (1%)	16 (8%)	12 6%)	167 (83%)	
Significance between totals of boot groups. FET = 22.13; p = 0.0002													
Specifications	Scale > 5 = tendency to develop more instrumental activities of daily living (IADL)												
	Depression-anxiety scale						Depression-anxiety scale					
	Nor	Slight	Anx	Significance between sexes			Nor		Slight	Sev	Anx	Significance between sexes
Feminine (n = 105) (53%)	80 (76%)	8 (8%)	17 (16%)	χ^2=5.01 p = 0.0814	Feminine (n = 128) (64%)		63 (49%)		34 (27%)	4 (3%)	27 (21%)	χ^2= 19.41 p = 0.0002
Masculine (n = 95) (47%)	59 (62%)	9 (10%)	27 (28%)		Masculine (n = 72) (36%)		58 (81%)		8 (11%)	0	6 (8%)	
Total (n = 200) (100%)	139 (69%)	17 (9%)	44 (22%)		Total (n = 200) (100%)		121 (60%)		42 (21%)	4 (2%)	33 (17%)	
Significance between totals of boot groups. FET = 17.41; p = 0.0006												
Specifications	*Nor* normal, *Slight* minimal; *Sev* severe, *Anx* anxiety											

According to the Lawton-Brody scale, no differences between sexes occurred in the scores for developing instrumental activities of daily living (IADL).

For the Calderon-Narvaez scale for depression-anxiety scale, masculine gender in the HTN group showed significant differences (higher frequencies of normality than the feminine gender).

At the bottom of each scale, in Table 2, we showed an overall significance in the scales, between the total groups of DM2 and HTN, frailty (has more frequency in DM2); Lawton Brady of IADL (a less frequency of develop IADL in HTN group); and Calderon-Narvaez, more depression in HTN.

Discussion

In the study, the groups are comparable in their variables of age, gender, occupation, and marital status, with the exception of lower schooling and higher BMI for persons with HTN, situations that could influence an increased frequency of the conditions tested.

Regarding the prevalence of frailty found based on the Fried scale criteria [4, 13]that were evident in the two groups studied (DM2 and HTN), there were frequencies higher than those originally identified by Fried of 6.4% [4], and on the other hand, our groups exhibited statistical differences between them, that is, 17% for DM2 and 8% in patients with HTN, and both figures are localized in an intermediate level of frailty, as indicated for the world's population by Choi et al. [18].

In our study, the patients are with frailty and with pre-frailty, in persons with DM2 representing a 17% and 42% respective and in the individuals with HTN 8% with frailty and 35% with pre-frailty. This relationship is similar to that mentioned by Liang et al. [19] in China, Tapia et al. in Chile [20], and Kang et al. in Korea [21].

The prevalence rates and percentages found for the condition of frailty and pre-frailty in patients with DM2 and HTN suggest the existence of a particular continuum of conditions, which reinforce the idea of gaps in the prevention of frailty [22]; the latter could favor an upward pattern in the step from pre-frailty to frailty (may be due to lack of detection and late registration). The situation appears more evident in the feminine gender (Table 2), in comparison to Canada [23] where there appears to be greater early detection.

For the functional dimensions studied, instruments applied to scales validated for Spanish-speaking individuals, scales, including PASE, Katz, Lawton-Brody, and Calderón-Narváez, possess reliability, validity, and proper agreements, ranging between 0.86 and 0.97 [24].

The physical dimension, in both study groups, was reflected in the same manner as that of the national figures in Mexico [1], where more than one half of persons do not perform any physical activity, a situation reflected and shown by the low punctuations of physical independence in the Katz scale; in $10\% + 8\% = 20\%$ patients with DM2 and $9\% + 4\% + 2\% = 15\%$ of those with HTN, this was somehow hampered as reflected with the results of the Lawton-Brody scale, where the percentage of dysfunctionality, $1\% + 2\% + 4\% = 7\%$ in DM2 and $2\% + 1\% + 8\% = 12\%$ in HTN, reinforces that the "resilience-frailty"-continuum patients with DM2 and HTN [25], principally in feminine gender, placing these in the stage of late frailty, of frailty going unnoticed or of frailty not served, in the early pre-frailty, because of frailty stages, where lack of activity or its (physical and social) dysfunction plays a harmful role in terms of the patient's care.

The authors, in relation to the authors in [20, 26], point out that the aging population reveals increased dependency and frailty, a binomial negative synergy for the elderly, which reduces their mobility. This situation, under conditions of limited social support, affects timely attendance at health care services, mainly if this corresponds to the case of poor people who are old and who live with chronic diseases such as diabetes mellitus and hypertension [27].

Regarding mental health, significant effects of anxiety and grades of depression (joint), were found in the group with DM2 ($9\% + 22\% = 31\%$) and in the HTN group ($21\% + 2\% + 17\% = 40\%$), which are possibly associated with conditions for the elderly, with their decline in the economic cycle, the family cycle, the reduction of social support, vitality, and relation spaces [28]. The previously mentioned material points to the need to evaluate frailty, along with psychosocial factors in daily living of the elderly [29].

But frailty is attributed to various physical factors, including slowing down and the lack or loss of range-of-motion, imbalance, and the decrease in strength and physical resistance. This situation is not spontaneous and is coupled with various psychosocial factors that produce social isolation, associated with reduction of physical mobility, possibly limiting access to health care, circumstances that affect health promotion and health education, and early detection of the frailty syndrome. The latter is even to a greater degree if we take into account that the line that divides the intersection between frailty, disability, comorbidity, and aging is tenuous and even imprecise [29].

Finally, we should consider, in future work, the qualitative research of the complex chronic condition, hypertension and/or diabetes mellitus—physical, mental, dysfunctionality, frailty, as a bio-socio-cultural process, the result of a biographical path, molded for welcome structures [30], such as family, neighborhood and services, with the linking of their roles in the determination of frailty [26].

The study has its limitations, including the operational definitions employed, their cross-sectional type for establishing causal directionality, and the studies including only patients attending outpatient services and belonging to the popular socioeconomic stratum in Guadalajara city.

We conclude that this type of knowledge will complement the traditional view of risk factors helping to understand why frailty dysfunction, associated with DM2 and HTN, among other aspects, appears to be more prevalent in females than in males and more prevalent in Latino than in Caucasian populations [31], which adversely affect the quality of life of these persons, thus supporting the establishment of strategies and policies to achieve the promotion of a happy old age and to prevent the syndrome.

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Compliance with ethical standards

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

Ethical approval The National Ethics Committee of IMSS approved the protocol with the admission number FIS/IMSS/PROT/PRIO/13/022 R-2012-785-099 2013-TE.

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LETTER TO THE EDITOR

Lockdown—the only solution to defeat COVID-19

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Sir/Ma'am,

An outbreak of COVID-19 caused by the zoonotic 2019 novel coronavirus (SARS-CoV2) has been declared a *pandemic* by the WHO [1]. It is well known that mortality associated with this condition is high among patients with diabetes mellitus.

As it is a new strain of coronavirus and little is known about its behaviour except its potential for transmission while being asymptomatic during an incubation period of up to 14 days and its sensitivity to heat, Sophie Bushwick, technology editor at *Scientific American*, a science magazine, stated on 20 March 2020 that asymptomatic people with COVID-19 have a higher viral load. (https://www.sciencefriday.com/articles/covid-19-questions-answers/) One infected person (even asymptomatic) can transmit the disease to more than 400 patients in 30 days. Because it is transmitted from host to host, keeping it away from a host will cause its own death (decay). This can be achieved through a lockdown.

A lockdown period depends upon the virus' faster decay rate which is directly related to the melting point of the outer protective lipid bilayers of SARS-CoV2.

According to virologist Thomas Pietschmann, the lipid layer of SARS-CoV2 is not heat resistant unlike that of other viruses, so it is quickly broken down when the temperature rises [2]. Results of a scientific data model indicate that there was a negative correlation in the predicted number of cases with temperatures from 1 °C and above (Fig. 1). An increase in the average temperature from 1 to 9 °C was associated with a decrease in predicted cases from 24 to 19. Similarly, an increase in the average temperature from 10 to 19 °C was associated with a decrease from 18 to 7 cases [3].

SARS-CoV2 is highly stable at 4 °C, but is sensitive to heat. At 4 °C, there was only a 0.7 log-unit reduction of infectious titres on day 14. With the incubation temperature increased to 70 °C, the time for virus inactivation was reduced to 5 min [4]. For most animals, the melting point of lipids is between 20 and 40 °C. In the case of SARS-CoV2 being zoonotic, it is presumed that the melting point of its lipid layer should be around 40 °C, resulting in its faster decay during the summer.

Outside a host, it is as good as non-existent. Because coronavirus is transmitted from host to host only, keeping it away from a host for longer than its incubation period through a lockdown can cause its own death and defeat COVID-19.

A 50% drop in the transmission doubling rate from 3 to 6.2 days in one week which was recently reported in India is an appropriate yardstick to judge the effectiveness of a lockdown. Many districts in India have become totally COVID free after a lockdown.

A lockdown is the only option to defeat COVID-19.

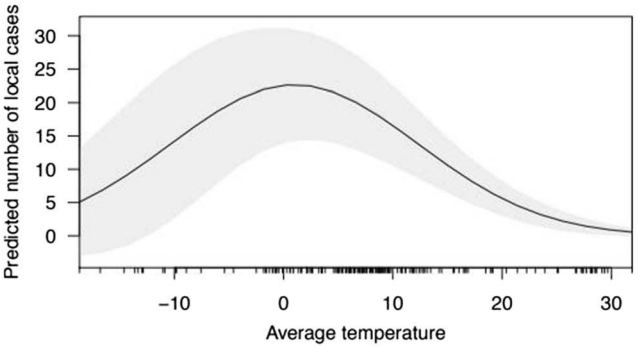

Fig. 1 Predicted number of cases of COVID-19 by sample report from 47 countries as a function of the average temperature during the period from the first reported case until 29 February 2020. The grey area represents the 95% confidence interval of the predicted values

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https://doi.org/10.1007/s13410-020-00811-w

CORRECTION

Correction to: The role of osteocalcin in mechanism of steroid induced diabetes mellitus

Holla Avinash[1] · Vidyasagar Sudha[1] · Bairy Laxminarayan[2] · B. Nandakrishna[1] · B. A. Shastry[1] · Kamath Asha[3] · Manjunath Hande[1] · Adiga Shalini[4]

Published online: 23 March 2020

Correction to: International Journal of Diabetes in Developing Countries https://doi.org/10.1007/s13410-019-00791-6

The manuscript used in the original version of this article was outdated. In this connection, the following errors were encountered.

1. Incorrect article title
2. Missing author Adiga Shalini

Because of this, the publisher regrets the error.
The original article has been corrected.

The online version of the original article can be found at https://doi.org/10.1007/s13410-019-00791-6

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VISION STATEMENT

To be recognized as a global leader for clinical care, education, training, research, advocacy and capacity building in the field of diabetes.

MISSION STATEMENT

1. Promotion of excellence in diabetes care to make India the Diabetes Care Capital
2. Empowerment of persons living with diabetes
3. Support for diabetes research
4. Dissemination of information and knowledge in diabetes care
5. Advocacy for the cause of diabetology

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- For providing research grants, RSSDI invites proposals from Indian scientists, interested in conducting original research in the field of diabetes mellitus. Furthermore, limited grants are also available for the students of medical colleges for smaller projects.
- There is no deadline for submission of the proposals, which can be sent throughout the year. These proposals may fall into one of the following three categories:

1. Projects involving funding up to Rs 40,000 per project (preference will be given to young scientists <40 years).
2. Projects involving funding up to 10 lakhs.
3. We also invite proposals for more than 10 Lakhs as major projects but these have to be preferably multicentric.

- The detailed proposals should include the following:

  ◊ Title, names of principal and co-investigators, summary, introduction/background, review of literature, aims, methodology, study design, and detailed plan of work and bibliography. Brief biodata of principal investigator and other co-investigators
  ◊ Importance of work in the context of national priorities. 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.
  ◊ Ethical committee clearance of the institution or other bonafide body.

TRAVEL GRANTS FOR YOUNG DIABETES RESEARCHERS TO ATTEND INTERNATIONAL CONFERENCES

Criteria for the travel grant are as follows:

- Applicant should apply 2 months in advance.
- Travel Grant is open only to the RSSDI members.
- Applicant should submit Oral paper / Poster acceptance document to RSSDI Secretariat.
- Applicant should submit Declaration that he/she has not receiving grant from any other agency / Organization – In case of receiving grant from any other Organization, RSSDI shall pay only the exceeding amount not covered by that agency.

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 carefully looked into all aspects of this course & has accredited & recognized 17 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.

List of RSSDI Accredited Centres

S.N.	Institute Name	Institute Location
1.	Diacon Hospital	Bengaluru, Karnataka
2.	North Delhi Diabetes Centre	New Delhi, Delhi
3.	Prithvi Hospital	Tumkur, Karnataka
4.	Totall 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.	G D Hospitals and Diabetes Institute	Kolkata, West Bengal
10.	Sunil's Diabetes Care N' Research Centre Pvt Ltd.	Nagpur, Maharashtra
11.	Marwari Hospital and Research Centre	Guwahati, Assam
12.	Down Town Hospital	Guwahati, Assam
13.	St.Theresa's Hospital	Hyderabad, Telangana
14.	Aegle Clinic	Pune, Maharashtra
15.	Tulip Hospital	Sonipat, Haryana
16.	Lilavati Hospital & Research Centre	Bandra West, Mumbai
17.	Srajan Hospital	Udaipur, Rajasthan
18.	Endeavour Clinic & Dr. Sambit's Centre of Diabetes and Endocrinology	Endocrinology N4/198, IRC Village Sapneswar Temple Lane, Nayapalli, Bhubaneswar, Odisha -751015
19.	ILS Hospital, Salt Lake	ILS Hospital, Salt Lake Cit$_y$
20.	Belle Vue Clinic	9, Dr. U N Brahmacahri Sreet (Formerly Loudon Street), Kolkata - 700017

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 preference.

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.

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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.

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