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EDITORIAL

Normoalbuminuric diabetic kidney disease: a distinct entity?

SV Madhu [1]

Published online: 22 May 2019

Diabetic kidney disease (DKD) is the major cause of renal disease due to hyperglycemia. Worldwide, approximately 40% of people with diabetes develop DKD. Presence of persistent albuminuria in urine is the initial clinical indication of DKD. Of the major pathways known to be involved in the development and progression of DKD, the renin-angiotensin-aldosterone system (RAAS) has been considered as the most important pathway as it plays a central role in maintaining blood pressure, glomerular pressure, and fluid and electrolyte balance via angiotensin II. During development of diabetic nephropathy (DN), there is increased formation of angiotensin II, by the action of ACE, which further results in renal vascular constriction via activation of its receptor ATR1. However, activation of ATR2 ensues beneficial effects including vasodilation, antiinflammatory, and antiproliferative actions [1].

Tsalamandris and his coworkers observed for the first time in 1994 that few type II DM patients displayed no significant proteinuria but had renal insufficiency and developed DKD (i.e., estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2) [2]. This has been described as normoalbuminuric diabetic kidney disease (NADKD) or diabetic kidney disease without proteinuria where albuminuria does not associate with impairment of kidney function. The ADA criteria for diagnosis of DKD now involve the presence of eGFR < 60 mL/min/1.73 m^2 or the presence of UAE > 30 mg/24 h [3]. In patients with nonalbuminuric diabetic kidney disease, risk factors include obesity, hypertension, high TG levels, sex, poor glycemic control, and glomerular hyperfiltration [4, 5] that may play a role in nephrosclerosis. Prevalence of NADKD varies from 14.29 to 56.6% among diabetic patients with different ethnicities [6, 7]. Macroangiopathy is found to be more prevalent in patients with NADKD [8]. Boeri et al. also observed that intrarenal arteriosclerosis is the main cause of renal impairment in NIDDM patients independent of albuminuria [9], and this may partly cause eGFR decline in these patients. Several studies also suggest that decline in renal function is mainly due to interstitial injury (a pathological change in DN) [10] as compared with glomerular injury [11]. Hence, eGFR decline may be a consequence of interstitial injury in DM patients and tubulointerstitial injury may be more important in the development of NADKD. Mesangial matrix proliferation, increased glomerular fibrosis, thickening of basement membrane, and increased type IV collagen in the interstitium and glomeruli are some pathological changes that were demonstrated in NADKD rats and patients [12]. Moreover, a faster eGFR decline in albuminuric DM patients was observed as compared with patients with NADKD [13]. Urinary NGAL and FABP are the important indicators of tubular injury in the kidney [14] that may be helpful in the diagnosis of NADKD.

Activation of ATR1 plays an important role in the pathogenesis of renal tissue injury. In an earlier study, it has been observed that hyperglycemia activates RAAS and contributes to renal fibrosis [15]. Ang II, the major mediator of kidney injury, displays majority of its deteriorating effects like generation of ROS, tissue inflammation, and fibrosis via activation of ATR1 [16]. Increased expression of ATR1 has been reported in the in vivo model of glomerular capillary hypertension in podocytes of the remnant kidney [17]. It has been observed that activated NF-κB complexes are largely situated in mesangial, tubuloepithelial, and endothelial cells [18] and promote renal inflammation by macrophage infiltration in a model of diabetic nephropathy [19] and treatment with ATR1 antagonist partly diminished NF-κB activation [20]. Also, treatment with ATR1 antagonist in kidney arterioles led to increased expression of protective ACE2 [21].

Among RAAS genes, ACE gene is the most extensively studied gene, and I/D polymorphism of ACE gene is found to be strongly associated with the activity of ACE [22] in DN patients. AGTR1 gene expression pattern also demonstrates significant association with DN [23]. Increased mRNA levels of ATR1 gene have been found to be associated with C allele of A1166C polymorphism [24]. Earlier studies have confirmed the significant association of A1166C polymorphism of ATR1

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gene with DN [25]. However, contrasting reports regarding association of ATR1 and DN also exist in literature [26].

In this issue, Viswanathan et al. [27] report the clinical and biochemical features of patients with NADKD from South India and their relation with ATR1 receptor polymorphisms. The finding of significant differences in clinical and biochemical profiles as well as higher cystatin C and a significant association of AGTR1 A1166C receptor polymorphism with normoalbuminuric DKD suggests that this entity could have a different underlying pathophysiology. It could also be possible that cystatin C or other biomarkers in the blood or the urine could serve as earlier indicators of DKD than albuminuria. Whether there is a distinct genetic basis for this condition is unclear and cannot be said with any degree of certainty from this study. Future prospective studies with much larger sample sizes might answer this question and need to be undertaken.

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LETTER TO THE EDITOR

Should we still collect blood glucose sampling in fluoride tubes? An evidence-based study

Mohini Bhargava[1] · Narinder Pal Singh[2] · Anish Kumar Gupta[2]

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Blood glucose can be measured in serum or plasma. If serum is let to stand in contact with RBC, the glucose level falls due to the glycolytic process occurring in the cytoplasm of the RBC. Fluoride, which blocks the glycolysis cycle, is being used as an additive in blood glucose collection tubes for decades. The use of these tubes was suitable for blood collection when there was a long delay (4 h or more) in separation of serum following blood collection. Recently with the availability of gel serum separator tubes (SST), serum no longer remains in contact with RBC as soon as the specimen is centrifuged. We hypothesized that the use of fluoride tubes can be eliminated if the glucose levels in gel tubes and fluoride tubes show no significant difference within 1 to 2 h of centrifugation and then after separation from RBCs for up to at least 4 h. Earlier study has shown that glucose is stable for 48–72 h, when separated from the red cells [1]. Some studies have also reported that there was no difference in glucose values for blood samples collected in plain gel tubes and fluoride tubes that were separated within 2 h of collection [2, 3], but the sample size was a limitation for valid statistical inference. American Diabetes Association (ADA) no longer recommends the use of fluoride to control glycolysis [4].

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To prove the hypothesis, we collected blood samples from 1000 subjects in both gel and fluoride tubes and centrifuged them at different time intervals (< 30, 30–59 and > 60 min respectively). Sugar estimation was done at 0, 1, 2, and 4 h after keeping the samples at room temperature. Laboratory assays were performed on the Cobas Integra 400 Plus, according to the manufacturer's instructions and using manufacturer-supplied quality control (QC) materials.

In the present study, we found that once the serum is separated from the red cells, the glucose level remained stable for at least 4 h (Fig. 1a). The difference was within the range of ± 6 mg/dl (standard acceptable range as per CLIA guideline) at all time intervals (Fig. 1b). In our study, there was some statistical difference in serum and plasma values if centrifuged with increasing lapse of time, the difference being more with increase in time gap (Fig. 1c). The longer the time gap, the more was the fall in glucose level. Though the difference was statistically significant, it was clinically non-significant (standard acceptable range as per CLIA guideline ± 6 mg/dl) even after more than 60 min. Bruns et al. also supports the use of SST in preference to sodium fluoride tubes [3].

It is our firm opinion that blood glucose measurement in serum can be done without the need for sodium fluoride if blood separation is achieved within reasonable time (less than 2 h). This will result in practical advantages including reduction in blood drawn, smoother work flow, better turn-around-time and lesser chances of hemolysis. It should be mandatory to have a centrifuge at all collection centers to achieve blood separation within reasonable time (within 1 to 2 h).

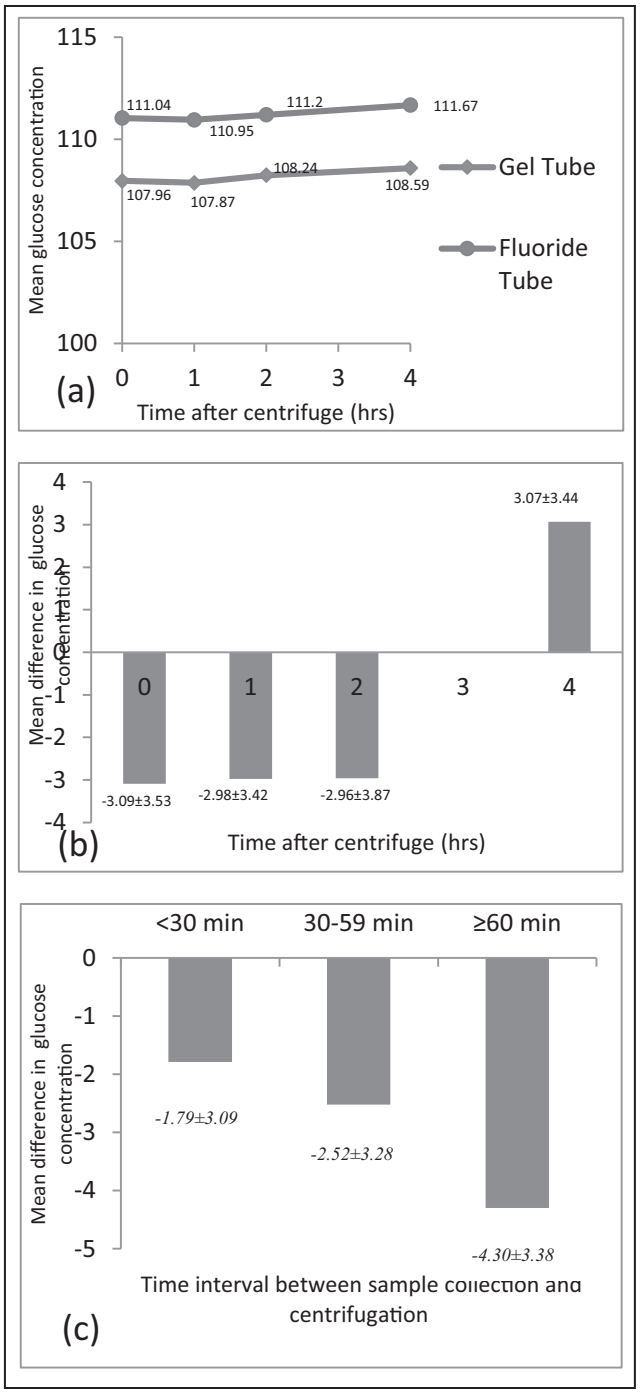

Fig. 1 Glucose concentration in gel (serum) vs fluoride (plasma) tubes

Compliance with ethical standards

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

Research involving human participants The study was approved by the Institutional Ethics Committee. All procedures performed in studies 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 For this type of retrospective study, formal consent is not required and consent waiver was obtained from the Institutional Ethics Committee.

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LETTER TO THE EDITOR

Maternal anxiety and competency of mothers of children with type 1 diabetes

Varsha Vartak[1] · Anuradha Khadilkar[1] · Vaman Khadilkar[1] · Shashi Chiplonkar[1] · Chittaranjan Yajnik[2] · Laila Garda[3] · Yasmeen Sethna[1] · Ujjwal Nene[3]

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Complexity of T1DM in children diagnosed under 5 years has been suggested to result in maternal anxiety. This may affect maternal competency in dealing with their child's diabetes [1]. High maternal anxiety is associated with maternal hostility and poor metabolic control in adolescents with T1DM [2]. Hence, assessing anxiety and competency in mothers of young diabetic children is critical. We aimed to explore anxiety and competency levels of mothers with a child with T1DM diagnosed under 5 years (T1DM < 5) and its interrelationships with child's metabolic control (HbA1c).

Families with children with T1DM < 5 attending diabetes clinics at three hospitals in Pune (India) were approached. Families received regular treatment, free medication, and guidance on diet and lifestyle. Sample size of 69 (80% power, 5% significance) was computed considering proportion of maternal anxiety as > 50% [1]. Eighty-six mothers having 1 child with T1DM < 5 (48 boys, 38 girls) gave consent and were randomly selected (November 2015 to January 2017). Medical history and HbA1c values were obtained from medical records. Mothers were administered Hamilton Anxiety Rating Scale (HAM-A) [3] and Parenting Sense of Competency (PSOC) [4] scale by trained psychologists. SPSS was used for analysis (SPSS, Chicago, IL, USA).

Families belonged to lower socio-economic strata, mean family size was 5, monthly income ≤ Rs. 20,000/month (290USD); 42% mothers reported family support for child management. Mild anxiety was reported by 32.5% mothers and 67.5% reported moderate-severe anxiety. Mean anxiety scores were lower and competency scores higher as duration of diabetes increased (Table 1). Competency score of 69% mothers was < 65, indicating average-low perceived competency in handling their child's condition.

Maternal anxiety and competency were negatively correlated ($r = -0.308$; $p = 0.004$); anxiety increased as competency reduced.

Children of mothers who reported average competency had lower HbA1c values than mothers reporting higher competency ($p = 0.03$). No differences in HbA1c concentrations of children were noted with varying maternal anxiety.

Thus, we report considerable anxiety among mothers of children with T1DM < 5; anxiety was not associated with child's metabolic control. Mothers perceived their competency in handling their child's condition to be average/low. Counselling along with diabetes education to reduce maternal anxiety and improve efficacy in dealing with the child's diabetes may have a positive outcome on T1DM in children.

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Table 1 Characteristics of children and their mothers across duration of diabetes

Parameters	Duration of disease (years)				Total
	< 1 year	1.1–2.5 years	2.6–4.5 years	> 4.6 years	
n	28	18	24	16	86
Children					
Age (years)	3.7 ± 1.1	4.6 ± 1.8	6 ± 1.5	10.2 ± 3.3*	5.7 ± 3
HbA1c	9.5 ± 2	9.1 ± 1.8	9.1 ± 1.8	9.9 ± 2.2	9.3 ± 1.9
		Mothers			
Age (years)	31.4 ± 5.4	28.9 ± 2.6	28.6 ± 4.8	32.5 ± 4.3	30.2 ± 4.7
Education[a]					
Less than std. 10	7 (26%)	5 (28%)	5 (24%)	5 (31%)	22 (27%)
Std. 10 and above	20 (74%)	13 (72%)	16 (76%)	11 (69%)	60 (73%)
Occupation[a]					
Housewife	20 (71%)	13 (72%)	18 (78%)	10 (63%)	61 (72%)
Working	8 (29%)	5 (28%)	5 (22%)	6 (37%)	24 (28%)
Anxiety score (HAM-A)	32 ± 13	34 ± 14	32 ± 14	28 ± 13	31 ± 13
Competency score (PSOC)	62 ± 8	61 ± 8	65 ± 8	64 ± 6	63 ± 8

*Significantly different across the disease duration categories ($p = 0.0001$)

[a] Results are presented as number of mothers (percentage)

Compliance with ethical standards

Ethical standards disclosure All procedures performed in this study 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.

Ethics approval Ethics approval was provided by the Ethics Committee Jehangir Clinical Development Centre Pvt. Ltd. Written informed consents were obtained from parents of subjects and written informed assents were obtained from subjects.

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

Clinical trial registration NA

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ORIGINAL ARTICLE

Validity study of the Turkish version of the barriers to insulin treatment questionnaire

Seyhan Citlik Saritas[1] · Behice Erci[2] · Ibrahim Sahin[3] · Evrim Timocin[4]

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Abstract
The purpose of this study is to test the validity and reliability of the Barriers to Insulin Treatment Questionnaire and adapt the questionnaire for Turkish society. This was a cross-sectional study. The population of this study consisted of adult patients with type 2 diabetes who were using insulin and being monitored in the Endocrine and Outpatient Clinics of Inonu University Turgut Ozal Medical Centre. The sample of the study consisted of 348 patients. The data were collected by using the Barriers to Insulin Treatment Questionnaire (BITQ) and a survey including demographic characteristics. The 14 items of the questionnaire are scored between 1 and 10. BITQ has five factors as fear of injections and self-testing, expectations regarding positive insulin-related outcomes, expected hardship from insulin treatment, stigmatization by insulin injections, and fear of hypoglycemia. In order to test the construct validity of the questionnaire, Exploratory Factor Analysis (EFA) and Confirmatory Factor Analysis (CFA) were utilized. The reliability of BITQ was evaluated by its Cronbach's alpha internal consistency coefficient, item total correlation, and test-retest reliability. It was determined that the Cronbach's alpha reliability coefficients of the questionnaire factors ranged between 0.70 and 0.82, the scale item factor loadings varied from 0.54 to 0.86, and the item total correlation coefficients were between 0.30 and 0.52. CFA supported the 5-factor scale structure obtained as a result of EFA. Good fit index values were obtained as a result of CFA. According to the obtained results, it may be asserted that BITQ is valid and reliable in terms of its application in Turkish society.

Keywords Barriers to insulin treatment questionnaire · Turkish adaptation · Validity · Reliability · Diabetes mellitus

Introduction

Diabetes is gradually increasing worldwide. Drug therapy in diabetic individuals varies according to the type of diabetes and presence of complications [1].

Studies anticipate that those with type 2 diabetes will become insulin-dependent in the next decade following the diagnosis [2–4]. Studies have revealed that insulin treatment is effective in slowing down the onset and progression of chronic complications of diabetes [5–7].

Many diabetic patients are concerned about self-injection and finger-lancing at the beginning, but they can learn to overcome these fears within a short time and make insulin injections and blood sugar measurements their daily routine. However, some diabetic patients are unable to perform daily insulin administration and blood sugar measurement and they feel permanent fear of insulin injection and finger-lancing. The prevalence of injection fear was found as 2% in diabetic patients and 1% in the general population; it is also reported that injection fear continues

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at the rate of approximately 1% even diabetic patients have received insulin treatment for a long time, and most of them have never shared this fear with their doctors and nurses; almost 5% of diabetic patients using insulin frequently experience stress while administrate the injection and hate self-injection [8, 9].

Khan et al. found that 83.7% of patients with type 2 diabetes did not want to administer insulin [10].

It is known that injection and insulin anxiety/fear affect patients' compliance in diabetes treatment, while these issues are widely seen and escape healthcare personnel's notice. However, it is seen that no universal definition has been made as a concept for them, and there are different approaches in both identification and the diagnosis. There is a need for valid and reliable tools for early detection of barriers existing in insulin treatment in a clinical setting. The universal acceptance of the barriers in the insulin usage brings the necessity of considering measurement tools to be used in screening this problem with universal characterization. It is necessary to evaluate whether or not the measurement tools to be used for this purpose are suitable for different cultural structures. Starting from this point of view, it was aimed to perform validity and reliability research of the Barriers to Insulin Treatment Questionnaire (BITQ) developed by Petrak et al. and adapt the questionnaire into Turkish [11].

Material and methods

Type of study

This questionnaire validation study was conducted to determine the validity and reliability of BITQ by adapting the questionnaire into Turkish.

Study design

This was cross-sectional study. Finally, those who agreed to participate were included in the study. The population of the study consisted of 388 adult patients with type 2 diabetes who were using insulin and being followed up in the Endocrine and Outpatient Clinics of Inonu University Turgut Ozal Medical Centre. Adult patients with type 2 diabetes who were literate, could communicate, had no psychiatric problems, and were using insulin were included in the study. Forty patients were not included in the study. It was planned to include the whole population. The study was completed by reaching 90% of the population with 348 patients.

Data collection tools

A survey form and BITQ were used to collect the data of the study.

Survey form The 5-item survey form prepared by the researcher includes the socio-demographic, medical, and physiological characteristics of the patients.

Barriers to insulin treatment questionnaire

The questionnaire was developed in 2007 by Petrak et al. [11]. It consists of 14 items, each of which is evaluated based on the Visual Analogue Scale (VAS) varying from 1 to 10 points. The questionnaire is assessed with five subscales that show barriers in the patient's insulin treatment. These subscales are fear of injections and self-testing (items 1, 2, 3), expectations regarding positive insulin-related outcomes (items 4, 5, 6), expected hardship from insulin treatment (items 7,8,9), stigmatization by insulin injections (items 10, 11, 12) and fear of hypoglycemia (items 13, 14). The questionnaire can be applied within 5–7 min. The Cronbach's alpha coefficient of the original scale developed by Petrak et al. [11] was 0.78–0.83. The Cronbach's alpha coefficients for five subscales were 0.85, 0.66, 0.81, 0.62, and 0.78, respectively.

Implementation of data collection tools

The data of the study were collected from the patients who were using insulin by the face-to-face interview method between 08:00 a.m. and 16:00 p.m. during the weekdays in the patient rooms at the Endocrine and Outpatient Clinics of Inonu University Turgut Ozal Medical Centre. The patients were asked to fill out the data collection tools. Data collection time was between 10 and 15 min for each participant.

Language validity

A scale developed in a particular culture and language has conceptualization and sampling characteristics specific to that culture. Translation of the scale that is developed in a foreign culture into another language changes the nature of that scale. This change arises from the differences in conceptualization and expressions [12, 13].

In the language translation of the Barriers to Insulin Treatment Questionnaire, the questionnaire was first translated from English to Turkish by the researchers. It was later translated back to English by a linguistics specialist. The researchers compared this translation to the original version, reviewed it, and the questionnaire was finalized.

Pilot implementation of the finalized BITQ was conducted with 15 type 2 diabetic patients who were using insulin. The results that were obtained from the pilot implementation were not included in the sample. As a result of the pilot implementation, it was determined that there was no misunderstood question in the questionnaire. Thus, the Turkish version of BITQ was finalized.

Validity and reliability of the barriers to insulin treatment questionnaire

The validity and reliability studies of the Barriers to Insulin Treatment Questionnaire were conducted in accordance with the related literature and expert opinions in this matter [14].

Psychometric testing of BITQ

Validity

Factor analysis was conducted to determine the construct validity of the questionnaire. Before the factor analysis, Kaiser Meyer Olkin (KMO) and Bartlett's tests were used to determine whether the sample size was suitable for factor analysis or not. In the literature, it is reported that the factor analysis can be continued if the value found as a result of KMO is higher than 0.50 [15]. Principal Component Analysis, one of the most common statistical techniques for factor analysis, was used to examine the factor structure of the BITQ. In order to obtain common factor variance values of the items among the results of principal components and interpretable factors, the results were examined via the "varimax" rotation technique. After Exploratory Factor Analysis, Confirmatory Factor Analysis (CFA) was carried out to support the results related to the subscales of the questionnaire. The X^2/sd rate of ≤ 5, RMSEA value of ≤ 0.07 and GFI, CFI, IFI values > 0.90 that were obtained as a result of CFA were accepted as the lower limit of the data-fit index of the model [16].

Reliability

Cronbach's alpha internal consistency coefficient is recommended for examining the reliability of Likert-type scales. Cronbach's alpha reliability coefficient is an indicator for the internal consistency and homogeneity of the items in a scale. The higher the Cronbach's alpha reliability coefficient of the scale is, the more consistent the items in the scale are with each other and the same feature is composed of the items examining the elements [17]. The measurement tool is sufficient to be used in studies in the case that the Cronbach's alpha scale reliability is 0.70 or higher [15, 17, 18].

The item-total correlation coefficients were taken into account to examine the relationship between the scores from BITQ test items and the total test score. A coefficient greater than 0.30 was considered acceptable while selecting the items [19].

For retest analysis, BITQ was reapplied to 348 type 2 diabetic patients who were using insulin after 2 weeks. The time invariance of the scale was assessed by test-retest correlation.

Data collection

The data were collected by the researchers who were conducting the face-to-face interviews between August 2016 and February 2017 after informing the participants in the Endocrine and Outpatient Clinics of Inonu University Turgut Ozal Medical Centre. The second interviews were carried out with all of the patients in the same sample for application of the retest 2 weeks later. It took 10–15 min to collect the data.

Data assessment

Cronbach's alpha reliability coefficient, Pearson Product-Moment Correlation, Factor analysis, Bartlett's test, Kaiser-Meyer Olkin Test, Correlation, and Number-percentage were used in the assessment of the data obtained as a result of the study. Level of significance was accepted as 0.05.

Results

Table 1 shows the socio-demographic, medical, and physiological characteristics of the patients who were included in the study.

It was determined that 50.6% of the patients were male, HbA1c levels of 45.1% were between 7 and 9, the mean age was 48.57 (14.72) years, the mean BMI was 24.63 (3.41), the mean duration of diabetes was 9.40 (4.69) years, and the mean duration of using insulin was 6.62 (3.24) years.

Validity

In the study, while the KMO coefficient was determined as 0.708, the X^2 value was determined as 1360 as a result of Bartlett's Test of Sphericity analysis. The test results were found to be significant on a level of $p < 0.001$. The result showed that the sample size was adequate and appropriate for the factor analysis.

As a result of the Exploratory Factor Analysis (EFA) conducted for the validity of BITQ, factor loading values were found between 0.77 and 0.86 in the fear of injections and self-testing subscale, between 0.73 and 0.81 in the expectations regarding positive insulin-related outcomes subscale, between 0.54 and 0.82 in the expected hardship from insulin treatment subscale, between 0.57 and 0.81 in the stigmatization by

Table 1 Socio-demographic, medical, and physiological characteristics of the patients who participated in the study ($n = 348$)

Characteristics	*N*	%
Sex		
Female	172	49.4
Male	176	50.6
A1C (/%)		
< 7	39	11.2
7–9	157	45.1
> 9	152	43.7
Age (years)	48.57 (14.72)	
BmI (kg/m^2)	24.63 (3.41)	
Duration diabetes (years)	9.40 (4.69)	
Duration using insulin (years)	6.62 (3.24)	

insulin injections subscale, and between 0.65 and 0.75 in the fear of hypoglycemia subscale. Additionally, it was determined that it accounted for 71.3% of the total variance, 72.4% of the variance of the fear of injections and self-testing subscale, 61.6% of the variance of the expectations regarding positive insulin-related outcomes subscale, 65.0% of the variance of the expected hardship from insulin treatment subscale, 58.5% of the variance of the stigmatization by insulin injections subscale, and 59.4% of the variance of the fear of hypoglycemia subscale (Table 2). Thus, the 14-item BITQ with five subscales was obtained.

The fit index values of CFA of BITQ were found as $X^2 = 243.50$, df = 67 ($p < 0.05$), $X^2/\text{df} = 3.63$, RMSEA = 0.087, GFI = 0.86, CFI = 0.86, and IFI = 0.86. In the evaluation, no good fit was determined in terms of X^2/df and RMSEA values. At this phase of the analysis, modification recommendations were examined and the error covariance between items 11 and 12 was determined to be high. A second CFA model was applied by correlating the error covariance related to the items in question. After the modification that was conducted, the CFA fit index values were found as; $X^2 = 185.786$ df = 65 ($p < 0.05$), $X^2/\text{sd} = 2.99$, RMSEA = 0.075, GFI = 0.92, CFI = 0.90, and IFI = 0.91. It was found that the model showed an acceptable fit. Figure 1 shows the CFA Path Diagram of BITQ after the second CFA model.

The Cronbach's alpha reliability coefficient of the Turkish version of BITQ was between 0.70 and 0.82. The Cronbach's alpha reliability coefficients of the subscales of the questionnaire (fear of injections and self-testing, expectations regarding positive insulin-related outcomes, expected hardship from insulin treatment, stigmatization by insulin injections, and fear of hypoglycemia) were determined as 0.82, 0.76, 0.72, 0.76, and 0.70, respectively (Table 2).

The item total correlation scores of the Turkish Version of BITQ were between 0.30 and 0.52 points. The total score correlation coefficients of all the items in the questionnaire were higher than 0.30. In this respect, the item total correlation values of the Turkish version of BITQ were on a proper

Table 2 Item-total score correlation coefficients, factor loadings, alpha coefficients, and explained variance of barriers to ınsulin treatment questionnaire

Scale items	Fear of injections and self-testing	Expectations regarding positive insulin-related outcomes	Expected hardship from insulin treatment	Stigmatization by insulin injections	Fear of hypoglycemia	Mean (SD)	Corrected item-total correlations	Cronbach's Alpha if item deleted
1	0.86					1.8 (2.1)	0.40	0.68
2	0.83					2.0 (2.3)	0.41	0.68
3	0.77					2.1 (2.3)	0.37	0.69
4		0.76				7.7 (2.9)	0.34	0.70
5		0.81				8.1 (2.7)	0.33	0.72
6		0.73				7.7 (2.9)	0.34	0.70
7			0.54			2.5 (2.9)	0.42	0.68
8			0.80			3.9 (2.5)	0.36	0.68
9			0.82			2.8 (2.6)	0.52	0.66
10				0.81		1.9 (2.3)	0.30	0.72
11				0.62		2.3 (2.5)	0.31	0.70
12				0.67		1.4 (1.5)	0.37	0.70
13					0.75	9.1 (1.7)	0.34	0.70
14					0.65	6.3 (3.6)	0.40	0.68
% Variance explained	72.4	61.6	65.0	58.5	59.4			Total = 71.3
Cronbach alpha	0.82	0.76	0.72	0.76	0.70			Total = 0 .70

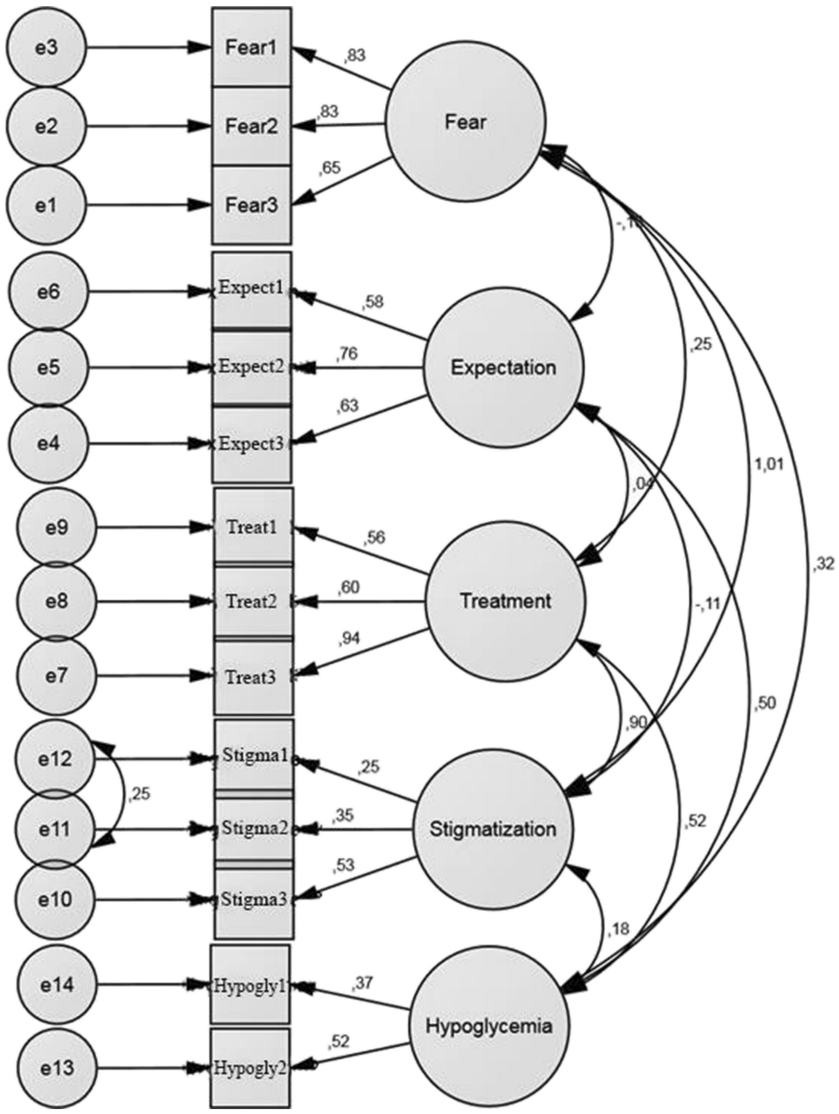

Fig. 1 CFA path diagram of BITQ

reliability level. In the analysis, it was found that the factor loadings of the Turkish version of BITQ were distributed between 0.54 and 0.86.

The correlation values between the average scores at the first and subsequent use (2 weeks later) of BITQ ranged from 0.998 to 1.00. The correlation between the BITQ total and subdimension average scores ($r = 0.83$–0.99) was positive and highly significant ($p < 0.001$).

Discussion

This study adapted the Barriers to Insulin Treatment Questionnaire developed by Petrak et al. [11] in 2007 into Turkish. As a result of the evaluation of the psychometric properties on a sample including type 2 diabetic Turkish patients who were using insulin, BITQ was determined to be a valid and reliable tool for all of the type 2 diabetic patients using insulin.

Validity

EFA and CFA were used to test the construct validity of BITQ adapted for Turkish patients who were using insulin. It was determined that the Turkish version was also gathered under five factors as in the original version of the questionnaire and explained 71.3% of the total variance. It is known that the factor structure of the scale is higher with higher variance rates, and variance rates ranging between 40 and 60% are accepted as sufficient in analyses that are conducted in social sciences [20]. It is stated in the literature that items whose factor load is lower than 0.30 should be excluded from evaluation [19, 21–24]. In the conducted analysis, it was observed that the factor loads of the Turkish version of BITQ ranged between 0.54 and 0.86 (Table 2). Therefore, none of the items in this questionnaire was excluded from the evaluation. The factor structure obtained with the factor analysis of the Turkish version of BITQ was determined to have construct validity.

CFA supported the 5-factor scale structure obtained as a result of EFA. Goodness of fit index was considered to evaluate whether or not the model established with CFA was appropriate for the data. In the evaluation, no good fit was determined in terms of the X^2/df and RMSEA values. In the literature, it is stated that while X^2/df values of ≤ 3 represent perfect fit, values between ≤ 3 and ≤ 5 represent good fit. RMSEA values that are equal to 0.08 or lower are acceptable values [16]. For the analysis conducted in this respect, X^2/df and RMSEA were observed to point out poor fit. The modification suggestions were examined, and a second CFA model was applied by correlating the error covariance. CFA fit index values after the conducted modification were found as; $X^2 = 185.786$, df = 65 ($p < 0.05$), X^2/sd = 2.99, RMSEA = 0.075, GFI = 0.92, CFI = 0.90, and IFI = 0.91. The model was determined to show an acceptable fit.

Reliability

The reliability of BITQ was evaluated with Cronbach's alpha internal consistency coefficient, item-total correlation, and test-retest analysis. The reliability coefficient, which can be considered sufficient in a measurement tool, should be as close to 1 as possible. In the literature, a reliability coefficient of lower than 0.40 is evaluated as that the measurement tool is not reliable, a coefficient between 0.40 and 0.59 is evaluated as that it is low in reliability, a coefficient between 0.60 and 0.79 is evaluated as that it is very reliable, and a coefficient between 0.80 and 1.00 is evaluated as that it is highly reliable [18, 25, 26]. The higher the Cronbach's alpha coefficient of the scale is, the more the items on the scale are consistent with each other, and the same property is composed of the items that check the elements or all the items function together to that extent [22]. In the original reliability and validity study of the questionnaire, the Cronbach's alpha coefficient was determined as 0.78–0.83 [11]. In this study, this coefficient's value was examined as an indicator of internal consistency and homogeneity of BITQ and determined to be between 0.70 and 0.82 (Table 2). It was observed that the internal consistency of BITQ was high and it had high reliability. This result indicated that the questionnaire is a reliable scale with internal consistency.

Item analysis refers to the relationship between the value of each item in the measurement tool and the total value taken from the overall measurement tool. If the items in the measurement tool have equal weight and are in the form of independent units, the relationship between the value of each item and the total value is expected to be high. It is decided that the scale items whose coefficients are low are not sufficiently reliable. When the item correlation coefficient falls below 0.30, it is reported that the reliability is insufficient [21]. The item total correlation scores of the Turkish Version of BITQ were between 0.30 and 0.52 (Table 2). Total score correlation coefficients of all of the items in the questionnaire were higher than 0.30. In this respect, the item total correlation coefficients of the Turkish Version of BITQ were on an appropriate reliability level.

If a measurement instrument is administered to individuals at different times and the individuals' responses at different times are consistent, this indicates the time invariance of the instrument [20]. When BITQ was applied to 348 type 2 diabetic patients who were using insulin at a 2-week interval for the test-retest analysis of the questionnaire, a positive and highly significant relationship was found for the entire questionnaire and for all subdimensions. This finding shows that the internal consistency of the questionnaire was high, and reliable results could be obtained in multiple applications.

Limitations of the study

The study has a limitation since it was conducted only on patients with type 2 diabetes. For this, it is recommended to conduct the study also on patients with type 1 diabetes. Finally, the psychometric suitability of the scale should be tested with larger populations.

Conclusion and recommendations

The results were consistent with the analysis results of the original version of the questionnaire as in the original version of the questionnaire, the 5-factor structure was confirmed. The Cronbach's alpha internal consistency coefficient, item total correlation, and test-retest analysis of the questionnaire had high correlation. These results showed that BITQ, on which we conducted a validity and reliability study, is a valid and reliable tool to assess the level of problems encountered in relation to insulin usage.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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

Ethical approval The population and sample of the study. The study was approved by the Scientific Research and Ethics Board of Inonu University Health Sciences Institute. Verbal information was provided to all participants in accordance with the principles of the Declaration of Helsinki. Additionally, the participants were informed about the research and notified that their personal data would be kept confidential.

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ORIGINAL ARTICLE

Clinical and biochemical characteristics and the association of angiotensin type 1 receptor with normoalbuminuric chronic kidney disease among South Indian type 2 diabetes population

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Abstract
The aim of the study was to determine the clinical and biochemical characteristics especially cystatin C and the association of angiotensin type I receptor (AT1R) gene polymorphism with normoalbuminuric chronic kidney disease (NACKD) among South Indian type 2 diabetes (T2DM) population.

Material and methods The study comprised of 308 (M:F 190:118) subjects with T2DM categorized into three groups. Group I: T2DM patients without albuminuria (NA) and estimated glomerular filtration rate (eGFR) > 90 ml/min/1.73m^2 ($n = 110$); group II: T2DM patients with albuminuric chronic kidney disease (CKD) with eGFR < 60 ml/min/1.73m^2 ($n = 98$); and group III: T2DM patients with normoalbuminuric CKD (NACKD) and eGFR < 60 ml/min/1.73m^2 ($n = 100$). The eGFR was estimated using the Modification of Diet in Renal Disease (MDRD) formula. Cystatin C was measured by particle-enhanced immunoturbidimetric assay. AT1R gene polymorphism was analyzed using the PCR-RFLP method.

Results The biochemical parameters urea, creatinine, post prandial glucose, HbA1c, triglycerides, cystatin C levels, erythrocyte sedimentation rate (ESR), and diastolic blood pressure were lower and the levels of calcium were higher in normoalbuminuric CKD subjects as compared to albuminuric CKD subjects. The AT1R genotypic distribution of AA, AC, and CC was 80.2%, 19.8%, and 0% in NACKD, and 95.1%, 4.9%, and 0% in CKD subjects. The distribution of allelic frequencies of A and C allele in NACKD was 90.1% and 9.9%, and in CKD, it was 97.6% and 2.4% respectively. The relative risk of AC ($p = 0.08$) genotype and C ($p = 0.01$) allele in NACKD was 4 times higher as compared to CKD.

Conclusion The present study highlighted that the clinical and biochemical parameters showed significant differences especially cystatin C whose levels increased in normoalbuminuric CKD subjects as compared to normoalbuminuric subjects. Significant association of AGTR1 A1166C polymorphism was observed in normoalbuminuric CKD subjects as compared to CKD subjects with T2DM.

Keywords Chronic kidney disease · Normoalbuminuria · Type 2 diabetes mellitus · AGTR1 · South India

Introduction

One of the major complications of type 2 diabetes mellitus (T2DM) is diabetic renal disease (DKD) that predisposes individuals to excess morbidity and mortality resulting in renal failure and cardiovascular disease [24, 31]. A recent study from India reported that patients with chronic kidney disease (CKD) spend three times more than those without any other diabetic complications [33]. The diagnosis of DKD is an important issue in people with long-term T2DM. Persistent albumin excretion has been considered to be the hallmark for DKD and is believed to be the earliest marker of glomerular disease [16]. However, it has been now established that a non-excretion of albumin is a type of kidney disease quite prevalent in T2DM [16]. Approximately, one-third to half of the T2DM patients have CKD without proteinuria may be due to atubular glomeruli, renal microvascular atherosclerotic disease, analgesics, etc. [29, 34].

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Studies have reported a prevalence of 17% and 21.8% of normoalbuminuria among diabetes subjects with CKD [1, 3, 14, 20]. Diagnosing renal impairment without the evidence of albuminuria is a critical issue. At this stage, the glomerular filtration rate (GFR) is believed to be preserved. However, some individuals with T2DM present reduced GFR level with little or no albuminuria [12]. Several studies in adults have shown that cystatin C is a more sensitive marker of changes in the GFR than serum creatinine [9, 4, 26]. Studies have demonstrated that serum cystatin C is an early renal marker in patients with diabetes [22, 27, 30].

In the last decade, the beneficial effect of blockade of the renin-angiotensin system has been demonstrated in a wide variety of cardiovascular disease, from heart failure to stable coronary artery disease and diabetic as well as non-diabetic chronic nephropathies [7, 28]. The angiotensin II type 1 receptor (AT1R) gene is located on chromosome 3q 21-25 and has a length of > 55 kb, composed of five exons and four introns. It is expressed mainly in the heart and kidney tissues and its products are predominantly found in the vascular smooth muscle cells, heart, adrenal gland, and kidney. AT1 plays an integral role in blood pressure control and is implicated in the pathogenesis of hypertension. A1166C polymorphism in AT1R gene has shown to be associated with an increased risk of urinary albumin excretion [11] and a faster progression of DKD in patients with T2DM [35]. Many studies have highlighted the role of AGTR1 (A1166C) gene polymorphism and the risk of DKD of which two were from India and the results were inconsistent across different populations [5, 6, 11, 23, 25, 35]. There is limited data available on this association among the South Indian population. The aim of the present study was to determine the clinical and biochemical characteristics especially cystatin C and the association of angiotensin type1 receptor (AT1R) gene polymorphism with normoalbuminuric CKD among south Indian subjects with T2DM.

Materials and methods

We conducted a cross-sectional study of subjects with T2DM who were attending a tertiary care center for diabetes in South India. A total of 1177 patients (M:F 542:635) with diabetes were screened from Feb 2015 to May 2016. At the end of the study period, a total of 308 (M:F 190:118) subjects were included in the study. A pilot study was first carried out using 30 subjects per group. Based on these preliminary results, with a confidence interval of 95%, a prevalence of 72%, a 5% type 1 error, an estimated p value < 0.05, and a power of 80%, the present sample size was derived. In this study, the inclusion criteria were T2DM subjects with proved kidney complication and categorized into three groups based on the urinary albumin excretion rate (UAER) and their eGFR levels. A total of 869 patients were excluded due to the history of intake of angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) in the last 6 months, eGFR > 60–89, T1DM, age > 65 years, DM duration < 5 years, and diabetic foot ulcer patients. Also, those who refused to participate or withdrew their consent were also excluded from the present study. The selected 308 subjects were categorized into 3 groups. Group I: T2DM patients without albuminuria (NA) UAER < 20 μg/mg creatinine and eGFR > 90 ml/min/1.73m^2 (n = 110); group II: T2DM patients with albuminuria (UAER > 20 μg/mg creatinine) and chronic kidney disease (CKD) eGFR < 60 ml/min/1.73m^2 (n = 98); and group III: T2DM patients with normoalbuminuria and CKD (NACKD) and eGFR < 60 ml/min/1.73m^2 (n = 100). CKD was based on eGFR < 60 ml/min/1.73m^2 for 3 months or more, irrespective of the cause. The eGFR was estimated with serum creatinine using the Modification of Diet in Renal Disease (MDRD) formula [17]. The written informed consent was obtained from all study participants in accordance with the principles of the declaration of Helsinki.

Anthropometric and biochemical investigations

Age, sex, height, and weight measurements were obtained, and body mass index (BMI) was calculated using the formula weight in kilograms divided by the square of height in meters. Blood pressure was recorded in the sitting position in the right arm to the nearest 2 mmHg with a mercury sphygmomanometer. The JNC VII criteria for hypertension were considered for classification of subjects having hypertension. Venous blood sample (whole blood, plasma, serum) was collected for biochemical investigations and genetic studies. Biochemical investigations were done on BS 400 auto-analyzer using DiaSys kits. Fasting plasma glucose (glucose oxidase-peroxidase [GOD-POD] method), serum cholesterol (cholesterol oxidase-phenol4-amino antipyrene peroxidase [CHOD-PAP] method), serum triglycerides (glycerol phosphatase oxidase−phenol4-amino antipyrene peroxidase [GPO-PAP] method), high-density lipoprotein cholesterol (direct method–polyethylene glycol-pre-treated enzymes), and low-density lipoprotein cholesterol (direct homogenous method) were measured using auto-analyzer. Glycosylated hemoglobin (HbA1c) was measured by high-performance liquid chromatography method using the Turbo Variant machine (Bio-Rad, Hercules, CA). The CV for in-house quality control was less than 2.5%. Serum creatinine was measured using the Jaffe method. Calcium was measured by arsenazo dye method. Urinary albumin excretion (UAE) was measured by immunoturbidimetric method. Serum cystatin C was measured by particle-enhanced immunoturbidimetric assay using DiaSys Cystatin C FS kit.

Isolation of genomic DNA from peripheral blood and PCR amplification

Genomic DNA was isolated from peripheral blood (anticoagulated EDTA-blood stored at − 20 °C) using standard phenol–chloroform extraction method with minor modification [19]. The isolated DNA was subjected to PCR amplification using T100 Thermal Cycler (Bio-Rad, USA). Genomic DNA (~ 50 ng) was incubated in a total reaction volume of 20 μL containing equal concentration of the forward primer 5′-ATA ATG TAA GCT CAT CCA CC-3′ and reverse primer 5′-GAG ATT GCA TTT CTG TCA GT-3′ (Bioserve Biotechnologies, India) ~ 10 picomoles and 2× solution of Prime Taq Premix (Genet Bio, Chungnam, Korea). DNA was initially denatured at 94 °C for 5 min prior to amplification. PCR amplification was accomplished using 35 cycles consisting of 1 min denaturation at 94 °C, 30 s annealing at 58 °C, and 45 Sec extensions at 72 °C. The final extension was carried out for 5 min at 72 °C. After PCR, 10 μL of the reaction mixture was digested with 4 U *DdeI* restriction enzyme (C*TNAG) (New England Biolabs) and Tris-HCl buffer (100 mM KCl, 1 mM DTT, 1 mM EDTA, pH 7.4 at 25 °C) in a 20 μL reaction volume for overnight at 37 °C. The digested products were subjected to electrophoresis on a 2.0% agarose gel and visualized under UV light using gel documentation system (BioRad).

Statistical analysis

All statistical analyses were performed using SPSS 20.0 version software (SPSS Inc., IL). Mean and standard deviation for continuous variables and proportions for categorical variables are reported. One-way ANOVA was used to test continuous variables and a chi-square test was used to compare categorical variables. Correlation analysis of cystatin C with clinical, anthropometric, and biochemical parameters was done in the study groups. A p value of < 0.05 was considered to be statistically significant. The relationship between genotypes and alleles was determined by obtaining the odds ratio (ORs) (OR, 95% confidence interval (CI)) using the Primer 6.0 software.

Results

Anthropometric, clinical, and biochemical characteristics of the study groups

The anthropometric, clinical, and biochemical characteristics of the study groups are shown in Table 1. As expected, significant differences were noted in age ($p < 0.001$), systolic blood pressure ($p = 0.01$), diastolic blood pressure ($p = 0.008$), duration of DM ($p < 0.001$), urea levels ($p < 0.001$), creatinine levels ($p < 0.001$), eGFR ($p < 0.001$), UAER ($p < 0.001$), uric acid levels ($p < 0.001$), hemoglobin levels($p < 0.001$), and erythrocyte sedimentation rate (ESR) ($p < 0.001$) in the CKD group as compared to NA group. In NACKD group, age ($p < 0.001$), duration of DM ($p = 0.03$), urea levels ($p < 0.001$), eGFR ($p < 0.001$), calcium levels ($p = 0.01$), uric acid levels ($p = 0.02$), hemoglobin levels ($p = 0.001$), and ESR ($p < 0.001$) showed significant differences as compared to NA subjects.

The subjects having history of hypertension was significantly higher in CKD ($p = 0.008$) and NACKD ($p = 0.03$) as compared to NA group. The duration of hypertension was significantly longer in NACKD ($p < 0.001$) and CKD ($p < 0.001$) groups as compared to NA group. The presence of dyslipidemia was not statistically significant among the groups but a higher percentage of subjects had dyslipidemia in NACKD (51.9%) group than NA (36.4%) and CKD (34.3%) groups. The presence of retinopathy was higher in CKD group ($p = 0.004$) than NA and NACKD groups ($p = 0.7$). The presence of cardiovascular disease was found to be non-significant among the groups.

The NACKD subjects were older ($p = 0.02$), and urea ($p < 0.001$), creatinine ($p = 0.001$), eGFR($p < 0.001$), UAER ($p = 0.002$), diastolic blood pressure ($p = 0.009$), post prandial glucose levels ($p = 0.04$), HbA1c % ($p = 0.05$), calcium levels ($p < 0.001$), triglyceride levels ($p = 0.04$), hemoglobin levels ($p = 0.001$), ESR ($p < 0.001$), and cystatin C levels ($p < 0.001$) showed significant differences when compared among NACKD and CKD subjects.

Determination of AT1R genotyping

The AA, AC, and CC genotypes were identified by PCR-RFLP and were visualized using gel documentation system as shown in Figs. 1 and 2. Table 2, 3 and 4 show the distribution of genotypic and allelic frequencies of AT1R SNP. In NA groups, 76.5% and 23.5% of the subjects had AA and AC genotype, respectively, while none of the subjects had CC genotype. On the other hand, subjects with CKD had 95.1% of AA genotype followed by 4.9% of AC genotype and 0.0% with CC genotype. The NACKD group had 80.2% of AA genotype and 19.8% of AC genotype and none of them had CC genotype. The distribution of allelic frequency of A and C allele in NA was 88.2% and 11.8%, respectively, 97.6% and 2.4% in CKD, and 90.1% and 9.9% in NACKD.

Levels of cystatin C in study groups

The cystatin C levels in NA, CKD, and NACKD were 1.06 ± 0.30, 2.70 ± 1.31, and 1.68 ± 0.58 mg/L respectively as shown in Fig. 3. The levels of cystatin C were significantly increased in CKD ($p < 0.001$) and NACKD ($p < 0.001$) compared to

Table 1 Biochemical and anthropometric parameters of study groups

Parameters	NA ($n = 110$)	CKD ($n = 98$)	NACKD ($n = 100$)
Male	72 (66%)	69 (70%)	49 (49%)
Female	38 (34%)	29 (30%)	51 (51%)
Age (years)	52.5 (13.2)	60.5 (11)***	63 (8)***#
Body mass index (kg/m^2)	26.1 (4.5)	26.0 (4.4)	26.4 (5.7)
Systolic BP (mm Hg)	120 (12)	130 (30)**	130 (30)**
Diastolic BP (mm Hg)	80 (10)	80 (20)	80 (10)
Duration of DM (years)	10 (7)	13 (10)***	10 (7)
Fasting plasma glucose (mg/dL)	154 (91)	152 (90)	125 (67)
Post prandial glucose (mg/dL)	214 (123)	247 (148)	190 (105)
Glycated hemoglobin (HbA1c) (%)	8.4 (2.7)	8.8 (2.4)	7.7 (2.2)#
Urea (mg/dL)	20 (7)	37 (38)***	41 (14)***###
Creatinine (mg/dL)	0.8 (0.2)	1.7 (0.8)***	1.3 (0.3)#
Albumin/creatinine ratio (mg/g)	7.5 (9.5)	52.5 (48.3)*	11 (10)#
eGFR (ml/min/1.73 m2)	95 (12)	42 (24)***	55 (9)***###
UAER μg/min	7 (4.2)	28 (4)***	7 (9)
Triglycerides (mg/dL)	123 (78)	119 (97)	113 (76)#
Total cholesterol (mg/dL)	165 (60)	143 (57)	145 (70)
HDL-cholesterol (mg/dL)	40 (13)	36 (12)	39 (12)
LDL-cholesterol (mg/dL)	94 (39)	80 (37)	78 (42)
VLDL-cholesterol (mg/dL)	30 (14)	28 (21)	25 (16)
Non-HDL cholesterol (mg/dL)	122 (53)	110 (56)	103 (60)
Calcium (mg/dL)	8.8 (7.6)	8.6 (0.9)	9.3 (0.6)**###
Uric acid (mg/dL)	4.6 (1.6)	6.2 (3.3)***	5.3 (3.9)**
Hemoglobin (g/dL)	13.3 (2.4)	10.8 (2.9)***	12.3 (2.3)***##
ESR (mm in 1st hour)	20 (31)	71 (65)***	38 (39)***###
History of hypertension (%)	26.6	54.1**	44*
Duration of hypertension (years)	1 (1)	1 (2)	2 (2)***
Retinopathy (%)	22.9	48.1**	31.9
History of dyslipidemia (%)	36.4	34.3	51.9
History of neuropathy (%)	45. 7	75.0***	68.8
History of CVD (%)	1.3	7.9	4

*($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) versus NA group. #($p < 0.05$), ##($p < 0.01$), ###($p < 0.001$) versus CKD group. *UAER*, urinary albumin excretion rate

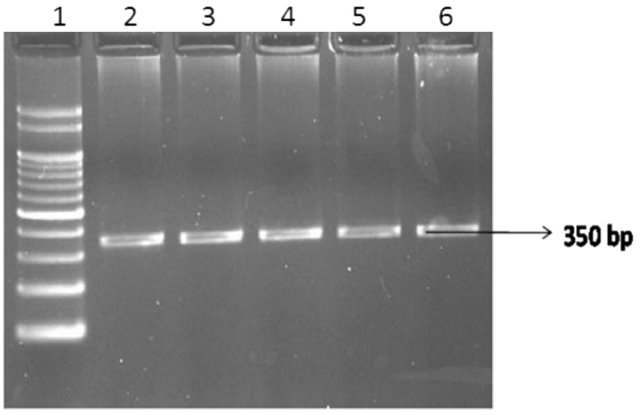

Fig. 1 PCR amplification of AT1R gene. 2% agarose gel electrophoresis of the amplified DNA of AT1R region. Lane 1, 100 bp DNA ladder, lanes 2–6 amplified 350 bp of AT1R region

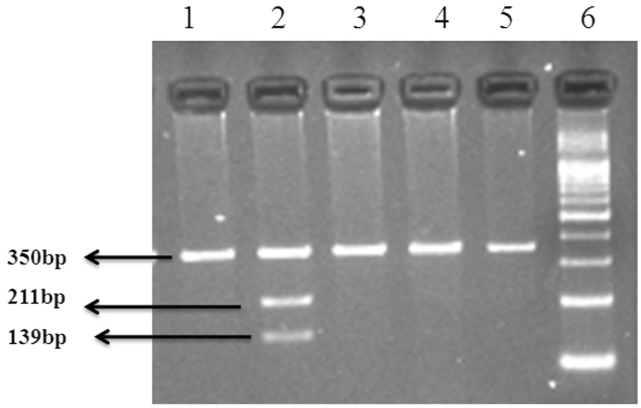

Fig. 2 A1166C polymorphism of the AT1R gene in 2% agarose gel. Lanes 1, 3, 4, and 5 show the presence of AA genotype (350 bp), lane 2 shows the presence of AC genotype (350, 211, and 139 bp), and lane 6 shows the 100 bp DNA ladder

Table 2 Odds ratio—relative risk between NA vs CKD. This table represents the comparison of genomic and allelic frequencies of NA and CKD

Genotype		NA (94) *n* (%)	CKD (82) *n* (%)	RR (95% CI)	*p* value
AA		72 (76.5)	78 (95.1)	5.9 (1.9–18.1)	0.001
AC		22 (23.5)	4 (4.9)	0.16 (0.05–0.5)	0.001
CC		0	0	–	–
Allele	A	166 (88.2)	160 (97.6)	5.3 (1.8–15.7)	0.02
	C	22 (11.8)	4 (2.4)	0.189 (0.06–0.6)	0.02

NA. The levels were significantly decreased in NACKD as compared to CKD ($p < 0.001$).

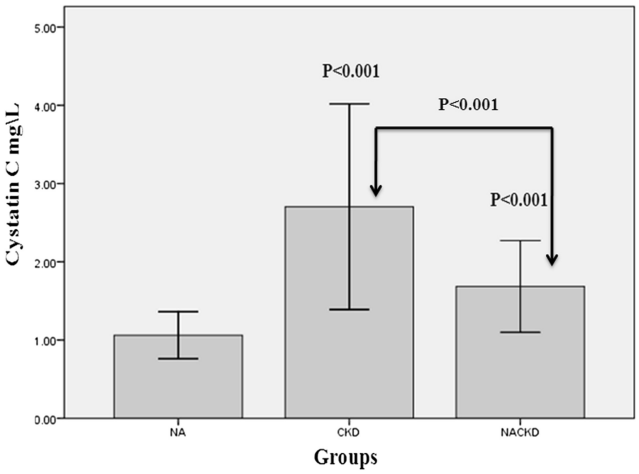

Fig. 3 The levels of cystatin C in study groups

Correlation of cystatin C with anthropometric and biochemical parameters

The levels of cystatin C were correlated with anthropometric and biochemical parameters. In NA subjects, cystatin C had a significant positive correlation with urea ($r = -0.227$, $p = 0.004$) and a significant negative correlation with fasting plasma glucose ($r = -0.269$, $p = 0.01$) and HbA1c ($r = -0.253$, $p = 0.02$) while other parameters did not show any correlation. In CKD subjects, there was a negative correlation between eGFR and cystatin C level ($r = -0.420$, $p < 0.001$). In NACKD subjects, none of the parameters correlated with cystatin C levels (Table 5).

Discussion

Normoalbuminuric renal impairment has much significance in the management of patients with T2DM. Only a limited number of studies have been reported on the clinical characteristics of T2DM subjects with reduced eGFR and normoalbuminuria. The main focus of the study was to determine the clinical and biochemical parameters and AT1R polymorphism association with normoalbuminuric CKD subjects in South Indian population.

Normoalbuminuric renal impairment has a different pathophysiology, in addition to the classical pathology. The presence of both kinds of pathology was associated with worse metabolic control, suggesting that hyperglycemia may cause different patterns of renal injury in T2DM subjects [10]. In addition to this, other possible causal factors associated with normoalbuminuric CKD are hypertension nephrosclerosis, renovascular disease, tubulointestinal fibrosis, cholesterol microemboli, and classical diabetic nephropathy effectively treated with ACE/ARBs [10]. In the present study, apart from their relatively low eGFR levels, normoalbuminuric CKD patients displayed a number of specific characteristics, they were older in age than NA and CKD groups, a higher prevalence of hypertension than NA group, a high prevalence of dyslipidemia than NA and CKD groups, a higher duration of hypertension than CKD and NA group, a greater duration of diabetes than NA group, a slightly higher levels of calcium than NA and CKD groups, a higher ESR than NA group, a higher level of uric acid than NA group, and a lower hemoglobin level than NA group.

In this regard, the lack of association of high blood pressure with NACKD could be related to extremely tight blood pressure control. The occurrence of dyslipidemia in NACKD is 51.9% which was higher than NA (36.4%) and CKD (34.3%)

Table 3 Odds ratio—relative risk between NA vs NACKD. This table represents the comparison of genomic and allelic frequencies of NA and NACKD

Genotype		NA (94) *n* (%)	NACKD (81) *n* (%)	RR (95% CI)	*p* value
AA		72 (76.5)	65 (80.2)	1.241 (0.6–2.6)	0.68
AC		22 (23.5)	16 (19.8)	0.80 (0.4–1.7)	0.68
CC		0	0	–	–
Allele	A	166 (88.2)	146 (90.1)	1.209 (0.6–2.4)	0.708
	C	22 (11.8)	16 (9.9)	0.827 (0.4–1.6)	0.708

Table 4 Odds ratio—relative risk between NACKD vs CKD. This table represents the comparison of genomic and allelic frequencies of NA and CKD

Genotype		NACKD (81) *n* (%)	CKD (82) *n* (%)	RR (95% CI)	*p* value
AA		65 (80.2)	78 (95.1)	0.2 (0.06–0.65)	0.008
AC		16 (19.8)	4 (4.9)	4.8 (1.53–15.0)	0.008
CC		0	0	–	
Allele	A	146 (90.1)	160 (97.6)	0.2 (0.07–0.7)	0.01
	C	16 (9.9)	4 (2.4)	4.3 (1.43–13.4)	0.01

Table 5 Correlation analysis of cystatin C with clinical parameters in the study groups

Clinical parameters	NA *r*	*n* = 80 *p*	CKD *r*	*n* = 66 *p*	NACKD *r*	*n* = 80 *p*
Age (years)	0.407	< 0.001	− 0.073	0.56	0.124	0.27
BMI (kg/ m^2)	− 0.020	0.864	− 0.045	0.719	0.131	0.245
Systolic BP (mmHg)	− 0.081	0.476	0.019	0.879	− 0.005	0.962
Diastolic BP (mmHg)	0.083	0.465	0.171	0.170	− 0.111	0.328
Duration of DM (years)	0.032	0.778	− 0.181	0.145	0.063	0.580
Urea (mg/dL)	0.227	0.04	0.352	0.004	0.19	0.07
Creatinine (mg/dL)	− 0.016	0.88	0.195	0.11	0.185	0.10
eGFR (MDRD)	− 0.041	0.717	− 0.420	< 0.001	− 0.181	0.108
FPG (mg/dL)	− 0.269	0.016	0.003	0.978	− 0.031	0.785
PPG (mg/dL)	− 0.115	0.367	− 0.031	0.828	− 0.037	0.752
HbA1c (%)	− 0.253	0.024	− 0.128	0.321	− 0.105	0.353
Tot cholesterol (mg/dL)	− 0.171	0.139	0.279	0.027	0.018	0.877
Triglycerides (mg/dL)	− 0.219	0.05	0.06	0.620	− 0.106	0.350
HDL cho (mg/dL)	− 0.132	0.255	0.085	0.505	0.041	0.719
Non-HDL cho (mg/dL)	− 0.130	0.265	0.264	0.037	0.075	0.506
LDL cho (mg/dL)	− 0.156	0.178	0.233	0.066	0.081	0.475
VLDL cho (mg/dL)	− 0.141	0.223	0.197	0.122	0.056	0.622
Calcium (mg/dL)	− 0.168	0.312	− 0.172	0.373	− 0.89	0.783
Uric acid (mg/dL)	0.225	0.163	0.095	0.588	0.379	0.120

*($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$)

groups. The cholesterol microemboli result in inflammation and in turn which elevates erythrocyte sedimentation rate (ESR) as reported in a study by Modi and Rao [21]; in the present study, ESR rate was significantly increased in NACKD group than NA.

Many studies have observed that HbA1c is an independent correlate of albuminuria, but not of impaired renal function, thus indicating that non-albuminuric renal disease is probably less related to hyperglycemia and microvascular disease than the classic proteinuric form of kidney disease [1, 29]. In the present study, NACKD subjects had lower HbAlc percentage and post prandial glucose levels than the subjects with albuminuric CKD. Serum creatinine was significantly lower in NACKD compared to CKD subjects. Similar results were obtained in a study done by et al. [3].

Lim et al. highlighted that low serum calcium level (< 9.0 mg/dL) is an independent prognostic marker for rapid renal function progression in CKD subjects in Taiwan [18]. In this study, we observed mean serum calcium levels approximately 9 mg/dL in normoalbuminuric CKD subjects and no significant difference in CKD group as compared to NA group, but the mean values of calcium in the study groups are lower than the upper limits (10.3 mg/dL) and seem to be within the normal range (8.6–10.3 mg/dL). So far, no studies have reported the levels of calcium in normoalbuminuric CKD subjects.

In the present study, the levels of serum cystatin C were significantly higher in NACKD subjects (1.68 ± 0.58 mg/L) when compared to normoalbuminuric subjects (1.06 ± 0.30 mg/L). In a recent report by Dayanidhi et al. [8], similar results were reported in microalbuminuria T2DM subjects (1.74 ± 0.66) and normoalbuminuric subjects (1.19 ± 0.62) ($p < 0.05$). The levels of serum cystatin C were significantly higher in CKD subjects (2.70 ± 1.31 mg/L) in our study. Similarly, levels of serum cystatin C (2.04 ± 1.19 mg/L) were observed in macroalbuminuric subjects in a Korean population in a study by Jeon et al. [15].

It has been also suggested that serum cystatin C is a more sensitive marker for detecting early changes in glomerular filtration in T2DM subjects than creatinine-based measurements [36]. In CKD subjects, the eGFR had a significant negative correlation with cystatin C. The correlation of cystatin C with eGFR confirms that the cystatin C could be one of the earlier markers representing CKD in T2DM subjects. In concordant to the above results, the urea levels also have a significant positive correlation with cystatin C level ($r = 0.22$, $p = 0.04$). It is known that serum cystatin C levels increase with advancing age and the serum cystatin C level has a significant positive correlation with age ($r = 0.40$, $p < 0.001$). There was no correlation found with serum cystatin C and biochemical parameters in NACKD subjects.

To our knowledge, the present study is first of its kind, to determine the polymorphism, in normoalbuminuric CKD subjects of AT1R in South Indian population. The relative risk of AC genotype ($p = 0.08$) and C allele ($p = 0.01$) in NACKD was 4 times higher compared to CKD which was significant.

There was a significant association of AGTR1 A1166C polymorphisms observed in normoalbuminuric CKD compared to CKD subjects. Ahluwalia et al. showed that the frequency of C allele of AGTR1 A1166C was higher in North Indian subjects with T2DM and DKD [2].

In this study, we found that the AA genotype was most frequent when compared to AC and CC genotypes in CKD (95.1%) and NACKD (80.2%) groups compared to NA (76.5%) group, while CC genotype was not found in any of the groups. Similar results were noted in the Chinese population without the frequency of CC genotype in normoalbuminuria, microalbuminuria, and macroalbuminuria subjects [37]. Jacobsen et al. reported that "A" allele of the AGTR1 A1166C polymorphisms is a risk allele in nephropathy subjects which was also confirmed in the survival analysis of time to doubling of serum creatinine or development of ESRD [13]. Similarly, AA genotype and "A" allele as a risk allele was observed in the present study as well in CKD subjects compared with normoalbuminuric subjects.

The conflicting results in the studies were ascribed to different ethnic population, difference in sample size, and a more complex model consisting of still poorly understood combinations of several AGTR1 gene variants that may affect disease susceptibility [25, 32]. Some of the limitations of this study include, owing to the cross-sectional design, it was difficult to clarify the causal relationship between the risk factors and the natural history of normoalbuminuric chronic kidney disease. Moreover, the subjects with normoalbuminuria and eGFR < 60 mL/min/1.73 m^2 might need more evaluation with other markers along with cystatin C to diagnose DKD. Second, the level of cystatin C was not measured in urine along with the albumin. The significant difference among the patient groups in terms of age can be seen as another factor which limits our study.

Conclusion

The present study highlighted that clinical and biochemical parameters showed significant differences especially cystatin C whose levels increased in normoalbuminuric CKD subjects as compared to normoalbuminuric subjects. Therefore, other markers, such as serum and urine NGAL, urine KIM-1, IgG, and transferrin, may add to the early diagnosis of diabetic kidney disease along with serum cystatin C. AGTRI, A1166C polymorphism showed significant association in normoalbuminuric CKD subjects. Relying on albumin excretion as first sign for renal involvement may be too late in diagnosing and modifying the progression of the disease and better markers for monitoring of renal function that can detect early renal damage are much needed. Early use of RAS blocking agents before the detection of urinary albumin excretion or renal impairment may be beneficial in preserving the renal function in diabetics.

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Authors' contribution Dr. Vijay Viswanathan conceived and designed the experiment, Ms. Anju Soni and Ms. Bliss screened and collected the samples, Ms. Ezhilarasi performed the experiment and drafted the manuscript, and Dr. Vijay Viswanathan and Dr. Satyavani contributed to the discussion and reviewed the manuscript.

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Compliance with ethical standards

The study protocol was approved by the institutional ethics committee (Ref ECR/51/Inst/TN/2013/MVDRC/30) and all methods were performed in accordance with the relevant guidelines and regulations of the institution. The written informed consent was obtained from all study participants in accordance with the principles of the declaration of Helsinki.

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

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ORIGINAL ARTICLE

Prevalence of thyroid disorders is not different in type 2 diabetes mellitus compared to nondiabetics in South India

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Abstract
Studies on thyroid disorders in type 2 diabetes mellitus (DM) are fewer than those in type 1 diabetes, but data from different parts of the world have shown conflicting results. To look at the prevalence of thyroid disorders in type 2 DM and nondiabetic controls separately in a community cohort and a hospital cohort from the same locality in South India. Thyroid function tests and thyroid autoantibodies were done in 986 people in the community (258 diabetics and 634 nondiabetics). In the hospital cohort of 194 people (147 diabetics and 47 nondiabetics), an ultrasonography of the neck was done in addition. Thyroid dysfunction was more common in the community compared to the hospital cohort 15.9 vs 9.6% but there was no difference in the proportion of people with thyroid dysfunction in diabetics and nondiabetics in both the community and the hospital cohorts. (15.6 vs 16.2% in the community and 9.6 vs 6.3% in the hospital in diabetics and nondiabetics respectively). Clinical goiter and sonographically detected goiter were also similar in diabetics and nondiabetics in the community and the hospital cohorts respectively. Females had more prevalence of goiter irrespective of the presence of diabetes. Commonest thyroid dysfunction was hypothyroidism and subclinical hypothyroidism in both cohorts. This unique study conducted in a community and hospital cohort from the same locality showed that the prevalence of thyroid disorders is similar in type 2 diabetic and nondiabetic subjects in our population.

Keywords Type 2 diabetes mellitus · Thyroid disorders · Prevalence

Introduction

Thyroid dysfunction and diabetes mellitus (DM) are the two most common endocrine disorders worldwide. The prevalence of DM is increasing worldwide and is projected to rise to 366 million in 2030, affecting 4.4% of all age groups. In India, various studies have shown diabetes prevalence of 15–20% in urban areas and a lesser but increasing prevalence in rural areas [1–3].

Data on the global prevalence of thyroid problems have considerable variations because of the different definitions of the conditions, particularly of subclinical thyroid dysfunction, and because of the varying design of the studies and varying study population. In population studies, prevalence of overt hypothyroidism or its subclinical manifestations range between 2–4% and 4–20%, respectively, both being significantly higher in women above the age of 60 years [4–6].

Many studies from western world have shown that patients with DM who are already having higher risk of cardiovascular disease [7] are at an increased risk of thyroid disease compared to the general population [8]. However, most of the studies on diabetes and thyroid were done in type 1 DM. Up to 50% of type 1 diabetes patients were reported to be positive for thyroid antibodies [9] and approximately 50% of them progress to develop clinical autoimmune thyroid disease. But in case of type 2 DM and thyroid, genetic links are less well characterized. Studies on thyroid problems in type 2 DM are fewer than those in type 1 diabetes, but data from different parts of the world showed conflicting results. Unrecognized thyroid dysfunction may impair metabolic control and add to cardiovascular disease risk in diabetic patients, and hence, it is really important to assess this problem in diabetic subjects. Recent evidence has shown that diabetics with thyroid disease will need initiation of insulin therapy earlier and those with higher TSH will progress from prediabetes to diabetes earlier

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Table 1 Baseline characteristics

	CDM (*N* 258)	CNDM (*N* 634)	*p* value	HDM (*N* 147)	HNDM (*N* 47)	*p* value
Age in years mean ± sd	52.8 ± 13.4	41.2 ± 14.7	< 0.001	54.78 ± 8.6	49.6 ± 8.2	0.0004
Male:female	1:2	1:1		4:1	1:1	
BMI kg/m^2 mean ± sd	24.2 ± 3.9	22.6 ± 4.3	0.047	24.95 ± 3.1	27.78 ± 4.15	< 0.001
FBS mg/dl mean ± sd	150 ± 64	87.4 ± 11.4	< 0.001	165.16 ± 61.26	99.3 ± 21.83	< 0.001
PPBS mg/dl mean ± sd	181 ± 81.6	97.2 ± 25.4	< 0.001	274.6 ± 85.2	128.5 ± 50.1	< 0.001
FT4 ng/dl mean ± sd	1.28 ± 0.22	1.27 ± 0.26	0.54	1.14 ± 0.18	1.05 ± 0.09	0.0012
TSH mIU/L median	1.7	1.9	NS	1.6	1.55	NS
Anti TPO IU/L median	18.2	16.6	NS	0.49	0.99	NS

CDM community diabetes mellitus, *CNDM* community nondiabetics, *HDM* hospital diabetes mellitus, *HNDM* hospital nondiabetics

[10, 11]. In India, only few old studies are available looking at the prevalence of thyroid problems in type 2 DM [12–16].

This study aims to study the prevalence of thyroid dysfunction, thyroid autoimmunity, and thyroid nodules in a cohort of type 2 DM patients and compare it with nondiabetic controls both at the community level and hospital level.

Methods

The study was conducted in two different populations one at the community level and the other at the hospital level in South India. Based on a previous study [17], studying the prevalence of thyroid disorders in diabetics vs nondiabetic controls the minimum sample size with 95% confidence and 20% allowable error was 191 in each group.

The community study is part of Amrita Diabetes and Endocrine Population Survey (ADEPS). Assuming a prevalence of 3–4% for common endocrine disorders, sample size came to 3000 for the whole study. Assuming an average of three adults per household, 350 houses from four areas were selected totaling 1050 households and approximately 3000 adults. In the second stage, wards were considered the primary sampling unit and these households were selected as per the sampling scheme suggested by WHO with modifications on the number of clusters to get the required sample size, i.e., 50 clusters of seven houses each. All subjects with type 2 DM were selected and were compared with nondiabetic subjects in this cohort. The hospital-based cohort of patients were selected from the executive health check-up clinic of a tertiary referral university teaching hospital in the same locality. T2 DM at community level was defined by WHO criteria using capillary glucose measurements. Subjects with fasting capillary glucose > 110 mg/dl and/or 2-h capillary glucose of > 200 mg/dl were classified as having DM. For the hospital-based cohort, a fasting plasma venous glucose more than 126 and/or RBS > 200 mg% and/or HbA1c more than 6.5 was taken as DM. Subjects were also considered to be diabetic if they reported history of diabetes diagnosed by a physician or if they were on anti-hyperglycemic agents irrespective of their blood glucose values. Subjects without any form of glucose intolerance were considered normoglycemic.

Both diabetics and nondiabetic controls had the following tests done: serum thyroid-stimulating hormone (TSH), free thyroxine (FT4), and antithyroid peroxidase antibody (Anti TPO antibody). The bloods were measured by electrochemiluminescence immunoassay (ECLIA) using Elecsys 2010 Roche for the community study and Abbott architect I 2000 sr for the hospital cohort. Hb A1C was measured by high-performance liquid chromatography (HPLC) using Biorad-D10 for the hospital cohort. Goiter was assessed clinically in the community and by ultrasound neck in the hospital cohort.

Subjects who were previously diagnosed to have hypothyroidism and were taking levothyroxine and those with serum FT4 less than lower limit and TSH more than upper limit were categorized as hypothyroid. Those with normal serum FT4 and TSH more than upper limit of normal were classified as having subclinical hypothyroidism. Subjects who had been diagnosed to have hyperthyroidism and were on treatment for this condition and those with serum FT4 above upper limit of normal and TSH less than lower limit of normal were considered as having primary hyperthyroidism. Subjects with normal serum FT4 and TSH less than lower limit of normal were considered as having subclinical hyperthyroidism. Those without any history of thyroid disease but having a thyroid

Table 2 Prevalence of thyroid dysfunction, goiter, and autoimmunity

	CDM	CNDM	HDM	HNDM
Hypothyroidism %	4.4	4	6.8	4.2
Subclinical hypothyroidism %	8.8	9.1	2.1	2.1
Hyperthyroidism %	1.2	1.3	0	0
Subclinical hyperthyroidism %	1.2	1.8	0.7	0
Total %	15.6	16.2	9.6	6.3
Goiter %	12	12	51.7	53.2
Anti TPO positivity %	20.2	23.2	17.4	24.4

Table 3 Ultrasonographically detected goiter in hospital cohort males vs females

USS diagnosis	HDM males 110	HNDM males 24	HDM females 37	HNDM females 23
NORMAL %	54.6	69.5	23.1	30.4
MNG %	34.2	22.2	51.9	42.7
SNT %	5.6	0	16.7	13.6
Thyroiditis %	5.6	8.3	8.3	13.3
Volume in cc	7.19 ± 5.15	3.52 ± 4.25	5.92 ± 3.19	5.00 ± 3.26

function test which is not classifiable into any of the above four categories were classified as abnormal thyroid function.

Statistical methods

Analysis was done using SPSS 20 version software. To compare the means, the two-tailed t test was done, and to compare proportions, chi-square test was done. $p < 0.05$ was taken as statistically significant. For finding correlation, Pearson product moment correlation coefficient was used.

Results

Baseline characters are shown in the Table 1.

From the community cohort of 986, 258 diabetic subjects and 634 nondiabetic normal subjects who had no impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) were included, and from the hospital cohort, 194 people (47 controls and 147 diabetics) were included.

The diabetics were compared with nondiabetics regarding prevalence of goiter (clinical in community and ultrasonographically in the hospital), thyroid dysfunction, and presence of auto antibody in each cohort separately. Diabetics were older than nondiabetics in both cohorts, whereas males predominated in the hospital cohort, females were more in the community cohort. In the hospital cohort, nondiabetics had significantly higher BMI compared to diabetic population. Majority of the population studied belonged to middle socioeconomic class, TSH was not different between diabetics and nondiabetics in both cohorts, whereas the free T4 levels were slightly more in diabetics compared to nondiabetics in the hospital cohort (1.14 vs 1.05 p value 0.0012). Overall, thyroid dysfunction was more common in community compared to the hospital cohort (15.9 vs 9.6%) because of the higher prevalence of subclinical thyroid disease in the community.

There was no statistically significant difference in the prevalence of clinical goiter in the community and ultrasonographically detected goiter in the hospital between diabetic and nondiabetic population as shown in Table 2. Also, there was no statistically significant difference in the prevalence of any form of thyroid dysfunction and autoantibody positivity between diabetic and nondiabetic population in both the community and hospital cohorts. Past history of thyroid dysfunction also was not statistically different in diabetics and nondiabetics in both study cohorts (6.2 vs 3.9% in community and 8.8 vs 2.1% in hospital cohort).

Since in general, goiter is more common in females compared to males; the difference in the prevalence of goiter in males and females was looked into which showed that that 70–75% of females had sonographically detected goiter in both diabetic and nondiabetic groups in the hospital cohort. The details are shown in Table 3.

There was no correlation between the Hba1c and thyroid dysfunction or antibody positivity in both community and hospital cohorts (Table 4). Thirty-seven out of 258 subjects in the community cohort and 113 out of 147 in the hospital cohort were on metformin. There was no relation between thyroid hormone levels and metformin intake in both community and hospital cohorts.

Discussion

In this unique case control study on the prevalence of thyroid disorders in type 2 DM and nondiabetic population conducted in a community and hospital cohort in the same geographical area, it was shown that there is no difference in the prevalence of goiter or thyroid dysfunction or autoimmunity in type 2 DM vs nondiabetics. The overall prevalence of thyroid

Table 4 Correlation of TFT and autoimmunity with glycemic control

r values	Hba1c CDM	FBS CDM	PPBS CDM	Hba1c HDM	FBS HDM	PPBS HDM
FT4	0.2368**	0.172*	− 0.144	0.001	0.011	0.089
TSH	− 0.013	0.058	− 0.039	− 0.05	− 0.032	− 0.042
Anti TPO	0.037	0.095	− 0.046	0.150	0.157	0.048

*Correlation significant at p value < 0.05

**Correlation significant at p value < 0.01

Table 5 Studies on thyroid disorders in type 2 diabetes mellitus

Study	*N* DM	*N* controls	Tests	Prevalence DM	Prevalence controls	Comments
Pimenta 2005 [17]	256	75	US, T3 T4 TSH, ANTI TPO TRAB	51.6%	38.7%	More in females. Family h/o thyroid disorder
Vondra, Schrner 2008 [21]	46	30	AITD	48%	Not significant	More in females. Family h/o thyroid disorder
Perros 1995 [22]	1310 (1 and 2)		T4 TSH	6.9% in males in type 2	More than in controls	Subcli hypo > hypo> hyper> subclihyper
Yasmin 2006 [23]	61	51	T4 TSH Anti TPO	42.3% positive anti TPO	12%	TSH low in type 2
Ishay 2009 [24]	410	125	TFT Anti TPO	5.4%	7.9%	Anti TPO more in subcli hypo pts
Smithson 1998 [25]	223		TFT	5.5% in community practice and 10.8% in general practice		5 hypo 4 subcli hypo 1 each hyper and subcli hyper
Radiaidah 2004 [26]	908	304	TFT antibodies	5.9% known 6.6% new overall 12.5%	6.6%	Subcli hypo commonest
Junik R 2006 [27]	98	50	USS	Volume more higher incidence of nodular goiter and parenchymatous goiter		
Gopinath 2008 [28]			TFT	7.1%	3.8%	Elderly
Badman Celany 2002 [29]	290		TFT	31.4%		More in females
Proces 2001 [30]	72	53				TSH lower in diabetics
Grazetto Nunez 2008 [31]	48	14				T3 lower in female DM and those with vascular disease
Gholampour Dehaki 2017 [32]	435	989	Follow-up tft for 11 years	14/1000PATIENT YEARS	21/1000 patient years	Abnormal thyroid function with higher degree of autoimmunity
Osei Sarfo-Kantanka [33]	302	310	Thyroid autoantibodies	12.2%	3.9%	Thyroid autoimmunity associated with poor glycemic control
Robin Maskey [34]	271		Thyroid function tests	4.5%		Dyslipidemia more in abnormal TFT
Vibha Uppal [14]	120		Thyroid function tests	24.5%		T3 low in DM
Ozair M [15]	250		Thyroid function tests	28%		Thyroid disorders more in females, dyslipidemia, retinopathy, and poor glycemic control
Jali MV [16]	713		Thyroid function tests	16.2%		More in females and those with poor glycemic control

dysfunction in the community was higher compared to that in the hospital cohort because of the higher prevalence of subclinical hypothyroidism in the community cohort.

The issue of whether all diabetic people require thyroid screening is still unsettled. The impact of thyroid alterations on glucose metabolism has been known for a long time. Thyrotoxic patients usually lose their glucose control, whereas hypothyroid patients encounter a reduction in insulin requirement and increased occurrence of hypoglycemia. Recent research has shown more extensive interaction between thyroid hormone and insulin action at molecular level [18]. Indices of insulin resistance as judged by the homeostatic model assessment (HOMA) are closely linked to thyroid hormone status even in euthyroid, eumetabolic subjects, where HOMA is related to the increase in thyroid hormone concentrations even within the normal range [19, 20].

Various studies which were done in the past looking at the prevalence of thyroid dysfunction in diabetics are summarized in Table 5 [14–17, 21–34].

Unlike in case of diabetes which is almost equally prevalent in both males and females, gender has significant influence on thyroid disorders, female gender having significantly higher prevalence of thyroid problems. When gender-specific prevalence of thyroid problems in total population was compared, it showed that goiter, thyroid autoimmunity, and increased TSH were more common in females than in males irrespective of the presence of type 2 DM.

Another important factor is the effect of poor diabetic control on thyroid hormone levels. In diabetic subjects, continuous variables of thyroid parameters were evaluated for linear association with parameters of glycemic control such as HBa1c and fasting and postprandial blood glucose values. Only free T4 levels had significant but mild positive correlations with Hba1c and FBS values but not with PPBS values.

Newer studies have shown that diabetic drugs which can reduce insulin resistance such as metformin can affect the thyroid function [35]. But this study did not show any significant difference in the thyroid function among the diabetic subjects on metformin.

This study is unique in nature given the fact that this has studied both community and hospital cohorts of type 2 DM and nondiabetics in the same geographical area which none of the previous studies have done. The hospital study is also unique in that it is one among the few studies which have done thyroid functions, thyroid antibodies, and ultrasound in all the participants.

There are few flaws in this study; males were more common in the hospital diabetic group compared to females which could have masked the difference in thyroid abnormality between diabetics and nondiabetics in the hospital cohort. But the community had more females compared to males which again showed no difference in prevalence of thyroid abnormalities between diabetics and nondaibetics. TSH increases with aging and hence incidence of subclinical hypothyroidism also increases with aging. TSH increases with increasing BMI also. Whether the difference in age and BMI between diabetics and nondiabetics negated the difference in thyroid dysfunction in diabetics vs nondiabetics needs to be looked into in future studies.

The assay used for the two population was different and whether this contributed to the higher prevalence of thyroid dysfunction in the community cohort is debatable. This issue needs larger studies to reach a conclusion. Another limitation is the lack of follow-up of these subjects to reassess the thyroid status especially in case of subclinical hypothyroidism.

Even though this study does not show difference in prevalence thyroid disorders in diabetics and nondiabetics, diabetic patients being more prone for macro vascular disease and dyslipidemia, screening for thyroid disease among diabetic subjects may be of more value than screening of general population, but this needs validation from future larger studies.

Conclusion

This study showed that the prevalence of thyroid disorders is similar in type 2 DM and nondiabetic subjects in a South Indian population. Commonest thyroid problem was subclinical hypothyroidism and overt hypothyroidism in both groups.

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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.

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

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Postprandial lipemia and its relation to TCF7L2 gene polymorphisms in normoglycemic first-degree relatives of type 2 diabetes patients

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Abstract
We compared postprandial triglyceride (PPTg) responses to fat challenge between risk allele and wild-type variant of two common TCF7L2 gene polymorphisms to ascertain if the risk of T2DM associated with this gene is related to its effects on PPTg metabolism. Postprandial triglyceride levels were evaluated in 71 NGT subjects with at least one first-degree relative with T2DM. Restriction fragment length polymorphism was performed for genotyping of SNP rs7903146 C/T and SNP rs12255372 G/T of TCF7L2 gene, and the PPTg levels were compared in the risk allele and wild-type variant of both these SNPs. Postprandial triglyceride responses were similar in wild and risk allele groups for both the SNPs. There was no significant difference in Tg-AUC (2027.04 ± 1313.54 vs 1853.58 ± 712.00, $p = 0.472$) and Peak Tg levels (343.95 ± 225.08 mg/dl vs 320.60 ± 137.49 mg/dl, $p = 0.591$) between CC and CT + TT of SNP rs7903146. Also, no significant difference was observed between Tg-AUC (1936.41 ± 1120.77 vs 1891.38 ± 812.69, $p = 0.845$) and peak Tg levels (324.89 ± 186.99 mg/dl vs 330.42 ± 158.64 mg/dl, $p = 0.894$) between GG and GT + TT of SNP rs12255372. The present study did not find any significant difference in PPTg responses between the risk allele and wild type of rs7903146(C/T) and rs12255372 (G/T) SNPs of TCF7L2 gene.

Keywords Postprandial triglyceride · TCF7L2 · Normal glucose tolerance

Abbreviations

PPTg	Postprandial triglyceride
DM	Diabetes mellitus
TCF7L2	Transcription factor 7-like 2
NGT	Normal glucose tolerance
SNP	Single nucleotide polymorphism

Introduction

Postprandial hypertriglyceridemia has been shown to be an independent risk factor for development of type 2 diabetes mellitus (T2DM) [1]. Also, it has been reported that prediabetic [2] and normoglycemic [3] first-degree relatives of type 2 diabetes patients tend to have higher postprandial triglyceride levels suggesting that this could be an inherited genetic defect. Transcription factor 7-like 2 (TCF7L2) gene has been found to be most consistently and strongly related to risk of type 2 diabetes. It has been reported that this gene contributes to 20% population attributable risk of type 2 diabetes [4]. Studies from India have shown that higher frequencies of T-alleles (TT + CT of rs7903146 C/T SNP and TT + GT of rs12255372 G/T SNP) of TCF7L2 gene are associated with high risk of type 2 diabetes [5–8]. People with these polymorphisms have impaired incretin-mediated insulin release [9], which may explain the higher risk of T2DM but the exact mechanism of action is still unclear. While studies in this context have been done mostly in reference to beta cell proliferation, incretins, and gluconeogenesis, the effects of TCF7L2 gene polymorphisms on lipid metabolism have not been explored much [10, 11]. Recently, it has been shown that people with rs7903146 polymorphism of TCF7L2 gene tend to have higher postprandial lipid levels when compared to people without the polymorphism, especially if they were consuming a high PUFA diet [12].

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Whether the risk of diabetes associated with TCF7L2 gene is related to its effects on postprandial triglyceride metabolism is not known. The current study was therefore undertaken to compare postprandial triglyceride responses to standard oral fat challenge test between risk allele and wild-type variant of rs7903146 and rs12255372 SNPs of the TCF7L2 gene in normoglycemic first-degree relatives of type 2 diabetes patients.

Subjects

Study was approved by institutional ethics committee-human research, University College of Medical Sciences, and written consent was taken from all the study participants. Apparently, healthy male normoglycemic individuals in the age group of 20 to 50 years with at least one first-degree relative with established type 2 diabetes were included in this study. Sample size was calculated based on a similar previous study [12], and after considering a difference of 75 mg/dl in postprandial triglyceride levels between risk allele and wild-type variant and with 80% power and 5% type 1 error, the sample size came out to be 27 in each group. Based upon the prevalence of gene polymorphism in North India [7], a sample size of 30 was required in each group but we have taken a total sample size of 70.

Materials and methods

Oral glucose tolerance test [13] was done in all subjects to identify normal glucose tolerance (NGT) subjects, and fasting lipid profiles were done to exclude those with baseline fasting serum triglycerides > 250 mg/dl. Individuals with hyperlipidemia, inherited disorders of lipoprotein metabolism, acute and chronic liver or kidney disease, known pancreatic disease, obstructive biliary disease, chronic diarrhea, malabsorption, hypothyroidism, Cushing's syndrome, and coronary heart disease were excluded from the study. Also, all individuals on drugs like beta blockers, diuretics, corticosteroids, and lipid-lowering agents as well as those consuming alcohol and smokers and those with a dietary PUFA intake of less than 10% were excluded from the study. Subjects with impaired glucose tolerance and overt diabetes were not recruited as this would have undermined the influence of genetic factors on postprandial lipemia.

A standardized oral fat challenge test [14] was carried out after an overnight fasting of minimum 12 h in all the subjects which was done 1 week after confirmation of their NGT status. The meal contained 729 kcal/m^2 BSA of calories, 65.2 g of fat, 24.75 g of carbohydrates, 5.3 g of protein, and 240 mg of cholesterol. It consisted of whipped cream, banana, and sugar and was consumed over a period of 10 to 15 min. Blood samples of 2 ml each were drawn at 0, 2, 4, 6, and 8 h for the measurement of post fat challenge lipid levels. After consumption of the test meal, subjects were not allowed to eat for entire study duration of 8 h. Whole blood was also collected and used to extract DNA by phenol-chloroform method. Restriction fragment length polymorphism was performed for genotyping of SNP rs7903146 C/T and SNP rs12255372 G/T of TCF7L2 [5] gene with the primers forward: 5′AAGAGAAGATTCCTTTTTAAATGGTG3′, reverse: 5′CCTCATACGGCAATTAAATTATACA-3′ and forward: 5′CTGGAAACTAAGGCGTGAGG3′, reverse: 5′GGGTCGATGTTGTTGAGCTT-3′ respectively. Polymerase chain reaction (PCR) amplification condition for both SNPs were as follows: denaturation 3 min at 94 °C followed by 40 cycles of denaturation for 30 s, annealing at 55.6 °C for 30 s, extension at 72 °C, and final extension at 72 °C for 9 min. HpyCH4III restriction enzyme was used for SNP rs7903146 C/T and Tsp509I for SNP rs12255372 G/T. 3.5% agarose gel was used for separation of fragments. Based upon the polymorphism status, two groups were derived with either polymorphism, i.e., risk allele group (homozygous and heterozygous) and wild genotype group (homozygous), and postprandial lipid responses were compared in the two groups by using Students unpaired *t* test by SPSS software version 20.

Results

The mean BMI and mean waist circumference of study subjects were 23.82 ± 3.84 Kg/m^2 and 86.97 ± 9.13 cm respectively. The mean fasting plasma glucose was 77.87 ± 11.76 mg/dl and 2 h postprandial plasma glucose was 109.90 ± 13.77 mg/dl. The mean fasting triglyceride levels were 136.49 ± 67.45 mg/dl. All the subjects were genotyped for two polymorphisms of TCF7L2 gene namely rs7903146 (C/T) and rs12255372 (G/T). For rs7903146 (C/T), incidence of wild genotype (CC), heterozygous mutant (CT), and homozygous mutant (TT) were 31%, 59%, and 10% respectively. For rs12255372 (G/T), incidence of wild genotype (GG), heterozygous mutant (GT), and homozygous mutant (TT) were 40%, 51%, and 10% respectively. The study population was divided into two groups, namely, wild genotype and risk genotype. On the basis of rs7903146 (C/T) SNP, the genotypes were CC for wild and CT + TT for risk variant. Similarly, based on rs12255372 SNP, the two genotypes were GG and GT + TT respectively. Comparisons between the different genotypes were done for baseline data and post fat challenge lipids (Tables 1, 2, 3, and 4).

Discussion

Our study found no significant difference in postprandial triglyceride responses between wild genotype and risk allele genotype among first-degree normoglycemic relatives of type

Table 1 Baseline clinical, anthropometric, and biochemical parameters of the study group for polymorphism—rs7903146 SNP

	CC genotype (mean ± SD) ($N = 23$)	TT + CT genotype (mean ± SD) ($N = 48$)	p value
Age (years)	29.26 ± 5.92	27.65 ± 4.71	0.218
Height (m)	1.68 ± 0.05	1.68 ± 0.08	0.914
Weight (Kg)	66.96 ± 9.46	69.38 ± 11.97	0.399
BMI (Kg/m^2)	23.30 ± 3.64	24.06 ± 3.95	0.440
Waist (cm)	84.83 ± 7.85	88.00 ± 9.59	0.172
FPG (mg/dl)	82.00 ± 10.71	75.90 ± 11.83	0.040
PPPG (mg/dl)	110.30 ± 15.62	109.71 ± 12.96	0.866
Fasting triglycerides (mg/dl)	134.04 ± 78.68	137.67 ± 62.23	0.834
Fasting total cholesterol (mg/dl)	169.00 ± 48.15	180.75 ± 47.35	0.834
Fasting HDL cholesterol (mg/dl)	40.70 ± 9.06	39.71 ± 6.74	0.608
Fasting LDL cholesterol (mg/dl)	101.50 ± 44.45	113.51 ± 44.62	0.291
Fasting VLDL cholesterol (mg/dl)	26.81 ± 9.46	27.53 ± 12.45	0.834

Table 2 Postprandial levels of triglycerides after standard oral fat challenge test for polymorphism—rs7903146 SNP

Parameter	CC genotype (mean ± SD) ($N = 23$)	TT + CT genotype (mean ± SD) ($N = 48$)	p value
Triglycerides AUC (mg dl^{-1} 8 h^{-1}) (area under curve)	2027.04 ± 1313.54	1853.58 ± 712.00	0.472
VLDL cholesterol –AUC (mg dl^{-1} 8 h^{-1})	405.41 ± 262.71	370.72 ± 142.40	0.472
Triglycerides 8 h (mg/dl)	179.22 ± 46.53	184.90 ± 46.80	0.633
Peak triglyceride value (mg/dl)	343.95 ± 225.08	320.60 ± 137.49	0.591

2 diabetes for both the SNPs vizrs7903146 and rs12255372. This is the first study which has evaluated the role of TCF7L2 gene polymorphism on postprandial triglyceride responses in the Indian context. Our findings on postprandial triglyceride levels are in contrast with an earlier study conducted by Warodomwichit et al. [12]. In this study, data was analyzed from European American subjects who participated in the "Genetics of Lipid Lowering Drug and Diet Network Study." The authors found that T-allele carriers (CT + TT) of rs7903146 SNP (C/T) who had ≥ 7.36% of energy intake from PUFA had higher levels of fasting plasma VLDL and postprandial triglyceride rich lipoproteins (TRLs). However, they did not find any abnormality in postprandial triglyceride metabolism with rs12255372 (G/T) SNP. The difference in findings between the two studies may be due to several factors. Firstly, it could be related to ethnic differences between

Table 3 Baseline clinical, anthropometric, and biochemical parameters of the study group for polymorphism—rs12255372

SLNO	Parameter	GG genotype (mean ± SD) ($N = 71$)	TT + GT genotype (mean ± SD) ($N = 9$)
1	Age (year)	28.79 ± 5.23	27.74 ± 5.10
2	Height (m)	1.67 ± 0.05	1.69 ± 0.07
3	Weight (Kg)	69.82 ± 12.22	67.73 ± 10.52
4	BMI (Kg/m^2)	24.69 ± 4.79	23.21 ± 2.93
5	Waist (cm)	87.93 ± 10.34	86.31 ± 8.26
6	FPG (mg/dl)	79.93 ± 10.10	76.45 ± 12.69
7	PPPG (mg/dl)	110.10 ± 14.59	109.76 ± 13.34
8	Fasting triglycerides (mg/dl)	134.10 ± 70.90	138.14 ± 65.77
9	Fasting total cholesterol (mg/dl)	177.83 ± 47.58	176.33 ± 48.16
10	Fasting HDL cholesterol (mg/dl)	40.69 ± 9.08	39.57 ± 6.28
11	Fasting LDL cholesterol (mg/dl)	110.32 ± 44.40	109.13 ± 45.27
12	Fasting VLDL cholesterol (mg/dl)	26.82 ± 14.18	27.63 ± 13.15

Table 4 Postprandial levels of triglycerides after standard oral fat challenge test for polymorphism—rs12255372 SNP

Parameter	GG genotype (mean ± SD) ($N = 62$)	TT + GT genotype (mean ± SD) ($N = 9$)	p value
Triglycerides AUC (mg dl^{-1} 8 h^{-1})	1936.41 ± 1120.77	1891.38 ± 812.69	0.845
VLDL cholesterol –AUC (mg dl^{-1} 8 h^{-1})	387.28 ± 224.05	378.28 ± 162.54	0.845
Triglycerides 8 h (mg/dl)	187.00 ± 44.95	180.33 ± 47.82	0.556
Peak triglyceride value (mg/dl)	324.897 ± 186.99	330.429 ± 158.64	0.894

Asian Indians and Europeans in the way TCF7L2 polymorphisms affect lipid metabolism. Secondly, ours was a very homogenous group of normoglycemic first-degree relatives of type 2 diabetic patients while the subjects in the study by Warodomwichit et al. [12] consisted of a heterogeneous group of individuals that included patients with overt diabetes also, who are known to display a much higher PPTg response to fat challenge. Thirdly, it could also be due to the significantly lower age of our study subjects (28.17 years vs. 49.4 years) that could have blunted PPTg responses and failed to demonstrate significant differences between the two groups. The reason for lower mean age in our study was because most of study subjects were children of patients with type 2 diabetes. Fourthly, the higher waist circumference reported in their study (101 cm vs 86.97 cm) could have led to greater postprandial response to fat challenge due to associated insulin resistance, and lastly, the smaller number of subjects in our study could also have contributed to the absence of significant PPTg differences in the two groups.

In our study, we did not find any difference in fasting triglycerides with respect to the two groups of both the polymorphisms. Our finding of normal triglyceride levels in persons with T-allele of rs7903146 (C/T) SNP was similar to an Italian study [15] which showed a favorable fasting lipid profile in the form of lower triglycerides and raised HDL cholesterol in an older group of patients with T-allele of rs7903146 (C/T) SNP. However, these workers did not asses the postprandial lipid responses.

The findings of the present study do not support the hypothesis that the effects of TCF7L2 gene for increasing the risk of type 2 diabetes are mediated by altered postprandial triglyceride metabolism as no difference in triglyceride responses to a fat challenge test could be demonstrated in subjects with and without T-allele genotype for both the SNPs studied. The results of our study show that the existing data regarding the role of rs7903146 SNP in postprandial lipid metabolism is still inconclusive and may involve more complex gene-environment interactions. Further studies are needed to elucidate the same.

In conclusion, our study did not find any significant difference in PPTg response to a standard fat challenge in normoglycemic first-degree relatives of type 2 diabetes patients between the risk allele and wild phenotype of rs7903146 SNP (C/T) and rs12255372 (G/T) of TCF7L2 gene.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics 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

Influence of non-surgical periodontal therapy on insulin resistance in chronic periodontitis subjects with prediabetes

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Abstract
Prediabetes (PRD) has been closely associated with chronic periodontitis (CP). Lately, studies have proposed the role of glutathione peroxidase (GPx), a ubiquitous antioxidant, in potentiating insulin resistance (IR), a significant factor in PRD. As overexpression of GPx is associated with CP as well as IR, the current study was undertaken to evaluate the influence of non-surgical periodontal therapy (NSPT) on GPx levels and hence the IR in CP subjects with PRD. Forty-seven subjects were divided into two major groups: healthy controls ($n = 11$) and CP ($n = 36$), according to probing depth (PD) and clinical attachment loss (CAL). The CP patients were subdivided into CP only ($n = 18$) and CP with PRD ($n = 18$) based on baseline FBS levels. Serum and crevicular GPx and malondialdehyde (MDA) were measured in all the subjects using spectrophotometry. The participants underwent NSPT at baseline and 3 months. At 6 months, all the parameters were re-recorded. At 6 months after NSPT, there was significant improvement in periodontal health ($p < 0.05$) in all the three groups which was associated with reduced GPx and MDA levels. Besides, a significant reduction in IR was noted in both CP and CP with PRD groups ($p < 0.05$). NSPT may be a vital tool in reducing IR in CP subjects with PRD. This was clearly evident as there was progressive decline in IR with GPx levels along with improvement in other parameters, specifically in the PRD group.

Keywords Chronic periodontitis · Glutathione peroxidase · Insulin resistance · Non-surgical periodontal therapy · Oxidative stress · Prediabetes

Introduction

Chronic periodontitis (CP) is a multifactorial disease of complex etiology which if left untreated results in tooth loss [1]. Lately, it is regarded as the sixth complication of type 2 diabetes mellitus (DM) [2]. The influence of diabetes on periodontal disease is mainly due to varied host inflammatory response, uncoupling of bone destruction and repair, and/or the effects of advanced glycation end products. Besides, systemic oxidative stress may play a vital role in linking the two conditions [3]. Even though there is immense data linking DM with periodontal disease, studies relating prediabetes (PRD), a precursor of established diabetes, with CP are scarce [4].

PRD has been defined by the American Diabetes Association (ADA) as "a condition in which the subjects have an impaired fasting glucose level of 100–125 mg/dl or an impaired glycated hemoglobin level of 5.7–6.4%" [5]. The global prevalence of PRD is rapidly increasing and by 2030, it is proposed that about 470 million people would be affected by it [6]. Studies have revealed a dose-dependent relationship between the parameters defining PRD and CP [7–9]. This includes an association between impaired fasting glucose, insulin resistance (IR), probing pocket depth (PD), and loss of attachment (CAL). It was suggested that chronic, low-grade inflammation as indicated by increased levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukins (IL)-1 and (IL)-6 played a significant role in both the conditions [10]. The former has already been reported as a connecting link between DM and periodontitis, as it increases IR [11]. Furthermore, some studies have reported oxidative stress as a forerunner of IR [12]. It was suggested that increased oxidative damage

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as revealed by elevated levels of lipid peroxides worsened both the conditions [13, 14].

Under normal physiological conditions, this reaction is counteracted by endogenous antioxidant enzymes like glutathione peroxidase (GPx). Its role in various metabolic processes is irrefutable. However, contrary to the conventional belief, it has been suggested that a small amount of reactive oxygen species (ROS) is needed for various cellular and insulin signaling pathways [15]. Overexpression of GPx results in a "reductive stress" characterized by a lack of oxidants and/or excess reducing equivalents (antioxidants) [16] (Fig. 1). This concept has been proposed lately, whereby lack of cellular oxidants can diminish cell growth responses. Besides, it leads to undesirable cellular and physiological effects like changes in protein disulfide bond formation, diminished mitochondrial function, and decreased cellular metabolism [16]. Although various physiological conditions that may create reductive stress have not been elucidated, conditions such as hypoxia, hyperglycemia, and other stresses that inhibit mitochondrial function are known to cause excess accumulation of cellular reducing equivalents [16]. Furthermore, studies have shown that excessive GPx in various chronic inflammatory disorders causes quenching of cellular oxidants like hydrogen peroxide (H_2O_2), an important signaling molecule for insulin sensitivity [17, 18]. Similarly, elevated GPx levels have been associated with progression of CP [19–21]. This was revealed in a study evaluating the gene expression of antioxidant enzymes in the gingival tissues of poorly and well-controlled type 2 DM with CP. The elevated levels of GPx were correlated with CP independent of glycemic status [18]. This could be attributed to excessive generation of lipid peroxides at the inflammatory sites which subsequently resulted in increased GPx levels [21].

As the excessive GPx levels play a significant role in the progression of both IR and periodontal disease, it may be hypothesized that its increased levels in CP may worsen IR [17, 19, 20]. Furthermore, non-surgical periodontal therapy (NSPT) in CP subjects with PRD may help in reducing the levels of GPx, which may prevent its progression to type 2 DM. With this background, the present study aimed to evaluate the role of NSPT in reducing IR through its influence on GPx in CP subjects with PRD.

Methods

Study population

Initially, about 150 patients who were referred to the Department of Periodontology, Manipal College of Dental Sciences, Manipal, between October 2015 and March 2016, were screened for inclusion in the study. The sample size was estimated at 80% power and 95% confidence interval. It resulted in a total of 47 subjects, with 18 each in chronic periodontitis group with and without PRD and 11 in healthy control group.

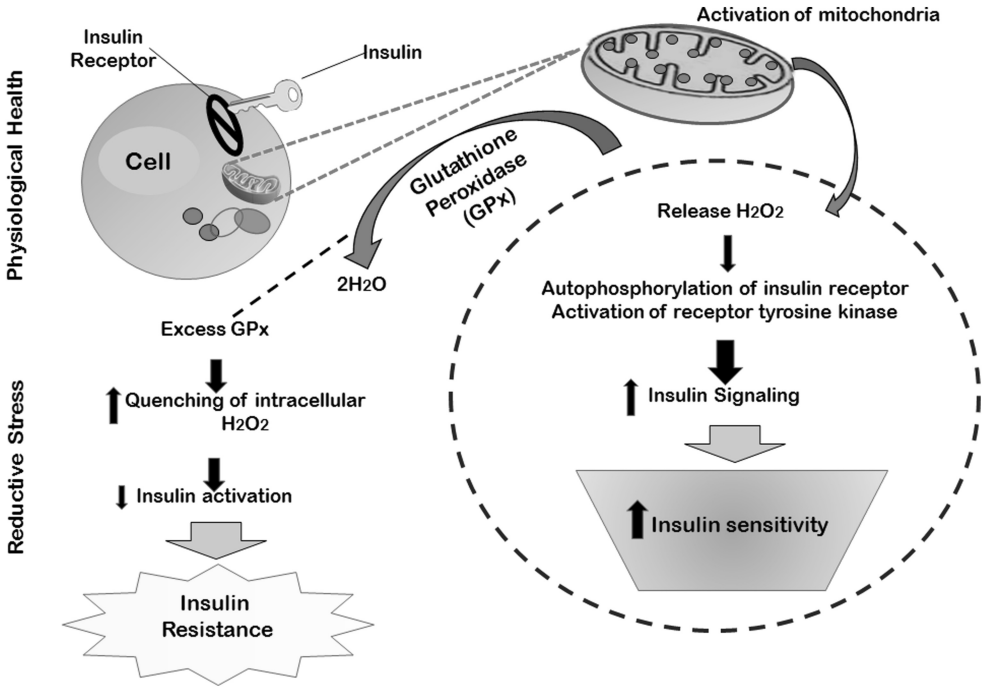

Fig. 1 Mechanism of reductive stress leading to insulin resistance

Inclusion criteria

Systemically, healthy subjects aged 18–45 years (both males and females), who fulfilled the inclusion criteria, were included in the study. Their medical records were validated to ensure that no systemic illnesses were present as it would influence the outcome of the study.

Exclusion criteria

The presence of any systemic diseases (e.g., DM, rheumatoid arthritis, obesity, and cardiovascular problems) or prescription of anti-inflammatory or antimicrobial drugs within past 3 months, regular users of mouthwash or vitamin supplements, special dietary requirements, or those who underwent periodontal therapy in the past 6 months, pregnant or lactating mothers, smokers and tobacco chewers were excluded from the study.

Measurement of periodontal parameters and subdivision into groups

A total of 47 subjects with minimum of 20 teeth underwent clinical periodontal examination for plaque index (PI), gingival index (GI), PD, and CAL [22, 23]. PD was measured as the distance in millimeters from the crest of marginal gingiva to base of pocket while CAL was measured as the distance from cemento-enamel junction to the base of the pocket. The CP group included subjects with moderate to severe periodontitis. All the subjects in this group were subjected to fasting blood glucose (FBS) examination at the initial visit to identify participants with PRD. PRD was recognized based on the criteria laid down by ADA viz. a fasting blood glucose (FBS) level of 100–125 mg/dl [5]. Subsequent division of subjects is shown in Fig. 2.

Sample collection for biochemical analysis

The biochemical parameters were analyzed in the gingival crevicular fluid (GCF) and serum. GCF was collected with the help of filter paper strips (Whatmann no. 3), 2 × 8 mm in dimension between 8.30 am and 10.00 am. A total of 6 strips were placed successively for 30 s at the entrance of the gingival sulcus or pocket and the fluid seeping out was collected [24]. The strips were stored in phosphate buffer saline (pH 7.4) until further analysis.

Additionally, 5 ml of blood was collected which was allowed to clot at room temperature. After 1 h, serum was extracted by centrifugation at 3000 rpm for 5 min. It was immediately stored at − 70 °C till further analysis. The biochemical markers estimated were malondialdehyde (MDA) and GPx enzyme in GCF (cMDA; cGPX), and serum (sMDA; sGPX) while insulin levels, IR and %S were measured in blood only.

Assay for GPx estimation

GPx was assessed by the continuous spectrophotometric rate determination method [25]. In this method, phosphate-buffered saline was mixed with ethylene di-amine tetra acetic

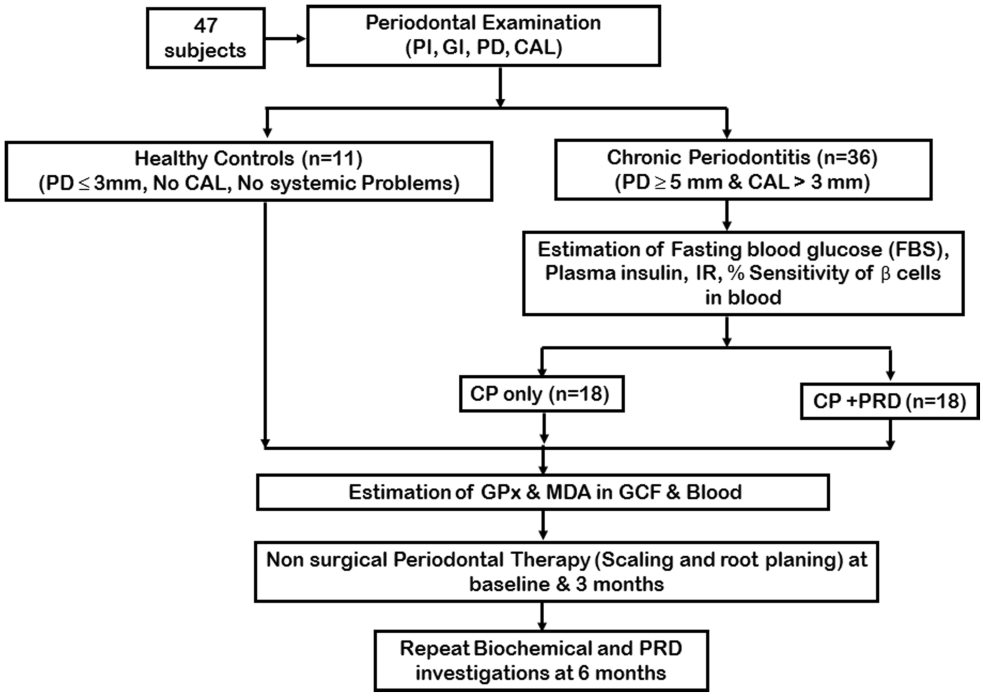

Fig. 2 Flowchart showing subdivision of subjects

acid (EDTA), which was further mixed with sodium azide and glutathione reductase. This mixture was incubated at 37 °C for 10 min, following which, reduced form of β-nicotinamide adenine dinucleotide phosphate (β-NADPH) was added. The final reaction was initiated with the help of H_2O_2. The optical density of the resultant solution was calibrated at 340 nm for 1 min, at an interval of 5 min.

Assay for MDA estimation

MDA, an oxidative stress marker of lipid peroxidation, was estimated using Kei Satoh's method [26]. The normal level of MDA in serum by this method is < 0.7 nmol/ml. For analysis, whole blood/GCF samples were centrifuged at 13,000 rpm for 30 min. The supernatant was stored at − 20 °C till MDA estimation using thiobarbituric acid (TBA) reaction. The serum lipid peroxide was measured by precipitating lipoporoteins with trichloroacetic acid (pH 2–3) and boiling with TBA. It reacted with MDA to form MDA-TBA2 (a pink colored complex) that was measured with the ultra violet spectrophotometer at 532 nm. The concentration of MDA was expressed in micrograms per milliliter.

Determination of IR

IR was calculated in all the CP patients with and without PRD with the help of the Homeostasis Model Assessment-IR (HOMA-IR) index as [27].

$$\mathrm{IR} = \text{fasting blood glucose (mmol/L)} \times \text{fasting plasma insulin (mU/L)}/22.5$$

Those subjects diagnosed with PRD were referred for further medical consultation regarding diet counseling and lifestyle changes.

Non-surgical periodontal therapy

The NSPT consisting of scaling and root planing (SRP) was done in all the subjects at baseline and 3 months. These procedures remove dental plaque biofilm, calculus, and necrotic cementum from both supra and subgingival areas as well as tooth-root to achieve a smooth, hard, and clean surface [28]. They were performed with powered scalers and Gracey curettes. The surfaces were instrumented till they were visually and tactilely free of all accretions.

Statistical analyses

Descriptive statistics were summarized using mean and standard deviation. Baseline differences between groups were evaluated using the ANOVA and paired *t* test. At 6 months after treatment, the intragroup comparisons were done with Wilcoxon signed rank test while intergroup comparisons were made using Kruskall-Wallis test and Mann-Whitney *U* test. All the tests were performed with the help of SPSS version 20 software and the values were considered as statistically significant if $p < 0.05$.

Results

Demographic characteristics of the study sample

Among the 47 subjects, 24 were females and 23 males. The mean age of the subjects in the control ($n = 11$), CP only ($n = 18$), and CP+PRD ($n = 18$) groups was 29.4 ± 3.09, 40.17 ± 5.79, and 39.61 ± 5.73 years respectively (Table 1). Intergroup analysis using ANOVA showed that there was no significant difference in the age of the subjects. The following observations were seen in the clinical and biochemical parameters recorded at baseline and 6 months after NSPT.

A. Clinical periodontal parameters

The clinical periodontal parameters recorded at baseline were PI, GI, and PD (Table 1). At baseline, intergroup comparison of these parameters using ANOVA revealed a statistically significant difference ($p < 0.05$). These results were further confirmed with a post hoc Games-Howell test which showed highest PI, GI, and PD in the CP+PRD group, followed by CP only and controls.

At 6 months after NSPT, intragroup analysis using Wilcoxon signed rank test showed that there was a highly significant reduction in the scores of PI, GI, and PD in all the three groups ($p < 0.001$) (Table 2). The intergroup analysis of the change in these parameters using Kruskall-Wallis test revealed a statistically significant ($p < 0.001$) reduction in the scores of PI and GI among the three groups (Table 3). However, there was no statistically significant difference in the change of PD among the three groups ($p > 0.05$).

B. Biochemical and PRD parameters

At baseline, the mean values of GPx in the GCF and serum were highest in controls, followed by the CP+PRD and CP only groups (Table 1). The intergroup analysis with ANOVA revealed a statistically significant difference in the levels of GPx in both the fluids ($p < 0.05$). Likewise, the mean levels of the oxidative stress marker, MDA, were highest in the CP only group followed by the CP+PRD group and controls respectively, in both GCF and serum. This difference was statistically significant among the three groups ($p < 0.05$).

The PRD parameters were recorded in the CP and CP+PRD groups only. At baseline, the mean FBS, plasma insulin,

Table 1 Comparison of baseline data between the controls and chronic periodontitis groups with and without pre-diabetes

Parameter	Groups			*p* value
	Controls (*n* = 11)	CP only (*n* = 18)	CP+PRD (*n* = 18)	
Age (years)*	29.09 ± 3.11	40.17 ± 5.79	39.61 ± 1.67	0.598
PI*	0.65 ± 0.18	1.81 ± 0.27	2.23 ± 0.53	< 0.001
GI*	0.67 ± 20	1.82 ± 0.27	2.26 ± 0.45	< 0.001
PD (mm)*	1.38 ± 0.49	6.30 ± 0.58	6.30 ± 0.78	< 0.001
cGPx (μg/ml)*	0.15 ± 0.10	0.07 ± 0.06	0.10 ± 0.1	0.048
sGPx (μg/ml)*	0.19 ± 0.10	0.09 ± 0.06	0.08 ± 0.03	< 0.001
cMDA (μg/ml)*	0.11 ± 0.05	0.31 ± 0.21	0.17 ± 0.07	0.001
sMDA (μg/ml)*	0.20 ± 0.16	0.34 ± 0.12	0.34 ± 0.09	0.003
FBS (mg/dl)#	–	92.33 ± 5.32	108.56 ± 6.90	< 0.001
Plasma insulin (μg/ml) #	–	12.00 ± 5.86	18.81 ± 5.53	0.001
IR#	–	1.55 ± 0.72	2.48 ± 0.72	< 0.001
% S#	–	77.31 ± 34.43	45.21 ± 21.34	0.002

*ANOVA; # paired *t* test; *p* is significant at < 0.05

and IR levels were higher in the CP+PRD group while %S was less in the same group (Table 1). The difference in the levels was highly statistically significant as revealed by paired *t* test ($p < 0.001$).

At 6 months after NSPT, there was a reduction in the mean levels of all the biochemical and PRD parameters (Table 4). Intragroup comparisons using Wilcoxon signed rank test showed a statistically significant reduction in all the groups ($p < 0.05$). Only cMDA in controls and FBS in the CP only group did not reveal a statistically significant reduction ($p > 0.05$) (Table 3).

Intergroup comparisons of biochemical parameters at 6 months using Kruskall-Wallis test revealed a statistically significant change in the levels of only sGPX and cMDA among the three groups ($p < 0.05$) (Table 4).

Additionally, there was no statistically significant difference in the change in the levels of PRD parameters between the CP and CP+PRD groups. Although, %S, increased significantly in the CP+PRD group after NSPT, as revealed by Mann-Whitney *U* test ($p = 0.007$) (Table 5). The effect size estimation of results between the CP and CP+PRD groups showed moderate clinical significance for the reduced levels

Table 2 Intragroup comparison of clinical periodontal parameters (mean ± SD) at 6 months after non-surgical periodontal therapy

Group	*N*	Parameter (mean ± SD)			Wilcoxon signed rank test *Z* value	*p* value
Controls	11	PI	Baseline	0.73 ± 0.25	3.72	< 0.001
			6 months	0.49 ± 0.21		
CGP	18		Baseline	1.81 ± 0.27	2.94	0.003
			6 months	1.36 ± 0.30		
PRD	18		Baseline	2.23 ± 0.53	3.38	< 0.001
			6 months	1.02 ± 0.50		
Controls	11	GI	Baseline	0.67 ± 20	3.72	< 0.001
			6 months	0.38 ± 0.20		
CGP	18		Baseline	1.82 ± 0.27	2.8	0.005
			6 months	1.16 ± 0.25		
PRD	18		Baseline	2.26 ± 0.45	3.68	< 0.001
			6 months	1.30 ± 0.45		
Controls	11	PD (mm)	Baseline	1.34 ± 0.51	3.52	< 0.001
			6 months	1.08 ± 0.46		
CGP	18		Baseline	6.30 ± 0.58	2.93	0.003
			6 months	5.84 ± 0.68		
PRD	18		Baseline	6.30 ± 0.78	3.68	< 0.001
			6 months	5.88 ± 0.68		

p is significant at < 0.05

Table 3 Intragroup comparison of biochemical and prediabetes parameters at 6 months after non-surgical periodontal therapy

Group	*N*	Parameter (mean ± SD)			Wilcoxon signed rank test *Z* value	*p* value
Controls	11	cGPx	Baseline	0.14 ± 0.11	2.39	0.017
			6 months	0.07 ± 0.07		
CGP	18		Baseline	0.07 ± 0.06	1.87	0.062
			6 months	0.03 ± 0.02		
PRD	18		Baseline	0.10 ± 0.10	3.72	< 0.001
			6 months	0.08 ± 0.18		
Controls	11	sGPx	Baseline	0.19 ± 0.11	2.31	< 0.001
			6 months	0.07 ± 0.07		
CGP	18		Baseline	0.09 ± 0.06	2.80	0.003
			6 months	0.04 ± 0.03		
PRD	18		Baseline	0.08 ± 0.03	3.64	0.001
			6 months	0.06 ± 0.10		
Controls	11	cMDA	Baseline	0.12 ± 0.05	0.57	0.570
			6 months	0.08 ± 0.03		
CGP	18		Baseline	0.31 ± 0.21	2.58	0.010
			6 months	0.19 ± 0.15		
PRD	18		Baseline	0.17 ± 0.07	3.64	< 0.001
			6 months	0.15 ± 0.10		
Controls	11	sMDA	Baseline	0.21 ± 0.17	2.15	0.031
			6 months	0.12 ± 0.10		
CGP	18		Baseline	0.34 ± 0.12	2.52	0.012
			6 months	0.28 ± 0.09		
PRD	18		Baseline	0.34 ± 0.09	2.77	0.006
			6 months	0.26 ± 021		
CGP	18	FBS	Baseline	92.33 ± 5.32	1.72	0.103
			6 months	90.44 ± 4.15		
PRD	18		Baseline	108.56 ± 6.90	3.98	0.001
			6 months	104.56 ± 7.29		
CGP	18	Plasma	Baseline	12.00 ± 5.86	4.35	< 0.001
		Insulin	6 months	8.58 ± 3.06		
PRD	18		Baseline	18.81 ± 5.53	5.94	< 0.001
			6 months	14.59 ± 4.94		
CGP	18	IR	Baseline	1.55 ± 0.72	3.72	< 0.001
			6 months	1.11 ± 0.39		
PRD	18		Baseline	2.4 ± 0.72	3.68	< 0.001
			6 months	1.94 ± 0.65		
CGP	18	%S	Baseline	77.31 ± 34.43	− 9.48	<0.001
			6 months	102.59 ± 39.93		
PRD	18		Baseline	45.27 ± 21.34	− 4.32	< 0.001
			6 months	60.22 ± 30.31		

p is significant at < 0.05

of sGPx and IR (= 0.3) while insulin sensitivity showed a large clinical significance (= 0.7) [29].

Discussion

The current study evaluated the influence of NSPT on IR through its effect on GPx in CP subjects with PRD. PRD is the precursor of diabetes and is a growing concern in both developed and developing nations [15]. Observational studies have revealed a plausible association between PRD and periodontitis [30, 31]. However, the exact mechanism of this linkage is yet to be determined. It has been suggested that in patients with periodontal diseases the incidence of PRD or undiagnosed type 2 DM is increased by 27–53% [32, 33].

Severe, untreated CP serves as a portal of entry for periodontopathogens and their products into systemic circulation. They stimulate immunoinflammatory cells to produce ROS and reactive nitrogen species (RNS) which may act as both toxic and beneficial compounds [34, 35]. There is a

Table 4 Intergroup comparison of the change in clinical periodontal and biochemical parameters at 6 months after non-surgical periodontal therapy

Group	*N*	Change in parameter (mean ± SD)		Median	Kruskall-Wallis test value	*p* value
Controls	11	PI	0.22 ± 0.11	0.180	17.77	< 0.001
CGP	18		0.45 ± 0.34	0.480		
PRD	18		1.22 ± 0.87	0.940		
Controls	11	GI	0.29 ± 0.22	0.210	16.12	< 0.001
CGP	18		0.66 ± 0.33	0.590		
PRD	18		0.96 ± 0.63	0.850		
Controls	11	PD	0.26 ± 0.22	0.190	4.16	0.125
CGP	18		0.46 ± 0.31	0.390		
PRD	18		0.42 ± 0.34	0.420		
Controls	11	cGPX	0.07 ± 0.15	0.088	0.315	0.207
CGP	18		0.05 ± 0.06	0.285		
PRD	18		0.01 ± 0.15	0.280		
Controls	11	sGPX	0.12 ± 0.07	0.090	6.608	0.037
CGP	18		0.05 ± 0.05	0.029		
PRD	18		0.02 ± 0.12	0.047		
Controls	11	cMDA	0.04 ± 0.04	0.160	14.948	0.001
CGP	18		0.12 ± 0.12	0.890		
PRD	18		0.07 ± 0.31	0.025		
Controls	11	sMDA	0.09 ± 0.12	0.019	1.296	0.523
CGP	18		0.06 ± 0.08	0.030		
PRD	18		0.09 ± 0. 25	0.125		

p is significant at < 0.05

delicate balance between their two antagonistic roles, whereby at low or moderate levels, they support cellular and immune functions while at high concentrations, they generate oxidative stress. The latter damages all cell structures and promotes development of chronic degenerative ailments, including diabetes [36].

The oxidative stress is countered with the help of either endogenous or exogenous antioxidants. These are essentially "free radical scavengers" that prevent and repair damages caused by reactive species and enhance immune defense. GPx is a major selenoprotein antioxidant enzyme directly involved in their neutralization. It specifically counteracts H_2O_2 which is transformed into water and oxygen [35]. This involves conversion of reduced glutathione (GSH) into oxidized glutathione (GSSG) with the help of H_2O_2.

The increase in IR in subjects with periodontitis may be due to a combination of factors [37]. The initial triggering mechanism involves accumulation of bacterial dental plaque rich in periodontopathogens like Porphyromonas gingivalis (P gingivalis) and Prevotella intermedia (P intermedia). Lately, a plausible dysbiosis of these gram-negative periodontopathogens was proposed in increased incidence of metabolic diseases [38]. Their lipopolysaccharides (LPS) produced "metabolic endotoxemia" and IR [39–41]. It was suggested that they may promote "metabolic inflammation" wherein the macrophages and

Table 5 Intergroup comparison of the change in prediabetes parameters at 6 months after non-surgical periodontal therapy

Group	*N*	Change in parameter (mean ± SD)		Median	Mann-Whitney test *p* value
CGP	18	FBS	1.89 ± 4.65	2.00	0.204
PRD	18		4.00 ± 4.26	3.00	
CGP	18	Plasma Insulin	3.42 ± 3.33	2.79	0.311
PRD	18		4.21 ± 3.01	4.16	
CGP	18	IR	0.44 ± 0.40	0.365	0.275
PRD	18		0.55 ± 0.39	0.530	
CGP	18	%S	− 25.28 ± 11.30	− 25.10	0.007
PRD	18		− 14.95 ± 14.67	− 8.50	

p is significant at < 0.05

T lymphocytes were recruited within metabolic tissues like liver and adipose tissues [42]. They enhanced the release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and plasminogen activator inhibitor (PAI)-11 that impaired the action of insulin [43]. Furthermore, elevated TNF-α stimulated cellular pathways like I-kappa-β (I-κ-β), nuclear factor-kappa-β (NF-κβ), and the protein C-Jun-N-terminal kinase (JNK) axes [37]. The IR developed secondary to the inflammatory process in which innate and adaptive immune responses promoted inflammatory reactions driven by gut microbiota [43, 44]. Additionally, some studies have suggested a plausible role of antioxidant GPx in promoting IR by causing reductive stress [17, 45]. They proposed that some amount of ROS was imperative for regulating insulin signaling pathways [17]. As a norm, when insulin is released from the β cells, it is taken up by various cells for metabolism [46]. It binds to a glycoprotein embedded in the cellular membrane which has an extracellular receptor domain, made up of two α-subunits. This binding to the α-subunit results in a conformational change in the membrane bound glycoprotein, which activates tyrosine kinase. The latter then undergoes auto-phosphorylation, which increases the insulin activity. It has been observed that insulin stimulation promotes production of intracellular H_2O_2. This step is critical in limiting the action of protein-tyrosine phosphatase, which controls the irreversible phosphorylation of tyrosine. In this manner, a small amount of ROS is necessary for optimal insulin signaling [45].

Although, high levels of GPx may quench ROS and produce reductive stress, some studies have regarded excessive production of GPx as an indirect marker of systemic oxidative stress [47]. In reductive stress, besides GPx, an abnormal increase of reducing equivalents like elevated ratio of reduced (GSH)/oxidized glutathione (GSSG) and NADPH/NADP are also seen [48–50]. In the present study, as only the levels of sGPx and cGPx were measured, development of reductive stress in CP subjects cannot be completely verified and requires further long term evaluation. Furthermore, the levels of GPx were higher in controls when compared to the diseased group. This could be attributed to the physiological process of health whereby optimal expression of GPx does not interfere with cellular signaling pathways [16]. The levels of both cMDA and sMDA were increased in the CP+PRD group, suggesting that the basal oxidative stress was elevated in this group. This could be related to inflammatory changes in the gingival microenvironment [20]. However, when the diseased groups were compared, the mean levels of GPx and IR were slightly higher in the CP+PRD group than in the CP group. This is in accordance with previous studies that reported that gene expression of plasma GPx was upregulated by H_2O_2 and other ROS in CP [20, 51, 52]. Additionally, periodontopathogens like P gingivalis, triggered production of GPx in early stages of infection as it invaded and survived within the epithelial cells [53, 54]. Subsequent, redox imbalance promoted progression of periodontitis and its co-morbidities [20].

An animal study has shown that prolonged expression of GPx may influence IR [17]. Although CP was not studied in these animals, it was hypothesized that higher amounts of GPx, quenched the intracellular ROS required for insulin signaling, resulting in compromised IR and obesity. Likewise, high GPx levels were directly correlated to type 2DM and IR in healthy pregnant women in other studies [18, 55].

In the present study, greatest amount of plaque, gingivitis and PD were observed in the CP+PRD group, followed by CP only and the controls. This suggests that subjects with PRD had worst periodontal health. Similar results were observed in earlier studies where increased bleeding on probing was correlated with greater impaired fasting glucose and/or duration of PRD [56–59]. Besides, markers of lipid peroxidation were significantly higher in diabetics and were correlated to degree of inflammation [60–63]. In the present study, NSPT reduced PI, GI, PD, and MDA to statistically significant levels in both CP and CP+PRD groups along with basal oxidative stress.

Since periodontopathogens have both direct and indirect role in producing oxidative stress and IR, their elimination through NSPT is justified. This may further prevent development and progression of PRD and type 2 DM. The primary objective of NSPT is to restore the gingival health by completely removing the elements associated with gingival inflammation, i.e., dental plaque, calculus, and endotoxins from the tooth surface [28]. Studies have reported a dramatic reduction in the numbers of periodontopathogens like Aggregatibacter actinomycetemcomitans, P gingivalis, and P intermedia and increase in coccoid cells in the subgingival environment following NSPT [28, 64, 65]. It has also been shown that treatment of periodontal diseases through NSPT may reduce glycosylated hemoglobin by 0.29% in patients with type 2 DM, over a period of 3–4 months [66]. This could be related to reduction of systemic oxidative stress following NSPT [51, 60, 67]. However, a recent study reported that although there was significant change in the oxidative stress balance following periodontal treatment at 3 months, the effects of NSPT on glycemic control were transient and required further investigation [68].

In the present study, NSPT reduced the levels of GPx in both serum and GCF. Furthermore, all the groups responded equally to the intervention as there was no significant difference in the change in the levels of GPx between them. This shows that GPx may be overexpressed

in inflammatory conditions like CP and PRD and may revert back to optimal levels after treatment, as a result of "antioxidant compensatory mechanisms" [69].

As IR is affected by high GPx levels, its reduction in serum and GCF may help in improving insulin sensitivity as explained earlier. This was seen in the present study, wherein reduction in GPx levels after NSPT significantly reduced the mean levels of FBS, insulin, and IR with a concomitant increase in %S. This further strengthens the hypothesis that high GPx levels influence IR. Furthermore, NSPT restores the optimal balance between ROS and GPx which subsequently improved %S. These findings were more prominent in the CP+PRD group and are in agreement with recent studies [58, 70, 71]. Therefore, NSPT in conjunction with diet counseling and lifestyle modifications may be regarded as an integral component of non-pharmacological management of PRD to prevent its progression to type 2 DM. Moreover, considering the cost-effectiveness of the procedure and clinical significance, the results may be beneficial for the general population. However, further long-term studies with larger sample size are required to gain a better insight into the complex mechanisms of IR in CP subjects with PRD.

Conclusion

The present study reaffirms the role of non-surgical periodontal therapy in reducing insulin resistance in chronic periodontitis subjects with prediabetes. It resulted in a progressive reduction in the levels of glutathione peroxidase and insulin resistance along with improvement in mean fasting blood sugar, percentage sensitivity of insulin, and clinical periodontal parameters, specifically in the prediabetes group. Accordingly, non-surgical periodontal therapy may be routinely considered as an adjunct in the management of prediabetes to prevent its progression to type 2 diabetes mellitus. However, further studies with larger sample size and long-term follow-up of subjects with chronic periodontitis and prediabetes are required to detail the exact mechanism of the influence of glutathione peroxidase on insulin resistance. Moreover, the influence of non-surgical periodontal therapy on insulin resistance through its effect on glutathione peroxidase levels needs further validation through longitudinal studies.

Compliance with ethical standards

Ethical approval was obtained from the institutional ethics committee of Kasturba Hospital, Manipal, prior to the commencement of the study. A written informed consent was taken from all the subjects.

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

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ORIGINAL ARTICLE

The all-cause mortality and risk factors for mortality within five years among prevalent Type 1 Diabetes Mellitus Patients

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Abstract
Despite new comprehensive approach in diabetes care, type 1 diabetes is still facing considerable premature mortality. This study aims to examine associated risk factors for all-cause mortality among prevalent patients with type 1 diabetes mellitus (T1DM) within 5 years' period and subsequently develop a logistic regression model to predict the outcome. This was a cohort study where prevalent patients diagnosed with T1DM were notified in a national diabetes registry. Patients' particulars were recorded between 1 January 2009 and 31 December 2009. Their records were matched with national death record at the end of year 2013 to determine the status of mortality within 5 years. The factors associated with mortality were investigated, and a prognostic model was developed based on logistic regression model. There were 665 patients included in the study, and 105 patients died within 5 years. The mortality rate was 1.6 persons per 100 person-years and the standardized mortality ratio was 10.04. Majority causes of death were due to circulatory system (33.8%) and infection (32.5%). Multivariate analysis suggested that gender, age group, and ischemic heart disease (IHD) were the major contributing factors towards the outcome. Elderly male with IHD has a significant risk of mortality within 5 years with probability of event of 0.755, while elderly female with IHD has probability of event of 0.612. The main causes of death among prevalent T1DM patients were heart disease and infection. Male gender, elderly age group, and having IHD were significant risk factors of mortality in prevalent T1DM patients within 5 years.

Keywords Associated factors · Diabetes type 1 · Mortality · Prognostic model

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic illness caused by the body's inability to produce sufficient insulin. This noncommunicable disease is usually diagnosed in children and young adults and was previously known as juvenile diabetes. There were wide variations existing with regard to the incidence rates of T1DM in different countries. The annual incidence rates for childhood T1DM are between 0.1 and 37.4 per 100, 000. The lowest incidences are in China and Venezuela with 0.1 per 100,000 per year, and the top highest incidences are in Finland and Sardinia with 40 per 100,000 per year [1–3]. Currently, there are no official statistics reported regarding incidence rate of T1DM in Malaysia.

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In the past, the survivals for T1DM patients were poor until insulin therapy was introduced since 1922 [4]. Since then, a marked decrease in mortality was observed. Although there was a reduction in mortality, T1DM still contributes to risk of death reported in developed countries [5]. However, diabetes mellitus has not been acknowledged as major disease that could lead to fatality. The reason might be because diabetes mellitus is known to be underreported on death certificates as an underlying or contributing cause of death [6, 7]. Hence, few studies have attempted to examine the survival of T1DM patients and specific cause of death [8–11]. In a Swedish study, patients with T1DM have twice the risk of death from cardiovascular event even with good glycemic control [11]. There were limited data on Malaysia's all-cause mortality on T1DM patients.

Studies regarding predictive modeling for mortality among T1DM patients are scarce. This study aims to investigate the associated factors towards mortality within 5 years among patients with T1DM at any point of disease. Following to that, a predictive modeling was developed as a screening tool to predict potential risk of dying within 5 years' period among patients with prevalent T1DM. The predictive modeling tool potentially can be used as screening tool to classify patients who are at risk that may require more attention and to increase the awareness among the public.

Methodology

This was a cohort study where patients' demographic and clinical details were captured from national registry, an Audit of Diabetes Control and Management (ADCM) between 1 January 2009 and 31 December 2009. Full details of the design, methods, and recruitment for the ADCM have been published elsewhere [12]. This clinic-based prospective cohort study examined 665 T1DM patients. The patients were T1DM with minimum age of 18 years notified from government health clinics throughout Malaysia. Demographic profile, risk factors, and clinical parameters were recorded according to a standardized protocol during the baseline of study. Their records were matched with National Death Record (NDR) at the end of year 2013 to determine status of mortality within 5 years and also the causes of death based on ICD-10, 2016. The study was registered under the National Medical Research Registry and has obtained approval from the Medical Research Ethics Committee (MREC).

Statistical analyses

Descriptive analysis was conducted to determine the incidence of cause of death based on ICD-10 code. Meanwhile, multivariate analyses were conducted to determine the major-associated factors contributing to mortality rate within 5 years. Following to that, a predictive model to predict mortality after 5 years was formulated using logistic regression model. The flow analysis was done as follows:

1. A univariate analysis based on Pearson's chi-square test was conducted to determine the associated factors towards risk of mortality within 3 years.
2. The significant factors from the univariate analysis were tested again using multivariate analysis based on logistic regression using forward likelihood ratio method. The cut off probability for variable selection was set at 0.05 for both inclusion and exclusion criteria. The coefficients, odds ratio with respective confidence interval, and p values were recorded.
3. The equation model was derived based on the selected variables' coefficients in the logistic regression model. Then, the z-score and probability of event were calculated for each combination among the significant variables in predicting the outcome. The probability of the outcome of interest, the Z value, was then transformed into the probability of event using the following link function: $P[\text{event}] = e^z/1 + e^z$. This probability value range from 0 to 1.

All analyses were carried out using SPSS (IBM Corporation, Released 2011, IBM SPSS Statistics for Windows, Version 20.0; Armonk, NY: IBM Corp.) and Microsoft Office Excel 2007.

Results

There were 70,279 records in the registry with only 665 patients recorded as T1DM, and thus, the prevalence of T1DM was 0.95%. There were 105 deaths (15.8%) out of T1DM patients where incidence of mortality within 5 years was 15.8% with average of 3.2% in each year. The incidence of mortality in 2009, 2010, 2011, 2012, and 2013 was 1.8%, 3.2%, 4.1%, 3.5, and 3.3%, respectively. The mortality rate was 1.6 persons per 100 person-years. The standardized mortality ratio (SMR) was 10.04 where male and female reported the SMRs with 13.38 and 8.00, respectively. Majority of patients died due to diseases of circulatory system (33.8%), infectious and parasitic diseases (32.5%), and respiratory system (6.5%). Majority of younger patients died due to infection and diabetes, whereas majority of elder patients died due to diseases circulatory system or heart disease (Table 1).

Out of 665 patients, majority were female (63.8%), between 40 and 64 years (61.9%) and Malay (87.0%). More than half have hypertension (58.1%) and 17.8% with dyslipidemia. (Table 2). Based on univariate analysis, age group at notification ($p < 0.001$), gender ($p = 0.001$), comorbidity such as hypertension ($p = 0.026$), diabetes complications such as

Table 1 Causes of death among T1DM patients based on age group

	18–39 years ($n = 6$)		40–64 years ($n = 50$)		≥ 65 years ($n = 49$)	
Cause of death	n	%	n	%	n	%
Diseases of the circulatory system	0	0.0	15	35.7	11	40.7
Certain infectious and parasitic diseases	4	50.0	14	33.3	7	25.9
Diseases of the respiratory system	1	12.5	2	4.8	2	7.4
Endocrine, nutritional, and metabolic diseases	2	25.0	5	11.9	0	0.0
Diseases of the genitourinary system	0	0.0	1	2.4	2	7.4
External causes of morbidity and mortality	1	12.5	0	0.0	1	3.7
Diseases of the nervous system	0	0.0	5	11.9	1	3.7
Diseases of the blood and blood-forming organs and certain disorders involving the immune m	0	0.0	0	0.0	1	3.7
Diseases of the digestive system	0	0.0	0	0.0	2	7.4
Symptoms, signs, and abnormal clinical and laboratory findings, not elsewhere classified	0		14		25	

*The percentage excludes the cause of death for symptoms, signs, and abnormal clinical and laboratory findings, not elsewhere classified

ischemic heart disease (IHD) ($p = 0.018$), and foot problem ($p = 0.018$) were associated with the outcome (Table 2, Table 3, and Table 4).

Multivariate analysis based on stepwise analysis suggested that only gender and age group were the major contributing factors towards the outcome. Further multivariate analysis was carried out to incorporate status of IHD in the analysis. Status of IHD was slightly more than $p = 0.05$, but the effect size based on odds ratio was large (Table 5). Thus, the prediction model was incorporating age group, gender, and status of IHD. Elderly with IHD has a significant risk of mortality within 5 years with probability of event of 0.612 and 0.755 for female and male, respectively. Regardless of age, patients without IHD have lower risk of dying within 5 years' period (less than probability of 0.5) (Table 6).

Table 2 Univariate analysis on patients' demographic characteristics

			Died		
Characteristics		N (%)	No	Yes	*p* value
Demographic					
Age group	18–39	85 (12.8)	79 (14.1)	6 (5.7)	< 0.001
	40–64	412 (61.9)	362 (64.6)	50 (47.6)	
	≥ 65	168 (25.3)	119 (21.3)	49 (46.7)	
Gender	Male	241 (36.2)	188 (33.6)	53 (50.5)	0.001
	Female	424 (63.8)	372 (66.4)	52 (49.5)	
Ethnicity	Malay	575 (87.0)	481 (86.5)	94 (89.5)	0.808
	Chinese	41 (6.2)	35 (6.3)	6 (5.7)	
	India	38 (5.7)	34 (6.1)	4 (3.8)	
	Others	7 (1.1)	6 (1.1)	1 (1.0)	
Comorbidity					
Hypertension	Yes	336 (57.0)	272 (55.1)	64 (67.4)	0.026
	No	253 (43.0)	222 (44.9)	31 (32.6)	
Dyslipidemia	Yes	105 (17.8)	90 (18.2)	15 (15.8)	0.571
	No	484 (82.2)	404 (81.8)	80 (84.2)	

Discussion

The mortality rate of T1DM was 1.6 persons per 100 person-years and is slightly higher compared with T2DM in the same

Table 3 Univariate analysis on patients' clinical characteristics

Clinical parameter		Died		
		No	Yes	*p* value
HbA1c	Good (≤ 7.0)	96 (29.7)	10 (17.2)	0.051
	Poor (> 7.0)	227 (70.3)	48 (82.8)	
Systolic BP	Good (≤ 130)	239 (48.5)	39 (41.5)	0.214
	Poor (> 130)	254 (51.5)	55 (58.5)	
Diastolic BP	Good (≤ 80)	304 (61.7)	55 (58.5)	0.565
	Poor (> 80)	189 (38.3)	39 (41.5)	
BMI	Underweight (< 18.50)	10 (2.1)	3 (3.2)	0.059
	Normal (18.50–24.99)	162 (33.4)	42 (45.2)	
	Overweight (≥ 25.00)	201 (41.4)	36 (38.7)	
	Obese (≥ 30.00)	112 (23.1)	12 (12.9)	
LDL	Good (≤ 2.6)	60 (18.5)	9 (16.7)	0.744
	Poor (> 2.6)	264 (81.5)	45 (83.3)	
HDL	Good (≥ 1.1)	244 (76.5)	35 (68.6)	0.226
	Poor (< 1.1)	75 (23.5)	16 (31.4)	
TG	Good (≤ 1.7)	192 (53.9)	31 (51.7)	0.745
	Poor (> 1.7)	164 (46.1)	29 (48.3)	

BMI, body mass index; *BP*, cerebrovascular disease; *HbA1c*, glycated hemoglobin; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *TG*, triglyceride

Table 4 Univariate analysis on patients' complications

Complications		Died		
		No	Yes	*p* value
Retinopathy	Yes	25 (6.1)	9 (12.3)	0.053
	No	387 (93.9)	64 (87.7)	
IHD	Yes	3 (0.7)	3 (4.0)	0.018
	No	410 (99.3)	72 (96.0)	
CVD	Yes	4 (1.0)	2 (2.6)	0.230
	No	417 (99.0)	76 (97.4)	
Nephropathy	Yes	21 (5.0)	5 (6.6)	0.577
	No	397 (95.0)	71 (93.4)	
Foot problem	Yes	8 (1.7)	5 (5.9)	0.018
	No	466 (98.3)	80 (94.1)	
Amputation	Yes	1 (0.2)	1 (1.0)	0.184
	No	559 (99.8)	104 (99.0)	
Duration	< 5 years	252 (51.0)	40 (42.1)	0.272
	5–10 years	193 (39.1)	43 (45.3)	
	> 10 years	49 (9.9)	12 (12.6)	

CVD, cerebrovascular disease; *IHD*, ischaemic heart disease

country [13]. The SMR reported in this present study was higher compared to that of other countries especially in developed countries [14]. The SMR ratio among female compared to male is 0.60, and the result was about similar to Finland and Taiwan [10, 15], while majorities reported SMR ratio among female compared to male was more than one especially in developed countries [14]. Thus in general, our population showed poorer survival among male compared with female patients.

The incidence of mortality among prevalent T1DM patients was relatively low with less than 5% in a year, and this shows that patients with T1DM have relatively good survival, although some studies showed that survival among individuals with T1DM has an increased age-adjusted mortality risk of death compared to the general population [16, 17]. This excess mortality risk is driven by the results of the development of complications due to T1DM in the population [18–20]. In other words, when there is no diabetes complication, some studies suggested that T1DM patients may have long-term survival comparable to the general population [18, 21]. From our findings, prevalent T1DM patients with no diabetes complications have probability of event with less than 0.5 in getting risk of mortality within 5 years.

Based on our data, the leading cause of death among patients with T1DM was due to circulatory system. All of them died after 40 years, and majority were at 65 years and above. Heart disease is a common and a leading cause of death in most countries including developed country like the USA [22]. In addition, heart disease or cardiovascular is also a leading cause of death among diabetes population [14]. This showed that our result is consistent with previous findings. Based on our data, only six patients reported to have diabetes complication of IHD. However, 26 patients died of heart disease within 5 years' period after notified into the registry. This can be explained by majority of them having also other

Table 6 Probability of event between gender, age group and status of IHD

Combination of factors	Male Prob (e)	Female Prob (e)
Adult and no IHD	0.072	0.038
Middle age and no IHD	0.161	0.089
Elderly and no IHD	0.328	0.241
Adult and yes IHD	Nil	Nil
Middle age and yes IHD	0.489	0.383
Elderly and yes IHD	0.755	0.612

IHD, ischaemic heart disease; *Prob (e)*, probability of event

Table 5 Risk factors towards mortality within 5 years, a multivariate analysis

Stepwise method: backward likelihood ratio						Enter method					
Factors		*B*	OR	95%CI	*p* value	Factors		*B*	OR	95%CI	*p* value
Constant		− 3.236				Constant		− 3.236			
Gender	Male	0.673	1.960	1.162, 3.304	0.012	Gender	Male	0.673	1.96	1.162, 3.304	0.012
	Female	Ref	Ref				Female	Ref	Ref		
Age	Adult	Re	Ref			Age	Adult	Ref	Ref		
	Middle age	0.915	2.497	0.737, 8.465	0.142		Middle age	0.915	2.497	0.737, 8.465	0.142
	Elderly	2.087	8.059	2.339, 27.759	0.001		Elderly	2.087	8.059	2.339, 27.759	0.001
						IHD	Yes	1.603	4.967	0.906, 27.237	0.065
							No	Ref	Ref		

B, logistic regression's coefficient; *CI*, confidence interval; *IHD*, ischaemic heart disease; *OR*, odd ratio; *Ref*, reference group

underlying comorbidity such as hypertension besides factor of aging. It has been reported that the survival rates for onset IHD were 25.0% and 38.0% in both men and women, respectively, with median survival of only between 2 and 3 years [23].

The second leading cause of death was infection. It is a fact that patients with diabetes are at risk of developing wounds and sores that are difficult to heal well [24]. If the wounds are not treated well, the patients are at high risk of developing infection. It was reported there seems to be reduced immune regulation in subgroup of individuals with T1DM [25]. Therefore, in poorly controlled diabetes, infections can get severe faster. In the worst case scenario, when infection overwhelms the body, patients develop sepsis which can lead to septic shock and eventually death.

In the univariate analysis, IHD and foot problem were also associated with mortality within 5 years. These two important factors were not significant in the multivariate analysis because these variables were confounded with age group. In other words, although these variables were dropped from the stepwise analysis, IHD and foot problem were still important predictors in relation to the outcome. Therefore, prevention is necessary to avoid developing early diabetes complications.

Other interesting finding in the analysis was dyslipidemia and its marker such as LDL cholesterol, HDL cholesterol, and triglycerides which had no significant association with the outcome. The cholesterol markers among the patients seemed well controlled; this may probably be due to patients taking oral lipid-lowering medications. Despite most patients taking oral antihypertension medication which lowers the blood pressure (systolic and diastolic), hypertension was still significantly associated with the outcome. Patients having both diabetes and hypertension were reported to be at high risk for all-cause mortality as compared to only diabetes [26]. However, so far there is limited evidence that relates dyslipidemia and mortality among T1DM patients. On the other hand, taking lipid-lowering agents are still necessary to prevent diabetes complications especially cardiovascular disease [27, 28].

This study had successfully developed a simple and quick prognostic model to predict prevalent T1DM patients who are at risk of mortality within 5 years. A risk model in predicting the mortality can be subjective because it varies between individuals. However, based on large pool of data, researcher could find some common characteristics in the population. Our study found that gender, age group, and IHD were the major contributors for risk of mortality within 5 years. This study is considered as preliminary work to develop a prognostic model for predicting mortality among prevalent T1DM patients based on logistic regression model. Logistic regression is a very useful statistical model that can be applied to predict an outcome provided the predictors have sufficiently large effect size [29].

Our predictive modeling in predicting mortality within 5 years' period among prevalent T1DM patients can act as a screening tool to classify patients with severe conditions. Our finding concluded that elderly patient with IHD has relatively shorter survival period which was within 5 years' period. Therefore, this group of patients requires closer attention. This finding can be used to increase awareness among patients, healthcare workers, and the public. Besides reporting the odds ratio, this study also reported the probability of event based on combination of the three factors. The model was simple as it only involved three factors, and thus, the model is quite practical to be applied to screen high risk patients in clinical setting.

One of the strengths of this study was because the study was conducted based on large sample size. Large sample size is necessary since T1DM patients have lower prevalence among diabetes patients as compared to that of T2DM patients. Our data at least reached more than 500 patients. Previous studies have showed that studies that have recruited large sample size such as with minimum 500 subjects, the statistical analyses are likely to represent the parameter in the intended population [30, 31]. Hence, the result of this study could be inferred to the larger population. Secondly, the factors in the final model have sizeable effect size that has increased the accuracy of the model.

This study has several limitations. Our study population only involved government health clinics all over Malaysia. No records were captured from private clinics. However, majority of patients in Malaysia with diabetes are being follow up in government health clinics since it is heavily subsidized. Hence, the result from this study is likely to reflect the general finding for T1DM in Malaysia. Besides that, the variables that were tested as risk factors were captured based on variables observed during the notification period, while there may be other important factors which have not been investigated. Thus, this could influence the accuracy of the prediction model.

In addition, there were 39 causes of death which were not verified. This was probably due to their cause of death which was not verified by medical officers and the failure to get consent among family members to conduct autopsy [32]. Due to the survival outcome of T1DM patients as considerably good, therefore, only small events (mortality) were observed, and this may influence the power of analysis for some analysis. Hence, some of the results need to be interpreted with caution. Some of the significant predictors were nonmodifiable and have limited clinical or public health intervention such as age and gender. However, the findings from this study are still important for health care personals to screen and identify groups of high risk T1DM patients who are more likely for mortality. This can help clinicians and public health workers to identify T1DM patients that require closer monitoring or more aggressive treatment to prevent this group

from developing diabetes complications and ultimately reduce the mortality rate.

In conclusion, this study found that the associated factors of mortality within 5 years' period among T1DM patients were gender, age group, and history of ischemic heart disease. We have developed a simple and quick prognostic model that can be used as a screening tool to predict risk of mortality within 5 years among T1DM patients at any duration of disease. Future studies are recommended to validate the model.

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Compliance with ethical standards

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

Ethical approval For this type of study, formal consent is not required. The study was registered under the National Medical Research Registry and has obtained approval from the Medical Research Ethics Committee (MREC).

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ORIGINAL ARTICLE

Diabetic retinopathy grade as a predictive marker of severity of cardiovascular disease and mortality: DIVERSE Study Group

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Abstract

Patients with diabetic retinopathy (DR) are more likely to have subclinical cardiovascular disease. Objective of this study was to assess the prevalence and association of DR grades along with comorbidities in patients with established cardiovascular disease (CVD); association of DR grades beyond ocular morbidity, i.e., long-term outcomes among established CVD; influence of DR grades on prognosis in patients undergoing coronary revascularization. This was a single center, retrospective, data analysis of T2DM patients with established CVD. Goodness of fit was analyzed using Pearson's chi-square test. DR was observed in 64% ($n = 2560$) of patients (non-proliferative diabetic retinopathy [NPDR] 37%; proliferative diabetic retinopathy [PDR] 27%). DR and CVD were strongly associated; highest association was observed for congestive heart failure (CHF) ($n = 1325$), followed by myocardial infarction (MI) ($n = 795$), unstable angina (UA) ($n = 275$), and cardiomyopathy ($n = 165$) ($p < 0.00001$). Patients with NPDR have greater risk of CHF, MI, UA, and cardiomyopathy vs. PDR [HR 1.32, 1.44, 1.2, and 1.75 respectively]. Five-year all-cause mortality was significantly higher in patients with DR (94.2%; $n = 766$) vs. that in patients with non-DR (5.78%; $n = 47$) ($p < 0.00001$). NPDR patients undergoing CABG ($n = 765$) had lower 5-year mortality than those underwent PTCA ($n = 615$) (8.36% vs. 25.85%, $p < 0.00001$).Three times lower 5-year mortality rate was noted in patients with PDR undergoing CABG vs. that in patients undergoing PTCA ($n = 265$) (25.3 vs. 72.2%, $p < 0.00001$). DR was strongly associated with CVD with highest association with CHF, followed by MI, UA, and cardiomyopathy. Cardiovascular events, cerebrovascular events, mortality, and all-cause mortality were higher in patients with DR. Higher risk of mortality was noted in NPDR patients who underwent PTCA than in NPDR patients who underwent CABG.

Keywords Diabetic retinopathy · Type 2 diabetes mellitus · Cardiovascular disease · Proliferative diabetic retinopathy · Non-proliferative diabetic retinopathy

Introduction

Coronary heart disease (CHD), a macrovascular complication, is a major cause of mortality in type 2 diabetes mellitus (T2DM) patients. Coronary microvascular dysfunction has an imperative role in CHD [1]. The Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) reported that both non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathies (PDR) were associated with a 30–90% excess risk of death in T2DM patients over 16 years of follow-up period [2].

Diabetic retinopathy (DR) is a microvascular complication of diabetes, and its severity is directly related to glycemia and duration of diabetes [3–5]. Patients with DR have a poor life expectancy and are known to have subclinical cardiovascular disease [6–10]. Reported evidence suggests that retinopathy predicts coronary artery disease (CAD)–associated deaths in

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individuals with T2DM who are free of cardiovascular disease (CVD) at baseline [11].

DR and CVD share similar pathophysiological milieu of vascular damage. Retinopathy has a direct effect on progression of atherosclerosis [11, 12] and has been linked with two-fold higher risk of CAD and myocardial infarction (MI), three-fold higher risk of fatal CAD, and fourfold higher risk of heart failure (HF) [13].

Diabetic patients have a more complex CAD characterized by small, diffuse, calcified, and multi-vessel diseases requiring coronary revascularization [14]. Studies have reported that patients with DR have more diffuse and severe coronary atherosclerosis as compared to diabetic patients without retinopathy. Retina is the only site where microcirculation can be imaged directly. It provides prospect to study in vivo structure and pathology of human circulation and may be helpful to detect microvascular changes with respect to the development of CVD [15, 16].

It is advisable that diabetic patients with retinopathy would benefit from coronary artery bypass grafting (CABG) as compared to percutaneous coronary intervention (PCI) [3, 17, 18].

The objective of the present study was to assess the prevalence and association of DR grades along with comorbidities in patients with established CVD and also to understand the association of DR grades beyond ocular morbidity, i.e., long-term outcome in patients with established CVD. We explored the influence of DR grades on survival benefits in patients undergoing coronary revascularization procedure as well.

Materials and methods

Study design

The present Diabetic Retinopathy Grade as a PredictiVeMarkEr of SeveRity of CardiovaScularDisEase and Mortality (DIVERSE study) study was a retrospective analysis of electronic health records of T2DM patients with established CVD attending a cardiovascular outpatient department in a tertiary care hospital in a real-life scenario.

Patient data

Data of electronic health records of patients with DR and established CVD with the following inclusion and exclusion criteria was selected.

Inclusion criteria: Patients with established CVD who have provided consent for using their health records; patients who have undergone dilated seven-field stereoscopic fundus photos examination and fluorescein in angiography, graded as per Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale by qualified ophthalmologist; availability of follow-up data for 5 years (4 visit in a year).

Exclusion criteria: Data of patients with microalbuminuria and/or eGFR < 60 ml/min/1.73 m^2; data of patients with history of stroke and all patients discharged from intensive care unit (ICU) in less than 6-month duration during the study period.

Outcome measures

Data for congestive heart failure (CHF), MI, unstable angina (UA), and cardiomyopathy was available and analyzed. Cardiomyopathy was considered left ventricular systolic dysfunction and ventricular dilatation. Patients with LVEF less than 50% in the absence of coronary artery disease and ventricular dilatation seen on echo were labeled as cardiomyopathy. 2-D echocardiography data was available for all patients. As this is a retrospective study, we relied on the diagnosis made by treating cardiologist in clinical notes. Data for seven-field fundus examination by qualified retinologist was available and analyzed. A dilated fundus examination was done with Carl Zeiss machine FF450 plus IR. The grading was done by a retinologist using the International Clinical Diabetic Retinopathy Disease Severity Scale which is based upon the findings of the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) and the Early Treatment Diabetic Retinopathy Scale (ETDRS).

Patients were assigned to three groups, namely, no retinopathy, NPDR, and PDR. Prevalence and association of DR grades along with comorbidities in established CVD patients were analyzed. Analysis was done by separating the 5-year data into three categories, i.e., CVD with no DR, CVD with NPDR, and CVD with PDR. Year-wise new CVD event data were segregated and status of DR was noted. Prognosis of coronary revascularization procedures (CABG vs. PTCA) in correlation with DR grades was also analyzed.

Compliance with ethics guidelines

This study was conducted in accordance with the principles of the Declaration of Helsinki, guidelines for good pharmacoepidemiology practice and local regulatory guidelines. Medical data of the patients who had given their prior consent was collected and analyzed.

Statistical analysis

In analyzing the differences of variables between groups, the goodness of fit was analyzed using Pearson's chi-square test. Two-sided p values of < 0.05 were considered indicative of a statistically significant difference.

Results

Data of 6800 patients was retrieved and analyzed between the timeline of 1 Jan. 2010 to 31 Dec. 2010 (Table 1). Data of 4000 patients was matched with inclusion and exclusion criteria and analyzed further.

Prevalence and types of CVD among study population

The study populations comprised of patients with established CVD namely patients with CHF 49.15% ($n = 1966$), MI 33.02% ($n = 1321$), UA 13.9% ($n = 556$), and cardiomyopathy 3.9% ($n = 157$) were noted.

Stages of DR among study population

From the data of 4000 patients with T2DM with established CVD, 36% ($N = 1440$) of patients did not have retinopathy and 64% ($N = 2560$) of patients had retinopathy. In the patients with retinopathy, 37% ($N = 1480$) were diagnosed with NPDR and 27% ($N = 1080$) were diagnosed with PDR.

Type of CVD and DR among study population

It was noted that DR was strongly associated with CVD. The association was strongest with CHF (47.98%, $n = 1325$), followed by MI ($n = 795$), UA ($n = 275$), and cardiomyopathy ($n = 165$). NPDR resulted in greater risk of CHF, MI, UA, and cardiomyopathy than PDR, hazard ratio (HR) 1.32, 1.44, 1.2, and 1.75 respectively (Table 2).

Duration and glycemic control of DM and grades of DR

Patients with established CVD having a duration of diabetes more than 10 years and HbA1c > 9% had a positive association with the grades of DR (Table 2).

Table 1 Demographic details

Total number of patients	4000
Sex, *n* (%)	
Male	2850 (71.25%)
Female	1150 (28.75%)
Duration of disease (years), *n* (%)	
< 5 years	466 (11.65%)
5–10 years	491 (12.28%)
11–15 years	1172 (29.30%)
> 15 years	1871 (46.78%)
Smoker, *n* (%)	1907 (47.68%)
History of hypertension, *n* (%)	2326 (58.15%)

Table 2 Type of CVD, duration of DM, glycemic control, ejection fraction, and DR grades among study population

	No DR ($N = 1440$)	NPDR ($N = 1480$)	PDR ($N = 1080$)	*p value
Cardiac disease				
CHF	618	755	570	< 0.00001
MI	370	470	325	
Unstable angina	255	150	125	
Cardiomyopathy	197	105	60	
Duration of DM (years)				
< 5	658	80	100	< 0.00001
5–10	342	208	129	
11–15	286	483	347	
> 15	154	709	504	
HbA1c (%)				
≤ 6.9	143	80	75	< 0.00001
7–7.9	251	114	147	
8–8.9	318	196	231	
9–9.9	358	360	288	
> 10	370	730	339	
Ejection fraction				
> 50	790	544	370	< 0.00001
41–50%	345	298	267	
31–40%	238	301	193	
21–30%	60	178	150	
< 20%	7	159	100	

CHF, congestive heart failure; *MI*, myocardial infarction; *DR*, diabetic retinopathy; *NPDR*, non-proliferative diabetic retinopathy; *PDR*, proliferative diabetic retinopathy; *CVD*, cardiovascular disease

Ejection fraction and grades of DR

It was noted that diabetic patients with ejection fraction < 50% had a higher prevalence of diabetic retinopathy especially NPDR as compared to diabetic patients with ejection fraction > 50% (Table 2).

Coronary angiographic profiles and grades of DR

Diabetic patients with multi-vessel diseases (two vessels and three vessels) had a higher incidence of DR. The prevalence of two-vessel and three-vessel involvement was higher in diabetic patients with NPDR as compared to that in diabetic patients with PDR (Table 3).

Type of CVD intervention and grades of DR

It was observed that patients who had undergone coronary revascularization (PTCA/CABG) had a higher prevalence of DR as compared to patients on medical management. Diabetic patients with NPDR had the highest interventions as

Table 3 Angiographic profile, type of CVD intervention(s), and grades of DR

	No DR ($N = 1440$, %)	DR ($N = 2560$, %)	*p value
Type of vessel blockage			
One-vessel disease	710 (62.55%)	425 (37.45%)	< 0.00001
Two-vessel disease	436 (33.59%)	862 (66.41%)	
Triple-vessel disease	294 (18.67%)	1273 (81.23%)	
Type of intervention(s)			
Medical management	318 (56.18%)	248 (43.82%)	< 0.00001
PTCA	520 (34.62%)	982 (65.38%)	
CABG	602 (31.16%)	1330 (68.84%)	

CVD, cardiovascular disease; *DR*, diabetic retinopathy

compared to diabetic patients with PDR. We believe that diabetic patients with multi-vessel diseases might have more diffuse coronary disease compared with diabetic patients with single-vessel disease (Table 3).

DR grades and CVD intervention 5-year follow-up

Follow-up data of 5 years was characterized by 698/4000 (17.45%) new inhospital cardiovascular and cerebrovascular events. These events were higher in patients with DR as compared to those in patients without DR. New CVD events were defined as new/repeat MI, CHF, and in-stent stenosis. New CVA events were defined as new ischemic or hemorrhagic stroke.

Causes of mortality

It was noted that patients with NPDR had a higher incidence of in-stent restenosis while patients with PDR had a higher incidence of CHF. Diabetic patients with retinopathy had more fatal events than diabetic patients without retinopathy. Overall mortality during the entire study was 813/4000 (20.32%). The 5-year all-cause mortality and CV mortality were significantly higher in patients with DR ($n = 766$, 94.2%) vs. those in patients with non-DR ($n = 47$, 5.78%; $p < 0.00001$) (Fig. 1).

T2DM patients with CVD and no DR-survival

Long-term survival among diabetic patients without DR was noted excellent particularly when they underwent coronary revascularizations (Fig. 2a).

T2DM patients with CVD and NPDR-survival

Diabetic patients with NPDR who underwent CABG ($n = 765$, 8.36%) had a lower 5-year risk of mortality than those who underwent PTCA ($n = 615$, 25.85%; $p < 0.00001$) (Fig. 2b).

T2DM patients with CVD and PDR-survival

Diabetic patients with PDR who underwent CABG ($n = 565$, 25.3%) also reported three times lower 5-year mortality risk as compared to diabetic patients who underwent PTCA ($n = 265$, 72.2%; $p < 0.00001$) (Fig. 2c).

Discussion

It has been speculated that the pathologic processes that occur at the microvascular level may contribute to the pathogenesis of macrovascular complication. Reported evidence suggests that cumulative burden of microvascular diseases significantly affects the risk of future CV diseases [19]. Long duration of diabetes leads to both microvascular and or macrovascular complications. Present study provides data on prevalence, stages, and outcomes associated with the presence of DR in patients with established CVD. Most of our results complied with the conclusions of formerly reported studies.

Prevalence of retinopathy was found to be 64% (NPDR 37% and PDR 27%) in our study. In DIAMOND study, it was observed that the higher incidence of DR may be attributed to the asymptomatic nature of the microvascular complication and the lack of awareness resulting in non-adherence to eye care guidelines and poor visual outcomes [20].

Retinal microvascular abnormalities may reflect early subclinical coronary or cerebral microvascular changes which may predispose to the development of clinical cardiovascular events [21]. DR which can be monitored non-invasively is a useful guide to measure diabetic microangiopathic burden,

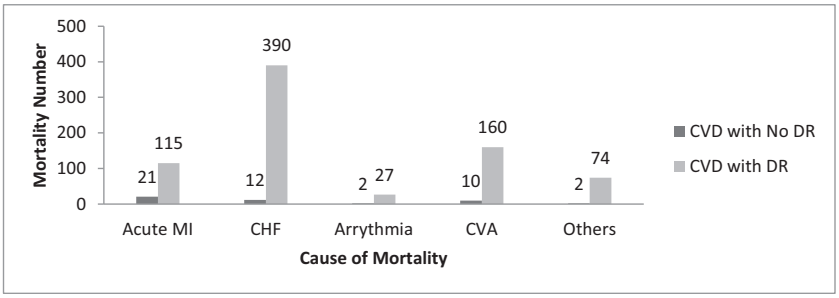

Fig. 1 Causes of mortality

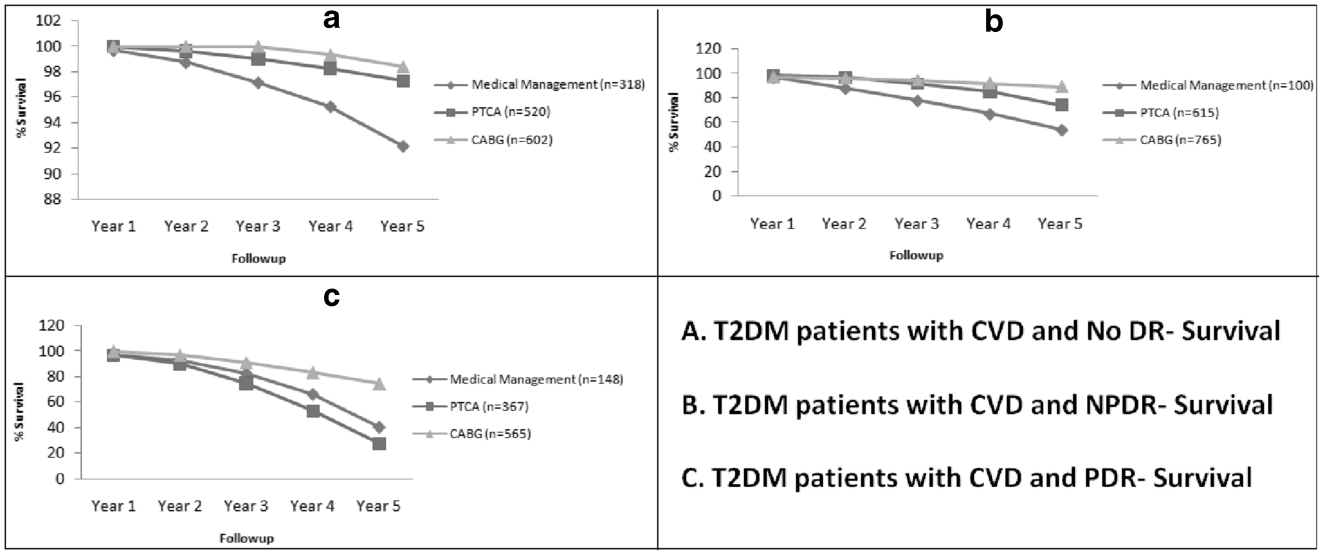

Fig. 2 T2DM patients with CVD and survival

which can serve as a possible biomarker to improve cardiovascular outcomes in patients with diabetes [13].

In our study, it was noted that there is a close relationship between DR and CVD. Highest association was observed for CHF (49.15%), MI (33.02%), and UA (13.9%) followed by cardiomyopathy (3.9%) ($p < 0.00001$). Some of the studies have concluded that DR is associated with decreased left ventricular function in T2DM patients. Low-grade inflammation and endothelial dysfunction are postulated as the association between DR and left ventricular dysfunction [22, 23]. Cheung et al. reported that the existence of DR signifies a surplus risk of HF. This further supports a contribution of microvascular disease to the development of HF in people with diabetes [24]. The patients with advanced DR are at a higher risk for CHF, suggesting that these patients have irreversible diabetic cardiomyopathy.

The major difference between the findings of previous studies and our 5-year follow-up study is that the presence of DR was associated with new CHD events (17.45%) independent of glycemic levels and CV risk factors. Patients with NPDR had a higher incidence of in-stent restenosis, whereas patients with PDR had a higher incidence of CHF and mortality [25–27]. In our study, patients with NPDR had the worst outcomes. Diabetic patients with NPDR are usually less vigorously treated which may lead to worst adverse outcomes. We feel NPDR has been overlooked in terms of its clinical significance and research attributes. Diabetic patients with PDR not only have poor general health but also have a poor life expectancy.

Ohno et al. predicted that retinopathy can be a prognostic predictor for diabetic patients undergoing revascularization procedure [27]. It has been reported that diabetic patients with DR has distinctive post-revascularization course as compared with diabetic patients without retinopathy. Diabetic patients with NPDR were more likely to have large number of diseased coronary vessels and implanted stents and to require the higher maximal pressure during procedure, suggesting that these patients had more complex, extensive, and diffuse coronary disease. Ohno et al. reported that diabetic patients with DR had a distinct post-CABG course as compared with diabetic patients without retinopathy. CABG conferred a survival advantage in diabetic patients with retinopathy [28]. It has been documented that CABG outcomes in advanced DR patients are suboptimal as compared to those in non-diabetic patients. This may be due to accelerated development of atherosclerosis [29]. More fatal events were reported in diabetic patients with retinopathy as compared to those in diabetic patients without retinopathy. Overall mortality during the study was 20.32%. Significantly high mortality rate was noted in patients with DR as compared to that in patients who were without DR (DR 94.2% vs. non-DR 5.78%; $p < 0.00001$). In our study, we have noted that patients who underwent revascularization (PTCA/CABG) had a higher prevalence of DR as compared to patients on medical management ($p < 0.00001$). In the present study, better survivor benefits were noted in diabetic patients with NPDR and PDR who underwent CABG compared to those in patients who underwent PTCA.

Large population-based cohort and detailed collection of data are some of the strengths of the present study. Longer duration of follow-up of the patients and best possible utilization of data are also a plus point of the present study. Also, this study succeeded to generate hypothetical link between DR, CVD, and atherosclerosis. Being a retrospective study, there are limitations, like data collected relies on accuracy of written record or recall of individuals. It is difficult to establish cause and effect of the results that are obtained after data analysis; study results are best at only generating hypothesis and cannot get the benefits that are obtained from randomization or blind study.

Conclusion

There is a close relationship between microvascular and macrovascular complications, and the relationship is in continuum and not distinct. This retrospective study highlights the importance of DR beyond ocular morbidity in patients of T2DM with CVD. CV events, cerebrovascular events, mortality, and all-cause mortality were higher in patients with DR as compared to those in patients without retinopathy. Retinal status may be used as a guide for treatment decision in diabetic patients needing revascularization, especially in diabetic patients with diabetic retinopathy.

Compliance with ethical standards

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

Ethical approval This study was conducted in accordance with the principles of the Declaration of Helsinki, guidelines for good pharmacoepidemiology practice and local regulatory guidelines. Medical data of the patients who had given their prior consent was collected and analyzed. Being a retrospective, non-interventional study, ethics committee approval was not needed and therefore not obtained.

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ORIGINAL ARTICLE

Clinical and angiographic profile of early-onset type 2 diabetes mellitus in acute coronary syndrome

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Abstract
Early-onset type 2 diabetes mellitus has been recognized as a distinct phenotype with a probable unique pathophysiology in recent years. Although well known to have an aggressive clinical and metabolic phenotype, with rapid development of vascular complications, studies regarding coronary artery disease in this group are sparse, especially in the Indian context. A cross-sectional study was conducted in Kasturba Medical College, Manipal, between October 2015 and May 2017. Sixty-three adult patients with early-onset type 2 diabetes mellitus presenting with an acute coronary syndrome were included. Early-onset type 2 diabetes mellitus (T2DM) was defined as onset of type 2 diabetes mellitus at less than 45 years of age. Fasting and post prandial sugar levels, fasting lipid profile, and HbA1c levels were obtained. All patients underwent conventional angiography using the Seldinger technique. Coronary artery disease was evaluated using the SYNTAX score. Mitral regurgitation and left ventricular dysfunction were assessed using conventional transthoracic echocardiography. Patients had a poor metabolic profile and clustering of cardiovascular risk factors, with high BMI, poorly controlled diabetes mellitus, and dyslipidemia. Majority of the patients had triple/multi-vessel disease, with moderate to high SYNTAX scores. A significant number of patients required were advised CABG. Significant number of patients had mitral regurgitation and left ventricular dysfunction. Early-onset type 2 diabetes mellitus is an aggressive phenotype. More studies are necessary to elucidate its impact on coronary vasculature and pattern of coronary artery disease.

Keywords Early-onset type 2 diabetes mellitus · Acute coronary syndrome · Angiographic profile in early-onset type 2 diabetes mellitus · Clinical profile of early-onset type 2 diabetes mellitus

Introduction

Diabetes mellitus is a major cause of morbidity and mortality due to its macrovascular complications such as coronary artery disease. Haffner et al. termed diabetes mellitus a coronary equivalent [1] and results of the Framingham study [2] observed multifold increase in the risk of cardiovascular disease in patients with diabetes mellitus.

Early-onset type 2 diabetes mellitus has been recognized as a distinct entity in the last few years, with a probable unique pathophysiology [3–5]. It has been shown to have an aggressive clinical and metabolic phenotype as compared to later onset diabetes [6–9]. It has also been shown to present earlier with vascular complications [10, 11].

In India, diabetes mellitus occurs in patients a decade earlier than other populations, and so does coronary artery disease [12]. Studies regarding the pattern and severity of coronary artery disease in early-onset type 2 diabetes mellitus are few in the Indian context. The increasing prevalence of early-onset type 2 diabetes, as well as coronary artery disease, prompted us to pursue this study.

Materials and methods

A cross-sectional study was conducted in Kasturba Medical College, Manipal, between October 2015 and May 2017. The study comprised of 63 adult patients with early-onset type 2 diabetes mellitus (T2DM), who had presented to Kasturba Hospital, Manipal, with an acute coronary syndrome (ACS). Early-onset T2DM was

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defined as onset of type 2 diabetes mellitus at less than 45 years of age. Patients with other forms of diabetes, chronic liver disease, chronic kidney disease, and hematological malignancy were excluded. Patients with congenital heart disease, valvular heart disease, and other pre-existing heart disease were also excluded.

Subjects were approached individually and explained about the study in their preferred language and a signed consent was taken from the subjects who were willing to participate.

Information pertaining to study was collected using a semi-structured proforma, which included age, sex, occupation, age at detection of diabetes mellitus, duration of diabetes mellitus, history of hypertension, dyslipidemia, and smoking. Chief complaints and presenting features were noted.

Height and weight were noted, and BMI was calculated. Laboratory investigations included fasting and post prandial sugar levels, HbA1c levels, fasting lipid profile, and renal function tests. All patients underwent conventional angiography using the Seldinger technique. The pattern of vessel involvement was evaluated using the SYNTAX score. The SYNTAX score [13] is an angiographic objective scoring system for estimating the severity of the coronary artery disease (CAD) and grading its complexity. It helps in estimating outcome inclusive of mortality, while simultaneously establishing the best way to treat CAD in a given patient. The score utilizes information such as the number of vessels involved, length of vessels involved, degree of luminal narrowing, presence of vessel occlusion, age of occlusion, presence of collaterals, and anatomical variations. The total number of patients advised/undergoing percutaneous coronary intervention (PCI)/percutaneous transluminal coronary angiography (PTCA) and coronary artery bypass graft (CABG) was noted.

Additionally, information regarding papillary muscle dysfunction was obtained in the form of presence and severity of mitral regurgitation, and left ventricular dysfunction by estimating ejection fraction, using conventional transthoracic echocardiography. Other factors assessed were development of cardiogenic shock, need for inotropes, and development of post ACS/post procedure pericarditis, post ACS arrhythmias, and need for a pacemaker.

Statistical methods

Continuous variables were compared with one-way ANOVA if the data was of normal distribution and with non-parametric tests if the data was skewed.

Categorical variables were compared with chi square test if the data was of normal distribution. Correlations were assessed using bivariate analysis. p values less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS version 20.

Results

Clinical and biochemical data are summarized in Table 1. A total of 63 patients were included in the study. Majority of the patients in the study were males. The median duration of early-onset type 2 diabetes mellitus prior to presentation with ACS was 3 years, with a range of newly detected diabetes up to 8 years.

History of hypertension was noted in 10 patients (15.87%). History of dyslipidemia as per ATP III guidelines was noted in three patients (4.76%). Twelve patients (19.04%) had a history of smoking. The mean BMI was 24.35 ± 2.03. As per Asia-Pacific guidelines, 42.85% were overweight (n = 27) and 33.33% were obese (n = 21). The mean glycosylated hemoglobin level was 8.78 ± 2.01%, indicative of poor glycemic control. Forty-one (65.07%) of the 63 patients had a HbA1c level > 7.5%. Thirty-three of these patients (52.38%) had glycosylated hemoglobin levels of > 8.5%. Fasting lipid profile levels post admission are expressed in Table 1.

Angiographic data is summarized in Table 2. All patients underwent conventional angiography using the Seldinger technique. Majority of the patients had triple vessel disease (n = 23, 36.5%). A significant number of patients had moderate to high SYNTAX scores (n = 27, 42.85%). Nearly 1 in every 4 patients was advised to undergo/underwent CABG.

In terms of morbidity incurred, 27 (42.85%) of the 63 patients had papillary muscle dysfunction in the form of mitral regurgitation. Left ventricular dysfunction, defined as ejection fraction (EF) < 60%, was present in 28 patients (44.44%). Data is summarized in Table 3.

Four patients (6.34%) developed cardiogenic shock. No patients developed arrhythmias or pericarditis. None of the patients required a pacemaker implantation.

Table 1 Clinical and biochemical characteristics of early-onset T2DM. (*Mean ± SD)

Variable	Value
Age (years)	42.6± 3.08
Male gender (%)	82.5
Duration T2DM prior to ACS (years)	3 years (IQR 2, 5)
History of hypertension	10 (15.87%)
History of dyslipidemia	3 (4.76%)
BMI (kg/m^2)	24.35 ± 2.03*
HbA1c (%)	8.78 ± 2.01*
Total cholesterol (mg/dl)	184.62 ± 49.47*
HDL (mg/dl)	37.56 ± 17.87*
LDL (mg/dl)	108.65 ± 48.01*

Table 2 Angiographic characteristics in ACS in early-onset T2DM

Vessel disease	Number of patients (%)
Normal vessels	12 (19.04)
Single vessel disease	11 (17.46)
Double vessel disease	14 (22.22)
Triple vessel disease/multi-vessel disease	23 (36.50)
Left main coronary artery involvement	3 (4.76)
SYNTAX score	
≤ 22	36 (57.14)
23–32	15 (23.80)
≥ 33	12 (19.04)
Procedure advised/undergone	
Medical management	12 (19.04)
PCI/PTCA	29 (46.03)
CABG	16 (25.39)
MVPCI/CABG	6 (9.52)

SYNTAX score groups as per SYNTAX trial

PCI percutaneous coronary intervention, *PTCA* percutaneous transluminal coronary angioplasty, *CABG* coronary artery bypass graft, *MVPCI* multi-vessel PCI

We analyzed the impact of duration of diabetes mellitus on the severity of SYNTAX score and number of vessels involved by CAD. No correlation was found between the duration of early-onset T2DM and severity of SYNTAX score (Pearson coefficient − 0.214, *p* value 0.09) (Fig. 1). The median duration of disease was 3, 2, and 2 years for the SYNTAX score groups of ≤ 22, 23–32, and ≥ 33 respectively (Table 4).

Similarly, data analysis to see if number of vessels involved by CAD increased with the duration of diabetes showed no statistical significance (*p* value 0.6).

We studied the correlation between HbA1c levels and severity of SYNTAX score and whether a higher HbA1c level was associated with more number of vessels involved by CAD. We found no correlation between HbA1c levels and severity of SYNTAX scores with a Pearson coefficient of 0.03 and *p* value 0.818. (Fig. 2).

Table 3 Morbidity in ACS in early-onset T2DM

Mitral regurgitation (MR)	Number of patients (%)
Mild MR	18 (28.57)
Moderate MR	7 (11.11)
Severe MR	2 (3.17)
Left ventricular dysfunction (EF < 60%)	
Mild (EF 50–59%)	10 (15.87)
Moderate (EF 40–49%)	8 (12.69)
Severe (EF < 40%)	10 (15.87)

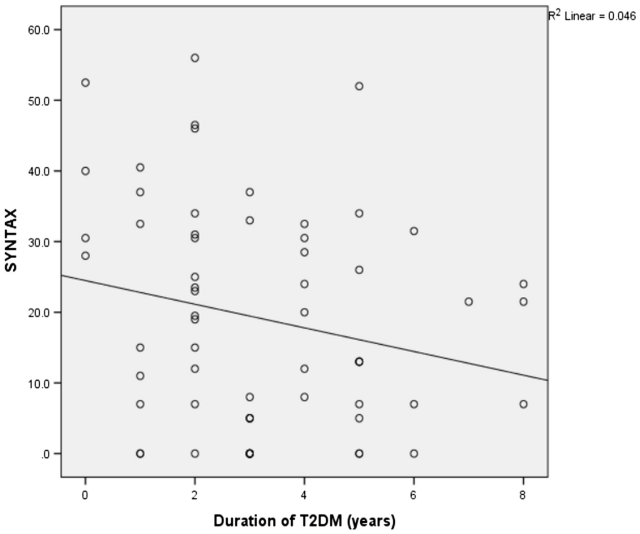

Fig. 1 The correlation between the duration of early-onset T2DM and the severity of SYNTAX score

HbA1c levels across the three SYNTAX score groups are shown in Table 5. No significant difference was noted between the three groups (*p* value 1).

Data analysis to assess if a higher HbA1c levels had more number of vessels involved by disease showed no significant difference in HbA1c values across the groups, with *p* value 0.09 (Table 6).

Discussion

Various studies have been conducted over in different parts of the world concerning clinical characteristics of early-onset type 2 diabetes mellitus. Age as a cutoff has been a point of disparity, with some studies considering the cutoff as onset at/before 40 years of age, while others considering the same as onset at/before 45 years of age [6–8]. In light of these studies, we took 45 years as our cutoff for early-onset type2 diabetes mellitus.

Risk factor analysis for the ACS in such a young group of diabetics showed that unlike prior studies, very few patients had a documented history of hypertension and dyslipidemia.

Table 4 Median duration of diabetes in different severities of vessel disease

Vessel involvement	Number of patients	Duration of diabetes (median, IQR 25–75)
Normal vessels	12	3 (2.25, 4.5)
Single vessel disease	11	3 (2, 7)
Double vessel disease	14	2 (2, 4)
Triple/multi-vessel disease	23	2 (2, 4)
Left main coronary artery involvement	3	5 (0, –)

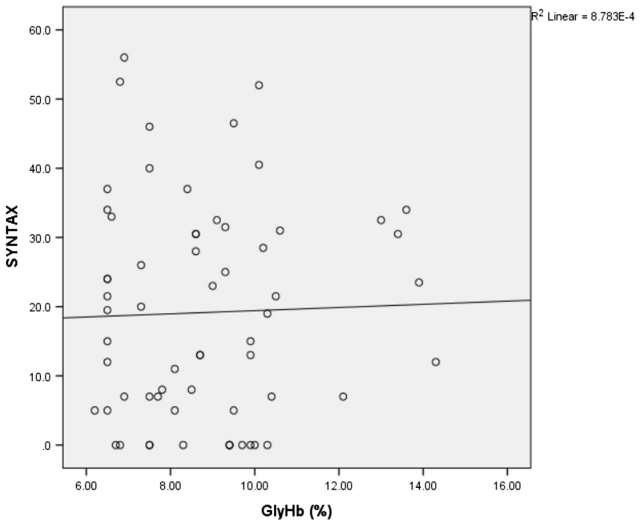

Fig. 2 The correlation between HbA1c levels and severity of SYNTAX scores

However, post admission for ACS, dyslipidemia was found to be prevalent with high total cholesterol and LDL levels that had probably remained undetected prior to presentation with ACS [6, 8, 15]. Smoking was not common, and we hypothesize that coronary artery disease observed in our study was due to factors other than smoking and that diabetes probably played a larger role in pathogenesis in this group.

As per Asia-Pacific guidelines, the average BMI for the cohort of early-onset type 2 diabetes mellitus was high and a significant number of people were overweight and obese, in keeping with previous data from Indian studies [14, 15]. Obesity may have caused insulin resistance which led to an atherogenic lipid profile and early atherosclerosis and CAD.

A poor glycemic control with high HbA1c levels was noted, as seen in both Indian and international studies [6, 7, 14]. Hyperglycemia can cause atherosclerosis by mechanisms such as endothelial dysfunction by AGE-RAGE pathway, ROS-mediated plaque instability, with increased propensity to rupture and increase expression of glycoprotein Ib which might explain the thrombogenic state in diabetics [16, 17]. In our study, the impact of duration of disease could not be assessed as the patients were young, follow-up was not involved, and majority had a similar duration of illness. However, the role of dysglycemia was evident by the high HbA1c levels.

On angiography, majority of the patients had triple vessel disease/multi-vessel disease. That early-onset type 2 diabetes mellitus as a phenotype bears a more aggressive nature and has a worse cardiovascular outcome has been described by Song et al. [10] where his cohort had similar level of CVD and microvascular complications around two decades earlier than their usual onset type 2 diabetes mellitus counterparts. Hillier and Pedula concluded a 14 times increased relative risk of cardiovascular mortality due to MI in the early-onset group with respect to age- and sex-matched non-diabetics in comparison to only a 4 times increased risk of cardiovascular mortality in usual onset T2DM w.r.t. age- and sex-matched controls. [11] However, major studies regarding the pattern of vessel involvement as well as severity of vessel involvement in early-onset type 2 diabetes mellitus are sparse.

Table 5 HbA1c levels in SYNTAX score groups

SYNTAX score	HbA1c (%)
≤ 22	8.60 ± 1.79
23–32	9.59 ± 2.30
≥ 33	8.33 ± 2.15

Table 6 HbA1c levels across different severities of vessel disease

Vessel involvement	HbA1c levels (mean ± SD)
Normal vessels	8.74 ± 1.30
Single vessel disease	8.40 ± 1.95
Double vessel disease	8.95 ± 2.47
Triple/multi-vessel disease	8.71 ± 1.95
Left main coronary artery involvement	10.16 ± 3.40

Between SYNTAX score and the number of vessels involved, SYNTAX scores give a better idea of the morbidity incurred, the effectiveness of therapy, and the prognosis of the CAD, as it assesses the complexity of lesions. As an example, a person with 60% block of the three main coronaries would be classified as a TVD, as would a person with 80–90% block.

A significant number of our young cohort (average age 42 years) had a SYNTAX score with moderate to severe disease, with duration of disease of only 2–3 years, suggesting an aggressive phenotype for early-onset diabetes mellitus. Higher SYNTAX scores signify more complex lesions, higher risk of ACS, and more need of intervention, even surgical. In keeping with the aforementioned points, nearly every 1 in 4 patients was advised to undergo or underwent a CABG in our study.

Incidence of mitral regurgitation and left ventricular dysfunction paralleled the severity of SYNTAX scores noted, suggesting larger infarcts involving papillary muscles.

We analyzed the data as to why this group of patients presented with ACS. We postulated that glycemic parameters like longer duration of diabetes mellitus and higher HbA1c levels may have translated into higher SYNTAX scores and worse CAD and more vessel involvement.

However, this was not so. The duration of early-onset type 2 diabetes mellitus and the SYNTAX score obtained had no correlation (Pearson coefficient − 0.214). One possible reason for the same could be that majority of the patients had a similar disease duration of 2–3 years, and this may have been a too narrow window to show any demonstrable difference due to glycemic exposure.

Further, the severity of disease had great heterogeneity, with SYNTAX scores ranging from < 10 to > 40. Duration

of disease may not be the sole determinant of CAD severity, which is rather a complex interplay of many factors. Hence, studies with larger number of patients may be needed to further elucidate the relationship.

HbA1c levels and SYNTAX scores did not show correlation (Pearson coefficient 0.03). This could be due to the fact that hyperglycemia is only one of the factors affecting atherosclerosis, as shown in the UKPDS. HbA1c also did not correlate with the number of vessels involved.

In conclusion, patients with early-onset type 2 diabetes mellitus even with a short duration of disease presented with an ACS. They had significant number of triple vessel and multi-vessel disease with high SYNTAX scores, with a high number of patients requiring CABG. They also had significant left ventricular dysfunction and mitral regurgitation. All these points suggest an aggressive underlying phenotype. Duration of disease and absolute HbA1c levels are not sufficient to explain the results obtained, and further studies are necessary to elucidate other determinants.

Strengths and limitations

Conventional coronary angiography was done for all the patients, with SYNTAX scores documented. Echocardiography was done for all patients. There was no missing data. It is one of the few studies in India in patients presenting with ACS in such a young diabetic patient pool.

The study has limitations of being cross sectional. Follow-up of the patients to understand long-term profile of the disease could not be done.

Compliance with ethical standards

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

Ethical approval All patients underwent conventional angiography as part of the evaluation for ACS. Ethical clearance was obtained from the IEC, Kasturba Hospital, Manipal, prior to initiation of the study. Informed consent was obtained from all individual participants included in the study. Ethics committee approval was obtained from the Institutional Ethics Committee of Kasturba Medical College, Manipal, prior to the commencement of the study.

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ORIGINAL ARTICLE

Genome-wide implicated risk variants of *TCF7L2* gene contribute to type 2 diabetes susceptibility by modulating serum lipids in Pakistani population

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Abstract
Diabetes is a socioeconomic burden worldwide as well as in Pakistan. International Diabetes Federation reported 7 million cases of diabetes and 86,384 deaths due to diabetes in Pakistan in 2015. Transcription factor 7 like 2 is a transcription factor encoded by *TCF7L2* gene and regulates several physiological processes in the cell. The aim of the current study was to determine the prevalence of two *TCF7L2* gene polymorphisms and analyze its effect on various anthropometric and biochemical parameters in a Pakistani cohort. We collected 926 samples, 500 healthy controls (fasting blood sugar < 99 mg/dL, random blood sugar < 126 mg/dL) and 426 cases with diabetes (fasting blood sugar > 99 mg/dL, random blood sugar > 126 mg/dL). The genotyping was done by RFLP-PCR and serum biochemical parameters were determined. The genotyping results of rs7903146 by RLFP-PCR showed the allelic frequency of $C = 0.64$ and $T = 0.36$ in controls while $C = 0.59$ and $T = 0.41$ in cases and genotypic frequency CC = 43.2%, CT = 41.6%, and TT = 15.2% in controls while CC = 34.03%, CT = 49.06%, and TT = 16.91% in cases. The minor T allele appeared to be a risk allele (OR = 1.25, CI = 1.03–1.51, $p = 0.016$), i.e., it increased the risk of diabetes in the selected study subjects. The genotyping results of rs12255372 showed allelic frequency $G = 0.75$ and $T = 0.25$ in controls while $G = 0.63$ and $T = 0.37$ in cases and genotypic frequency GG = 54.2%, GT = 42.2%, and TT = 3.6% in controls, while GG = 40.14%, GT = 46.24%, and TT = 13.62% in cases. The minor T allele appeared to be a risk allele for this SNP as well (OR = 1.77, CI = 1.44–2.16, $p = 1.9 \times 10^{-8}$). The biochemical parameters and general characteristics were checked for association with the *TCF7L2* variant rs7903146, showed no association with all tested parameters. While rs12255372 had significant association with fasting plasma glucose, triglycerides, HDLC, LDLC, and leptin, but no association with total cholesterol, age, height, weight, and BMI. However, the multivariate analysis showed significant association of lipid parameters only when homozygous risk genotype of rs12255372 co-existed with all genotypes of rs7903146. In conclusion, the polymorphisms in *TCF7L2* gene can increase the susceptibility to diabetes and the effect may involve modulating certain serum traits.

Keywords Diabetes · Transcription factor 7-like 2 (*TCF7L2*) · rs7903146 · rs12255372 · Pakistan

Introduction

Type 2 diabetes is a metabolic disorder characterized by chronic hyperglycemia and insulin resistance [1]. It is a serious health issue worldwide as well as in Pakistan. It is widespread in approximately all the regions of Pakistan, with an overall occurrence of 22.04% in urban and 17.15% in rural areas. The genderwise division of diabetes in different provinces of Pakistan is: Punjab; males 16.6%, females 19.3%, Khyber PakhtoonKhuwa; 11.1% both sexes, Balochistan; 10.8% both sexes, Sindh; males 16.2%, females 11.7% [2].

TCF7L2 (transcription factor 7-like 2) is a Wnt signaling-associated transcription factor [3]. It is expressed in various tissues like gut and pancreas. It is involved in the development of pancreatic cells, induces the expression of glucagon-like peptide 1 (GLP-1) encoding genes and exocytosis of insulin granules [4]. *TCF7L2* gene is located on the long arm of chromosome 10q25.3. In the past, TCF7L2 was also known as TCF-4 [5].

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The product of *TCF7L2* gene is a high mobility box-containing transcription factor important in signaling. The major function of this pathway is the induction of proliferation of pancreatic cells, lipid, and glucose metabolism [6]. Variations in this pathway lead to the decreased GLP-1 production, disturbed insulin secretion, and ultimately impaired blood glucose homeostasis [7]. Genome-wide association studies have identified several loci of *TCF7L2* showing significant association with type 2 diabetes [8].

TCF7L2 gene polymorphisms rs7903146 (C/T) and rs12255372 (G/T) have previously been reported to show strong association with T2DM in subjects from Colombia [9], Brazil [10], Mexico [11], and Asia [12]. However, there is no report available on the pattern of association of this variant in the Pakistani subjects. The purpose of the current study was, therefore, to compare the association pattern of the selected *TCF7L2* gene polymorphisms with diabetes in the diabetic patients and healthy controls belonging to Pakistan, and find out if these SNPs have any effects on selected anthropometric and serum biochemical traits.

Methods

Subject recruitment

A total of 926 subjects (426 diabetic cases and 500 normal controls) were collected from the local population. The subject recruitment, inclusion, and exclusion criteria have been described previously [13].

Blood sampling

Five milliliter venous blood was collected from the median cubital vein via aseptic procedures. Half was poured in an EDTA vial for DNA isolation while the rest half was poured in a gel clot activator containing vial for biochemical analysis.

Biochemical parameters' determination

Gel clot activator containing vial was centrifuged to separate out plasma for determination of biochemical parameters. Serum was screened for HBV, HCV, and HIV. Serum fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), and high density and low density lipoprotein cholesterol (HDL-c, LDL-c) levels were determined using commercially available kits (Spectrum Diagnostics, Egypt).

Genotyping

Genomic DNA was isolated manually by salting out method and results were checked on 1% agarose gel. The DNA was quantified by measuring the optical density and was standardized to a final concentration of 100 ng/μl. PCR was done with the following sets of primers, for rs7903146 polymorphism: forward primer 5′-AAGAAGATTCCTTTTTAAATGGTG-3′ and reverse primer 5′-CCTCATACGGCAATTAAATTATACA-3′; for rs12255372 polymorphism: forward primer 5′-CTGG AAACTAAGGCGTGAGG-3′ and reverse primer 5′-GGGT CGATGTTGTTGAGCTT-3′. PCR consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, extension at 72 °C for 45 s and a final extension at 72 °C for 10 min.

Advanced Primus 96 (PeqLab) thermal cycler was used for amplification. For rs7903146, 251 bp PCR product was subjected to restriction digestion using restriction enzyme *Taa*1 at 30 °C for 6 h [14]. It cuts the product in wild type state producing fragments of 139 bp and 112 bp. PCR and digestion products were analyzed on 2% agarose gel. While for SNP rs12255372, the product size was 346 bp. Restriction digestion was done by using *Tsa1* and incubated at 30 °C for 6 h [15]. *Tsa1* cuts the product in wild type state and producing three fragments of different length 143 bp, 126 bp, and 104 bp respectively.

Statistical analysis

The statistical analyses were done using statistical package for social sciences (SPSS) IBM, version 22. The quantitative variables (e.g., age, blood lipid levels) were checked for normality and were compared between the cases and the controls using independent sample *t* test. Allele and genotype frequencies were tested for Hardy Weinberg equilibrium. The risk allele frequencies (RAFs) were compared between the cases and the controls using χ^2 test. Since diabetes is a binary variable, the association of the SNPs with diabetes was examined using binary logistic regression. The association of the selected SNPs with diabetes was also adjusted for the confounding variables like age, sex, blood lipids, and hypertension. Quantitative variables were expressed as mean ± standard deviation (SD) and categorical variables were expressed as percentage. The differences of the demographic, clinical, and biochemical characteristics between the cases and the controls were estimated using the chi-square test (for categorical variables) and Student *t* test (for continuous variables). Linear regression analysis was applied to find the association between the SNPs and continuous lipid (Total cholesterol, TG, LDL, and HDL) values when adjusted for confounding factors (age and sex). Non normal variables (TG and HDL) were log transformed where appropriate. Due to inclusion of two SNPs at a time, values were considered statistically significant at a corrected *p* value of < 0.025 ($0.05/2 = 0.025$).

Results

The general characteristics of the subjects have been described elsewhere [13] (Table 1). The fraction of diabetic patients suffering population from other disorders, e.g., cardiovascular disease, hypocholesteremia, hypertension, nephropathy, and retinopathy was higher in the cases than controls (Table 2). The average blood pressure of the diabetic patients was normal 120/80 $mmHg^{-1}$; however, the patients suffering from cardiovascular disease and nephropathy showed high blood pressure 140/80 $mmHg^{-1}$. The mean BMI (Kg/m^2) of diabetic subjects was 22.7 ± 0.45 and of non- diabetics was 21.6 ± 0.43. The SNP genotyping call rate was > 90%, and the selected SNPs were not in LD as tested by the pairwise LD test available at http://archive.broadin stitute.org/mpg/snap/ldsearchpw.php. The study power was 91.9% assuming a standard deviation of 1 at 95% confidence level, as tested by statistical power calculator available at https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx.

Allele/genotype frequencies of TCF7L2 variants

After genotyping, we calculated the allele genotype *TCF7L2* polymorphisms. The genotyping results of rs7903146 showed allelic frequency of $C = 0.64$ and $T = 0.36$ in controls while $C = 0.59$ and $T = 0.41$ in cases and genotypic frequency CC = 43.2%, CT = 41.6%, TT = 15.2%, in controls while CC = 34.03%, CT = 49.06%, TT = 16.91% in cases (OR = 1.25, CI = 1.03–1.51, $p = 0.016$). The genotyping results of rs12255372 showed allelic frequency G = 0.75 and $T = 0.25$ in controls while G = 0.63 and $T = 0.37$ in cases and genotypic frequency GG = 54.2%, GT = 42.2%, TT = 3.6% in controls while GG = 40.14%, GT = 46.24%, TT = 13.62% in cases (OR = 1.77, CI = 1.44–2.16, $p = 1.9 \times 10^{-8}$) (Table 3). The number of cases and controls along with the OR, CI, and p values were checked across the combined genotypes and are presented in Table 4.

Table 1 Demographic characteristics of the study subjects

Parameters		Diabetic ($n = 426$)	Non-diabetic ($n = 500$)	p value
Gender	Male	55%	53%	0.5
	Female	45%	46.7%	0.318
Age (yr)		47.55 ± 12.3	35.78 ± 13.4	0.0001*
Height (ft)		5.50 ± 0.3	5.49 ± 0.59	0.756
Weight (kg)		68.6 ± 13.83	65.38 ± 13.7	0.0004*
BMI (kg/m^2)		22.7 ± 5.6	21.67 ± 5.3	0.0043*

BMI body mass index, *n* total number

*Indicates significant differences

Table 2 Prevalence of comorbidities

Disorders	Frequency (%)	
	Cases ($n = 426$)	Controls ($n = 500$)
Cardiovascular disease	8.1%	2%
Nephropathy	7%	0
Retinopathy	3.4%	0
Foot ulcer	3.8%	0
Hypocholesteremia	7%	1%
Hypertension	8.9%	2.3%

Effect of the TCF7L2 variants on anthropometric parameters

Anthropometric parameters were checked by one way ANOVA. In rs7903146, the mean height was CC = 5.52 ± 0.03, CT = 5.52 ± 0.03, and TT = 5.51 ± 0.04 (p value 0.952), the mean weight was CC = 61.63 ± 1.18, CT = 63.21 ± 1.17, and TT = 60.84 ± 1.63 (p value 0.427), and the average BMI value was CC = 21.73 ± 0.49, CT = 22.43 ± 0.48, and TT = 21.96 ± 0.71 (p value 0.610). In rs12255372, the mean height was GG = 5.53 ± 0.02, GT = 5.52 ± 0.03, and TT = 5.43 ± 0.07 (p value 0.384), the mean weight was GG = 61.73 ± 1.03, GT = 62.29 ± 1.17, and TT = 63.64 ± 2.65 (p value 0.770), and the average BMI value was GG = 21.52 ± 0.43, GT = 22.33 ± 0.5, and TT = 24.06 ± 0.9 (p value 0.069) (Supplementary Table 1).

Effect of the TCF7L2 variants on biochemical parameters

Biochemical parameters were checked across the three genotypes for both SNPs. In rs12255372, the mean value of glucose was GG = 158.56 ± 5.14, GT = 177.19 ± 7.47 and TT = 190.17 ± 15.32 (p value 0.0350); the mean triglycerides value was

Table 3 Allelic and genotypic frequency in study subjects.

SNP	Allele/ Genotype	Controls	Cases	Odds ratio	95% CI	p value
rs7903146	C	640	500	1.25	1.03–1.51	0.016*
	T	360	352			
	CC	216	145			
	CT	208	209			
	TT	76	72			
rs12255372	G	753	539	1.77	1.44–2.16	1.9×10^{-8}*
	T	247	313			
	GG	271	171			
	GT	211	197			
	TT	18	58			

CI confidence interval

*Shows significant association

Table 4 Diabetes by combined genotype groups

rs7903146	rs12255372	*N* controls/cases	OR (95% CI)	*p* value
CC	GG	63/38	1.00 (0.21–1.22)	0.11
	GT	61/52	1.01 (0.44–2.01)	0.75
	TT	10/18	2.75 (0.33–4.25)	0.28
CT	GG	142/107	1.05 (0.89–2.11)	0.18
	GT	108/44	1.31 (0.75–2.61)	0.12
	TT	14/16	1.32 (0.32–2.71)	0.41
TT	GG	65/86	1.21 (0.75–1.75)	0.34
	GT	31/44	1.34 (0.95–2.21)	0.01
	TT	6/21	1.29 (0.33–2.61)	0.35

GG = 5.00 ± 0.07, GT = 5.06 ± 0.07, and TT = 5.53 ± 0.15 (*p* value 0.019); the mean total cholesterol was GG = 2.30 ± 0.05, GT = 2.38 ± 0.05, and TT = 2.56 ± 0.11) (*p* value 0.165; the mean LDLC was GG = 1.43 ± 0.03, GT = 1.42 ± 0.03, and TT = 1.25 ± 0.03 (*p* value 0.046); and the mean HDLC was GG = 2.54 ± 0.04, GT = 2.62 ± 0.04 and TT = 2.99 ± 0.12 (*p* value 0.001). For rs7903146, none of the tested biochemical parameters showed significant association (supplementary Table 2). Although the univariate analysis revealed some significant associations, we replicated the analysis for association of combined genotypes with lipid parameters in multivariate analysis using general linear model. The combined effect of both SNPs on was examined where common homozygous genotype of rs12255372 was examined against common homozygous, heterozygous and risk homozygous genotypes of rs7903146. The results of this analysis are shown in Table 5. The table shows that the presence of homozygous risk genotype of rs12255372 in any combination with rs7903146 (TT + CC, TT + CT, TT + TT) had greatest effect on the change of lipid parameters (significant rise in TC, TG, LDL-C and decrease in HDL-C).

Discussion

Diabetes is a serious health problem and poses a huge socio-economic burden worldwide [16, 17]. Diabetes mellitus is characterized by insulin deficiency, insulin resistance, β cell damage in pancreas, infection and impaired glucose secretion by pancreas [18]. The treatments are immediately required to subordinate downward the after effects and to stop the further complications of diabetes [19]. The identification of *TCF7L2* gene polymorphisms associated with T2DM was a major advancement in the field of diabetes genetics. For the first time, Grant et al. identified that *TCF7L2* gene variants increased the risk of diabetes among Icelandic population in 2006 [20]. Biochemical profiling is an important part of diabetes diagnosis and evaluation. The elevated total cholesterol, triglycerides, and LDLC result in a disturbed lipid profile that further contributes to the complications of diabetes. Our cases had dyslipidemic profiles in concordance with the previously reported findings from a similar ethnic group [21].

In the current study, the two SNPs of *TCF7L2* gene showed strong association with diabetes as tested by logistic regression analysis. Previously, a strong association of rs7903146 with diabetes has been reported in various populations like Lebanese [22], Urban Ghana [23], Iranian Kurdish [24], Omanis [25], and China [26]. Similarly, a strong association of rs12255372 with diabetes has been observed among different populations as Asian Indian [27], Japanese [28], Mexican [29], African-American [30], and Finnish populations [31].

The minor allele frequency (MAF) was higher in the cases than the controls as compared to the major allele (C for rs7903146, G for rs12255372). The odds ratio is higher for risk allele ($p = 0.017$) rs7903146 and for rs12255372 as well ($p = 7.03 \times 10^{-5}$). Similarly, the genotypes bearing the risk allele, i.e., CT/TT for rs7903146 and GT/TT for rs12255372, were higher in the cases than the controls. Odds ratio for rs7903146 shows there is a 1.81-fold increased risk of diabetes in our population (95% CI 1.35–2.34). Similarly, odds ratio for rs12255372 showed there is a 1.32-fold increased risk of diabetes in our population (95% CI 1.03–1.69).

The results of the current study showed a diverse pattern of association; age, height, weight, and BMI were significantly different between the cases and the controls. For biochemical parameters, fasting plasma glucose, triglycerides, total

Table 5 Lipid profile parameters by combined genotype groups

rs7903146	rs12255372	N	TC (mmol/L)	TC (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
CC	GG	101	4.91 ± 1.42	2.16 ± 0.73	1.60 ± 0.53	2.30 ± 0.53
	GT	113	5.06 ± 1.38	2.21 ± 0.70	1.46 ± 0.47	2.49 ± 0.65
	TT	28	4.97 ± 0.79	2.39 ± 0.68	1.40 ± 0.53	2.68 ± 0.68
CT	GG	249	4.86 ± 1.35	2.20 ± 0.77	1.55 ± 0.47	2.37 ± 0.67
	GT	152	5.06 ± 1.35	2.13 ± 0.69	1.54 ± 0.48	2.35 ± 0.54
	TT	30	5.51 ± 1.57	2.76 ± 1.14	1.26 ± 0.29	2.57 ± 0.82
TT	GG	151	5.20 ± 1.57	2.34 ± 0.85	1.42 ± 0.39	2.47 ± 0.64
	GT	75	5.05 ± 1.34	2.50 ± 0.87	1.27 ± 0.41	2.48 ± 0.59
	TT	27	4.83 ± 1.19	2.40 ± 0.72	1.21 ± 0.37	2.48 ± 0.79

cholesterol, leptin, LDLC, and HDLC showed significant deviation from normal ranges in the cases compared to the controls. Similarly, the genotyping results showed a highly significant association of rs7903146 and 12255372 with diabetes.

The limitations of this study include a relatively small sample size, samples restricted to the Punjab province only, and relatively high prevalence of comorbidities in the cases. The results should therefore be validated by future larger studies with subjects included from all provinces of Pakistan. However, despite these limitations, the results from the current study demonstrate the role of common variants in the progression of complex diseases like diabetes in the Pakistani population, which is a less studied but unique ethnic groups due to religious, cultural, and social restrictions.

Conclusion

In conclusion, these findings suggest that the presence of common polymorphisms may contribute to increase the risk of type 2 diabetes in the Pakistani population. The allele as well as genotype frequencies for both the SNPs in the *TCF7L2* gene were higher in cases compared to controls and the observed associations with some serum traits indicate the multifactorial nature of complex diseases like diabetes.

Author contributions SS performed the experiments, analyzed the results, and wrote the manuscript. Shabana designed and supervised the study, proofread the manuscript, and provided technical support.

Compliance with ethical standards

Conflicts of interest All authors declare that they have no conflict of interest.

Ethical approval All the study procedures were according to the Helsinki declaration and ethical approval was obtained from the institutional ethical board.

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.

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

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ORIGINAL ARTICLE

Association of apolipoprotein A-V with mRNA expression of IL-6 and NF-κB genes in type 2 diabetes with hypertriglyceridemia: a possible link with inflammation

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Abstract

Background Hypertriglyceridemia and inflammation are implicated in cardiovascular complications in type 2 diabetes mellitus (T2DM). While some studies have evaluated apolipoprotein A-V protein (Apo A-V) in diabetes and its effect on triglycerides (TG) levels, only a few have explored its relation with inflammatory markers.

Objectives To evaluate the expression of mRNA genes involved in inflammatory response (IL-6 and NF-κB) and to study their association with Apo A-V in patients of T2DM, with and without hypertriglyceridemia.

Methods Two groups of T2DM patients, comprising of 40 participants each, were constituted according to NCEP ATP III criteria for CVD risk: group 1/controls (TG ≤ 1.65 mmol/l) and group 2/cases (TG ≥ 2.2 mmol/l). Serum levels of Apo A-V, free fatty acids (FFA) and IL-6 were estimated. Fold change in mRNA expression of IL-6 and NF-κB (p65) gene in blood was calculated by ΔΔCT method.

Observations The mRNA expression of IL-6 and p65 was higher in cases. Significant inverse association of Apo A-V levels was observed with expression of p65 gene ($p = 0.000$) and IL-6 gene ($p = 0.003$) in all subjects. FFA levels also correlated inversely with the expression of p65 ($p = 0.001$) and IL-6 ($p = 0.005$) mRNA. The association of FFA levels and ApoA-V with mRNA expression of IL-6 and p65 genes was independent of each other.

Conclusion This study highlights that inflammatory pathways are unregulated in hypertriglyceridemia in T2DM. Also, apart from its association with TG levels, Apo A-V may have an anti-inflammatory role as evident from its inverse association with the expression of IL-6 and NF-κB expression. Thus, Apo A-V may provide an important link between TGs, inflammation, and vascular complications in T2DM.

Keywords Apolipoprotein av., human · Hypertriglyceridemia · Type 2 diabetes mellitus · Free fatty acids · Nf kappa b p65 · il6

Introduction

Various apolipoproteins play an important role in metabolism of circulating triglycerides (TGs), such as apolipoproteins A-II, C-II, and C-III. Apolipoprotein A-V protein (Apo A-V), a new addition to the family of apolipoproteins is believed to have a role in metabolism of TGs. Apolipoprotein A5 (*ApoA5*) gene was discovered when comparative genomic analysis was carried out in the *ApoA1/ApoC3/ApoA4* gene cluster [1]. Apo A-V is an exchangeable apolipoprotein found to be associated with very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), and chlyomicrons in circulation. Since its discovery, Apo A-V is believed to be a key regulator of plasma TGs in spite of its very less concentration in circulation [1, 2]. There have been conflicting reports regarding the mechanisms by which it regulates the concentration of TGs. While some reports suggest that it inhibits the assembly and secretion of VLDL, others have attributed its role to the activation of lipoprotein lipase (LPL) [2].

Apo A-V concentrations have been found to be affected in type 2 diabetes mellitus (T2DM), a disease which currently affects 382 million people worldwide [3–5]. T2DM is

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associated with a constellation of metabolic abnormalities, including hyperglycemia, low levels of HDL-cholesterol, elevated levels of atherogenic, small dense LDL, and hypertriglyceridemia [6]. These metabolic aberrations in the pathways of lipid and glucose metabolism arise from low levels of insulin and/or resistance to the action of insulin in target tissues, which mainly include skeletal muscles, adipose tissue, and liver. Microvascular and macrovascular complications associated with diabetes add to both morbidity and mortality associated with this disease [6].

TGs are important mediators of inflammation in T2DM [7]. TG levels are raised in the disease due to an alteration in insulin-mediated modulation of lipoprotein lipase (LPL) [6]. In addition, de novo synthesis of TGs in liver also contributes to high circulating levels of TGs. These levels are an established marker for the increase in risk of cardiovascular diseases (CVD). According to the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP ATP III criteria), TG levels ≥ 2.2 mmol/L are believed to increase the risk substantially [8].

Chronic low-grade systemic inflammation plays an important role in the pathophysiology of development of T2DM as well as its associated vascular complications [9–12]. Pro-inflammatory factors like cytokines (interleukin-1b, interleukin–6), products of phospholipid hydrolysis (phosphotidylcholine, lysolecithin), tumor necrosis factors, etc. are believed to contribute to systemic inflammation, which is the hallmark of T2DM [12]. The importance of the role played by these inflammatory mediators in the diabetes complication has been proven by interventional studies, which have demonstrated beneficial effects of the drugs that inhibit these cytokines [13]. Earlier study has also demonstrated the effect of inflammatory mediators such as IL-6 and TNF-α in altering the expression of Apo A5 gene [14].

Nuclear factor kB (NF-κB) is a master regulator (transcription factor) which orchestrates the production of pro-inflammatory cytokines [15]. Activation of NF-κB is implicated in chronic disorders such as diabetes mellitus (DM) and its complications. Various studies conducted on humans and animals have reported that NF-κB (p65) level is increased in DM [16]. The pro-inflammatory cytokine, IL-6 is a downstream target for NF-κB and plays an important role in the development of insulin resistance. Its role in the pathogenesis of dyslipidemia in obesity has already been established [17].

Different studies conducted until now have reported either a mild to moderate positive or a negative correlation between Apo A-V and TG levels in diabetes patients [4, 18]. In most studies, Apo A-V has been studied in groups of patients of T2DM with widely varying levels of TG. DM is often but not always associated with hypertriglyceridemia. This would mean that the risk of inflammation and thus vascular complications would be different in those diabetic patients in whom hypertriglyceridemia is also present. In an effort to understand the pathophysiology of TG dysregulation and vascular complications, we evaluated the expression of pro inflammatory gene NF-κB and its downstream target IL-6 gene as well as the association of these genes with APOA-V in the groups categorized on the basis of their TG levels [8].

Methods

Study design and subjects We recruited 80 patients of T2DM in the age group of 35–60 years for a cross-sectional study. Cases were selected from the outpatient department of the Centre for Diabetes, Endocrinology and Metabolism at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Diagnosis of diabetes was made for each patient, as per the American Diabetes Association (ADA) criteria. [19] Patients of both genders were recruited for the study. Patients were only recruited in the study if they did not have any active infection, hypothyroidism, and were not taking drugs, such as insulin, hypolipidemic drugs, thiazide diuretic, and hormone replacement therapy. Patients with a past medical history of cerebrovascular accidents and cardiovascular disease were also excluded. General physical and systemic examination was carried out and anthropometric data was collected. A sample of blood was collected after overnight fasting for estimation of routine biochemical parameters and molecular studies. Patients who had deranged liver and renal function tests parameters were excluded from the study. Based on the levels of serum TGs, the patients were then divided into two groups with 40 participants each: group 1/controls: T2DM with TG < 1.65 mmol/l ($n = 40$), and group 2/cases: T2DM with TG ≥ 2.2 mmol/l ($n = 40$).

Power of study The power of study was calculated using the study conducted by Dalinga et al to evaluate Apo A-V levels in patient with normal and abnormal levels of TGs [4]. To detect a difference of 13 μg/100 ml in Apo A-V in patients of both groups, the sample size of 40 subjects in each group was sufficient with 80% power and 95% confidence level, with standard deviation of 20 μg/100 ml in both the groups.

Laboratory methods Blood was collected from each patient for biochemical analysis and genetic analysis, after an overnight fast. Serum was separated within 4 h, using a refrigerated centrifuge, followed by processing for routine biochemical analysis. An aliquot of serum was stored at – 80 °C for estimation of serum Apo A-V protein, IL-6, FFA levels. Biochemical parameters (such as serum urea, creatinine, AST, ALT, total bilirubin, TC, HDL, VLDL, TG, and plasma glucose) were estimated using system packs on DXC 800 Synchron (Beckman Coulter, USA). HbA1c was evaluated using HPLC (BIORAD D-10, USA). Fasting levels of Apo A-V (BT Biotech, China) and IL-6 (Komabiotech, Korea) were estimated using commercially available ELISA kit and following the manufacturer's protocol. Serum free fatty acid

(FFA) levels were estimated using fluorometric assay kit (Cayman, USA) following manufacturer's instructions.

mRNA expression analyses of IL-6 and p65 genes For RNA extraction, 250 μl of whole blood was stored with 750 μl of TRIZOL LS reagent (Invitrogen, USA). RNA was separated from the stored whole blood sample following the manufacturer's protocol. An absorption ratio of 1.8 (260 nm: 280 nm) was used as criteria for the purity for mRNA isolation. From the extracted mRNA, cDNA was synthesized using cDNA synthesis kit, following the manufacturer's protocol (Fermantas Inc., USA) in Thermocycler (Eppendorf Master CyclerGradient-5331, Germany). The cDNA was used as template sample for qPCR. REL-A gene that codes for p65 subunit was used for expression analysis of NF-κB. Specific primers were used for qPCR of IL-6 and p65 mRNA expression as well as for β-2 microglobulin (B2M) and 18 s which were used as housekeeping genes. The primer sequences are given in Table 1. Real-time PCR was carried out in a Thermocycler (QIAGEN Rotor-Gene Q, Netherlands). The reactions were set up in duplicates. Each reaction volume of 20 μl comprised of Maxima Hot start PCR Mix 2× (Fermantas Inc., USA), Syto9 dye (Invitrogen), primers (forward and reverse), cDNA, and nucleus free water (NFW). NFW was used instead of cDNA for nontemplate control (NTC). The PCR protocol included an initial activation temperature of 95 °C (4 min) followed by 35 cycles of amplification (denaturation for 15 s at 95 °C, annealing for 15 s at 54 °C, and extension for 30 s at 72 °C). The fluorescence was acquired at 72 °C. The results were analyzed as per described protocols.

Fold change in the expression of IL-6 and p65 was calculated using ΔΔ CT method taking B2M and 18 s as normalizers (housekeeping genes). ΔCT value was calculated by subtracting the CT value of the target gene from the CT value of the normalizer gene. Average ΔCT was then calculated for each group. ΔΔ CT value was calculated from the average ΔCT values by subtracting the average ΔCT of cases from the average ΔCT of controls. The fold chain was calculated as follows:

$$2^{-\Delta\Delta CT}.$$

Statistical analysis Data was analyzed using SPSS software, version 20.0 (Illinois, USA). The comparison of variables between groups was carried out using Student's *t* test for parametric data and Mann-Whitney *U* test was applied where the data was nonparametric. Correlation studies were carried out to evaluate the relationship between different parameters. ΔCT was used for mRNA expression as variable in correlation analysis. Pearson correlation coefficients were calculated where the data was parametric and Spearman rho's correlation was calculated for nonparametric data. Bivariate regression and partial regression were also carried out wherever required. Log transformations were done where the data did not show normal distribution. A p value less than 0.05 was considered statistically significant.

Results

Demographic and clinical and biochemical profile The recruited patients comprised of two groups, group 1/controls: patients of T2DM with TG ≤ 1.65 mmol/L ($n = 40$) and group 2/ cases: patients of T2DM with TG ≥ 2.2 mmol/L mg/dl ($n = 40$). Of the 40 patients in control group, 18 (45%) were male and 22 (55%) were female, whereas in cases group there were 14 (35%) male and 26 (65%) female. The mean age of patients in the control group (51.17 ± 10.42 years) was not statistically different ($p = 0.294$) from that of cases (48.68 ± 10.40 years). Demographic, anthropometric, and clinical data for both groups is given in Table 2 along with routine biochemical investigations. All the patients in the study were on oral hypoglycemic agents except one subject in the cases group who was being managed on diet alone. Six patients in each group were on antihypertensive medications. Obesity was diagnosed as per the criteria of International Diabetes Federation using ethnic specific waist circumference as the marker [20]. There were 33 obese patients in the control group and 38 obese individuals in the cases group. None of the routine biochemical parameters were significantly different between the groups except the levels of total cholesterols and TGs. Levels of serum Apo A-V protein were compared using Mann-Whitney *U* test. Apo A-V levels were significantly lower ($p = 0.04$) in cases group as compared to that in controls.

Expression of mRNA for IL-6 and p65 Fold change in mRNA expression of IL-6 and p65 genes was calculated from the CT values obtained from qPCR. Average ΔCT values for each gene, using 18 s and B2M as normalizer are depicted in Table 3. The mRNA expression of both IL-6 and p65 genes was higher in cases as evident from Table 3.

Table 1 Sequence of primers used in the study

GENE	Forward	Reverse
IL-6	CAGCCCTGAGAAAGGAGACAT	AGCCATCTTTGGAAGGTTCA
p65	CCTGGAGCAGGCTATCAGTC	ATGGATGAGAAAGGACAGG
B2M	TAGCTGTGCTCGCGCTACT	TCTCTGCTGGATGACGTGAG
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 2 Demographic and clinical profile of study patients [21]

Variables	Group 1 ($n = 40$)	Group 2 ($n = 40$)	p value
BMI (kg/m^2)	28.12 ± 4.94	28.52 ± 4.03	*0.696*
Weight (kg)	67.88 ± 13.33	67.07 ± 12.27	*0.782*
Waist circumference (cm)	99.71 ± 10.93	102.22 ± 10.57	*0.310*
Systolic BP (mm of Hg)	131.20 ± 17.17	131.86 ± 16.65	*0.862*
Diastolic BP (mm of Hg)	80.61 ±11.93	84.14 ± 11.00	*0.181*
AST (IU/L)	29.5 ± 14.0	27.38 ± 7.72	*0.67*
ALT (IU/L)	34.7 ± 19.2	28.81 ± 18.06	*0.16*
Creatinine (μmol/l)	65.41 ± 19.44	60.11 ± 14.14	*0.15*
Urea (mmol/l)	9.38 ± 2.23	9.6 ± 2.80	*0.56*
TC (mmol/l)	4.36 ± 0.89	5.7 ± 0.59	*0.000*
HDL-C (mmol/l)	8.85 ± 2.33	9.75 ± 2.71	*0.128*
LDL-C (mmol/l)	29.80 ± 7.82	32.25 ± 10.08	*0.238*
TG (mmol/L)	1.04 ± 0.27	2.71 ± 0.67	*0.000*
FPG (mmol/l)	7.79 ± 3.07	8.53 ± 2.97	*0.287*
PPG (mmol/l)	11.41 ± 2.83	12.57 ± 5.05	*0.188*
HbA_{1C} (%)	7.74 ± 1.49	5.44 ± 0.39	*0.32*
Apo A-V (ng/ml)	466.9 ± 271.8	369.6 ± 68.57	*0.040*
FFA (μM)#	1.32	1.38	*0.291*
IL-6 (pg/ml)#	0.35	0.21	*0.338*

Values are expressed as mean ± SD, $p < 0.05$ is significant

AST aspartate transaminase, *ALT* alanine transaminase, *FPG* fasting plasma glucose, *PPG* postprandial glucose, *HbA1C* glycated hemoglobin, *FSI* fasting serum insulin, *HOMA-IR* homeostatic model for insulin resistance, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglycerides

#Mean from log transformation of data

Correlation analysis Correlation analysis between variables was carried out for all patients ($n = 80$) and then in each group ($n = 40$). A significant correlation was observed between the mRNA expressions of p65 and IL-6 genes. ($r = 0.759$, $p = 0.000$) when analysis was carried out for all subjects ($n = 80$). However, no significant correlation was observed between the levels of IL-6 and the expression of IL-6 gene ($r = -0.009$, $p = 0.940$).

Apo A-V levels had a highly significant inverse association with the expression of p65 gene ($r = -0.457$, $p = 0.000$) and IL-6 gene ($r = -0.268$, $p = 0.030$) in all subjects. FFA levels also showed significant negative correlation with the mRNA expression of p65 ($r = -0.382$, $p = 0.001$), and IL-6 ($r = -0.328$, $p = 0.005$) genes. Significant correlation was observed between levels of TG and the mRNA expression of p65 gene ($r = 0.270$, $p = 0.030$) only, TG levels did not correlate with the mRNA expression of IL-6 gene ($r = 0.191$, $p = 0.119$).

Correlation analysis of Apo A-V levels and FFA levels with expression of p65 and IL-6 genes was carried out in individual groups as well and the result is given in Table 4.

Since both FFA and Apo A-V protein levels correlated significantly with the mRNA expression of p65 and IL-6, we performed a bivariate regression analysis to find out whether the association of Apo A-V and FFA levels with mRNA expression were dependent on each other. Association between FFA and mRNA expression was carried out keeping Apo A-V as constant. The results are depicted in Table 5a, b. It was observed that after adjusting Apo A-V, the inverse correlation between FFA levels and mRNA expression of p65 was significant. This indicates that the association of FFA levels with the mRNA expression of p65 is independent of the association of Apo A-V. Similar results were obtained upon regression analysis for FFA levels and Apo A-V with expression of IL-6, keeping Apo A-V as constant.

Our study also revealed that p65 expression correlated positively with the waist circumference in all subjects ($r = 0.257$, $p = 0.031$). However, this correlation was not observed in case of IL-6 gene. ($r = 0.103$, $p = 0.394$).

Discussion

Diabetes mellitus is a chronic progressive disease, with serious social and economic impact. T2DM is associated with vascular complications like CAD, nephropathy, and retinopathy. These complications have been attributed to chronic, low-grade inflammation that occurs in the disease. T2DM is often also associated with hypertriglyceridemia, although not all patients develop TG derangement. Since hypertriglyceridemia is also considered an important risk factor for the development of CAD, this study was planned to analyze the interplay of Apo AV, TGs, and pro-inflammatory genes (IL-6 and NF-κB)

Table 3 Fold change in mRNA expression of the IL-6 and p65 genes

	IL-6 gene			p65 gene		
	Controls	Cases	Fold change	Controls	Cases	Fold change
ΔCT keeping 18s as normalizer	− 6.9 ± 3.6	− 5.5 ± 3.5	2.71	− 3.2 ± 3.9	− 1.4 ± 3.4	3.41
ΔCT keeping B2M as normalizer	− 1.5 ± 3.3	− 0.4 ± 3.5	2.06	2.1 ± 3.8	3.8 ± 4.2	3.20

ΔCT values are expressed in mean ± SD for IL-6 and p65 expression in both groups. Fold change is calculated by 2 -ΔΔ CT. Expression of IL-6 was found to be higher in cases. It was 2.71 times higher when 18s was used as a normalizer and 2.06 times when B2M was kept as normalizer. mRNA expression of p65 was 3.41 times higher in cases when 18s was kept as normalizer and 3.20 times when B2M was used as normalizer

Table 4 Correlation analysis of Apo A-V level and FFAs levels with mRNA expression of IL-6 and p65 gene

	Apo A-V*				Free fatty acid#			
	Controls		Cases		Controls		Cases	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
p-65m RNA expression levels	− 0.457	0.005	− 0.399	0.026	− 0.538	0.001	− 0.289	0.092
IL-6m RNA expression levels	− 0.166	0.349	− 0.392	0.029	− 0.477	0.003	− 0.153	0.381

Apo A-V levels correlate negatively with expression of p-65 gene in both groups but with IL-6 gene expression in cases only. Significant inverse correlation of FFA levels is observed with expression of both genes in controls only

*Pearson's correlation for Apo A-V with expression of IL-6 and p65 mRNA

#Spearman's rho correlations for FFA and TG with mRNA expression of IL-6 and p65, p value < 0.05 taken as significant

in T2DM. For this purpose, patients of T2DM were grouped based on the risk of CAD according to TG levels as per NCEP ATP -III criteria [8].

In the present study, we have demonstrated for the first time that mRNA expression of IL-6, and p65 is higher in patients with moderate hypertriglyceridemia. Earlier studies have also indicated that TGs are a risk factor for activation of systemic inflammation in T2DM although expression of genes involved in inflammation were not studied [7]. The results of our study indicate that systemic inflammation is higher in diabetic patients with hypertriglyceridemia owing to an increase in the expression of pro-inflammatory cytokine IL-6 and its regulator NF-κB in blood cells. Our findings are also supported by a study conducted by Norata et al. [22] In their in vitro study, they demonstrated that hypertriglyceridemia induces pro-inflammatory response via involvement of NF-kB in endothelial cell of nondiabetic patients. Thus, apart from diabetes per se, hypertriglyceridemia is an additional risk factor for the development of chronic inflammation and thus complications like CAD in diabetes patients. One of the underlying mechanisms for this risk may be the increased expression of pro-inflammatory cytokines in circulating cells in such patients. In addition, we observed that there was a strong correlation between the expression of p65 and IL-6. Since it is well-known that NF-κB is a master regulator of pro-inflammatory cytokines, our report is also consistent with previous observations that NF-κB increases the expression of IL-6 [15].

In the study, circulating IL-6 levels did not correlate with its mRNA expression. This may be explained from the fact that the sources of IL-6 in blood include inflammatory cells as well as adipose tissue [23]. Previous studies have already reported that secretion of IL-6 from adipose tissue is increased in T2DM [17]. However, we analyzed the expression of IL-6 gene only in whole blood. It would therefore be worthwhile to analyze the expression of IL-6 in adipose tissue also in a similar setup to explain the overall relationship between IL-6 levels and mRNA levels of IL-6 genes in blood.

This study also evaluated the relationship between ApoA-V levels and the mRNA expression of pro-inflammatory cytokines. We observed a strong negative correlation between Apo A-V levels and the expression of NF-κB and IL-6 genes in hypertriglyceridemia. From this study, it is evident that Apo

Table 5 Regression analysis of Apo A-V protein and Free fatty acid (FFA) levels with respect to mRNA expression of NF-kB (a) and IL-6 gene (b)

	Unstandardized coefficients		Standardized coefficients	Significance
	B	SE	Beta	
(a)				
mRNA expression of p65 gene	− 1.369	0.390	− 0.401	0.001
Apo A-V protein levels	− 0.003	0.007	− 0.054	0.639
(b)				
mRNA expression of IL-6 gene	− 1.441	0.404	− 0.405	0.001
Apo A-V levels	− 0.002	0.007	− 0.028	0.807

Dependent variable for regression analyses is free fatty acid levels; Apo A-V levels are taken as constant; $p < 0.05$ is taken as significant. (a) On keeping Apo A-V levels as constant, association between expression of p65 and FFA levels is significant ($p = 0.001$). (b) After keeping Apo A-V levels as constant, association between expression of IL-6 and Constant: Apo A-V levels is significant ($p = 0.001$)

(a) R square: 0.159; adjusted R, 0.133; intercept, 22.802 (3.265); constant: FFA levels

(b) R square, 0.163; adjusted R, 0.138; intercept, 16.224 (4.049); constant: FFA levels

A-V may have some anti-inflammatory role. This finding is further corroborated by the observation that Apo A-V levels were higher in patients with normal TG levels, who are considered to be at a lower risk for the development of vascular complications in contrast to patients with hypertriglyceridemia. The anti-inflammatory role may be independent of its effect on TG metabolism. Our previous study indicated that Apo A-V may have a minimal role in TG metabolism [20]. Earlier study conducted by Genoux et al. has also tried to establish a link between Apo A-V expression and inflammation and has indicated that pro-inflammatory cytokines IL-1 and TNF-α decrease the expression of Apo A-V [14]. They demonstrated the effect of IL-1 and TNF-α on expression of APOA5 in in vitro experiments and on transgenic mice. However, our study has evaluated the expression of inflammatory genes (IL-6 and NF-kB) in patients of T2DM. The inverse relationship of Apo A-V with both IL-6 and NF-kB mRNA levels in our study indicates that the effect of Apo A-V may have been mediated either directly or through NF-κB. However, a clear understanding of the cause and effect relationship between Apo A-V and inflammation requires more detailed studies. Our findings, therefore, indicate that Apo A-V may provide an important link between inflammation and the complications associated with hypertriglyceridemia.

FFA has been implicated in the inflammatory pathways [12]. Therefore, we expected to find a direct positive association of FFA with inflammatory markers. However, we observed a significant *negative* correlation of the expression of NF-kB and IL-6 with FFA levels. It has been postulated that IL-6 affects the metabolism of TGs by inhibiting LPL [22]. Loss of this association in hypertriglyceridemia further adds to the evidence that IL-6 from circulating cells may have an additional role in maintaining TG levels. In our study, we also found a positive correlation between NF-κB (p65) expression and waist circumference. Since NF-κB is an important transcription factor that increases the expression of various cytokines, our finding suggests that obesity also has an important role to play in systemic inflammation, as has been proven in previous studies [24].

Our study is the first to evaluate mRNA expression of IL-6 and p65 genes in relation to hypertriglyceridemia in diabetes, and we have also attempted to establish their relationship with Apo A-V and FFA. The major strength of this study is the categorization of patients of T2DM on the basis of their TG levels. For predicting the risk of CAD, TG levels are regarded as an important criterion for assessment. However, while evaluating the role of Apo A-V, the previous studies did not compare the variables according to the degree of derangement in TG levels. This is the first study to evaluate Apo A-V levels and inflammatory markers (IL-6 and NF-kB) in groups with normal and moderate risk of CAD as per NCEP ATP III criteria.

This study indicates that diabetic patients with hypertriglyceridemia have a higher expression of genes involved in inflammatory pathway in blood as compared to those with normal TG levels. Moreover, our observations also indicate that Apo A-V protein may have actions beyond the regulation of TG and may be an important link between lipid metabolism and inflammatory pathways in diabetes. In addition, the study also supports the role of IL-6 in TG metabolism. Further studies are needed in order to develop a clear understanding of role of Apo A-V in systemic inflammation in T2DM and the part it plays beyond its role in the homeostasis of TG. Such studies may open up new avenues for the better understanding of the pathophysiological basis of chronic systemic inflammation in T2DM which results in the complications associated with this disease.

Limitations of our study include small sample size. Although power of study for comparison of ApoA-V levels was 80%, the same could not be calculated for the analysis of correlation between variables. In addition, the participating population is heterogeneous regarding obesity. However, owing to small sample size, subgrouping and multiple regression analysis was not carried out. Therefore, the findings need to be ascertained in a larger sample size where concerns related to statistical precision can be addressed.

Conclusion

Patients of T2DM with hypertriglyceridemia exhibit higher expression of IL-6 and NF-kB genes. By virtue of its negative association with mRNA expression of IL-6 and NF-kB, Apo A-V seems to have some relationship with inflammatory pathways. Low levels of ApoA-V in patients with hypertriglyceridemia indicate its role in pathogenesis of complications associated with hypertriglyceridemia. We propose that Apo A-V has a potential to be developed as a surrogate marker for systemic inflammation as well as therapeutic target for prevention and treatment of complications of T2DM.

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Glossary

Apo A-V Apolipoprotein A-V protein
Apo A5 Apolipoprotein A5 gene
TG Triglycerides
T2DM Type 2 diabetes mellitus
IL-6 Interleukin 6
NF-κB Nuclear factor-kappa B
P65 Nuclear factor-kappa B gene
FFA Free fatty acids

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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.

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

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ORIGINAL ARTICLE

Comparison of intake of food groups between participants with normoglycemia, impaired fasting glucose, and type 2 diabetes in PURE Poland population

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Abstract
According to the World Health Organization, diabetes could be responsible for 1.5 mln deaths a year and prevalence of diabetes is still increasing. Improper diet is one of modifiable risk factors of type 2 diabetes. Because diabetes is a major health burden, research recognizing factors contributing to increased risk of type 2 diabetes is important. The aim of the study was conducting the comparison of intake of food groups between participants with normoglycemia, impaired fasting glucose (IFG), and type 2 diabetes of Prospective Urban and Rural Epidemiology (PURE) Poland population. Assessment of intake of food groups was conducted with the use of validated Food Frequency Questionnaire (FFQ) among 1654 participants of PURE Poland—baseline (2007–2009). Assessment of the differences between groups had been performed with the use of the Kruskal-Wallis test. The significance level was established to be $p \leq 0.05$. Participants with IFG in comparison to participants with diabetes consumed significantly more fruit juices, beverages with added sugar, sweets, honey, and sugar. Participants with IFG in comparison with normoglycemic participants consumed significantly more refined grains, fruit juices, lean meat, and processed meat and less nuts and seeds. Participants with diabetes in comparison to normoglycemic participants consumed significantly more lean meat and processed meat and less tea and coffee, alcohol, dried fruit, honey, sugar, and nuts. Especially participants with IFG, who consumed more products of high glycemic index should be the subject of intensive counseling and other prophylactic measures to reduce the risk of progression to type 2 diabetes.

Keywords Diabetes · Impaired fasting glucose · Diet · Food groups

Introduction

According to the World Health Organization (WHO), more than 422 mln people worldwide have diabetes and the prevalence of this disease have rapidly risen in the last decades [1]. Diabetes is the main cause of kidney failure, blindness, lower limb amputation, and stroke and contributes strongly to other cardiovascular diseases [1]. Impaired fasting glycemia (IFG) and impaired glucose tolerance (IGT)—conditions preceding full-symptomatic type 2 diabetes—are considered

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independent risk factors for cardiovascular diseases [2], though according to recent meta-analyses, this association is rather moderate in comparison with diabetes [3]. There have been evidence that endothelial dysfunction caused by hyperglycemia and macrovascular lesions occur much earlier than previously thought—endothelial dysfunction develops already in prediabetic state [4]. According to the WHO, the modifiable risk factors that contribute to the development of diabetes can be included: tobacco smoking, overweight and obesity, low physical activity, and improper diet [1]. So-called *western diet*, rich in, e.g., saturated fatty acids, trans-unsaturated fatty acids, and refined grains, is associated not only with cardiovascular diseases but also with type 2 diabetes [5, 6], whereas Mediterranean diet is associated with much lower risk of those conditions [7]. Progression of impaired fasting glycemia and impaired glucose tolerance to full-symptomatic type 2 diabetes is not inevitable. Early behavioral intervention, like improving physical activity and changing dietary habits, can slow down or even cease the process of progression to type 2 diabetes [8]. Standards for diabetes management, not only Polish [9] but also international (e.g., Standards of American Diabetes Association [10]), emphasize the need for implementing intensive counseling regarding changing the lifestyle in prediabetic patients. Nutritional intervention includes, e.g., changing the quality of dietary fat, increased intake of wholegrain, high-fiber carbohydrate products and decreased intake of additionally sweetened beverages, sweets, and high processed products. The influence of consumption of individual food groups on the risk of type 2 diabetes differ from one group to another, as it was observed in many studies [11]. The aim of the study was to compare the intake of groups of dietary products between individuals with normoglycemia, impaired fasting glucose, and type 2 diabetic patients in the population of Prospective Urban and Rural Epidemiology (PURE) study.

Material and methods

Prospective Urban and Rural Epidemiology study engages 21 countries of different status of economic development and more than 150,000 participants worldwide. The study is designed to collect data in 3 years and intervolves and is planned for 12 years in total [12]. The Polish arm of the PURE study was established in 2007 at Wroclaw Medical University. The baseline cohort included 2036 participants, aged between 30 and 85 years, both from urban and rural area. All participants were examined according to the international PURE project protocol [13]. The paper presents the results of PURE Poland study—baseline, covering the group of 1654 participants, who completed the Food Frequency Questionnaire (FFQ) and had the blood test performed.

The Food Frequency Questionnaire consists of 154 dietary products, characteristic for Polish dietary habits. The FFQ was collected by trained staff and referred to dietary habits of 1 year prior to the interview. The FFQ was specially developed and validated for Polish population [14]. One hundred fifty-four dietary products were divided in 26 food groups (Table 1). The criteria for such specific division were not only dietary recommendations for general Polish population [15] but also standards for nutrition therapy in diabetes [9, 10]. We had taken into consideration, e.g., the glycemic index of the dietary products, the content of fiber, and quality of dietary fat. We gathered soups in one independent food group, because of their traditional and Polish-specific ingredients and way of preparation. Other traditional Polish dishes were included in the "mixed dishes" group. Because of different glycemic index and fiber content, we divided grains into "whole grains" and "refined grains," similarly, fruits and vegetables were divided into "raw," "cooked," "dried," and "juices." Due to the quantity and quality of dietary fat, but also recommendations for Mediterranean diet, we divided dairy products into "low-fat" and "full-fat," moreover, meats were divided into "lean meat," "red meat," and "processed meat." Participants whose calorie intake assessed by FFQ was < 500 kcal/day or > 5000 kcal/day were excluded from analysis due to presumably unreliable interview.

Overall analysis concerned 1654 eligible participants: 992 with normoglycemia, 464 with IFG, and 198 with diabetes. People, whose fasting plasma glucose was between 100 mg/dL (5.6 mmol/L) and 125 mg/dL (6.9 mmol/L) were included into the group of participants with IFG. In the group of diabetes, there were included people whose fasting plasma glucose was ≥ 126 mg/dL (7.0 mmol/L), or those who had already been diagnosed with diabetes in the past and had been undergoing treatment ever since. Participants with normal fasting plasma glucose (70–99 mg/dL, 3.9–5.5 mmol/L) and who were declared with no diabetes diagnosed in the past were included into the group of normoglycemia. A trained health professional, who performed examination, verified whether fasting prior to the examination lasted at least 8 h. Enzymatic hexokinase method was used to measure the fasting plasma glucose from venous blood with the use of Cobas machine and reagents (Roche Diagnostics).

Statistical analysis was performed with the use of Statistica 12.0 PL computer program. Assessment of the differences between groups had been performed with the use of the Kruskal-Wallis test. The significance level was established to be $p \leq 0.05$.

Results

Study population consists of 1654 eligible participants: 36.2% of men and 63.8% of women (Table 2). Overall, 992

Table 1 Characteristic of food groups

Lp.	Food groups	FFQ dietary products
1.	Low-fat dairy products	Milk 1–2% fat
		Buttermilk 0.5% fat
		Cocoa w/ milk 1–2% fat
		Cottage cheese
		Low-fat yoghurt
		Kefir
2.	Full-fat dairy products	Milk 3.2% fat
		Milk 3.2% fat (from mixed dish—oatmeal w/ milk)
		Feta Greek cheese
		Granulated cottage cheese w/ sour cream
		Cheese
		Edam cheese
		Fromage cheese
		Yoghurt 2–8% fat
		Sour cream 12% fat
		Sour cream 18% fat
		Sour cream 18% fat (from mixed dish—salad w/sour cream)
3.	Whole grains	Whole-meal rye bread
		Mixed bread w/ rye and wheat flour w/ sunflower seeds
		Buckwheat groats, boiled
		Barley groats, boiled
		Pasta w/ durum, boiled
		Oatmeal (from mixed dish—oatmeal w/ milk)
4.	Refined grains	Wheat bread, white
		White rice, boiled
		Wheat roll, white
		Mixed bread w/ rye and wheat flour, white
		Cornflakes
5.	Fats w/o oils	Butter
		Lard
		Fat spread w/ butter
		Mayonnaise
		Margarine, soft
6.	Raw fruit	Apple
		Banana
		Grapefruit
		Grapes
		Mandarin
		Strawberries
		Kiwi fruit
		Lemon
		Orange
		Pear
		Peach
		Prunes
		Raspberries
7.	Fruit juices	Orange juice
		Raspberry juice
		Carrot juice
		Apple juice
		Grapefruit juice
		Black currant juice
		Multifruit juice (local fruits)
		Multifruit juice (exotic fruits)
8.	Raw vegetables	Cabbage red, raw
		Chinese cabbage, raw
		Cabbage white, raw
		Carrot, raw
		Cauliflower, raw
		Chives
		Cucumber, raw
		Garlic cloves, raw
		Salad, leaves
		Onion, raw
		Parsley, leaves
		Horseradish
		Red pepper, raw
		Radish
		Tomato, raw
		Sauerkraut salad
		Chinese cabbage salad w/ mayonnaise
		Salad (from mixed dish—salad w/ sour cream)
9.	Cooked vegetables	Kidney beans, cooked
		Beetroot, cooked
		Broccoli
		Cabbage white, cooked
		Carrot, cooked
		Cauliflower, cooked, w/butter
		Mushrooms, fried
		Red pepper, cooked
		Tomatoes, cooked
		Tomato passata
		Spinach, cooked
		Zucchini, cooked
		Green beans, cooked
		Corn, canned
		Peas, canned
		Salad of mixed cooked vegetables w/ mayonnaise
10	Potatoes	Potatoes, boiled
		Potatoes, mashed
		French fries
11.	Lean meat	Chicken w/ skin boiled/fried
		Chicken w/o skin boiled/fried

Table 1 (continued)

Lp.	Food groups	FFQ dietary products
		Turkey, roasted
12.	Red meat	Beef, cutlets
		Beef ham, boiled
		Pork, bacon
		Cutlets of ground beef and pork, fried
		Offal
13.	Meat w/ breadcrumbs	Chicken nuggets
		Pork chops, w/ breadcrumbs
14.	Processed meat/charcuterie	Chicken ham
		Sausage
		Luncheon meat, pork
		Pork ham
		Sausage, pork, smoked (traditional polish)
		Sausage, mixed beef/pork, smoked (traditional polish)
		Sausage, pork, white, boiled (traditional polish)
		Turkey ham
		Turkey sausage ham
		Brawn
		Chicken pâté
15.	Eggs	Eggs, boiled/fried
16.	Fish	Codfish, fried, w/ breadcrumbs
		Herring, w/ cream
		Mackerel, smoked
17.	Mixed dishes	Baked beans w/ tomato sauce
		Meat and rice stuffed cabbage w/ tomato sauce
		Dumplings w/ meat, boiled
		Dumplings w/ potatoes and cottage cheese, boiled
		Sauerkraut and meat stew
18.	Beverages w/ added sugar	Fruit drink
		Soft drink w/ added sugar
19.	Low-calorie beverages	Low-calorie soft drink
20.	Tea, coffee	Coffee
		Tea, black
		Tea, green/herb
21.	Alcohol	Beer
		Wine
		Vodka
22.	Sweets	Milk chocolate
		Dark chocolate
		Tea biscuit
		Yeast cake
		Shortbread cake
		Gingerbread
		Pound cake
		Cheesecake
		Halvah
		Caramel candy
		Other sweets
		Candy
		Ice cream
23.	Honey and sugar	Honey
		Sugar
24.	Dried fruits	Raisins
25.	Nuts and seeds	Walnuts
		Other nuts
		Seeds
26.	Soups	Broth
		Sour rye soup
		Vegetable soup
		Barley soup
		Tomato soup
		Bean soup
		Sauerkraut soup

w/, with; *w/o*, without

participants had normoglycemia (34.6% of men and 65.4% of women). Overall, 464 participants had IFG (37.1% of men and 62.9% of women). Overall, 198 participants had diabetes (41.9% of men and 58.1% of women).A total of 17.5% of participants overall were < 45 years old (73.4% and normoglycemia, 21.7% had IFG, and 4.8% had diabetes). Age group between 45 and 64 years old was the largest group: 66.9% of participants overall (57.8% with normoglycemia, 29.6% with IFG, and 12.6% with diabetes). Of the participants, 15.5% were older than 64 years (54.1% with normoglycemia, 28.4% with IFG and 17.5% with diabetes). A total of 55.9% of participants were urban dwellers and 44.1% were rural dwellers. Among urban dwellers, 69.0% had normoglycemia, 22.7% had IFG, and 8.3% had diabetes. Among rural dwellers, 48.6% had normoglycemia, 34.8% had IFG, and 16.6% had diabetes. Overall, 15.3% participants had primary education (46.6% with normoglycemia, 31.2% with IFG, and 22.1% with diabetes). Overall, 16.6% of participants had vocational education (53.5% with normoglycemia, 32.4% with IFG, and 14.2% with diabetes), while 39.0% of participants had secondary education (60.2% with normoglycemia, 28.8% with IFG, and 11.0% with diabetes). Total of 29.1% of participants had university degree (70.5% with normoglycemia, 22.9% with IFG, and 6.7% with diabetes).

Average BMI in groups of normoglycemia, IFG, and diabetes was 27.2, 29.2, and 31.6 kg/m^2 respectively. The analysis revealed statistically significant differences in distribution of body weight between participants with normoglycemia, IFG, and diabetes ($p < 0.000$) (Table 3). The largest group

Table 2 Characteristics of 1654 participants of PURE Poland study population

	Total		
Men *n* (%)	598 (36.2%)		
Women *n* (%)	1056 (63.8%)		
	Normoglycemia (70–99 mg/dL)	Impaired fasting glucose (IFG) (100 and 125 mg/dL)	Type 2 diabetes (≥ 126 mg/dL or previously diagnosed)
Total *n* (%)	992 (59.9%)	464 (28.1%)	198 (11.9%)
Men *n* (%)	343 (57.4%)	172 (28.8%)	83 (13.9%)
Women *n* (%)	649 (61.5%)	292 (27.7%)	115 (10.9%)
Age			
< 45 years	213 (73.4%)	63 (21.7%)	14 (4.8%)
45–64 years	640 (57.8%)	328 (29.6%)	139 (12.6%)
> 64 years	139 (54.1%)	73 (28.4%)	45 (17.5%)
Place of residence			
Urban *n* (%)	638 (69.0%)	210 (22.7%)	77 (8.3%)
Rural *n* (%)	354 (48.6%)	254 (34.8%)	121 (16.6%)
Education level			
Primary *n* (%)	118 (46.6%)	79 (31.2%)	56 (22.1%)
Vocational *n* (%)	147 (53.5%)	89 (32.4%)	39 (14.2%)
Secondary *n* (%)	388 (60.2%)	186 (28.8%)	71 (11.0%)
Higher *n* (%)	339 (70.5%)	110 (22.9%)	32 (6.7%)
BMI			
Average BMI [kg/m^2]:			
M ± *SD*	27.2 ± 4.7	29.2 ± 5.1	31.6 ± 5.9
Min-Max	15.0–47.1	17.8–46.9	19.8–49.2

with normal body weight was participants with normoglycemia (34.3 vs. 20.3% participants with IFG and 12.1% participants with diabetes) (Table 3). Obesity was more prevalent in participants with IFG (40.3%) and diabetes (57.6%), than in participants with normoglycemia (23.8%).

The analysis revealed statistically significant differences in consumption of 12 out of 26 food groups (Table 4). Participants with IFG in comparison to participants with diabetes consumed statistically significantly more fruit juices, beverages with added sugar, sweets, and honey/sugar. Participants with IFG in comparison with normoglycemic participants consumed significantly more refined grains, fruit juices, lean meat, and processed meat/charcuterie and significantly less nuts/seeds. Participants with diabetes in comparison to normoglycemic participants consumed significantly more lean meat, processed meat/charcuterie, and soups, while on the other hand, significantly less tea/coffee, alcohol, dried fruit, nuts/seeds, and honey/sugar. Simplified results of post hoc analyses are presented in Table 5.

Discussion

The comparison between consumption of food groups between participants with normoglycemia, IFG, and type 2 diabetes revealed significant differences in 12 out of 26 food groups.

Participants with IFG consumed more refined grains than normoglycemic participants. Refined grains are characterized by high glycemic index and deprivation of most of the dietary fiber and are not recommended, especially in the prevention and treatment of diabetes. According to the guidelines, whole grains should be the main source of carbohydrates [10]. Alminger et al. [16] observed that the intake of whole grains in comparison to refined grains is associated with much lower rise of postprandial glucose and insulin level. According to Wirström et al. [17], consumption of whole grains lowers the risk of development of prediabetes. Meta-analysis conducted by Yao et al. [18] concludes that increased intake of dietary fiber is associated with lower risk of development of type 2 diabetes. In a randomized, double-blind study conducted by Dainty et al. [19], participants who consumed bagels rich in resistant starch in comparison with participants who consumed regular bread were characterized by lower fasting and postprandial insulin levels and lower fasting insulin resistance. Participants with IFG consumed more fruit juices than either normoglycemic or diabetic participants.

It is already known that higher consumption of fruit and vegetables is associated with lower risk of metabolic diseases. Recently published meta-analysis, by Wang et al. [20],

Table 3 Population characteristics according to BMI

	Underweight (BMI < 18.5 kg/m^2) *n* (%)	Normal body weight (BMI 18.5–24.9 kg/m^2) *n* (%)	Overweight (BMI 25.0–29.9 kg/m^2) *n* (%)	Obesity (BMI > 30.0 kg/m^2) *n* (%)	*p* value*
Normoglycemia (70–99 mg/dL)	9 (0.9)	340 (34.3)	407 (41.0)	236 (23.8)	0.000
Impaired fasting glucose (IFG) (100 and 125 mg/dL)	3 (0.6)	94 (20.3)	180 (38.8)	187 (40.3)	
Type 2 diabetes (≥ 126 mg/dL or previously diagnosed)	0 (0.0)	24 (12.1)	60 (30.3)	114 (57.6)	

*With exclusion of underweight group due to insufficient number of participants

concludes that higher intake of raw fruit and vegetables (especially green leafy, yellow, and cruciferous vegetables) is associated with lower risk of type 2 diabetes. On the other hand, fruit juice is characterized by higher content of monosaccharides and lower content of fiber than raw fruit, which also results in higher glycemic index. Moreover, fruit juice frequently contains artificially added glucose-fructose syrup (or high-fructose corn syrup), which is cheaper than saccharose, in order to improve the taste of the juice. Meta-analysis performed by Imamura et al. [21], who assessed impact of consumption of soda and fruit juices on the risk of type 2 diabetes, adjusted for adiposity and calorie intake, concluded that both soda and fruit juices increase the relative risk for diabetes. In fact, increasing the intake of fruit juice by one serving a day was associated with 7% greater risk of type 2 diabetes. On the contrary, meta-analysis recently conducted by Wang et al. [22] provides no hard evidence for association between consumption of fruit juices and diabetes. Either way, overconsumption of fructose could be deleterious for the metabolic health. Although fructose has lower glycemic index than glucose, it is speculated that its consumption promotes deposition of lipids in visceral abdominal tissue and decreases glucose tolerance and insulin sensitivity especially in overweight persons [23]. To sum up, excessive consumption of fruit juices, especially those with added sugar or glucose-fructose syrup, should not be recommended in patients with metabolic disorders. Consumption of highly sweetened beverages is one of the risk factors for overweight and obesity and is strongly unrecommended [1]. The herein analysis revealed increased consumption of beverages with added sugar by participants with IFG in comparison to diabetic patients. Meta-analysis of 11 cohort studies, performed by Malik et al. [24], concludes that among participants who consumed the highest amounts of sweetened beverages (one to two servings a day), the risk of development of type 2 diabetes was higher by 26%. Moreover, the Framingham Offspring Cohort study provided an observation that consumption of sweetened beverages increased the risk of development of prediabetes and insulin resistance by 46% in comparison to the risk of those who did not consume such beverages [25]. In the presented paper, participants with IFG consumed more sweets and honey/sugar than diabetic patients. Because of increasing consumption of sugar worldwide and association of higher consumption of sugar with increased risk of overweight/obesity and non-communicable diseases, the World Health Organization strongly recommends to limit consumption of free sugars to 10% of total energy intake (and states that ideal intake is below 5% of total energy intake) [26].

Either participants with IFG or diabetes consumed more processed meat and charcuterie than normoglycemic participants. Meta-analysis conducted by Feskens et al. [27] concluded that increased consumption of red meat and processed meat increases the risk of type 2 diabetes. According to the meta-analysis conducted by Micha et al. [28], every additional serving of processed meat per day increases the risk of diabetes by 19%. Possibly, the high content of saturated fatty acids and trans-unsaturated fatty acids in processed meat is a factor responsible for such association. Analysis performed by Guess et al. [29] concludes that higher consumption of saturated fatty acids is associated with higher fasting plasma glucose, hepatic insulin resistance, and higher plasma glucose after 2 h in oral glucose tolerance test (OGTT). Further non-nutritive compounds (differentiating processed meat from red meat), like sodium or nitrosamines, are candidate factors affecting metabolic disorders.

According to the herein findings, diabetic participants consumed significantly less alcohol than normoglycemic participants. As stated in recommendations for diabetic patients by the American Diabetes Association [10], excessive alcohol consumption should be strictly avoided (no more than two servings per day for men and no more than one serving per day for women). The findings presented in this paper suggest that it is possible that counseling introduced after diagnosis of diabetes influenced decreased consumption of alcohol. Excessive consumption of alcohol can increase the risk of delayed hypoglycemia in diabetic patients, especially those who use insulin [10]. Excessive consumption of alcohol should be avoided not only by diabetic patients but also by healthy population. According to findings from the Northern Swedish Cohort study [30], a 27-year prospective cohort study, increased consumption of alcohol and binge drinking

Table 4 Differences in consumption of food groups between participants with normoglycemia, IFG, and type 2 diabetes—result of analyses with the use of the Kruskal-Wallis test

Food groups	Normoglycemia[a]			Impaired fasting glucose (IFG)[b]			Diabetes[c]			*p*
	Mean [g/day]	Median	SD	Mean [g/day]	Median	SD	Mean [g/day]	Median	SD	
Low-fat dairy products	147.08	82.29	169.74	134.60	81.35	141.05	129.78	77.30	149.10	0.500
Full-fat dairy products	120.95	69.42	139.79	139.16	71.59	172.88	132.50	68.32	151.43	0.490
Whole grains	62.53	47.40	48.10	61.75	47.40	43.61	70.22	48.08	57.10	0.560
Refined grains	75.56	59.91[b]	63.95	87.56	83.57[a]	64.15	78.59	78.66	68.72	0.001
Fats w/o oils	20.33	15.98	16.29	21.48	17.14	16.31	21.23	17.82	16.22	0.170
Fruit raw	300.15	235.41	211.56	285.18	223.91	205.97	288.86	234.17	217.65	0.310
Fruit juices	152.69	107.14[b,c]	148.88	173.83	137.00[a,c]	162.11	138.18	84.89[a,b]	163.70	0.000
Vegetables raw	172.45	142.05	115.25	162.72	137.04	100.26	167.45	141.84	105.35	0.670
Vegetables cooked	148.92	120.82	116.45	134.97	117.28	90.44	141.19	121.30	99.89	0.430
Potatoes	87.24	71.82	58.95	93.34	78.71	58.85	94.25	78.71	52.24	0.017
Lean meat	19.78	15.08[b,c]	15.96	21.82	15.08[a]	16.79	21.98	16.25[a]	14.50	0.003
Meat w/ breadcrumbs	21.97	20.84	15.31	22.86	20.84	15.95	22.56	20.84	15.65	0.750
Red meat	22.89	17.70	20.61	24.70	19.88	22.14	21.51	18.06	16.10	0.120
Processed meat/charcuterie	60.02	47.90[b,c]	44.92	68.46	52.95[a]	47.47	75.09	63.36[a]	49.88	0.000
Eggs	15.29	19.28	13.65	16.79	19.28	16.49	16.90	19.28	17.56	0.560
Fish	13.49	13.11	10.98	14.35	13.11	10.29	14.72	13.11	10.95	0.035
Mixed dishes	25.71	26.22	18.49	26.79	26.22	18.96	27.69	26.22	17.80	0.180
Beverages w/ added sugar	40.32	0.00	93.04	45.51	0.00[c]	100.86	36.24	0.00[b]	89.11	0.027
Low-calorie beverages	156.47	0.00	343.44	122.07	0.00	320.84	146.07	0.00	374.32	0.050
Tea, coffee	970.88	910.71[c]	463.22	939.58	891.39	439.76	866.33	883.19[a]	491.06	0.035
Alcohol	56.23	12.13[c]	127.30	49.59	9.31	104.04	52.64	0.00[a]	144.12	0.006
Sweets	42.87	35.40	35.20	45.16	38.16[c]	32.15	37.37	31.08[b]	33.18	0.004
Honey, sugar	17.94	15.71[c]	16.39	17.32	15.42[c]	15.53	12.03	3.90[a,b]	15.08	0.000
Dried fruit	5.68	4.91[c]	10.86	4.57	4.91	9.31	2.95	0.00[a]	4.44	0.000
Nuts, seeds	9.22	5.57[b,c]	15.13	8.19	5.44[a]	14.60	7.14	4.91[a]	12.22	0.000
Soups	242.61	221.53[c]	140.61	252.75	228.57	138.02	270.27	250.11[a]	134.40	0.003

Results of post hoc analyses were marked with the upper indexes [a], [b], and [c]. Upper indexes indicate between which groups significant differences occurred

IFG, impaired fasting glucose; *w/o*, without; *w/*, with; *[g/day]*, grams per day; *SD*, standard deviation

behavior were associated with higher values of fasting plasma glucose among adult women. These findings were consistent with findings obtained by Baliunas et al. [31] in the meta-analysis of cohort studies. According to this meta-analysis, moderate consumption of alcohol decreases the risk of type 2 diabetes among women, but excessive consumption of alcohol significantly increases the risk of diabetes.

In the herein study, it was also observed that participants with diabetes consumed less tea and coffee than normoglycemic participants. There is evidence that drinking unsweetened tea and coffee can improve health status, presumably due to the high content of antioxidative compounds. However, the association between consumption of tea and coffee and incidence of type 2 diabetes needs further investigation, but there have been some observations that have emerged. Meta-analysis conducted by Yang et al. [32] concluded that consumption of at least three cups of tea per day can contribute to lower risk of type 2 diabetes. According to van Dieren et al. [33] and the Dutch arm of EPIC-NL study, which investigated more than 40,000 participants over a 10-year period, consuming at least three cups of tea or coffee decreased the risk of type 2 diabetes by 42%.

According to our findings, participants with diabetes consumed significantly less nuts and seeds than normoglycemic participants. It is not clear why diabetic participants consumed less of those products, but one of the reasons for such behavior could be an attempt to reduce the overall energy intake, since nuts and seeds are rather calorie-dense products. On the other hand, such behavior can deprive of some essential nutrients provided by nuts and seeds, e.g., polyunsaturated fatty acids.

Table 5 Summary of results of post hoc analyses

Groups of participants	Results
IFG vs. normoglycemia	↑Refined grains
	↑Fruit juices
	↑Lean meat
	↑Processed meat and charcuterie
	↓Nuts and seeds
IFG vs. diabetes	↑Fruit juices
	↑Beverages w/ added sugar
	↑Sweets
	↑Honey and sugar
Diabetes vs. normoglycemia	↑Lean meat
	↑Processed meat and charcuterie
	↑Soups
	↓Tea and coffee
	↓Alcohol
	↓Dried fruit
	↓Nuts and seeds
	↓Honey and sugar

Results obtained within the Nurse's Health Study [34] suggest that higher consumption of nuts is associated with lower risk of type 2 diabetes among adult women, though findings from the Physician's Health Study found no such association among adult men [35]. A meta-analysis conducted by Wu et al. [36] also failed to find an association between consumption of nuts and risk of type 2 diabetes. On the other hand, the role of polyunsaturated fatty acids prevalent in nuts and seeds in lowering the risk of cardiovascular diseases is well established [37]. Since diabetic patients are at high risk of developing cardiovascular complications, including nuts and seeds in the diet is recommended in order to lower the risk of metabolic complications [38].

The participants with IFG consumed more food groups characterized by higher glycemic index in comparison with the participants with diabetes (fruit juices, beverages with added sugar, sweets) and normoglycemia (refined grains, fruit juices). On the other hand, diabetic participants were characterized by lower consumption of high glycemic index food groups, e.g., alcohol. These findings can suggest that dietary counseling introduced after diabetes diagnosis is—at least partially—effective and influences dietary choices of the participants. There is evidence in the literature that dietary counseling is effective not only in promoting diabetes management but also preventing progression to full-symptomatic diabetes [8]. According to the standards of diabetes care, both Polish [9] and American [10], intensive dietary and behavioral counseling should be introduced in prediabetic patients as soon as possible in order to prevent deterioration of glucose tolerance. Nevertheless, not all prediabetic patients are referred to dietary counseling. According to Ginde et al. [39], only 10% of the patients with incidentally diagnosed IFG or IGT in emergency ward received information about their condition and 6% were referred for further management. According to the results of NHANES study [40], 43.6% of patients with prediabetes and 10% of normoglycemic patients were informed about health risks of type 2 diabetes.

What is more, analysis revealed that obesity was more common in participants with IFG and diabetes than in normoglycemic participants, in fact there was a gradual increase in percentage of obese individuals along with deterioration of glucose metabolism (23.8% of individuals with normoglycemia, 40.3% with IFG, and 57.6% with diabetes were obese). Obesity is a major risk factor of type 2 diabetes, cardiovascular diseases [41], and cancer [42]. Both higher consumption of unrecommended food groups and high prevalence of obesity in IFG participants are alarming risk factors of metabolic deterioration.

Conclusion

The diets of participants with IFG were characterized by higher content of unrecommended food groups. The prevalence of obesity was much higher among participants with IFG and diabetes than participants with normoglycemia. Because of high risk of further metabolic deterioration, individuals with IFG should be the group of the highest priority for dietary and behavioral counseling in order to prevent progression to full-symptomatic type 2 diabetes.

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Study limitations Food Frequency Questionnaire is an acknowledged method of assessment of nutritional habits and intake of nutritional products but has its limitations. FFQ assesses the intake of nutrients retrospectively and depends entirely on participant's memory and judgment, which supposedly can contribute to overestimation of consumption of recommended food groups and underestimation of consumption of unrecommended food groups. Categorization of participants into groups of normoglycemia, IFG, and diabetes was made only on the basis of fasting glucose levels (in the case of diabetes, also on self-reported disease and taking glucose-lowering agent). Mentioned methodology could possibly cause some underestimation in IFG and diabetes prevalence (neither hemoglobin A_{1c} was measured nor OGTT was performed), but this is a methodology implemented in the PURE study worldwide [43]. Dietary data was only recorded, and participants did not receive any dietary counseling following the examination.

Author's contributions AB – manuscript preparation, literature search, manuscript editing
DR –concept and design, manuscript review
KPZ – statistical analysis
MW – data acquisition
AS – manuscript final approval
KZ – concept and design, manuscript review, manuscript guarantor
All authors read and approved the final manuscript.

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Compliance with ethical standards

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

Informed consent Informed consent was obtained from all individual participants included in the study. All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki (Positive opinion of bioethical committee of Wroclaw Medical University nr KB-443/2006).

Abbreviations IFG, impaired fasting glucose; FFQ, Food Frequency Questionnaire; WHO, World Health Organization; IGT, impaired glucose tolerance; PURE, Prospective Urban and Rural Epidemiology study; OGTT, oral glucose tolerance test

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ORIGINAL ARTICLE

Prevalence of diabetic micro vascular complications at a tertiary care unit of Karachi, Pakistan

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Abstract
To determine the prevalence of microvascular complications and associated risk factors among subjects with type 2 diabetes reported at a tertiary care unit of Karachi, Pakistan. This retrospective observational study was carried out in the outpatient department of Baqai Institute of Diabetology and Endocrinology (BIDE), a tertiary care diabetes center of Karachi from January 2005 to April 2016. Data records of patients with type 2 diabetes at presentation were analyzed. Selected data was extracted from Health Management System (HMS) including basic demographics, anthropometric measurements, biochemical results, medical information, and microvascular complications results. Data analysis was performed on Statistical Package for Social Sciences (SPSS) version 20.0. Overall, prevalence of at least one microvascular complication was 56.9%, retinopathy 15.8%, nephropathy 31.0%, and neuropathy 48.7% were noted. Male gender, age ≥ 40 years, duration of diabetes > 10 years, obesity, hypertension, HbA1c > 7%, and low HDL were found to be significant risk factors for microvascular complication. Hypertriglyceridemia and hypercholesterolemia were significantly associated with nephropathy and neuropathy, whereas no significant association of high LDL was found with any complication. High prevalence of microvascular complications was observed among type 2 diabetic subjects visited first time at a tertiary care hospital. Early identification and effective management are required at primary and secondary care levels to combat this situation.

Keywords Complication · Retinopathy · Nephropathy · Neuropathy · Diabetes

Introduction

Diabetes mellitus is an important global health issue as the number of people with diabetes are rising every year, particularly of type 2 diabetes [1]. Pakistan had an estimated population of 7.5 million diabetics as of 2017, and this population is predicted to near 16.1 million in 2045 [2]. The recent National diabetes survey of Pakistan is just completed and reports are sent for publication. People with type 2 diabetes mellitus have a considerably higher risk of both macrovascular complications (peripheral vascular disease, coronary artery disease, and cerebrovascular disease) and microvascular complications (nephropathy, neuropathy, and retinopathy) [3]. These complications result in significantly high morbidity and mortality. People with long duration of diabetes or uncontrolled disease are usually affected by microvascular complications. However, the complications can already be present at the time of diagnosis [4].

The increasing trend in the prevalence of microvascular complications of type 2 diabetes has been observed in various epidemiological studies [5–8]. Joint Asia Diabetes Evaluation (JADE) reported that in seven countries of Asia, retinopathy, neuropathy, and nephropathy affected 20.4, 15.0, and 7.5% of diabetic subjects, respectively [9]. Previous studies identified that the prevalence of microvascular complications was more common in Asians as compared to Europeans [10, 11] and Caucasians [8]. Data on prevalence rate of microvascular complications in

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Pakistan is scarce. However, a few small hospital-based studies highlighted high prevalence of microvascular complications ranged from 12 to 31% for retinopathy, 28 to 56% for nephropathy, and 36 to 68% for neuropathy [12–17]. The issue of rising type 2 diabetes in young individuals makes it significantly more important to identify and treat the disease earlier as the long-term microvascular complications are related with hyperglycemia and duration of the disease. Thus, the present study aims to assess the prevalence of microvascular complications and associated risk factors among subjects with type 2 diabetes reported at a tertiary care unit of Karachi, Pakistan.

Methodology

This retrospective study was conducted at Baqai Institute of Diabetology and Endocrinology (BIDE). Ethical approval for the study was obtained from the institutional review board (IRB) of BIDE. For this study, data records of the first visit of type 2 diabetic subjects (the time when the patient first consulted our institute for management) who attended outpatient department (OPD) of the institute from January 2005 to April 2016 were analyzed without any breach of confidentiality. Subjects of type 1 diabetes and gestational diabetes were not included in the study. Data obtained from computerized hospital management system (HMS) included risk factors of diabetes along with its microvascular complications. For screening of diabetic retinopathy, fundus was examined using Vista 20 direct ophthalmoscope by a diabetologist. The retinopathy was classified as normal background (presence of microdots and hard exudates), pre-proliferative and proliferative (presence of soft exudates and new vessels), or maculopathy [12, 13]. It also included subjects who had prior laser photocoagulation for diabetic retinopathy. Peripheral neuropathy was defined as absent touch or vibratory sensations of the feet. Touch sensation was assessed by 10 g monofilament and vibration sensation by 128 Hz tuning fork [12, 13]. Nephropathy was classified as protein > 1 plus on dipstick (Combur 10, Rouche Diagnostics) with no other abnormal findings on urinary examination [12, 13]. In case of urine dipstick negative, microalbuminuria was done by quantitative analysis. A result of more than 30 mg on at least two consecutive occasions is considered as positive for nephropathy [13].

Biochemical parameters were measured by standard laboratory test methods. A triglyceride > 150 mg/dl was considered to be hypertriglyceridemia. LDL cholesterol concentration was stated to be abnormal if it was > 100 mg/dl. Low HDL was considered at < 40 mg/dl for male and < 50 mg/dl for female. Hypercholesterolemia was defined as cholesterol over 160 mg/dl [18, 19].

HbA1c value of ≤ 7% and > 7% were taken as good and poor control. Body mass index (BMI) was calculated by the standard formula and obesity was taken as BMI > 25 kg/m^2 [12, 13].

Statistical analysis

Descriptive analysis included the estimation of mean values and standard deviations for continuous variables. Prevalence or frequencies were expressed in terms of percentage. The Student's t-test (for continuous variables) and chi-square test (for categorical variables) were employed to compare differences among the subgroups of the population. Association between microvascular complications and risk factors were examined using logistic regression. Variables significant at p value ≤ 0.25 in an initial univariate regression analysis were entered in a multiple regression model. Backward stepwise regression analysis was undertaken for model building. Odd ratios and 95% confidence interval were generated as a measure of association. The level of significance was set at p value < 0.05. All descriptive and comparative analyses were conducted in SPSS version 20.

Results

A total of 28,601 type 2 diabetic subjects were included in the study. Among them, 56.9% subjects had at least one microvascular complication. Subjects with microvascular complications were older than subjects without microvascular complication (54.4 ± 11.1 vs 50.1 ± 11.1 years) with longer duration of diabetes (9.8 ± 7.7 vs 6.0 ± 6.0 years) and increased BMI (28.3 ± 5.2 vs 27.8 ± 5.6 kg/m^2). Significant differences were found between the groups in biochemical parameters except for triglycerides (Table 1).

Figure 1 showed prevalence of various microvascular complications. Among the studied subjects, 15.8% had retinopathy, 31% had nephropathy, and 48.7% had neuropathy. High prevalence of all three microvascular complications was seen in subjects of older age and subjects who had poor glycemic control and longer duration of diabetes (Table 2).

Table 3 shows the results of univariate regression analysis demonstrating relationship between microvascular complications and their risk factors. Significant differences were observed between all the risk factors and microvascular complications except retinopathy which was not significantly associated with components of dyslipidemia.

Potential risk factors which were significantly associated in univariate analysis were considered for inclusion in a multivariate logistic regression analysis. In multivariate analysis, the risk factors for having at least one microvascular complication were found to be male gender, age ≥ 40 years, duration of diabetes > 10 years, obesity, hypertension, HbA1c > 7%, and low high-density lipoprotein.

Table 1 Association between variables (demographics, anthropometrics, biochemical, and clinical parameters) and diabetic complication

Variables	No Complication	At least one complication	*p* value	Overall
n	12,327 (43.1%)	16,274 (56.9%)		28,601
Male	7903(48.6%)	8129(65.9%)	< 0.0001	16,032(56.1%)
Female	8371(51.4%)	4198(34.1%)		12,569(43.9%)
Age	50.1 ± 11.1	54.4 ± 11.1	< 0.0001	52.5 ± 11.3
Weight (kg)	73.3 ± 14.8	72.3 ± 15.4	< 0.0001	72.7 ± 15.1
Height (cm)	160.9 ± 9.6	161.2 ± 9.7	0.049	161.0 ± 9.7
BMI (kg/m^2)	27.8 ± 5.6	28.3 ± 5.2	< 0.0001	28.1 ± 5.4
Systolic BP (mmHg)	127.6 ± 18.0	132.8 ± 20.8	< 0.0001	130.6 ± 19.8
Diastolic BP (mmHg)	81.1 ± 9.81	81.7 ± 10.7	< 0.0001	81.4 ± 10.3
Duration of DM	6.0 ± 6.0	9.8 ± 7.7	< 0.0001	8.2 ± 7.5
Marital status				
Married	11,545(93.9%)	10,333(92.7%)	0.001	21,878(93.3%)
Single	436(3.5%)	464(4.2%)		900(3.8%)
Widow	315(2.6%)	353(3.2%)		668(2.8%)
Smoking habit				
Ex-smoker	528(4%)	899(7.6%)	<0.0001	1427(5.7%)
Non-smoker	11,591(87.3%)	9538(80.9%)		21,129(84.3%)
Current smoker	1155(8.7%)	1360(11.5%)		2515(10%)
Drinking habit				
Ex-drinker	32(0.2%)	39(0.3%)	0.26	71(0.3%)
Non-drinker	13,100(99%)	11,601(98.8%)		24,701(98.9%)
Current drinker	106(0.8%)	107(0.9%)		213(0.9%)
Fasting blood sugar (mg/dl)	180.5 ± 68.5	198.43 ± 80.66	< 0.0001	189.27 ± 75.2
Random blood sugar (mg/dl)	259.3 ± 105.0	268.67 ± 109.76	< 0.0001	264.42 ± 107.71
HbA1c (%)	9.3 ± 2.2	9.9 ± 2.32	< 0.0001	9.63 ± 2.28
Creatinine (mg/dl)	1.0 ± 1.5	1.2 ± 3.5	< 0.0001	1.2 ± 2.9
Cholesterol (mg/dl)	181.3 ± 46.3	174.3 ± 48.9	< 0.0001	177.3 ± 47.9
Triglyceride(mg/dl)	179.2 ± 135.7	174.5 ± 142.6	0.08	176.4 ± 139.7
Low-density lipoprotein(mg/dl)	110.5 ± 35.5	105.6 ± 36.7	< 0.0001	107.7 ± 36.3
High-density lipoprotein (mg/dl)	38.6 ± 9.0	35.9 ± 9.8	< 0.0001	37.1 ± 9.5

Data presented as mean ± SD or *n*(%), *p* value < 0.05 was considered statistically significant

Nephropathy was found to be significantly associated with male gender, duration of diabetes > 10 years, hypertension, hbA1c > 7%, hypercholesterolemia, and low high-density lipoprotein. Additionally, male gender, age > 40 years, duration

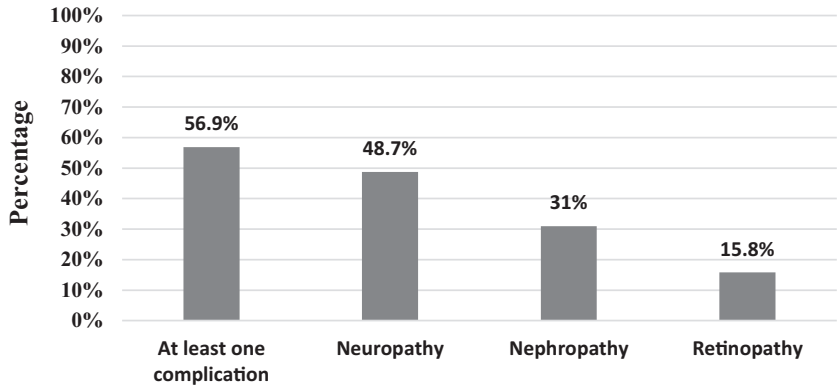

Fig. 1 Prevalence of microvascular complications at baseline

Table 2 Prevalence of microvascular complications according to different subgroups

Variables	Retinopathy	Neuropathy	Nephropathy
Age < 40 years	7%	26%	25.6%
Age ≥ 40 years	17%	51.5%	31.7%
Female	14.3%	46.2%	29.9%
Male	17%	50.5%	31.9%
Duration of DM < 10 years	10.4%	40%	27.1%
Duration of DM ≥ 10 years	30.2%	67.3%	39.6%
HbA1c ≤ 7%	8.8%	41.5%	22.6%
HbA1c > 7%	16.5%	50.2%	29.6%

of diabetes > 10 years, obesity, hypertension, and HbA1c > 7% were found to be significant predictors of developing retinopathy. Moreover, it was observed that presence of neuropathy was associated with male gender, age ≥ 40 years, duration of diabetes > 10 years, obesity, hypertension, HbA1c > 7%, hypercholesterolemia, hypertriglyceridemia, and low HDL (Table 4).

Discussion

The present study analyzed the microvascular complication profile of T2DM subjects at presentation for the first time attending a tertiary care unit of Karachi, Pakistan. The occurrence of any one of the microvascular complications in this study was 56.9% which was comparable to a study by Santos AD et al. in which they observed microvascular complications in 53.8% of type 2 diabetics [20]. Current study observed similar prevalence of microvascular complications as reported by us from the same tertiary care unit 13 years back except for neuropathy which is slightly higher as compared to previous one [12, 14–16]. According to our findings, neuropathy was the most common complication as compared to other complications. Similar outcomes were observed in previous studies from India and other countries from the region [21, 22]. However, some studies conducted in Hong Kong, Jammu and Kashmir, India reported nephropathy as the most common microvascular complication [23, 24].

Retinopathy seems to be the least common microvascular complication in this study population, and the findings are consistent with the earlier study done in Faisalabad, Pakistan [25]. In contrast, a study by Sohail observed higher prevalence of retinopathy (56.9%) among Pakistani individuals [26]. More sensitive screening tool along with the involvement of a trained ophthalmologist could have a better screening result for future studies.

Poor glycemic control, hypertension, gender, and longer duration of diabetes were found to be prominent risk factors of microvascular complications in this study. Comparable findings have been recorded where these factors were reported as contributing factors in progression of microvascular complications [7, 16, 27–29].

Results of the present study provide evidence that older people were at greater risk for the development of diabetes complications particularly retinopathy and neuropathy as reported by other researchers [5, 28, 30, 31]. Previous studies examined positive relationship between obesity and microvascular complications in type 2 diabetes [32, 33]. Similar outcomes were observed in current study. Previously, it was well

Table 3 Univariate regression models

Risk factors	At least one complication		Nephropathy		Neuropathy		Retinopathy	
	COR (95% CI)	*p* value	COR (95% CI)	*p* value	COR (95% CI)	*p* value	COR (95% CI)	*p* value
Gender (male)	1.16(1.11–1.22)	< 0.0001	1.09(1.02–1.16)	0.004	1.18(1.12–1.25)	< 0.0001	1.22(1.10–1.36)	< 0.0001
Age ≥ 40 years	2.27(2.09–2.46)	< 0.0001	1.35(1.22–1.50)	< 0.0001	3.01(2.73–3.31)	< 0.0001	2.71(2.18–3.38)	< 0.0001
Duration of diabetes > 10 years	2.69(2.51–2.85)	< 0.0001	1.76(1.65–1.88)	< 0.0001	3.09(2.91–3.28)	< 0.0001	3.74(3.36–4.17)	< 0.0001
Obesity	1.30(1.23–1.38)	< 0.0001	1.13(1.04–1.21)	0.001	1.43(1.35–1.52)	< 0.0001	1.67(1.49–1.87)	< 0.0001
Hypertension	1.39(1.32–1.47)	< 0.0001	1.44(1.35–1.54)	< 0.0001	1.27(1.20–1.34)	< 0.0001	1.45(1.30–1.62)	< 0.0001
HbA1c > 7%	1.61(1.47–1.77)	< 0.0001	1.44(1.28–1.62)	< 0.0001	1.42(1.28–1.56)	< 0.0001	2.05(1.63–2.57)	< 0.0001
Hypercholesterolemia	1.23(1.13–1.33)	< 0.0001	1.17(1.06–1.29)	0.001	1.29(1.18–1.41)	< 0.0001	1.15(0.97–1.35)	0.091
Hypertriglyceridemia	1.16(1.08–1.25)	< 0.0001	1.02(0.94–1.12)	0.003	0.75(0.69–0.81)	< 0.0001	1.09(0.93–1.26)	0.255
High low-density lipoprotein	0.77(0.71–0.84)	< 0.0001	1.20(1.09–1.33)	< 0.0001	1.33(1.21–1.46)	< 0.0001	0.85(0.71–1.01)	0.072
Low high-density lipoprotein	1.37(1.25–1.5)	< 0.0001	1.37(1.23–1.53)	< 0.0001	1.35(1.22–1.49)	< 0.0001	1.16(0.97–1.39)	0.093

Obesity: BMI > 25 kg/m^2, hypertension: (SBP/DBP: 130/85 mmHg), hypercholesterolemia > 160 mg/dl, hypertriglyceridemia > 150 mg/dl, high low-density lipoprotein > 100 mg/dl, low high-density lipoprotein < 40 mg/dl for male; < 50 mg/dl for female, p value < 0.05 was considered statistically significant

COR crude odds ratio

Table 4 Stepwise multivariate logistic regression models

Risk factors	At least one complication		Nephropathy		Neuropathy		Retinopathy	
	AOR (95% CI)	*p* value	AOR (95% CI)	*p* value	AOR (95% CI)	*p* value	AOR (95% CI)	*p* value
Gender (male)	1.19(1.09–1.31)	< 0.0001	1.20(1.08–1.33)	< 0.0001	1.18(1.07–1.30)	0.001	1.15(1.02–1.31)	0.024
Age ≥ 40 years	1.72(1.50–1.98)	< 0.0001	–	–	2.14(1.82–2.51)	< 0.0001	1.70(1.32–2.18)	< 0.0001
Duration of diabetes > 10 years	2.48(2.24–2.76)	< 0.0001	1.71(1.54–1.90)	< 0.0001	2.61(2.36–2.89)	< 0.0001	3.31(2.92–3.76)	< 0.0001
Obesity	1.18(1.06–1.30)	0.001	–	–	1.20(1.09–1.33)	< 0.0001	1.52(1.33–1.74)	< 0.0001
Hypertension	1.34(1.22–1.46)	< 0.0001	1.44(1.30–1.59)	< 0.0001	1.22(1.11–1.34)	< 0.0001	1.33(1.17–1.52)	< 0.0001
HbA1c > 7%	1.54(1.35–1.75)	< 0.0001	1.49(1.27–1.76)	< 0.0001	1.36(1.18–1.56)	< 0.0001	1.86(1.46–2.37)	< 0.0001
Hypercholesterolemia	–	–	1.18(1.01–1.37)	0.035	1.16(1.01–1.34)	0.033	–	–
Hypertriglyceridemia	–	–			0.86(0.78–0.94)	0.002	–	–
High low-density lipoprotein	–	–	–	–	–	–	–	–
Low high-density lipoprotein	1.38(1.24–1.53)	<0.0001	1.32(1.17–1.50)	<0.0001	1.44(1.29–1.61)	<0.0001	–	–

Obesity: BMI > 25 kg/m^2 , hypertension: (SBP/DBP 130/85 mmHg), hypercholesterolemia > 160 mg/dl, hypertriglyceridemia > 150 mg/dl, high low-density lipoprotein > 100 mg/dl, low high-density lipoprotein < 40 mg/dl for male; < 50 mg/dl for female, *p* value < 0.05 was considered statistically significant

AOR adjusted odds ratio

defined that dyslipidemia causes risk of developing microvascular complications [5, 28, 34]. Our findings also showed significant association of hypercholesterolemia, hypertriglyceridemia, and low HDL with neuropathy and nephropathy. In contrast, no significant association of LDL cholesterol was found with any microvascular complication.

Limitations of this study include its retrospective design focusing only microvascular complications. Another limitation was the diagnosis of retinopathy, based on fundoscopy instead of using more sensitive screening tool like fundal photography. Additionally, it was single tertiary care center-based study and not a community survey as prevalence of diabetic complications may vary in a community-based survey.

Conclusion

High prevalence of microvascular complications was observed among type 2 diabetic subjects who visited for the first time in a tertiary care hospital. Male gender, age ≥ 40 years, duration of diabetes > 10 years, obesity, hypertension, HbA1c > 7%, and low HDL were strongly associated with any type of microvascular complication. These outcomes need to be validated by multicentered or larger-scale community-based studies. Early identification and effective management are required at primary and secondary care levels to combat this situation.

Compliance with ethical standards

Ethical approval for the study was obtained from the institutional review board (IRB) of BIDE.

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

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ORIGINAL ARTICLE

Prevalence of type 2 diabetes mellitus among adult population of District Srinagar

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Abstract
Over the previous 30 years, the status of diabetes has changed from being considered as a mellow issue of the elderly to one of the significant reasons for morbidity and mortality influencing the young and moderately aged individuals. The aim of the study was to estimate the prevalence of type 2 diabetes mellitus (T2DM) and impaired fasting glycemia (IFG) in the adult population, age ≥ 20 years of District Srinagar and determine its sociodemographic and lifestyle correlates. The study was conducted among the adult population of District Srinagar with a sample size of 580. A three-stage cluster random sampling design was used, and study participants were selected using a Kish grid method. Sociodemographic data, anthropometric data, and other data were collected as per the pretested semi-structured proforma. The participants were then subjected to fasting venous blood glucose estimation. The American Diabetes Association (ADA) criteria 2015 were used for classifying the subjects as normal, pre-diabetic, and diabetics. The prevalence of T2DM was 9.8% and that of IFG was 22.2%,the majority of them (9.1%) had known T2DM. The prevalence of IFG and T2DM was statistically associated with increasing age ($p < 0.001$), marital status ($p < 0.001$), educational status ($p < 0.001$), high chocolates and carbonated drinks consumption ($p < 0.001$), level of physical inactivity ($p < 0.05$), body mass index ($p < 0.001$), family history of T2DM ($p < 0.001$), and smoking behavior and hypertension ($p < 0.001$). Our analysis suggests an increase in the prevalence of T2DM in District Srinagar.

Keywords Type 2 diabetes mellitus · Diabetes risk factors · Prediabetes · Diabetes prevention · Diabetes screening · Diabetes prevalence

Introduction

In the course of the recent years, the status of diabetes has changed from being considered as a mild disorder of the elderly to one of the significant reasons for mortality and mortality influencing the adolescent and moderately aged individuals [1]. It is reported that the rise in the predominance of T2DM is found in every one of the six continents of the globe [2]. It is assessed that in the year 2014, 66.8 million individuals aged 20–79 years living with diabetes and 35.4 million with undiscovered diabetes in India [3]. This number is estimated to rise to 101.2 million by 2030 [4]. Further, 77.2 million individuals in India are said to have prediabetes [5]. A most exasperating pattern observed in the past decade is the shift in the age of the beginning of diabetes from the elderly to a more youthful age. Indians get diabetes by and large 10 years sooner than their Western counterparts[6]. Obesity and physical inactivity are the two most imperative modifiable risk factors for T2DM [7]. Lifestyle intervention has been shown to reduce the incidence of T2DM, thus justifying the implementation of population-based strategies for identifying and treating high-risk individuals.

Diabetes is considered to be an epidemic of the twenty-first century and the primary driver of the epidemic of diabetes is the rapid epidemiological transition associated with the changes in dietary patterns and decreased physical activity as evident from the higher prevalence of diabetes in the urban population [8]. This rapid increase is mostly attributed to lifestyle transitions resulting in obesity and physical inactivity, population aging, and urbanization [9]. A study demonstrated that a

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low-fiber diet with a high glycemic index was related to a higher prevalence of T2DM in the study subjects [10]. A study from India demonstrated that over half of the individuals with diabetes have poor glycemic control, uncontrolled hypertension, and dyslipidemia, and a vast number have diabetic vascular complications [11]. Another study on the Indian population demonstrates that the basic factors responsible for diabetes development and its complications are hypertension, poor metabolic control, smoking, and dyslipidemia [12]. However, most of the factors for the development of T2DM vary on geographic and ethnic background [12] and this forms the basis of this investigation, to estimate the prevalence of the T2DM and IFG in this part of the Indian subcontinent.

An intensive literature search uncovers the absence of information on the prevalence of T2DM in Kashmir. The latest archived study was reported by Zargar et al. 2008 [13] among adult population aged (20–40 years) and Zargar et al. 2000 [14] among individuals (> 40 years), where they evaluated the prevalence of T2DM as 2.4 and 6% respectively. Owing to the increased susceptibility towards the contributing factors of the disease and the changing lifestyle over the last decade, we have conducted this study to estimate the prevalence of T2DM and IFG among the adult (≥ 20 years) population of the District of Srinagar. The findings of this study will be useful to formulate policies for the control of T2DM in Kashmir and implement programs for the early detection and prevention of this disease among the adult population of Kashmir valley.

The aim of the study was to estimate the prevalence of T2DM and IFG in the adult population, age ≥ 20 years of the District Srinagar and study its sociodemographic and lifestyle correlates.

Methods

The study was conducted by the Department of Community Medicine, Government Medical College, Srinagar, in the District Srinagar, the summer capital of Jammu and Kashmir State from August 2016 to July 2017. Adult population > 20 years of age and permanent residents of the District of Srinagar who have been residing in the district for the last two decades were included in the study. Pregnant females and patients with known type 1 diabetes mellitus were excluded from the study. In this study, the sample size was calculated using the estimated previous prevalence of 6.1% reported by Zargar et al. [14], absolute precision at 2.5%, design effect of 1.5%, non-response rate of 10%, and confidence level at 95%, the sample size came out to be 580. A three-stage cluster random sampling design was used for the study.

Stage I Selection of clusters: In the first stage, 30 clusters were selected by using the probability proportionate to size sampling (PPS) method to achieve the required sample size.

Stage II Selection of households: In the second stage, households were selected by using a systematic random sampling. The sampling was done by selecting a random house as a starting point for the study from each selected cluster and then from there moved in a predetermined random direction and selected every tenth house in order to achieve the cluster size of 20 households. Only one adult from each household was included in the study. In case the house was locked, the adjacent household was chosen.

Stage III Selection of study participant: The persons in the household were explained with the objectives of the study. The study participant was selected using the Kish Grid method [15]. A proper written informed consent was taken from the selected participant in a household. Then the participant was subjected to a pre-tested, semi-structured questionnaire for the identification of data, and to collect anthropometric and clinical data. Flowchart of the sampling method is illustrated in Fig. 1.

The subjects were then submitted to a medical examination including the measurement of blood pressure (BP) and anthropometric data (weight and height). Subjects were weighed using a standardized weighing scale which was placed on a flat surface and set to zero. All values were documented on the recording sheet. Waist and hip circumference were measured using standard techniques and the mean of two measurements was taken for calculating the waist-hip ratio (WHR). Then, the participants were asked to fast overnight for at least 8 h before blood collection scheduled for the next morning. Fasting was confirmed verbally by the participants immediately before collecting the blood sample. If fasting could not be confirmed, then a new specimen for repeat testing was obtained at a later date.

The blood sample was collected in labeled tubes containing citrate buffer, sodium fluoride, and disodium EDTA, which stops glucose consumption by red blood cells [16]. The samples were then sent to a certified biochemical laboratory at G. B Pant Hospital using the fastest available transport in a cold box within 2 h of sample collection.

Definition and specific description of variables used in study

a) Known diabetes mellitus: Participants with known diabetes are those who had been told by a physician that they had the condition and/or were being treated with insulin or oral antidiabetic agents [17].
b) Newly diagnosed diabetes mellitus: Participants with newly diagnosed diabetes had a fasting blood plasma glucose

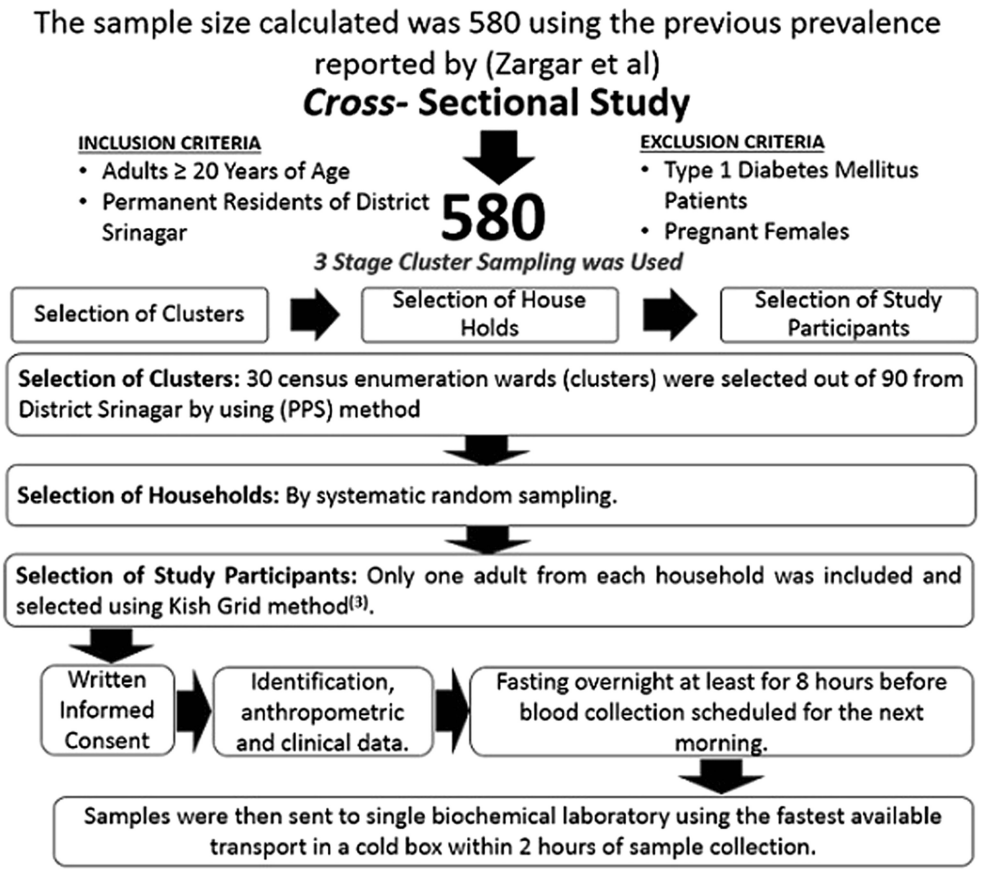

Figure 1: Process of Sampling used for the study

Fig. 1 Flowchart showing sampling process of the study

of ≥ 126 mg/dL during the study and had not previously been told that they had diabetes. A repeat sampling was implemented to reconfirm the test results [17]. Those with fasting blood plasma glucose ≥ 126 mg/dL on the second instance were referred to the endocrinologist for treatment.

c) Impaired fasting glycemia: IFG was defined as a fasting plasma blood glucose of 101–125 mg/dL [17]. Patients with IFG were educated about the risk factors of diabetes mellitus, life style interventions, and role of physical activity in the prevention of disease.

Statistical analysis: Data was entered in a Microsoft Excel spreadsheet. Continuous variables were summarized as mean and standard deviation. Categorical variables were summarized as percentages. Chi-square test was used to test independence between two categorical variables. A p value of less than 0.05 was considered statistically significant. All statistical analysis was done using SPSS 20.0.

Results

Diabetic status of the study participants is shown in Fig. 2, 129 (22.24%) of the study participants had fasting blood glucose levels between 100 and 125 mg/dl (IFG), while 57 (9.8%) had fasting blood sugar levels more than 126 mg/dL (T2DM).

In this study, the prevalence of IFG among the study participants increased from 20 to 23.6% between age groups [21–40 years and 41–60 years]. Moreover, the prevalence of T2DM increased from 0.5 to 14.8% between age groups [21–40 and 41–60 years] respectively. There was a further increase in the prevalence of T2DM to 16.9% among the geriatric population (61–80 years). In our analysis, age was found to be an important factor predisposing to IFG and T2DM and the association was statistically significant. Furthermore, T2DM and IFG were found to be more prevalent among study participants having education up to the primary, middle, and high school levels respectively. In this study, low education status was found to have an impact on the prevalence of IFG and T2DM and the association was statistically significant. In this study, the socioeconomic status of the study participants was found to have no role in the development of IFG and T2DM. We also found that those participants who used to smoke any form of tobacco too have a higher prevalence of IFG and T2DM with significant p value (0.018). [Table 1].

In our study, we found that those study participants who used to consume carbonated drinks, chocolates, or candies

more than 1–3 times in a usual week were having a higher prevalence of IFG. As the majority of the diabetics found in this study were already diagnosed cases of T2DM, they used to avoid carbonated drinks, chocolates, and candies as part of their dietary modification treatment recommended by their physicians. In this study, it was found that physical inactivity among study participants was responsible for the development of IFG and T2DM and the association was found to be statistically significant. Similarly, higher BMI scores, history of essential hypertension among the study participants, and family history of diabetes were an important factor to be associated with the development of IFG and T2DM among them [Table 2].

In Table 3, the results of a multiple logistic regression analysis in the District of Srinagar is shown in which diabetes was the dependent variable. Age, obesity, increased body mass index, hypertension, family history of diabetes, smoking behavior, and fasting blood sugar levels of the subjects were independent risk factors for diabetes. High socioeconomic status and physical activity score of the study subjects were not related to diabetes in this analysis.

Discussion

This was a community-based study to estimate the prevalence of T2DM among adults (≥ 20 years) in the District of Srinagar. This study estimated the overall prevalence of diabetes in the District of Srinagar to be 9.8% [95% CI 7.4–12.0] and the prevalence of prediabetes to be 22.2% [95% CI 18.8–25.5] (ADA criteria). Most (93%) of all cases of T2DM were already a known case or on treatment. The rest (7%) of T2DM cases were newly diagnosed. This district-wide community-based study was done in one of the largest districts of Kashmir division. There have been few large community-based studies looking at the prevalence of diabetes and prediabetes in Kashmir Division. Zargar et al. (2001, 2008) [13, 14] reported a prevalence of T2DM and IFG (6.1 and 8.09% in 2001 and 2.4 and 11.1% in 2008) among adults > 40 years and 20–40 years respectively. The National Urban Diabetes Survey showed the prevalence of 12.1% for diabetes and 14% for impaired glucose tolerance in six large metropolitan cities of India [9]. Recently published INDIAB phase 2 study by ICMR, New Delhi, estimated prevalence of T2DM and IFG in 15 states of India which included both urban and rural areas. They reported a significantly higher prevalence of T2DM and IFG in urban areas. [18]. The study estimated the prevalence of diabetes in different states of India which varied from 4.3% in Bihar to 10.0% in Punjab and was higher in urban areas (11.2%, and 10.6–11.8) than in the rural areas (5.2%, 4·9–5·4) [18].

The above estimates clearly indicate that there is a difference in the prevalence of T2DM and IFG between different regions of the Indian states and might be explained by factors such as the difference in socioeconomic status, obesity prevalence, physical activity, dietary pattern, and possibly genetic variations. Our study adds to the growing body of evidence suggesting that the prevalence of T2DM has increased since 2001 from 6.1 to 9.8% [ADA criteria] and IFG from 8.1 to 12.1% [WHO criteria] in the present scenario. This can be explained by several factors including the adoption of new lifestyles in parallel with the strong economic growth our state has experienced in the past 20 years. An important finding of this study was that using the study conducted by Zargar et al. [13], conducted in 2000 as a reference, the estimated prevalence of T2DM in the region has increased by 3.55% and that of IFG by 1.55% among adults > 40 years of age since 2001. Thus, even though the prevalence of T2DM in the District of Srinagar is lower than the other Indian states [Tripura 15.5%, Andhra Pradesh 12.6%, Assam 12.4%, Punjab 12.0%, Karnataka 11.1%, and Bihar 10.8%] [18], there seems to be an increasing trend in T2DM prevalence and is certainly quite high and warrants immediate attention. This is thus the right time to accelerate efforts to prevent further increase in the prevalence of T2DM.

In our study, we found that 20 and 0.5% of study subjects with age 21–40 years have IFG and T2DM respectively. With an increase in age, the prevalence of IFG and T2DM increased significantly ($p < 0.05$), the maximum

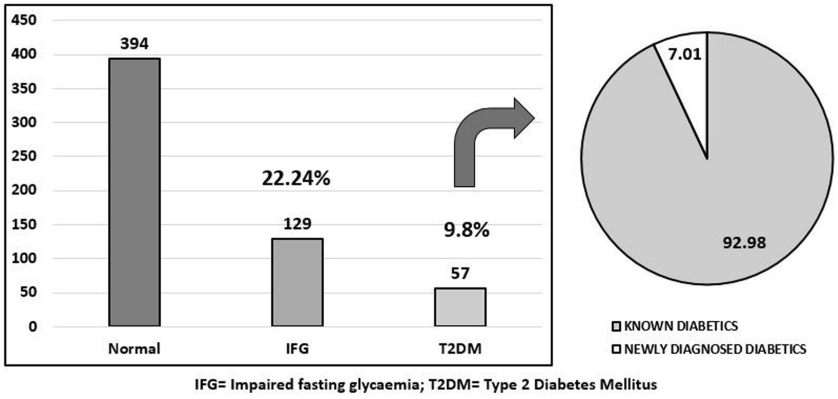

Fig. 2 Diabetic status of study participants

Table 1 Demographic characteristics of study subjects and their relation with outcome variable

Variable	Frequency (%)	Outcome variable categories			Statistical analysis
		Normal (*n*)	IFG (*n*)	Diabetic (*n*)	
Age group (in years)					$\chi2 = 36.833$
21–40	210 (36.2)	167	42	1	df = 4
41–60	305 (52.6)	188	72	45	p value < 0.05*
61–80	65 (11.2)	39	15	11	
Gender					$\chi2 = 3.041$
Male	289 (49.8)	189	73	27	df = 2
Female	291 (50.2)	206	56	30	p value 0.219
Marital status					
Currently married	457 (78.8)	96	14	0	$\chi2 = 26.341$
Unmarried	110 (19.0)	291	110	56	df = 2
Widow	12 (2.1)	1	0	0	p value < 0.05*
Divorced	1 (0.2)	6	5	1	
Educational status ¥					$\chi2 = 45.947$
Illiterate	125 (21.6)	79	26	20	df = 8
Primary school	21 (3.6)	14	1	6	p value < 0.05*
Middle school	38 (6.6)	19	9	10	
High school	58 (10.0)	34	22	2	
Higher secondary	112 (19.3)	76	30	6	
Graduate	217 (37.4)	164	40	13	
Post-graduation and above	9 (1.6)	8	1	0	
Socioeconomic status ¥					$\chi2 = 2.054$
Upper (I)	5 (0.9)	5	0	0	df = 2
Upper middle (II)	445 (76.7)	307	97	41	p value 0.35
Lower middle (III)	89 (15.3)	53	24	12	
Upper lower (IV)	41 (7.1)	29	8	4	
Occupation¥					$\chi2 = 20.855$
Unemployed	115 (19.8)	84	21	10	df = 12
Unskilled	17 (2.9)	8	6	3	p value 0.05*
Semi-skilled	26 (4.5)	14	7	5	
Skilled	183 (31.5)	123	40	20	
Clerical, farmer	106 (18.4)	69	32	5	
Semi-professional	101 (17.4)	68	21	12	
Professional	32 (5.5)	28	2	2	
Smoking history					$\chi2 = 8.068$
Yes	135 (23.3)	82	42	11	df = 2
No	445 (76.7)	312	8	46	p value 0.018*

IFG, impaired fasting glycaemia; χ^2 = chi-square test; df, degree of freedom

¥ As per Kuppuswamy's Socioeconomic scale 2016

* p value < 0.05 is considered significant

being in the age group of 61–80 years where the T2DM prevalence was 16.9% and that of IFG in the age group of 41–60 years, where it was 23.6%. This is in accordance with the results published by Seclen et al. [17], Y Yin et al. [19], and Suastika K et al. [20] who reported an increased prevalence of T2DM and IFG with increasing age. One of the important findings in our study was the 20% prevalence of IFG in subjects who were less than 40 years of age. This finding is worrying because of the additional burden this group will add in the future as studies have documented early onset of T2DM in younger age groups in south Asian people compared with other populations [21], the strain on the country's healthcare system is likely to be immense.

In our study, the gender of the participants had no relationship with their diabetic status ($p > 0.05$), these findings are

Table 2 Relationship of independent and dependent variables

Variable	Frequency	Outcome variable categories			Statistical analysis
		Normal	IFG	Diabetic	
Carbonated drinks consumption/week					$\chi^2 = 117.7$ df = 4 p value < 0.05*
Never	109 (18.8)	52	16	41	
1–3 times	359 (61.9)	257	88	14	
4–6 times	111 (19.1)	84	25	2	
> 6 times	1 (0.2)	1	0	0	
Candies/chocolate consumption					$\chi^2 = 19.16$ df = 2 p value < 0.05*
Yes	92 (15.9)	79	13	0	
No	488 (84.1)	315	116	57	
Physical activity#					$\chi^2 = 6.05$ df = 2 p value 0.048*
Physically active	454 (78.3)	297	109	48	
Physically not active	126 (21.7)	97	20	9	
Body mass index					$\chi^2 = 52.3$ df = 6 p value < 0.05*
Under weight (<18.5)	3 (0.5)	3	0	0	
Normal (18.5–22.9)	192 (33.1)	152	36	4	
Over weight (23.0–24.9)	214 (36.9)	150	48	16	
Pre-obese (25.0–29.9)	143 (24.7)	76	35	32	
Type1 obese (30.0–40.0)	28 (4.8)	13	10	5	
History of hypertension					$\chi^2 = 62.42$ df = 2 p value < 0.05*
Yes	66 (11.4)	26	16	24	
No	514 (88.6)	368	24	33	
Family history of diabetes mellitus					$\chi^2 = 79.31$ df = 2 p value < 0.05*
Yes	78 (13.4)	23	29	26	
No	502 (86.6)	371	100	31	

IFG = Impaired fasting glycaemia; χ^2 = Chi-square Test; df = Degree of freedom

*p value < 0.05 is considered significant

An equivalent of 150 min of moderate-intensity physical activity/75 min of vigorous-intensity physical activity in a usual week

similar to the one reported by A. Kautzky Willer et al. [22] and L. Arnetz et al. [23] in their studies respectively. P. Ballotari et al. [24], however, have reported a significant difference in the prevalence of T2DM among males and females.

In our study, 217 (37.4%) of the study participants being graduate, the educational status had a significant association ($p < 0.05$) with the diabetic status of the study participants. These findings are similar to the one reported by S. Duke et al. [25] and X. Shang et al. [26] in their studies. This could be attributed that a participant who had an education has a good chance to modify their lifestyle and their health status by finding different alternatives for checkups. Another important finding in our study was the association of the occupation of subjects with T2DM, showing more prevalence of T2DM in subjects who belong to the clerical, shop owner, semi-professional, and professional occupations with statistically significant association (p value < 0.05). This can be attributed to the fact that most of the people have adapted unhealthy lifestyle behaviors and lack of physical activity in their day-to-day life. These findings are in accordance with the studies conducted earlier [27, 28].

Diabetes prevalence and IFG were also seen higher in the more economically developed group and common in

Table 3 Multiple logistic regression with diabetes as the dependent variable in study a population

Variable	Odds ratio (95% CI)	p value
Age (in years)	1.04 (1.04–1.05)	< 0.001*
Body mass index	2.11 (1.87–2.38)	< 0.001*
Family history of diabetes mellitus	2.13 (1.87–2.54)	< 0.001*
History of hypertension	1.66 (1.50–1.84)	< 0.001*
Fasting blood sugar	1.87 (1.47–2.10)	< 0.001*
Smoking (present)	1.20 (0.98–1.32)	< 0.05*
Socioeconomic status	0.74 (0.45–0.90)	0.28
Physical activity score	0.56 (0.34–0.67)	0.25

*p value < 0.05 is considered significant

individuals of medium or high socioeconomic status than in individuals of low socioeconomic status, which agrees with results from a study in India by E E Agardh et al. [29]. This finding suggested that the diabetes epidemiological transaction is advanced in the urban Srinagar District affecting the highly productive group of the society. INDIAB phase 2 studies reported less advanced epidemiological transaction in which a higher socioeconomic group in rural areas and low socioeconomic group in urban areas were affected with diabetes mellitus which contradicts with our findings [18].

Furthermore, the consumption of carbonated drinks and chocolates by the study subjects was also associated with the development of T2DM and IFG and the relationship was statistically significant ($p < 0.05$). These findings are in accordance with the published literature [30]. The consumption of carbonated drinks and chocolates raises blood glucose levels and can provide several hundred calories in just one serving which eventually with continuous consumption over a period of time causes weight gain and insulin insensitivity leading to the development of IFG and T2DM.

One of the important risk factors of T2DM is physical inactivity, and studies have proved the protective role of physical activity in preventing and treating IFG and T2DM [31–33]. In our study, majority 297(65.4%) of the study subjects who were physically active had normal diabetic status, while 109 (24%) had IFG. We had already 53 known cases of T2DM and they were currently on treatment with drugs and lifestyle modification regimen. The association between physical activity and the diabetic status of the study participants was statistically significant (p value = 0.048).

Generalized obesity/high BMI and abdominal obesity were independently associated with T2DM and IFG which is similar to the results in most other studies [34–38]. Indians have a lower BMI than those of European descent. However, the risk of diabetes increases at very low levels of BMI for Indians [39]. Family history of T2DM is a strong predictor of the disease which is supported by most other studies [34, 40–43]. In this study, there was a positive association between a family history of diabetes mellitus with IFG and T2DM. These findings also support previous studies demonstrating that family history is a strong and independent risk factor for diabetes [34, 40, 41]. However, familiar risk must be evaluated in the context of other known or suspected risk factors.

History of hypertension is also an established risk factor for T2DM [44]. In our study, hypertension among study subjects was associated with the development of IFG and T2DM. The association was statistically significant. These findings were also reported by many studies [39, 45–48].

In some studies, smoking did not seem to independently increase the risk of diabetes mellitus but in our study, smoking was associated with the diabetic status of the study subjects and these findings were reported by many earlier studies [23, 29, 49–51]. Although differences in patterns of use (e.g., type and quantity of smoking) might help to account for these differences in different people, further studies are needed to explore these hypotheses.

All the abovementioned parameters clearly indicate deviation from healthy lifestyles to the adoption of a new lifestyle behavior by Kashmiri population. The main factors driving the diabetes epidemic in the District of Srinagar are older age, higher socioeconomic status, low education level, higher consumption of carbonated drinks and chocolates, physical inactivity, obesity/higher BMI, associated hypertension, smoking behavior, and positive family history of diabetes.

The prevalence estimates of our study of prediabetes or IFG were high, exceeding those of diabetes and implying the existence of a huge number of individuals who could conceivably develop T2DM in the near future. In this study, the diabetes-to-prediabetes ratio of almost 1:2 suggests that there continues to be a large pool of individuals at risk of developing T2DM in the District of Srinagar. A higher ratio known to newly diagnosed diabetes, as shown in the results of this study suggests better awareness of diabetes in the population. This improved awareness and diagnosis are possibly the results of concerted efforts by the Government (through programs such as the National Programme for Control and Prevention of Cancer, Diabetes, Cardiovascular Disease and Strokes) and non-governmental organizations.

Strengths of the study

It is the first study on diabetes to include the whole of the District of Srinagar, Jammu and Kashmir, and both rural and urban populations, and it is the largest epidemiological community-based study on T2DM in the state. In this study, a representative sampling frame and robust methods, with venous fasting blood glucose tests used for the detection of diabetes in a sample of 580 people have been used. In spite of the fact that obtaining venous blood samples from the subjects in epidemiological studies is difficult, blood samples were drawn from the subjects after explaining the objectives of the study and informed consent. All the blood tests for venous fasting blood glucose estimations were performed in a single quality-controlled laboratory with same methods with the Glucose Estimation Criteria 2015 recommends repeating blood tests for the diagnosis of diabetes, we were able to do so following the study protocol. Door-to-door assessment was done and from each household, we randomly selected one individual, in accordance with the WHO Kish method, thereby avoiding selection bias with respect to sex and age. Moreover, the study was done by a single investigator using the same methods and standardized techniques with stringent

quality control, so any differences in prevalence noted probably cannot be attributed to this methodological limitation.

Limitations of the study

The prevalence of diabetes based on HbA1c (now an accepted diagnostic tool) could not be estimated because of the high costs associated with its testing. The cross-sectional nature of the study does not allow for inferences of causality to be made. Our results also do not provide information on the prevalence of diabetes in individuals younger than 20 years because this was beyond the scope of the study.

Conclusion

Our analysis suggests an increase in the prevalence of diabetes in the District of Srinagar. Risk factors for T2DM and IFG include increasing age, higher socioeconomic status, low literacy levels, obesity, higher consumption of junk food and carbonated drinks, hypertension, and positive family history of diabetes. People should meet with professional dieticians to plan an individualized diet within the general guidelines that take into consideration their own health needs. Consumption of junk food, chocolates, carbonated drinks, and smoking in any form should be avoided. People should try to achieve a healthy weight, control of blood pressure, and normal blood glucose levels.

Compliance with ethical standards

Ethical approval Ethical clearance was obtained from the institutional ethical committee before the commencement of the study. The committee permitted the same as per protocol presented and described in the proposal vide no: Acad/6579–87/MC dated 09/03/2016.

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ORIGINAL ARTICLE

Dietary zinc reduces endoplasmic reticulum stress and autophagy to protect against diabetic renal damage in streptozotocin-induced diabetic rats

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Abstract
Most of the patients with type 2 diabetes mellitus suffer from renal injury. The current study explored the protective effects of dietary zinc against diabetic renal injury and the underlying molecular mechanisms. In experiments, $ZnSO_4$ at 15 mg/kg was intragastrically administrated into type 2 diabetes mellitus-like Wistar rat models daily for continuously 53 days. Biochemical assay was performed to detect serum fasting blood glucose, blood urea nitrogen (BUN), creatinine, and insulin. The H&E staining, immunohistochemical assay, and Western blotting were performed to detect the changes in kidney tissues. In results, dietary zinc reduced the lipid peroxidation malondialdehyde levels and enhanced the total anti-oxidation capacity levels. The pathological changes in diabetic kidney were improved. The percentage of autophagy-associated LC3-II positive cells was lowered. Western blot levels of LC3-II proteins and endoplasmic reticulum (ER) stress-associated GRP78 proteins were lowered. In conclusion, dietary zinc can relieve ER stress and autophagy in diabetic kidney to improve kidney conditions of type 2 diabetes mellitus-like rat models. These findings may be useful to treat patients with diabetic renal injury.

Keywords Zinc · Diabetes mellitus · Renal injury · Metallothionien · Protein kinase B · Endoplasmic reticulum stress · Autophagy

Introduction

Most of the patients with type 2 diabetes mellitus (T2DM) suffer from chronic renal disease due to cellular oxidative damage, endoplasmic reticulum (ER) stress, autophagy dysfunction, renal interstitial fibrosis, and even renal failure [1–5]. Insulin resistance and β cell dysfunction contribute to the pathological mechanisms of T2DM with deficiency of insulin. Lack of zinc over time may affect insulin activity leading to insulin resistance in T2DM [6]. Zinc supplementation can protect against the diabetic renal injury through multiple mechanisms, inhibition of renal LOX-1-mediated ICAM-1 expression [1], downregulation of profibrosis mediator connective tissue growth factor (CTGF) [7], upregulation of antioxidant metallothionein (MT) [7–10], downregulating P38 MAPK-mediated inflammation [7, 11], reducing renal cortical apoptosis [12], and upregulation of Nrf2, NQO1, SOD1, SOD2, Akt phosphorylation, and GSK-3β phosphorylation [13]. The mechanisms that zinc protect diabetic kidney may be complicated further.

Recently, ER stress is found to play an important role in the pathogenesis of diabetes to worsen islet β cell loss and insulin resistance [14, 15]. A molecular chaperone glucose-regulated protein78 (GRP78/Bip) with positive response to ER stress can be an indicator for ER stress in cells [16]. Autophagy is a featured phenomenon in eukaryotic cells to involve in cell death of diabetic rats [17, 18]. Microtubule-associated protein light chain 3 (LC3-II) levels, with proportion to autophagic vacuoles in mammalian cells, can be considered to determine the autophagy status [19]. Studies have shown that zinc supplementation can downregulate both ER stress and autophagy in the heart and the liver of rats with T2DM to relieve diabetic injury [17, 18]. These may be likely referenced to treat diabetic renal damage.

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Zinc-binding protein metallothionein (MT) plays an important role as potent antioxidants to prevent various oxidative damages in diabetes mellitus [15, 20, 21]. Serine/threonine protein kinase B (PKB/Akt) mediates PI3K-controlled insulin signaling events to involve in the insulin signal pathway of diabetes mellitus [22, 23]. Therefore, MT and PKB are inevitable molecules for studying diabetic organ injury.

The current study intended to explore the effect of dietary zinc on diabetic renal damage and the underlying mechanisms through reducing the ER stress and autophagy status in the T2DM rat kidneys. The involved MT and PKB expression levels were also detected to evaluate the diabetes condition. We expected to verify the new putative mechanisms protecting against diabetic renal damage.

Materials and methods

Animal modeling

Animal models were prepared as references [17, 18]. Briefly, totally 39 male Wistar rats weighing 180–220 g were kept at a constant temperature of 22 ± 2 °C in The Laboratory Animal Center of Jilin University. The animal experiments were approved by The Jilin University Animal Ethics Committee. Type 2 diabetes mellitus (T2DM)-like rat models were prepared by a single intraperitoneal administration of streptozotocin (STZ, 40 mg/kg weight) following 35-days high-fat dieting.

Treatment and sampling

The rat models were treated as reference [18]. Briefly, three days following STZ injection, the fasting blood glucose (FPG) was tested by the serum biochemical assay described in the next section, and then the rat models with FPG ≥ 16.7 mmol/l were determined as T2DM rats to be included in the following experiments. Subsequently, for the DM + Zn (diabetes with Zn supplementation) group, T2DM rats underwent intragastric (ig) administration of 15 mg $ZnSO_4$ in 5 ml double-distilled water per kilogram bodyweight daily until sacrificed. Diabetes-free normal control (NC) and T2DM model control (MC) groups were treated with equivalent volumes of double-distilled water. Rats in the NC group always took normal diet, and rats in the DM + Zn group and the MC group always took high-fat diet. The body weight was recorded every 7 days. At 53 days following Zn supplementation, rats were narcotized by intraperitoneal injection of 3% pentobarbital sodium at 30 mg/kg weight and then fixed at the supine position. Blood was collected by abdominal vein. The whole blood was placed at 4 °C for 1.5 h and then centrifuged at 3000×*g* for 15 min at 4 °C to obtain serum for inspection. Kidneys were removed and placed rapidly into precooled 0.9% NaCl solution at 4 °C for rinsing off the blood. After dried by filter papers, kidneys were weighed and assayed.

Serum biochemical assay

Automatic biochemical analyzer (7170A, Hitachi Inc., Tokyo, Japan) was used to test the FPG, blood urea nitrogen (BUN), and creatinine (CRE). Serum insulin was assayed by a radioimmunoassay method using the insulin radioimmunoassay kit (Northern Institute of Biotechnology, Beijing, China) according to the manufacture's instruction. The radioactivity was detected by SN-695 gamma radioimmune counter (Hesuo Rihuan Photoelectric Instrument Co, LTD, Shanghai, China).

Hematoxylin and eosin staining

Kidneys were cut into pieces and fixed in 10% neutral formaldehyde solution. Fixed kidney pieces were embedded in paraffin, sectioned into slices of 4 μm thick, dewaxed by xylene, and dehydrated by a series of graded ethanol. A portion of dehydrated slices was conventionally stained with hematoxylin and eosin (H&E).

Immunohistochemical assay

For immunohistochemical assays, dehydrated slices were placed in 3%H_2O_2 for 10 min to block endogenous peroxidase and boiled 10 min in 0.01 mol/l sodium citrate buffer (pH = 6.0). Then, slices on the slides were blocked 15 min with anti-species serum following washing by 1 mol/l PBS (pH = 7.4). Goat anti-rat p-Akt (phosphorylated Akt/PKB) and anti-rat LC3-II polyclonal antibodies (Beyotime Institute of Biotechnology, Najing, Jiangsu, China) were added at 4 °C overnight. Biotinylated secondary antibody rabbit anti-goat IgGs were added for 20 min at 37 °C. Streptavidin and biotinylated horseradish peroxidase were added for 20 min at 37 °C, followed by color development using the DAB (3,3-diaminobenzidine) kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Brown stains indicated positives. Microscopic examination (Olympus PM-10A0, Olympus, Beijing, China) was done by two separately blind pathologists. Each pathologist counts the positive cells from randomly selected five microscopic fields (× 400) of each slice and calculated their percentage: < 5%, 5~25%, 25~50%, and 50~75% graded ±, +, ++, and +++, respectively.

Detection of redox in tissue homogenates

Kidneys were put in precooled 10 mmol/l Tris-HCl buffer (pH = 7.4, at 4 °C) with a mass/volume ratio of 1 g/4 ml, homogenized on ice bath, and centrifuged at 10,000×*g* for 10 min at 4 °C. Lipid peroxidation product malondialdehyde

(MDA) in supernatants was assayed by the thiobarbituric acid (TBA) method [24] using the MDA detection kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Total anti-oxidation capacity (T-AOC) was also detected using the T-AOC detection kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). MT protein content was determined by the ^{109}Cd hemoglobin saturation method as referenced to the literature [25]. Briefly, supernatants were boiled for 2 min and centrifuged at 10,000×*g* for 2 min to remove heat-precipitated proteins. An aliquot of 200 μl supernatants was transferred into an Eppendorf tube, and 200 μl of radioactive cadmium (2.0 mg $^{109}CdCl_2$/ml with radioactivity of 1.0 μCi/ml from Sigma, USA) was added. The mixture was incubated for 10 min at room temperature. Free cadmium was thoroughly removed by a repeated procedure of 2% bovine hemoglobin binding. The gamma-ray radioactivity of cadmium-binding MT was assayed using Wizard 1470 Gamma Counter (Perkin–Elmer Corporation, Shanghai, China). MT protein concentration was calculated by a ratio of 1 μmol MT to 6 μmol cadmium.

Western blotting of tissue homogenate proteins

LC3-II and GRP78 levels in kidney tissues were detected by Western blotting. Briefly, total protein was extracted with RIPA lysis buffer adding to abovementioned tissue homogenates. Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL) according to the manufacturer's instructions. Proteins were separated by 12% (*w*/*v*) SDS-PAGE and electroblot to PVDF membranes. Membranes were blocked for 2 h in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, 1 vol.% Tween 20) containing 5% skimmed milk powder and 0.5% BSA at room temperature. Anti-GRP78 and anti-LC3-II polyclonal antibodies (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) were used with a dilution 1:1000 at 4 °C overnight, respectively. After washed by TBS buffer, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted by 1:1000, Zhongshan GoldenBridge Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. Color was developed using an enhanced chemiluminescence (ECL) detection kit (Pierce Chemical Company, Rockford, IL). β-actin was inner reference. Relative blot grayscales were determined by a ratio of test protein to β-actin grayscales using UVP BioImaging and Analysis Systems (UVP, LLC, Upland, CA).

Statistical analyses

SPSS12.0 software was used for statistical analyses. Data were expressed as mean ± SEM. One-way ANOVA with Dunn' ad hoc test and Wilcoxon rank sum test (Mann–Whitney *U* test) were used to compare between multiple groups. $p < 0.05$ was statistically significant.

Results

General data

At 35 days following high-fat dietary, the hyperlipemia was successfully prepared in rats. Then, three days following STZ injection, FPG was detected. The rats with FPG ≥ 16.7 mmol/l were used in the subsequent experiments. Finally, 15 T2DM-like rats were qualified, 7 and 8 rats for the MC (diabetes model control) and DM + Zn (diabetes with Zn dietary) groups, respectively. Nine rats served as the diabetes-free normal control (NC). General data of rats including body weight, blood glucose, and insulin were shown in Table 1.

Kidney mass, function, and redox indices

Kidney mass (kidney/body weight), kidney function (BUN and CRE), lipid peroxidation (MDA), and anti-oxidation (T-AOC) indices at 53 days following Zn dietary were shown in Table 1. Upon kidney mass, BUN levels and MDA levels, the MC and DM + Zn groups were significantly higher than the NC group ($p < 0.05$). The BUN, CRE, and MDA levels in the DM + Zn group were reduced significantly than those in the MC group ($p < 0.05$). Anti-oxidation T-AOC levels in the NC and DM + Zn groups were significantly higher than the MC group ($p < 0.05$). Both CRE and T-AOC levels in the DM + Zn group were equivalent to those in the NC group ($p > 0.05$).

Kidney MT content

MT content was detected by the ^{109}Cd hemoglobin saturation method. The MT content of the MC group was 0.86 ± 0.19 μg/g kidney, lower than 1.09 ± 0.13 μg/g of the NC group ($n = 5$, $p > 0.05$). The DM + Zn group 1.68 ± 0.26 μg/g was significantly higher than the MC group and the NC group ($n = 5$, $p < 0.05$).

H&E staining of kidney tissues

Pathological changes in kidney tissues were observed under the microscopic examination (Fig. 1). Upon the NC group, the glomerular volume was moderate, no hyperemia was seen, the glomerular cyst lumen was obviously seen, and the glomerular basement membrane was not thickening. Neither vacuolar changes nor tubular lesions were seen in the tubular epithelial cells. There was no apparent change in renal interstitium. Upon the MC group, the glomerular volume was reduced, a part of the glomeruli was divided into lobes, the mesangial area was enlarged, the mesangial cell hyperplasia was

Table 1 Kidney mass, function, and redox indices 53 days after zinc supplementation

	Number	Kidney/body weight (mg/g)	BUN (mmol/l)	CRE (μmol/l)	MDA (nmol/ml)	T-AOC (U/ml)
NC	9	7.02 ± 0.29	7.20 ± 1.09	49.32 ± 6.24	4.1 ± 0.6	10.7 ± 0.9
MC	7	10.12 ± 1.79 [a]	16.51 ± 3.26 [a]	61.50 ± 6.07 [a]	5.9 ± 0.8 [a]	5.9 ± 0.9 [a]
DM + Zn	8	9.99 ± 1.88 [a]	10.30 ± 2.36 [b]	51.60 ± 4.88 [b]	4.8 ± 0.7 [a, b]	9.4 ± 1.1 [a, b]

Data were expressed as mean ± SEM. One-way ANOVA with Dunn' ad hoc test. [a] $p < 0.05$ vs. the NC group, [b] $p < 0.05$ vs. the MC group. NC, normal control ($n = 9$); MC, diabetes model control ($n = 7$); DM + Zn, diabetes with Zn supplementation ($n = 8$)

obvious, and the mesangial matrix accumulated. A large number of renal tubule epithelium cells was swollen with vacuolar-like degeneration, the tubule lumen was narrowed, low-to-moderate transparent tubules were seen, and a part of the renal tubular epithelial cell was necrosis and peeling. There was interstitial fibrosis hyperplasia. In the DM + Zn group, the glomerular volume was slightly enlarged with hyperemia, and the tubule cyst lumen was slightly narrower than the NC group. The mesangial region was slightly widened, the glomerular basement membrane was seen to be thickened, and the mesangial matrix accumulated. Renal tubule epithelial cells were hypertrophy, with partially vacuolar-like change, and the tubule lumen was narrowing.

Immunohistochemical staining of kidney p-Akt and LC3-II

Table 2 showed the immunohistochemical grade of positive kidney cell percentages. The staining images were shown in Fig. 1. The percentage of p-Akt positive cells in the DM + Zn group was significantly more than the MC group ($p < 0.05$), demonstrating an elevated insulin-like effect. Those in the MC and DM + Zn groups were significantly more than the NC group ($p < 0.05$). The percentage of LC3-II positive cells in the DM + Zn group was significantly lower than the MC group ($p < 0.05$), indicating a lowered autophagy in rat diabetic kidneys.

Western blots of GRP78 and LC3-II

The GRP78 and LC3-II protein blots were shown in Fig. 2. The NC group had no blot signals. Both GRP78 and LC3-II protein blot relative grayscales in the DM + Zn group were significantly lower than the MC group ($n = 3$, $p < 0.05$), indicating a lowered ER stress and a decreased autophagy again in rat diabetic kidneys. Those in either the MC group or the DM + Zn group were significantly higher than the NC group ($n = 3$, $p < 0.05$).

Discussion

Most of the patients with diabetes suffer from sustained high blood glucose, oxidative damage, and lipid peroxidation

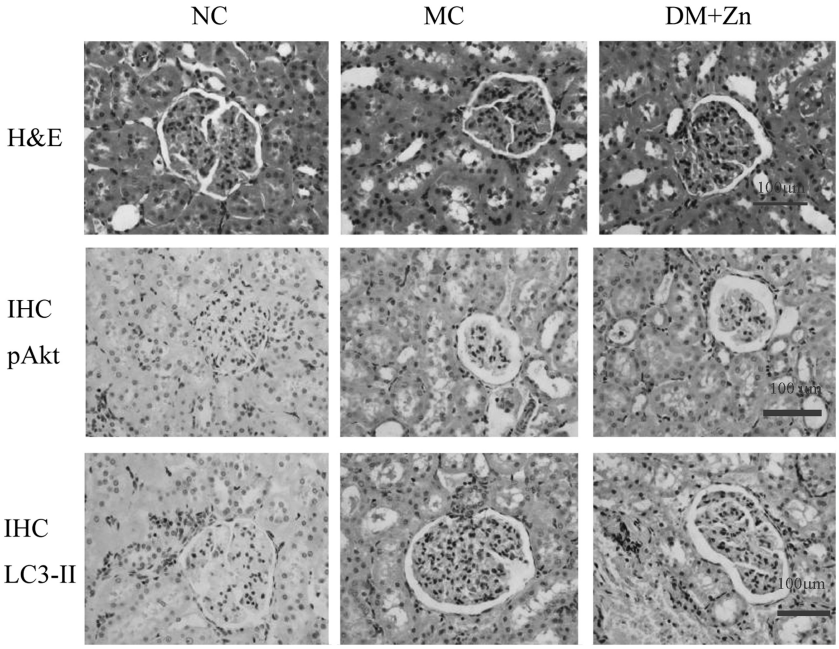

Fig. 1 H&E staining and immunohistochemical (IHC) staining (×400 magnification) of rat kidney 53 days after Zinc supplementation. NC, normal control; MC, diabetes model control; DM + Zn, diabetes with Zinc supplementation. pAkt, phosphorylated Akt/PKB

Table 2 Immunohistochemical grade of positive renal cell percentages of p-Akt and LC3-II 53 days after zinc supplementation

Grade		±	+	++	+++
p-Akt	NC	30	0	0	0
	MC [a]	14	14	2	0
	DM + Zn [a, b]	12	12	6	0
	$\chi^2 = 61.617$, $p = 0.001$				
LC3-II	NC	28	2	0	0
	MC [a]	0	16	14	1
	DM + Zn [a, b]	10	11	9	0
	$\chi^2 = 99.601$, $p = 0.001$				

Randomly five microscopic viewfields of each slice, six slices for each group. Wilcoxon rank sum test (Mann–Whitney *U* test) was used to compare between multiple groups. $p < 0.05$ was statistically significant. [a] $p < 0.05$ vs. the NC group, [b] $p < 0.05$ vs. the MC group. NC, normal control ($n = 9$); MC, diabetes model control ($n = 7$); DM + Zn, diabetes with Zn supplementation ($n = 8$)

product MDA in kidney. In the current study, T2DM-like rats had a significant increase in kidney mass index and kidney function (BUN/CRE) levels, indicating the diabetic rat kidneys were damaged severely. Zinc supplementation improved the kidney conditions substantially. With increased insulin levels and decreased blood glucose levels (Table 1), the kidney mass index and kidney function were improved, even CRE levels recovered to a normal condition, demonstrating that the diabetic kidney damage was relieved substantially. This recovery was confirmed by the H&E staining results (Fig. 1). The lipid peroxidation MDA was decreased apparently, and anti-oxidation T-AOC levels recovered up to the normal condition, indicating the oxidative stress levels were reduced obviously.

MT with anti-oxidation activity is the core node linking intracellular zinc to redox-mediated signaling [26]. Upregulation of MT has been found to protect against oxidative damage of diabetic kidney [7–10]. The current study got consistent results with these literatures. Zinc supplementation promoted MT synthesis, subsequently promoted the antioxidant effect and reduced lipid peroxidation, and finally mitigated oxidative damage of diabetic kidney. Zinc is insulinomimetic and Akt/PKB involved in the insulin signaling [27]. Akt phosphorylation is one of the mechanisms reducing diabetic kidney damage [13]. In the current study, zinc supplementation markedly upregulated pAkt/PKB levels to reduce diabetic kidney damage. Studies have also found that zinc can protect against the diabetic renal injury through inhibition of renal LOX-1-mediated ICAM-1 expression [1], downregulation of profibrosis mediator connective tissue growth factor (CTGF) [7], downregulating P38 MAPK-mediated inflammation [7, 11], reducing renal cortical apoptosis [12], and upregulation of Nrf2, NQO1, SOD1, SOD2, and GSK-3β phosphorylation [13].

In the current study, we have explored a novel mechanism by which dietary zinc can mitigate diabetic renal damage. Both ER stress and autophagy status were mitigated apparently, by which zinc reduced pathological damage in diabetic kidneys. At the same time, both GRP78 blots and LC3-II levels of the DM + Zn group were still higher than the normal control, demonstrating that zinc supplementation is conducive to ameliorating ER stress and autophagy in diabetic kidney partially. Akt/PKB is involved in the T2DM-associated ER stress through three signaling pathways IRE-1α-JNK [28, 29], CHOP [30], and GSK3β [22]. Akt/PKB is also involved in the autophagy pathways [31, 32]. MT upregulation induced by dietary zinc is able to activate Akt/PKB in diabetic cells. In the current study, dietary zinc upregulated MT expression in kidneys, subsequently or simultaneously activated Akt/PKB, thus reducing ER stress and autophagy in the diabetic kidneys. These may be the underlying mechanisms by which dietary zinc functioned in the diabetic kidney, similar to those in the diabetic heart damage [17] and diabetic liver damage [18]. These findings suggest that dietary zinc can protect multiple organs comprehensively against diabetic injury.

In conclusion, dietary zinc can downregulate GRP78-linked ER stress and LC3-II-linked autophagy to improve rat T2DM kidney damage to some degree.

Compliance with ethical standards

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

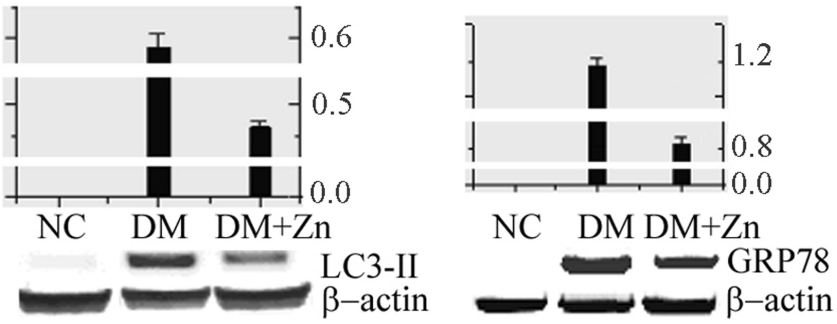

Fig. 2 Western blots of LC3-II and GRP78 proteins of T2DM rat kidney 53 days after Zinc supplementation. β-actin is inner reference

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal experiments were approved by The Jilin University Animal Ethics Committee (Changchun 130012, Jilin province, China). This article does not contain any studies with human participants performed by any of the authors.

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ORIGINAL ARTICLE

Prevalence and determinants of high blood glucose in urban and rural Indonesian adult population

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Abstract

Introduction World Health Organization (WHO) estimates the prevalence of people with diabetes increased from 4.1% (1980) to 8.6% (2014) in the Southeast Asia region. A few studies have examined impaired fasting glucose (IFG) as the main factor precedes diabetes while nutritional status, imbalanced diet, and physical activity as risk factors of IFG. A cross-sectional study was conducted to assess the prevalence and determinants of high blood glucose (IFG and diabetes mellitus) among urban and rural Indonesian adults population. The study included 385 adults 19–64 years of age from urban Umbulharjo sub-district (n = 195) and rural Minggir and Turi sub-districts (n = 190). We collected information on social, food consumption, and physical activity, and measured weight, height, waist circumference, and body fat mass.

Results The prevalence of high blood glucose in the urban area was higher (55.4%) compared with the prevalence in the rural area (28.4%). Waist circumference and visceral fat (%) were associated with high blood glucose.

Conclusions Social, food pattern, and physical activity were not associated with high blood glucose. In this study, we reported that male and female adults living in the urban area had a higher risk for high blood glucose compared with those who lived in rural area.

Keywords Pre-diabetes · Urban · Rural · Adults

Introduction

Globally, it has been reported that the prevalence of diabetes among adults was 382 million (8.3%) in 2013 and is predicted to reach 592 million (8.8%) by 2035 [1, 2]. WHO estimates prevalence and number of people with diabetes among adults above 18 years of age have risen two times from 4.1% (1980) to 8.6% (2014) in Southeast Asia region [3]. In Indonesia, it has been estimated that people with diabetes were 6.9% (12 million people) in 2013 while the other 36.6% (65 million people) were living with impaired fasting glycemia (IFG) [4]. The main factor predisposing them to diabetes and cardiovascular disease is IFG or pre-diabetes [5]. The previous study had found 5–10% of people per year with IFG would progress to diabetes [6]. This condition 20–30% potentially changes to diabetes over the next 5–10 years if the medical and nutrition management is not proper [7].

Few studies have explained IFG/pre-diabetes are associated with some factors such as obesity and abdominal obesity, increasing body fat mass, dietary pattern, physical activity, and socioeconomic factor [8–11]. This study also stated there is different prevalence of diabetes and pre-diabetes where the prevalence in urban is dramatically higher than the prevalence in rural. A study from China has explained the characteristic life in the urban area which being less physically active, having more smokers, alcohol users, overweight, and having a higher intake of animal food was associated with a potentially higher risk of pre-diabetes in adult population [12].

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However, the progression from pre-diabetes to diabetes can be delayed or prevented [7]. Early identification of the risk factors affecting pre-diabetes is essential for proper planning of health actions and preventing of negative clinical consequences and other complications. Due to the reasons above, we conducted this study to investigate the prevalence of high blood glucose (IFG and diabetes) and determine the risk factors of high blood glucose among urban and rural adults population in Yogyakarta, Indonesia. Several previously known factors such as body composition, social status, food consumption, and physical activity between urban and rural area were also evaluated.

Materials and methods

The study design and populations

Between June and October 2016, we conducted this cross-sectional study in the region of Yogyakarta, Indonesia, which consists of five districts representing the urban and rural area. The district of Yogyakarta represents the urban area and consists of 13 sub-districts. We chose Umbulharjo sub-district, which represents the most populated area in the region. For the rural area, we assigned the district of Sleman and two sub-districts namely Turi, representing the highest-land area (mountainous area), and Minggir, representing the lowest-land area. We selected the participants by a proportionate random sampling based on age and sex. The age was grouped into four categories according to the Indonesian recommendation dietary allowance (I-RDA) as follows:

> 19–29 as young adult, 30–39 as adult, 40–49 as older adult, and 50–64 as elderly [4].

The inclusion criteria for this study are healthy adult, aged 19–64 years old, and able to follow this study by signing the informed consent. The exclusion criteria are the subject who was getting pregnant or breastfeeding. The minimum sample size was 384 using WHO "Sample Size Determination in Health Studies" [13] for a cross-sectional study with a prevalence rate of 15% for impaired fasting glucose (IFG) [6] and *d* 0.05%.

Interview for data collection of social status, physical activity, and food consumption

We collected information about individual-level social status, family history of diseases, nutritional status, dietary pattern, fasting glucose, and physical activity. Social status, client history, and physical activity information were collected using a questionnaire by the trained field workers. Prior to this study, the social scale was pre-tested in order to obtain validity. Current smokers were defined as those who smoked at least one cigarette per day or those who stopped smoking for less than a year, and non-smokers were defined as those who either were former smokers or never smoked a cigarette in their lives. A participant was also asked about family diseases histories such as diabetes, hypertension, cardiovascular diseases, or stroke. Physical activity was measured using the global physical activity questionnaire (GPAQ) version 2 which was already translated and validated for Indonesians [14]. Data are presented as METs (metabolic equivalent of tasks)—minute/week.

Information on food consumption was collected using two times the 24-h dietary recall. The dietary recall was collected in 2 separate days which include 1 working day and 1 weekend day. Consumption of foods was grouped into six food groups based on Health Basic Research by Indonesia Ministry of Health [4]: (1) rice; (2) wheat-based; (3) fried foods; (4) animal-protein source; (5) plant-protein source; (6) fruits and vegetables. Those food groups were analyzed based on typical food consumption of people who lived in the region.

Anthropometric measurements

To obtain nutritional status, we measured height without shoes to the nearest 0.1 cm using a standardized constructed height stick. Body weight and body fat mass were measured in light clothing to the nearest 0.1 kg using a bio-impedance analyzer (Karada Scan HBF-358, Omron, Japan) digital weighing scale while, in the same setting, waist circumference was measured to the nearest 0.1 cm using a standardized measuring tape. All the measurements were examined two times. The mean value of the two measurements was considered as the participant's value. All the tools mentioned above have been calibrated and standardized by Yogyakarta Agency of Metrology.

The WHO definitions of threshold values were used for classifying body mass index (BMI) and waist circumference (WC). The body mass index (kg/m^2) was calculated as weight (kg) divided by the square of the height (m^2) and classified into four categories: < 18.5 kg/m^2 is underweight, 18.5–24.9 kg/m^2 is normal, 25.0–29.9 kg/m^2 is overweight, and ≥ 30 kg/m^2 is obese [15]. The waist circumference was defined as abdominal obesity if ≥ 90 cm for male or ≥ 80 cm for female and normal if < 90 cm for male or < 80 cm for female [14].

Biochemical measurement

We determined pre-diabetes as an asymptomatic condition where blood glucose, including impaired fasting glucose (IFG), is elevated and precedes the onset of diabetes [16]. Diabetes, IFG, and normal glucose were defined by fasting blood glucose concentration 126 mg/dL or above, 100–125 mg/dL, and less than100 mg/dL, respectively [3]. Subjects were grouped as high blood glucose when fasting blood glucose was 100 mg/dL or higher, this includes IFG and diabetes mellitus. Fasting blood glucose was performed through venous blood samples from all participants after an overnight fast (8 h at least) by a trained laboratory worker, and the samples were analyzed directly. Fasting blood glucose levels were determined using the system from fluoride plasma, which operates via cation exchange high-performance liquid chromatography from whole blood ethylene diamine tetra acetic acid (EDTA) treated samples.

Data analysis

Data were explained and presented as the mean and standard error of the mean for the continuous variables. The Kolmogorov-Smirnov test was performed to obtain a normally distributed data. The independent *t* test was used to compare differences in anthropometric measures, food consumption, physical activity, and fasting blood glucose among urban and rural. We also analyzed the association between anthropometric measures, food consumption, physical activity, and high blood glucose specific for rural and urban area using the independent *t* test. A chi-square test was used to assess the association between social status and the occurrence of high blood glucose in all subjects. A linear regression test was performed for identifying the determinants of high blood glucose controlling by age, gender, body weight, and living area (urban and rural). The correlation between age, physical activity, food consumption, and waist circumference was examined using a Spearman correlation test and linear regression test. A p value < 0.05 was considered statistically significant, and all analyses were done in the two-tailed test. All data analyses were conducted using JASP software version 0.8.3.1 (The University of Amsterdam).

Results

Descriptive characteristics of study participants

There were 385 male and female adults who participated in this study (Graph 1). The proportion of male and female participants were 46.8% ($n = 180$) and 53.2% ($n = 205$), respectively. The age range between individuals was between 19 and 64 years. The distribution of age in this study was 25.5% for those between 19 and 29 years old, 50.0% for those between 30 and 49 years old, and 24.5% for those between 50 and 64 years old.

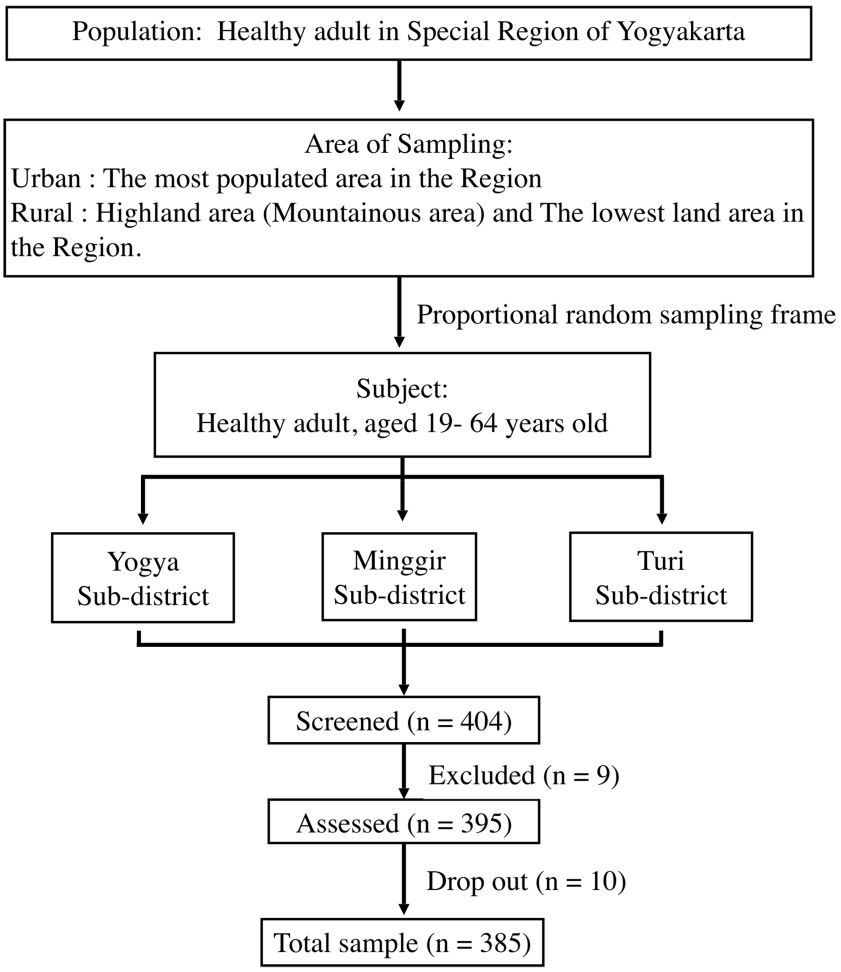
The characteristics of the subjects in the rural and urban areas are presented in Table 1. In this study, we reported that there were no age differences between those who lived in the rural and urban areas. The proportion of male and female subjects were similar to those who lived in the rural (male:female = 90:100) and urban (male:female = 90:105) areas. Subjects who lived in the urban area significantly had higher body weight, waist circumference, hip circumference, waist-to-hip ratio, and fasting blood glucose. We reported that there were no differences in BMI between subjects in the rural and urban areas. Living in the urban area was significantly associated with higher fasting blood glucose.

Lifestyle factors including food consumption and physical activity were compared between those who live in the rural area and those in the urban area. There were no differences in physical activity between subjects in the rural and urban areas. In addition, we also reported that there were no differences in the consumption of rice, fried foods, plant-based protein source, fruits, and vegetables between rural and urban. Subjects in the urban area consumed more wheat-based food products and animal-based protein sources compared with those who live in rural area.

The prevalence of adult individuals with diabetes, IFG, and normal glucose in this study was 11.95%, 30.1%, and 57.9%, respectively. The prevalence of high blood glucose in the urban area was higher (55.4%) compared with the prevalence in the rural area (28.0%). Table 2 shows the association between social status and high blood glucose. In this, we showed that gender, type of job, smoking status, and family history of non-communicable diseases were not associated with high blood glucose (all $p > 0.05$), whereas the living area was related to pre-diabetes ($p < 0.001$).

In Table 3, we reported the association between anthropometric measures, food consumption, and pre-diabetes. In this study, we showed that there was no association between food consumption and pre-diabetes. Physical activity was also not associated with pre-diabetes ($p = 0.794$). Additionally, we also analyzed the association between anthropometric measures and pre-diabetes status. In this study, we showed that subjects with pre-diabetes status had significantly higher visceral fat ($p = 0.003$) and waist circumference ($p = 0.027$) compared with those without pre-diabetes. The association between physical activity, food consumption, and pre-diabetes was also not significant after the separate analysis based on the location of residency.

The correlation between physical activity, food consumption, and fasting blood glucose was presented in Table 4. In this study, we showed that none of those variables were correlated with fasting blood glucose after correction for age, gender, and body weight. The correlation between physical



Graph 1 Schematic flow of subjects' participation

activity, food consumption, and fasting blood glucose was also not significant after separate analysis based on the location of residency (Table 5).

An additional analysis was done to evaluate the factors associated with waist circumference because waist circumference was an important factor that increases the risk for pre-diabetes. Age was significantly associated with waist circumference ($r = 0.204$, $p < 0.001$). Physical activity was negatively correlated with waist circumference even after corrected for age, gender, and body weight. By contrast, in this study, we showed that food consumption was not correlated with waist circumference.

Discussion

In this study, we aimed to investigate the interaction between residential area and lifestyle factors on pre-diabetes in male and female adults living in Indonesia. The prevalence of pre-diabetes was higher in the urban area than in rural area. Furthermore, we investigated several factors including lifestyle and social that might explain this association. There was no difference in body mass index between those who live in the urban and those in the rural area, but those who live in the urban area had a higher waist and hip circumference. We also showed that those who lived in the urban area had a higher intake of wheat-based food products and animal-protein food source. There was no association between social status, physical activity, and pre-diabetes in this study.

The main finding of this study shows that living in the urban area was associated with increased risk for pre-diabetes in Indonesian male and female adults. This finding consistent with other findings in India showed that the prevalence of diabetes was higher in those living in urban area [17]. The mean age of subjects in this study was 40.0 years which was comparable with those analyzed by Anjana et al. [17] (mean age 41.3 years old). However, the prevalence of IFG in this study was 30.1%, and this was three times higher than those found in India (10.3%). It is important to note that the standard of IFG in this study was 100 mg/dL which is higher from the cut-off used by Anjana et al. (110 mg/dL). The reasons of using this standard were because this standard was used by the national surveillance of diabetes and pre-diabetes in Indonesia [4] and this was previously reported to be more sensitive in predicting diabetes mellitus [16]. It has been reported that there was an increasing prevalence of

Table 1 Characteristics of subjects based on the location of residence (rural and urban)

	High blood glucose ($n = 162$)	Normal glucose ($n = 223$)	Mean difference (95% CI)	p*	Rural ($n = 190$)^	Urban ($n = 195$)^	Mean difference (95% CI)	p*
Age (years)	41.0 ± 0.9	39.3 ± 0.8	1.7 (−0.8–4.2)	0.173	40.7 ± 0.9	39.4 ± 0.9	1.3 (− 1.1–3.8)	0.298
Anthropometric measures								
Body weight (kg)	59.4 ± 1.0	57.1 ± 0.7	2.3 (− 0.0–4.7)	0.049	56.4 ± 0.8	59.6 ± 0.9	− 3.2 (− 5.5–− 0.9)	0.013
Body mass index (kg/m^2)	24.0 ± 0.4	23.6 ± 0.3	0.4 (− 0.6–1.4)	0.438	23.5 ± 0.4	24.0 ± 0.3	− 0.4 (− 1.4–0.6)	0.161
Body fat (%)	26.4 ± 0.7	26.2 ± 0.5	0.3 (− 1.5–2.0)	0.784	25.7 ± 0.6	26.9 ± 0.6	− 1.2 (− 3.0–0.5)	0.071
Fat-free mass (%)	73.6 ± 0.7	73.8 ± 0.5	− 0.3 (− 2.0–1.5)	0.784	74.3 ± 0.6	73.1 ± 0.6	1.2 (− 0.5–3.0)	0.071
Visceral fat (%)	8.3 ± 0.4	6.9 ± 0.3	1.5 (0.5–2.5)	0.003	7.2 ± 0.4	7.9 ± 0.4	− 0.8 (− 1.7–0.2)	0.129
Waist circumference (cm)	81.8 ± 0.9	79.2 ± 0.7	2.6 (0.3–4.8)	0.027	78.0 ± 0.8	82.5 ± 0.8	− 4.6 (− 6.8–− 2.3)	<0.001
Hip circumference (cm)	93.5 ± 0.9	93.1 ± 0.5	0.5 (− 1.5–2.4)	0.658	91.9 ± 0.6	94.6 ± 0.8	− 2.8 (− 4.7–− 0.8)	<0.001
Waist-to-hip ratio	0.92 ± 0.05	0.85 ± 0.01	0.1 (− 0.0–0.2)	0.114	0.85 ± 0.01	0.91 ± 0.04	− 0.1 (− 0.1–0.02)	0.047
Fasting blood glucose (mg/dL)	123.9 ± 36.6	88.2 ± 7.1	35.7 (30.8–40.7)	< 0.001	96.8 ± 1.6	109.4 ± 2.5	− 12.3 (− 18.2–− 6.4)	<0.001
Physical activity (METS-min./week)	1068 ± 80.6	1097 ± 72.3	− 28.5 (− 243.1–186.1)	0.794	1182.1 ± 81.6	989.7 ± 70.0	192.4 (− 18.6–403.5)	0.184
Foods consumption								
Rice (g/day)	369.7 ± 14.7	379.8 ± 11.9	− 10.1 (− 47.1–26.8)	0.590	380.0 ± 12.9	371.2 ± 13.3	8.7 (− 27.8–45.1)	0.652
Wheat-based food products (g/day)	63.9 ± 3.7	64.7 ± 3.3	− 0.8 (− 10.6–9.0)	0.873	55.5 ± 3.3	73.1 ± 3.5	− 17.5 (−27.0–− 8.0)	<0.001
Fried foods (g/day)	57.7 ± 4.4	54.0 ± 3.5	3.7 (− 7.3–14.7)	0.510	51.1 ± 3.8	59.9 ± 4.0	− 8.9 (− 19.7–2.0)	0.094
Animal-based protein sources (g/day)	75.2 ± 4.0	74.2 ± 3.4	1.0 (− 9.3–11.2)	0.849	67.7 ± 3.5	81.3 ± 3.7	− 13.6 (− 23.6–− 3.6)	0.011
Plant-based protein sources (g/day)	80.8 ± 5.1	71.0 ± 3.4	9.8 (− 1.8–21.5)	0.098	75.4 ± 4.1	74.9 ± 4.2	0.5 (− 11.1–12.1)	0.800
Fruits and vegetables (g/day)	111.8 ± 7.0	119.0 ± 6.3	− 7.2 (− 25.9–11.5)	0.448	119.5 ± 6.8	112.5 ± 6.5	7.0 (− 11.4–25.5)	0.538

METs metabolic equivalent of tasks

*Independent *t* test

^Presented as mean ± standard deviation

Table 2 Association between social status and IFG

	High blood glucose^ (%)	Normal glucose (%)	OR (95%CI)	p*
Gender			1.37 (0.91–2.05)	0.133
Men ($n = 180$)	46.1	53.9		
Women ($n = 205$)	38.5	61.5		
Type of job based on physical activity			1.45 (0.81–2.60)	0.212
Sedentary ($n = 326$)	44.5	55.5		
Active ($n = 58$)	34.5	65.5		
Family history of NCDs			0.98 (0.54–1.80)	0.952
Without a family history of NCDs ($n = 228$)	59.2	60.8		
With a family history of NCDs ($n = 157$)	50.0	50.0		
Smoking status			1.42 (0.93–2.17)	0.105
Smoking ($n = 133$)	47.4	52.6		
No-smoking ($n = 252$)	40.0	60.0		
Living area			0.32 (0.21–0.49)	< 0.001
Rural ($n = 189$)	28.4	71.6		
Urban ($n = 196$)	55.4	44.6		

*Chi-square test, fasting blood glucose 100 mg/dL or higher

diabetes and pre-diabetes especially for those living in the urban area of South Asia [18, 19]. This data was then confirmed by other studies conducted in Bangladesh [20], Turkey [21], and Congo [22]. Several factors have been evaluated to understand the risk factors associated with high blood glucose especially in the urban area including financial

Table 3 The association between anthropometric, dietary intake, and pre-diabetes

	Rural			Urban		
	High blood glucose ($n = 54$)	Normal glucose ($n = 136$)	p	High blood glucose ($n = 108$)	Normal glucose ($n = 87$)	p
Age (years)	42.7 ± 1.7	39.9 ± 1.1	0.174	40.2 ± 1.1	38.3 ± 1.3	0.274
Anthropometry						
Body weight (kg)	57.2 ± 1.5	56.1 ± 0.9	0.549	60.5 ± 1.2	58.5 ± 1.1	0.251
BMI (kg/m^2)	23.4 ± 0.6	23.5 ± 0.4	0.844	24.3 ± 0.5	23.6 ± 0.4	0.362
Body fat (%)	24.7 ± 1.4	26.0 ± 0.7	0.361	27.3 ± 0.8	26.4 ± 0.9	0.479
FFM (%)	75.3 ± 1.4	74.0 ± 0.7	0.361	72.7 ± 0.8	73.6 ± 0.9	0.479
Visceral fat (%)	8.1 ± 0.8	6.7 ± 0.3	0.053	8.5 ± 0.5	7.1 ± 0.4	0.064
Waist circ. (cm)	78.3 ± 1.5	77.9 ± 0.9	0.787	83.5 ± 1.1	81.3 ± 1.2	0.178
Hip circ. (cm)	91.9 ± 1.1	91.8 ± 0.7	0.974	94.3 ± 1.2	95.0 ± 0.9	0.683
WHR	0.85 ± 0.01	0.85 ± 0.01	0.827	0.95 ± 0.1	0.85 ± 0.1	0.239
Physical activity*	1210.1 ± 162.1	1171.0 ± 94.5	0.830	997.2 ± 89.5	980.4 ± 111.4	0.906
Food consumption^						
Rice	371.9 ± 24.2	383.2 ± 15.3	0.695	368.6 ± 18.5	374.6 ± 19.1	0.823
Wheat-based foods	50.5 ± 6.1	57.4 ± 3.9	0.349	70.6 ± 4.6	76.1 ± 5.5	0.439
Fried foods	45.5 ± 7.4	53.3 ± 4.4	0.357	63.8 ± 5.4	55.1 ± 5.8	0.276
Animal protein	63.8 ± 6.5	69.2 ± 4.2	0.488	80.8 ± 5.0	81.9 ± 5.6	0.887
Plant protein	81.8 ± 9.5	72.8 ± 4.4	0.331	80.3 ± 6.0	68.1 ± 5.6	0.144
Fruits and vegetables	111.5 ± 12.2	122.7 ± 8.2	0.461	111.9 ± 8.6	113.2 ± 9.8	0.920

BMI body mass index, *FFM* fat-free mass, *WHR* waist-to-hip ratio, *METs* metabolic equivalent of tasks

*In METS, minute/week

^ In g/day

Table 4 The linear regression analysis between physical activity, food consumption, and fasting blood glucose

	All subjects		Urban		Rural	
	*B**	*p*	*B**	*p*	*B**	*p*
Physical activity (METs, minute/week)	− 0.010	0.841	0.052	0.473	− 0.080	0.295
Food consumption						
Rice (g/day)	− 0.087	0.091	− 0.092	0.213	− 0.070	0.338
Wheat-based food products (g/day)	− 0.012	0.819	− 0.038	0.606	− 0.078	0.286
Fried foods (g/day)	0.048	0.349	0.069	0.340	− 0.029	0.684
Animal-based protein sources (g/day)	0.063	0.224	0.051	0.486	− 0.005	0.946
Plant-based protein sources (g/day)	0.041	0.427	0.072	0.326	− 0.014	0.852
Fruits and vegetables (g/day)	− 0.026	0.618	− 0.082	0.258	0.071	0.332

METs metabolic equivalent of tasks

*Linear regression test with correction for age, gender and body weight

income, physical inactivity, graduate education, and office-based education. Unfortunately, those factors were not associated with pre-diabetes in this study. It has also been argued that it was also due to the interaction between environmental factors (diet and physical activity) and genetic predisposition [23–26].

There are several factors that might help explain the reason why there is a high prevalence of diabetes and pre-diabetes in urban area. One of the interesting findings in this study was that although subjects in rural and urban area had a comparable BMI, subjects who live in urban area had higher visceral adiposity which was shown by the higher waist and hip circumference. Additionally, we also showed a direct association between visceral fat mass (%) and waist circumference on pre-diabetes. It has been argued whether a specific type of fat accumulation or all fat accumulation was responsible for the development of pre-diabetes and diabetes in adults. In a meta-analysis study of 40 investigations in 56 populations, it was revealed that all fat depot measurements (total body fat, visceral fat, body mass index, waist circumference) were correlated with insulin resistance but visceral fat mass had the strongest correlation [27]. It has long been proposed that the link between adiposity and hyperglycemia was through inflammation.

The excess of adipose tissue mass, which has a common feature of obesity and visceral adiposity, was associated with increased production of pro-inflammatory cytokines [28]. In addition, there is a remarkable reduction in anti-inflammatory immune cells in obese adipose tissue. Those combinations lead to an increment of systemic inflammation in the individuals with obesity [28]. The increasing volume of adipose tissue was also

Table 5 The relationship between physical activity, food consumption, and waist circumference

	All subjects (Spearman correlation)^		All subjects (Linear regression)*	
	r^	*p*	*B**	*p*
Age (years)	0.204	< 0.001		
IPAQ (METS, min/week)	− 0.132	0.009	− 0.100	0.002
Food consumption				
Rice (g/day)	− 0.087	0.089	− 0.020	0.539
Wheat-based products (g/day)	0.014	0.788	0.011	0.734
Fried foods (g/day)	− 0.013	0.805	0.018	0.579
Animal protein sources (g/day)	− 0.087	0.088	0.001	0.972
Plant protein sources (g/day)	− 0.008	0.868	− 0.002	0.940
Fruit and vegetables (g/day)	− 0.020	0.695	0.019	0.568

Data are presented from two analyses

METs metabolic equivalent of tasks

^ The Spearman correlation test for non-corrected analysis and

*linear regression test for correction of confounding factors

*Linear regression value after correction for age, gender and body weight

^ *r* value for the Spearman correlation analysis

associated with a condition called metabolic overload which leads to an increasing release of free fatty acid. This condition increases cellular stress which will induce a release of reactive oxygen species and adipokine. The accumulation of pro-inflammatory signals, ROS, free fatty acid, and adipokine will in turn reduce cellular insulin sensitivity [29].

Because the importance of waist circumference in the development of pre-diabetes, additional analysis to evaluate factors associated with waist circumference was done. In this study, we showed that physical activity, but not food consumption, was negatively correlated with waist circumference. The importance of physical activity on the regulation of visceral adiposity was supported by the previous studies. In a 10-year follow-up of adults in Whitehall II cohort study, it was shown that physical activity was consistently associated with waist circumference than with BMI [30]. In a clinical trial, it has been reported that although increasing physical activity by exercise alone does not induce weight loss; this intervention was associated with a significant reduction in visceral adiposity [31]. A clinical trial conducted in overweight/obese adults with high fasting blood glucose showed that 8 weeks of aerobic and resistance exercise was associated with reduction visceral adiposity and blood glucose simultaneously [32].

There were several limitations in this study. First, there are huge variations in age, gender, and body mass index, which might be able to induce potential bias in data analysis. Second, because the nature of this study is cross-sectional, we cannot analyze the causal effect of our variables. In addition, there are several risk factors of IFG such as blood pressure ethnic group, self-reported comorbidities, and lipid status were not explored in this study [33–36]. We suggest for further study in order to break our limitations so that it can emphasize the result and represent more about IFG in adults.

Conclusions

In this study, we reported that male and female adults living in urban area were associated with an increasing risk for high fasting blood glucose or pre-diabetes. In this study, we suggested that this was because individuals who live in urban area had higher waist circumference. Social status, physical activity, and food consumption were not associated with pre-diabetes. Physical activity was correlated with waist circumference, which is an important determinant factor of pre-diabetes in adults. Further study is needed to evaluate factors associated with increased risk for pre-diabetes in urban area.

Author contribution For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "S.S., E.H. and B.J.I.K conceived and designed the experiments; S.S. and F.F. performed the data collection; S.S. and H.F.L.M analysed the data; S.S., F.F. and H.F.L.M wrote the paper." Susetyowati Susetyowati (SS); Emy Huriyati (EH), BJ; Istiti Kandarina (BJIK); Harry Freitag Luglio Muhammad (HFLM); Farah Faza (FF).

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Compliance with ethical standards

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

Ethical approval Each participant gave written informed consent before participating in this study. The present study was conducted according to the Declaration of Helsinki principles [37]. This study was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada, Indonesia.

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ORIGINAL ARTICLE

Left atrial diameter is associated with target organ damage in patients with type 2 diabetes mellitus

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Abstract
This paper aims to investigate whether there is a relationship between left atrial diameter (LAD) and target organ damage (TOD) in patients with type 2 diabetes mellitus (DM). Two-hundred-and-eleven patients with type 2 DM were recruited. Data on left ventricular mass index (LVMI), diabetic retinopathy, carotid intima–media thickness/carotid plaque, micro-albuminuria, and serum creatinine levels were collected to determine whether TOD occurred in patients with type 2 DM. Age, body mass index, waist-hip ratio, a history of DM, Framingham Score, and 10-year risk were used to assess cardiovascular disease risk. Patients were divided into four groups: zero TOD (group I, $n = 50$), one TOD marker (group II, $n = 76$), two TOD markers (group III, $n = 51$), and at least three TOD markers (group IV, $n = 34$). Using multivariate regression analyses, age, body mass index, waist-hip ratio, a history of DM, Framingham Score, and 10-year risk were significantly associated with LAD. LAD was associated with an increased number of markers for TOD. Univariate analyses demonstrated significant relationships between LAD and TOD in the context of serum creatinine and urinary albumin creatinine ratio ($r = 0.292$, $p < 0.001$), creatinine ($r = 0.346$, $p < 0.001$), carotid intima–media thickness ($r = 0.128$, $p = 0.032$), and LVMI ($r = 0.399$, $p < 0.001$). Multivariate regression analyses also determined that LVMI and creatinine were independent predictors of LAD enlargement. LAD may be associated with cardiovascular disease risk. LAD enlargement could be an effective indicator of TOD, particularly renal impairment and left ventricular hypertrophy. Screening for LAD may offer a new and rapid approach for evaluating the severity of DM.

Keywords Diabetes mellitus · Left atrial · Cardiomyopathy left ventricular mass · Echocardiography

Introduction

Left atrial diameter (LAD) is related to incidence of cardiovascular disease and is a predictor of stroke, atrial fibrillation, and death [1]. Increased left atrial (LA) size was observed in patients with type 2 diabetes mellitus (DM) [2]. In patients with stage 3 chronic kidney disease with either hypertension, DM, or both, LA alterations were independent echocardiographic markers for cardiovascular involvement [3].

Markers of target organ damage (TOD) in patients with type 2 DM have included left ventricular hypertrophy (LVH), diabetic retinopathy (DR), carotid wall thickening, carotid plaque, micro-albuminuria, and mildly increased serum creatinine levels [4–8]. Presence of TOD markers correlated with adverse prognostic outcomes in diabetic patients [9]. However, little is known about the relationship between LAD and TOD in patients with type 2 DM. So, LA size was an effective, convenient, and widely used indicator in clinical practice and research studies [10, 11]. Here, associations between LAD and markers of TOD were investigated in cases of type 2 DM in which patients did not have significant co-morbidities. Use of this technique could offer a new and rapid approach for evaluating the severity of DM.

Wei He and Wenhui Zhu contributed equally to this work.

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Materials and methods

Patients

This was a cross-sectional study. Two-hundred-and-eleven (99 males and 112 females) consecutively enrolled (from June to August in 2016) patients with type 2 DM were recruited from the outpatient clinic of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The study protocol was approved by the ethics committee of the institution, and all study patients signed informed consent documents. OGTT detection was also performed when the patients were diagnosed with diabetes.

Exclusion criteria included a history of cardiac valve disease, cardiomyopathy, congenital heart disease, chronic heart arrhythmia, bundle branch block [evidenced by electrocardiogram (ECG) testing], heart failure, ischemic stroke, transient ischemic attack, cerebral hemorrhage, severe renal impairment (diagnosed as a serum creatinine > 133 mmol/L in men, > 124 mmol/L in women), peripheral artery disease, and advanced malignancy. A total of 220 patients were screened; among them, 5 cases were unable to complete the retinal examination and 4 cases showed the left ventricular ejection fraction (LVEF) < 50%.

Blood glucose levels were measured after fasting (8 h), as well as 2 h after oral glucose challenge (administration of 75 g of glucose). DM was defined as either a fasting blood glucose level of > 7.0 mmol/L, or a 2-h glucose level of > 11.1 mmol/L, or both. Patient histories, physical examinations, and blood tests were also conducted to assess the presence of additional cardiovascular risk factors. For each participant, data were collected regarding age, gender, smoking status, waist-hip ratio (WHR), body mass index (BMI), duration of DM, systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), serum total triglyceride levels (TG), serum total cholesterol levels (TC), low-density lipoprotein cholesterol levels (LDL-C), high-density lipoprotein cholesterol levels (HDL-C), intima–media thickness (IMT) or common carotid artery plaque formation, serum creatinine, and urinary albumin creatinine ratio (UACR).

Definition of TOD

Participants were considered TOD if they exhibited any of the following criteria: micro-albuminuria (UACR > 30 mg/g creatinine), modest increased plasma creatinine (115–133 mmol/L in men or 106–124 mmol/L in women), changed vasculature as detected by ultrasound imaging (evidenced by carotid artery plaque or IMT > 0.9 mm), LVH (defined as a left ventricular mass index (LVMI) > 115 g/m^2 in men or > 95 g/m^2 in women) [12, 13], or DR.

Cardiovascular disease risk

Cardiovascular disease risk was evaluated by Framingham Score and10-year risk for coronary heart disease rate, and Framingham Score includes gender, age, smoking, diabetes, blood fat, and SBP.

DR

Evidence of DR was assessed by an ophthalmologist and a medical retinal specialist. Patients with both DR and diabetes mellitus were subdivided into five grades using standards that were previously described: (1) non-proliferative DR, (2) mild non-proliferative DR, (3) moderate non-proliferative DR, (4) severe non-proliferative DR, and (5) proliferative DR [14].

Echocardiography

LAD was measured from the anteroposterior diameter of the parasternal long-axis view [12]. Transmitral measurements were taken of *E* peak velocity (cm/s measured in early diastole) and *A* peak velocity (cm measured in late diastole). All of the images were obtained using a Philips IE 33 ultrasound machine(Bothell, WA, USA)with a 2–4-MHz transducer for transthoracic echocardiography and 3–7-MHz linear array transducer for carotid examinations. All the data were measured by a single, blinded ultrasound technician.

Statistical analyses

Statistical results were presented throughout the manuscript as the mean ± standard deviation (SD). Spearman's rank correlation tests were used to determine whether statistical relationships existed between LAD and DR. Pearson correlation analyses were used to identify potential relationships between LAD and both TOD markers, and cardiovascular risk factors. An multiple regression analysis was used to evaluate contributions of variables on LAD including age, gender, BMI, WHR, DM, PP, SBP, serum creatinine levels, UACR, IMT, LVMI, and DR. One-way analysis of variance (ANOVA) was used to compare LAD among patients that exhibited four different levels of TOD (ranging from 0 to 3 TOD markers). $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, USA).

Results

Demographics

In this study, 211 patients were recruited, of whom 112 (53%) were women and 99 (47%) were men. The mean ages of the

patients were 55.8 ± 16.7 and 55.4 ± 13.7 years for women and men, respectively. When considering the entire patient population, 36 (17.1%) had hypertension, 20 (9.5%) smoked, and 68 (32.2%) had a family history of DM. Fifty participants had no evidence of TOD, while 76, 51, and 34 participants exhibited one, two, or at least three markers of TOD, respectively (Table 1). Every patient in the cohort had received insulin therapy for at least 3 months.

Cardiovascular risk scoring systems

To further explore the relation between LAD and cardiovascular disease risk, we analyzed the Framingham Score and10-year risk for coronary heart disease rate. The results indicated that with the increased number of markers for TOD, Framingham Score and 10-year risk for coronary heart disease rate were significantly increased (Table 1). Our results also revealed that LAD was significantly correlated with Framingham Score ($r = 0.403$, $p < 0.001$) and 10-year risk for coronary heart disease rate ($r = 0.316$, $p < 0.001$).

Distribution rates of individual TOD markers

Prevalence of IMT/carotid plaques, LVH, micro-albuminuria, changes in serum creatinine levels, and DR were measured in the entire cohort. IMT/carotid plaques were found in 55 (26.1%) patients. LVH was found in 65 (30.8%) patients. Micro-albuminuria and moderate increases in serum creatinine were found in 62 (29.4%) and 26 (12.3%) patients, respectively. DR occurred in 77 (36.4%) participants. DR was further evaluated by a grading system in which grade 1 was considered non-proliferative DR and grade 5 was proliferative DR (see the "Materials and methods" section for more details) [12]. The prevalence of the DR five grades was 134 (63.5%) patients with grade 1, 24 (11.4%) patients with grade 2, 17 (8.1%) patients with grade 3, 12 (5.7%) patients with grade 4, and 24 (11.4%) patients with grade 5 disease.

Correlation of LAD with clinical variables

Next, cardiovascular risk factors were analyzed as potential variables contributing to LAD in this patient population. The following cardiovascular risk factors correlated with LAD: age ($r = 0.322$, $p < 0.001$), BMI ($r = 0.430$, $p < 0.001$), WHR ($r = 0.443$, $p < 0.001$), SBP ($r = 0.218$, $p = 0.001$), PP ($r = 0.232$, $p < 0.001$), and the duration of DM ($r = 0.369$, $p < 0.001$). There were no significant correlations between LAD and gender, DBP, *E*/*A*, HbA1c, either fasting and 2-h-challenge glucose measurements, or levels of TG, TC, LDL-C, or HDL-C. Interestingly, some TOD markers significantly correlated with LAD, including UACR ($r = 0.292$, $p < 0.001$), serum creatinine ($r = 0.346$, $p < 0.001$; Fig. 1), IMT ($r = 0.128$, $p = 0.032$), LVMI ($r = 0.399$, $p < 0.001$; Fig. 2), and DR ($r = 0.110$, $p = 0.056$). Variables that significantly predicted enlarged LAD included LVMI ($\beta = 0.250$, $p < 0.001$), creatinine ($\beta = 0.158$, $p = 0.008$), age ($\beta = 0.145$, $p = 0.028$), BMI ($\beta = 0.259$, $p < 0.001$), WHR ($\beta = 0.138$, $p = 0.044$), and the duration of DM ($\beta = 0.137$, $p = 0.042$) (Table 2).

Increased counts of TOD markers correlated with LAD

As discussed above, patients were separated into four groups denoted by ascending prevalence of TOD markers (zero to at least three). One-way analysis of variance was used to compare these groups ($F = 5.12$, $p = 0.002$). Mean LAD size was significantly increased in the group with at least three markers of TOD (group IV 35.9 ± 4.9 mm), when compared to participants with two markers of TOD (group III 33.9 ± 4.2 mm; $p < 0.05$), one marker of TOD (group II 33.5 ± 5.2 mm; $p < 0.05$), and zero markers of TOD (group I 31.8 ± 4.2 mm; $p < 0.05$; Fig. 3).

Discussion

Patients who experience a prolonged clinical course of type 2 DM have increased incidence of various diabetic complications. Previous studies have shown that patients with type 2 DM had increased LA volume [2], and enlargement of LA size was a strong predictor of cardiovascular disease [1]. Thus, investigation elucidating the relationship between left arterial size and TOD in type 2 DM patients is essential.

In a cohort of early adults, cardiovascular risk factors such as higher blood pressure and BMI predicted LA enlargement over a 20-year follow-up period [15]. Another study investigated a large cohort (1849 men and 2152 women) and demonstrated that higher SBP and PP were significantly associated with LA enlargement [16]. Another report of 112 patients (mean age of 14.2 years) showed that the LA enlargement was prevalent in children and adolescents with hypertension. Weight loss and control of high blood pressure may prevent LA enlargement in this population [17]. Furthermore, Zoppini et al. found that duration of DM was strongly associated with increased LA size in men with type 2 DM [18]. The present study confirmed that LAD enlargement correlated with age, BMI, WHR, duration of diabetes, systolic blood pressure, pulse pressure, TG levels, and blood glucose measurements 2 h after glucose challenge. Age, BMI, WHR, and a history of DM were significantly associated with increased LAD, as indicated by multivariate linear regression models. These findings were consistent with the previous studies and supported the hypothesis that LA enlargements associate with cardiovascular risk factors.

Framingham Score is a main method of cardiovascular disease event, which has been used in patients with diabetes and rheumatoid arthritis in different countries and ethnic

Table 1 Characteristics of the type 2 DM patient population

Characteristics	Zero TOD ($n = 50$)	One TOD ($n = 76$)	Two TOD ($n = 51$)	≥ Three TOD ($n = 34$)
Age (years)	45.2 ± 16.0	54.9 ± 12.6*	59.6 ± 13.8*, +	65.2 ± 13.5*, +
Gender, F (%)	25 (50)	38 (50)	29 (57)	20 (59)
Smoking, *n* (%)	1 (2)	9 (12)	12 (24)	8 (13)
BMI (kg/m$^{2)}$	23.8 ± 4.2	24.1 ± 4.6	23.6 ± 4.7	24.7 ± 3.0
WHR	0.90 ± 0.06	0.91 ± 0.07	0.94 ± 0.06*, +	0.97 ± 0.08*, +
HbA1c (%)	8.9 ± 3.6	8.6 ± 2.3	8.9 ± 2.6	8.8 ± 3.0
Glucose (mmol/L)	7.3 ± 3.4	7.2 ± 2.8	7.6 ± 3.4	8.0 ± 3.7
Glucose 2 h (mmol/L)	14.3 ± 3.8	15.0 ± 3.2	15.5 ± 3.7	15.6 ± 2.7
Hypertension, *n* (%)	5 (10.0)	11 (14.5)	11 (21.6)	9 (26.5)
SBP (mmHg)	119.4 ± 16.1	126.4 ± 21.4	130.7 ± 22.3*	133.9 ± 24.4*
DBP (mmHg)	76.1 ± 11.3	77.7 ± 11.2	77.4 ± 12.5	75.6 ± 11.2
PP (mmHg)	43.2 ± 12.0	48.7 ± 16.3*	53.3 ± 14.9*	58.2 ± 22.5*, +
TG (mmol/L)	2.0 ± 2.8	1.6 ± 1.3	1.6 ± 0.9	1.6 ± 1.1
TC (mmol/L)	5.1 ± 1.6	5.0 ± 1.2	5.3 ± 1.4	4.8 ± 1.5
HDL-C (mmol/L)	1.2 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	1.1 ± 0.3
LDL-C (mmol/L)	3.0 ± 0.7	3.1 ± 1.0	3.4 ± 1.1	2.9 ± 1.1
Framingham Score	5.2 ± 2.1	6.9 ± 1.9*	7.6 ± 2.1*	8.3 ± 2.1*, +
10-year risk for coronary heart disease rate	3.0 ± 2.3	5.2 ± 4.4*	7.5 ± 7.6*, +	8.9 ± 7.0*, +
E	71.4 ± 26.7	71.6 ± 17.0	72.2 ± 16.0	72.6 ± 18.4
A	69.2 ± 19.5	78.0 ± 21.1	84.0 ± 17.4*	91.9 ± 21.3*, +
E′	9.2 ± 3.1	8 ± 3.1	8.5 ± 2.9	7.6 ± 4.2*
Diabetic treatments				
Insulin	0 (0.0)	11 (0.14)	26 (0.51)	34 (1.0)
Metformin	39 (0.78)	39 (0.51)	15 (0.29)	0 (0.0)
Acarbose	6 (0.12)	5 (0.07)	0 (0.0)	0 (0.0)
Gliclazide	5 (0.1)	21 (0.28)	10 (0.2)	0 (0.0)
Antihypertensive				
ACEI, *n*(%)	2 (40.0)	5 (45.5)	4 (36.4)	4 (44.4)
ARB, *n*(%)	0 (0.0)	1 (9.1)	1 (9.1)	0 (0.0)
CCB, *n*(%)	3 (60.0)	6 (54.5)	7 (63.6)	6 (66.7)
Diuretics, *n*(%)	0 (0.0)	1 (9.1)	0 (0.0)	2 (22.2)
β-blocker, *n*(%)	2 (40)	3 (27.3)	5 (45.4)	4 (44.4)

A a peak velocity in cm/s (peak of transmitral atrial filling velocity during late diastole), *ACEI* angiotensin-converting enzyme inhibitors, *ARB* angiotensin receptor blockers, *BMI* body mass index, *CCB* calcium channel blockers, *DBP* diastolic blood pressure, *DM* diabetes mellitus, *E E* peak velocity in cm/s (peak of early transmitral filling velocity during early diastole), *E′* tissue Doppler of the lateral wall, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *M/F* males/females, *PP* pulse pressure, *SBP* systolic blood pressure, *TC* total cholesterol, *TG* triglyceride, *WHR* waist-hip ratio

*$p < 0.05$ for comparisons between zero TOD markers and one, two, or at least three TOD markers; +$p < 0.05$ for comparisons between one TOD marker and other groups; &$p < 0.05$ for comparisons between two TOD markers and other groups. *p* values were adjusted for multiple pairwise comparisons using the Bonferroni correction

populations [19–21]. In our study, Framingham Score and 10-year risk for coronary heart disease rate were significantly associated with the increased LAD. Therefore, we demonstrated that LAD may be in connection with cardiovascular disease risk.

Previous work has shown that obesity, LVH, IMT, and metabolic syndrome were all independent predictors of LAD in patients with hypertension [22]. Furthermore, Paoletti et al. demonstrated that LA size was related to renal outcomes [23]. In this study, a positive correlation was observed between LAD enlargements and increased counts of TOD markers in patients with type 2 DM. Significant independent relationships were found through modeling experiments between LAD and UACR, creatinine, IMT, and LVMI. Correlations

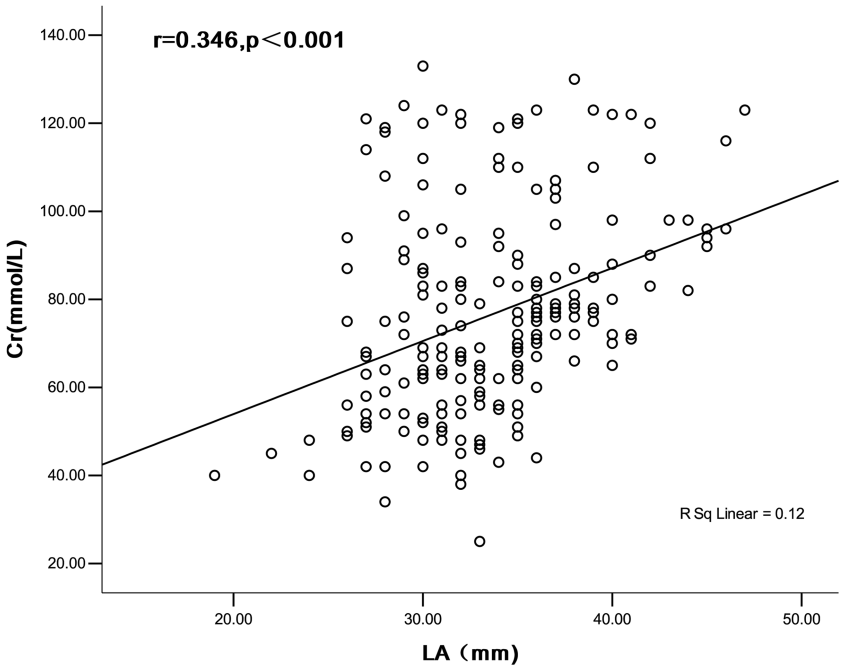

Fig. 1 The relationship between LAD and serum creatinine (Cr) levels in type 2 DM patients

were the strongest between LAD and both LVMI and creatinine. No evidence of a significant relationship was found between LAD and DR in this patient population.

Taken together, this study revealed that correlations existed between LAD and increased presentation of TOD markers in patients with type 2 DM. LVMI, creatinine, age, BMI, WHR, and a history of type 2 DM were significantly associated with LAD enlargements. LA size was significantly increased in patients who experienced the most severe TOD symptoms. Thus, LA enlargements could be effective indicators of TOD in patients with type 2 DM, particularly patients with renal impairment and LVH.

Conclusion

In our study, we have indicated that LAD was significantly associated with age, body mass index, waist-hip ratio, and history of DM, which are the risk factors of cardiovascular disease. In addition, we found that LAD was significantly

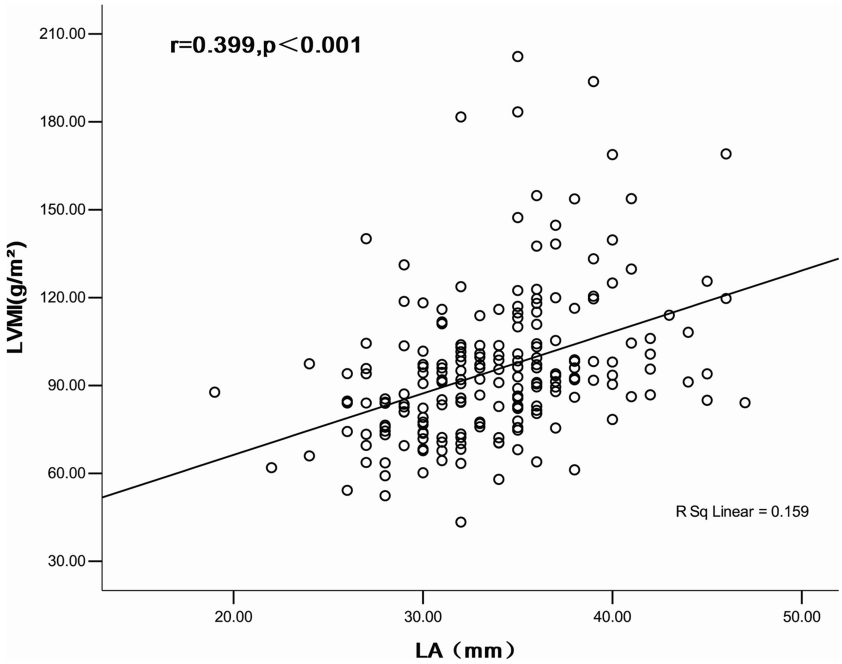

Fig. 2 The relationship between LAD and LVMI in type 2 DM patients

Table 2 Multiple regression analyses of independent LAD covariates

Variables	B	*p* value
Age	0.145	0.028
Gender	− 0.050	0.367
BMI	0.259	< 0.001
WHR	0.138	0.044
Duration of DM	0.137	0.042
SBP	0.053	0.634
PP	− 0.070	0.547
UACR	0.058	0.343
Cr	0.158	0.008
IMT	0.021	0.715
LVMI	0.250	< 0.001
DR	− 0.034	0.555

$p < 0.05$ was considered statistically significant

Cr serum creatinine, *DM* diabetes mellitus, *DR* diabetes retinopathy, *IMT* intima–media thickness, *LAD* left atrial diameter, *LVMI* left ventricular mass index, *PP* pulse pressure, *SBP* systolic blood pressure, *UACR* urinary albumin-creatinine ratio, *WHR* waist-hip ratio

closely related to Framingham Score and 10-year risk for coronary heart disease rate. Therefore, we suggested that LAD may be associated with cardiovascular disease risk. In addition, we also have demonstrated that LAD was significantly associated with an increased number of markers for TOD. Multivariate regression analyses also determined that LVMI and creatinine were independent predictors of LAD enlargement. LAD enlargement could be an effective indicator of TOD, particularly renal impairment and left ventricular hypertrophy. Therefore, screening for LAD may offer a new and rapid approach for evaluating the severity of DM.

Limitations

Some limitations existed in this study. First, this was a cross-sectional study design. Second, 17% of the diabetic participants also had hypertension. The proportion of patients experiencing this was relatively small, and deemed insignificant to the results. Third, a single LAD was obtained for each participant using M-mode echocardiography and measuring the anterior-posterior diameter. While this may not be the most effective way to measure LA chamber size, obtaining this measurement is consistent and replicable, and is therefore a standard echocardiographic measurement. LAD has been regarded as a valid measurement to determine LA size [18], and diastolic dysfunction is strongly correlated with LAD [24]. It is important to note that Doppler imaging of cardiac tissue and LA size are both used to evaluate left ventricular diastolic function. So, the relation between Doppler imaging of tissue, *E* peak, and *A* peak were not analyzed evaluated here. Finally, data found in this study could be limited by the relatively small sample size of this population.

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Critical revision of article: Haoyu Wang, BSc, Fengjuan Yao.

Running title: Left atrial diameter and target organ damage.

Approval of article: Wei He, Wenhui Zhu,Yanqiu Liu, Min Ye, Haoyu Wang, BSc, Wei Li, Hong Lin, Donghong Liu, Fengjuan Yao.

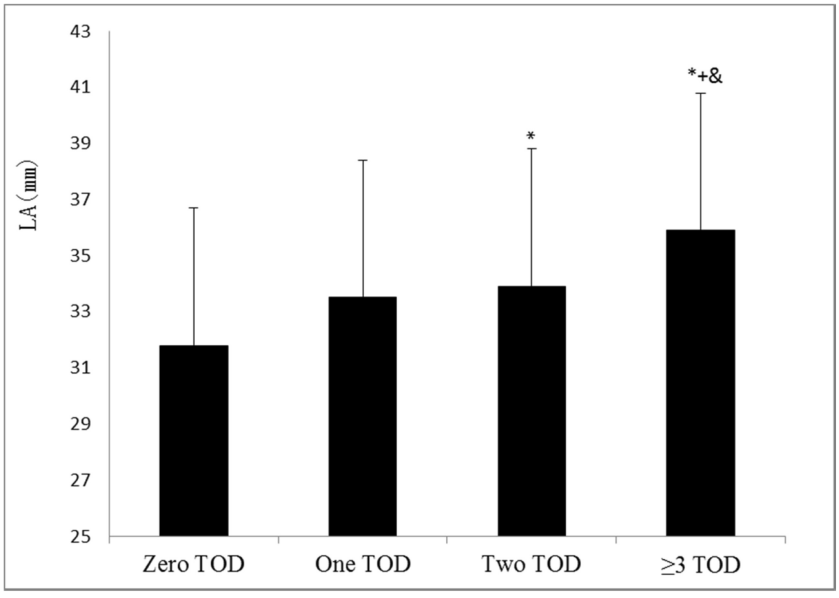

Fig. 3 LAD of type 2 DM patients, stratified by number of TOD markers. LAD increased in proportion to increased incidence of TOD markers. $^{*}p < 0.05$ for the comparisons between patients with zero TOD markers and patients with one, two, and at least three TOD markers; $^{+}p < 0.05$ for comparisons made between one TOD and at least three TOD markers; $^{\&}p < 0.05$ for comparisons made between two TOD and at least three TOD markers

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and 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

Ferritin and serum iron as surrogate markers of poor glycemic control and microvascular complications in type 2 diabetes mellitus

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Abstract
This study was designed to find the correlation of iron indices with HbA1c levels and microvascular complications among patients with type 2 DM. Results were consistent with our hypothesis that high iron indices (serum iron and serum ferritin) not high enough to cause hemochromatosis but were still associated with poor glycemic control and its complications. The mean age of study group was 58.57 ± 3.17 years; whereas, the mean age of control group was 53.95 ± 4.43. The mean HbA1c of study group was 9.46 ± 1.31; whereas, the mean HbA1c of control group was 6.42 ± 0.28. The duration of diabetes in study group was 9.69 ± 2.69 years; whereas, it is 5.26 ± 2.81 years in control group. The mean serum iron level in study group was 155.08 ± 22.13 μg/dl; whereas, it is 88.81 ± 38.04 μg/dl in control group. The mean serum ferritin level in study group was 284.79 ± 50.06 ng/ml; whereas, it is 181.31 ± 54.08 ng/ml in control group. The mean serum transferrin saturation in study group was 30.25 ± 9.94; whereas, it is 28.92 ± 6.03 in control group. Out of the 100 patients in the study group, 40 patients had nephropathy, 33 patients had retinopathy, and 31 patients had neuropathy. In 100 patients in the control group, 12 patients had nephropathy, 11 patients had retinopathy, and 12 patients had neuropathy. On applying Pearson's coefficient of correlation, a moderately significant correlation was obtained between serum iron, ferritin, and HbA1c in study group. However, no significant correlation was obtained with transferrin saturation. On applying regression analysis among HbA1c, serum ferritin, and serum iron, it was observed (the sum of squares of the group was 30.5) that variation is not due to chance.

Keywords Microvascular complications · Serum iron · Ferritin · Surrogate markers

Introduction

We have come a long way as far as our understanding about pathophysiology of type 2 diabetes is concerned from the core defects of impaired insulin secretion from β cells of pancreas and increasing peripheral insulin resistance to De Fronzo's famous ominous octet [1] and further to "Dirty Dozen" [2] involving a delayed incretin response from the gut, increased glucagon production from the α cells as well as insulin resistance in the brain and increased glucose reabsorption in the kidneys all playing a crucial role in worsening of hyperglycemia.

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The fact that the frequency of diabetes is increased in classic hereditary hemochromatosis gives us the clue that systemic iron overload could contribute to abnormal glucose metabolism [3].

The contribution of iron in the pathogenesis of diabetes is suggested by the following: (1) diverse causes of iron overload have been found to be associated with an increased incidence of type 2 diabetes and (2) improvement in glycemic burden with a reduction in iron load was achieved using either phlebotomy or iron chelation therapy.

It is recognized recently that increased body stores of iron have association with the development of insulin resistance syndrome, glucose intolerance, gestational diabetes, and type 2 DM [4–8]. Reduction in iron stores by repeated blood donations leads to decrease in postprandial hyperinsulinemia and improvement in insulin sensitivity [9]. Phlebotomy leads to fall in serum glucose, cholesterol, triglycerides, and improvement in both beta cell secretion

as well as peripheral insulin action in type 2 DM [10, 11]. Epidemiological studies also suggest the same correlation [12, 13]

Recent in vitro studies have shown that H-ferritin mRNA is four- to eightfold higher in rat islets treated with 20 mmol/l glucose than in islets treated with 1 mmol/l glucose [14]. The fact that beta cells are particularly sensitive to oxygen radicals and ferritin has some potential to exhibit antioxidant properties explains increased levels of ferritin in beta cells [15].

Study patients of DM have hyperferritinemia which corelates with diabetic retinopathy, diabetic nephropathy, and vascular dysfunction [16–18]. It is important to realize that raised levels of iron above physiological requirement do not seem to serve any useful purpose in these patients. Although few indirect evidences from the western region do suggest that iron overload influences DM in a negative way, but overall there is paucity of literature especially from our country showing any direct evidence between increased iron load and control of diabetes mellitus. Moreover, finding out such correlation in Indian population carries great clinical significance as anemia has very high prevalence in Indian population, and continuous efforts are being made at physician, community, and government level to prevent and treat anemia which might influence the coexisting diabetic state. Hence, the present comprehensive study was planned to find out the relationship between iron indices (serum iron, serum ferritin, and transferrin saturation) with HbA1c in type 2 DM and to study the influence of body iron stores on diabetic microvascular complications in patients coming to OPD and IPD of our hospital.

Aims and objectives

1. To study the correlation between serum ferritin, serum free iron, and transferrin saturation with HbA1c in patients of type 2 diabetes mellitus
2. To study the correlation between serum ferritin, serum free iron, and transferrin saturation with microvascular complications (neuropathy, nephropathy, retinopathy) in patients of type 2 diabetes mellitus

Sample size of 200 was calculated using the epi-info statistical software for cross-sectional comparative study. Taking reference from Mukesh Gohel study [19], using proportion of patients in each group with raised serum iron level, the sample size came out to be 84 in each group at 5% precision, but taking attrition into consideration, we took 100 patients in each group.

One hundred patients of type 2 diabetes mellitus with HbA1c greater than seven constituted study population. One hundred patients of type 2 diabetes mellitus with HbA1c less than seven constituted control population. Study duration was from March 2014 to November 2015.

Blood investigations

1. Glycated hemoglobin (normal range, < 6.5) was estimated by high performance liquid chromatography (HPLC).
2. Serum iron (normal range, 41–130 μg/dl) was estimated by *Bathophenthroline disulphonate assay* (BPS).
3. Transferrin saturation (normal range, 16–45%) was estimated by photometric color method.
4. Serum ferritin (normal range, males 29–248 μg/dl and females 10–150 μg/dl) was measured by CLIA (chemiluminescence immuno assay).

Tests for Micro vascular complications

Retinopathy was screened by fundus examination.

Nephropathy screening was done by *urine for albuminuria*. Patients were taken as positive for microalbumin (a ratio of albumin (mcg/l) to creatinine (mg/l) of less than 30 was taken as normal; a ratio of 30–300 signified microalbuminuria) if found in this range on two occasions separated by 3 months.

Neuropathy screening was done by bed side criteria which have been validated by Chawla et al. [20] using the following:

A) NSS (neuropathy symptom score)
B) NDS (neuropathy disability score)

A) *Diabetic neuropathy symptom score* (*NSS*): The questions should be answered "yes" (positive, 1 point) if a symptom (as mentioned in the table below) occurred more than two times a week during the last 2 weeks or "no" (negative, no point) if it did not.

Symptoms	No	Yes
Unsteadiness in walking	0	1
Burning feet	0	1
Numbness or tingling of hands and/or feet	0	1
Fatigue, cramping, aching, or nocturnal exacerbations	0	2

B) *NDS* (*neuropathy disability score*) is calculated as mentioned below.

Neuropathy disability score (NDS)

Vibration perception threshold using 128 Hz tuning fork Normal = can distinguish vibration	Normal = 0 Abnormal = 1	Right foot	Left foot
Temp perception on dorsum of foot Using cup full of cold or warm water or thermal tip	Normal = 0 Abnormal = 1	Right foot	Left foot
Pin prick Apply prick proximal to big toe nail just enough to deform the skin Trial pair = sharp/blunt Normal can distinguish sharp/not sharp	Normal = 0 Abnormal = 1	Right foot	Left foot
Achilles reflex	Present = 0 With reinforcement = 1 Absent = 2	Right foot	Left foot
	NDS total out of = 10	Right foot	Left foot

Statistical analysis

Data was entered into MS Excel and analyzed using the SPSS version 17. Descriptive statistics in the form of mean and standard deviations or proportions were used to characterize the study sample. For qualitative data, chi-square or Fisher's exact test was used to observe difference between proportions for independent groups. For continuous variables, student's *t* test was used to compare the two groups. Pearson correlation coefficients were calculated between the outcome and quantitative independent demographic and clinical factors. *p* value of less than 0.05 was considered to be statistically significant. Linear regression analysis was done to determine the models contributing to HbA1C/predictors of HbA1C.

Results

The present comparative study evaluated the relationship between iron indices, Hb1Ac levels, and microvascular complications among patients with type 2 diabetes mellitus. This cross-sectional comparative study comprised of 200 patients (100 patients each categorized under the control and study type 2 diabetes). The overall statistics of the study subjects are shown in Tables 1 and 2.

Serum Iron and HbA1c

The present study states that there is a significant positive correlation of serum iron and HbA1c ($r = 0.46$; $p = 0.001$*) in the study diabetic group (Table 3). Similar results have been found in a study conducted by Shetty et al. [21] where a positive correlation between free iron and HbA1c was found in uncontrolled type 2 diabetes ($r = +0.513$; $p < 0.01$)

Table 1 Overall sample statistics

	Mean	SD	N
Hb1Ac	7.943	1.7911	200
IRON	121.95	45.466	200
Ferritin	233.05	73.432	200
Transferrin saturation	29.58	8.219	200

Serum ferritin and HbA1c

Our study shows that there is a significant positive correlation between serum ferritin levels with HbA1c ($r = 0.43$; $p = 0.001$*) which coincides with a study conducted by Sumeet et al. [22] (Fig 1). Their study showed significant positive correlation between serum ferritin with HbA1c ($p = 0.04$) and also positive correlation between raised ferritin levels in study diabetics. In a study conducted by Sun et al. [23], elevated-circulating ferritin concentrations were associated with higher risk of type 2 diabetes and metabolic syndrome in middle-aged and elderly Chinese independent of obesity, inflammation, adipokines, and other risk factors which support our association of ferritin with HbA1c.

Microvascular complications

In the present study, there is a significant positive correlation between serum iron, serum ferritin levels, and microvascular complications; nephropathy, retinopathy, and neuropathy ($p = 0.001$) (Tables 4, 5, 6, 7, and 8). HbA1c and duration of diabetes also correlated well with microvascular complications ($p = 0.001$) which were significantly higher in the study diabetes group in comparison to the control diabetes group (Table 9). Correlation with HbA1c and duration is a well-known fact, and our study also showed the same consistent results. Dymock et al. [10] reported influence of the increase body iron stores on diabetic nephropathy and vascular dysfunction. In a study by Canturk et al. [16], patients of diabetes had hyperferritinemia, and they found correlation between ferritin levels and diabetic retinopathy. These findings are in accordance with our study.

Table 2 Evaluation of study parameters among the control and study diabetics

Parameter		Study	Control	Statistic
Male	*N*	56	62	Fisher exact = 0.744;
	Percent	47.5	52.5	*p* value = 0.388
Female	*N*	44	38	
	Percent	53.7	46.3	
OHA (oral hypoglycemic agent)	*N*	73	97	Fisher exact = 22.588;
	Percent	42.9	57.1	*p* value = 0.001
OHA + INS (oral hypoglycemic agent + insulin)	*N*	27	3	
	Percent	90.0	10.0	
Iron (normal)	*N*	14	81	Fisher exact = 90.095;
	Percent	14.7	85.3	*p* value = 0.001
Iron (increased)	*N*	86	19	
	Percent	81.9	18.1	
Transferrin saturation (normal range)	*N*	95	99	Fisher exact = 7.749;
	Percent	49.0	51.0	*p* value = 0.097
Transferrin saturation (increased)	*N*	5	1	
	Percent	83.3	16.7	
Ferritin (normal)	*N*	16	68	Fisher exact = 55.501;
	Percent	19.0	81.0	*p* value = 0.001
Ferritin (increased)	*N*	84	32	
	Percent	72.4	27.6	

Transferrin saturation and HbA1c

We also observed the relationship of transferrin saturation with HbA1c; however, we found weak negative correlation in the study diabetic group which was insignificant ($r = -0.05$; $p = 0.6$) consistent with a study conducted by Montonen et al. [3]. But in study conducted by Thomas et al [24], they observed that higher transferrin saturation was observed in diabetics compared to healthy individuals.

Fernandez-Real et al. [25] documented in general population increased body iron stores associated with increased occurrence of glucose intolerance, type 2 diabetes, and gestational diabetes which is consistent with our study.

Elevated iron and diabetes

In the last few decades, the impact of transition metals, and iron in particular, on human physiology has been explored. Iron, being a first-line prooxidant, seems to regulate the clinical manifestations of various systemic diseases, including diabetes and atherosclerotic vascular diseases. Iron regulation of the cell oxidative stress can explain, to some extent, its close association with abnormalities in insulin sensitivity.

Table 3 Correlation values among study diabetes correlations

		Iron	Ferritin	Transferrin sat
Hb1Ac	Pearson correlation	0.462	0.436	− 0.050
	p value	0.001	0.001	0.620

The mechanisms by which elevated iron stores may induce diabetes include oxidative damage to pancreatic beta cells, impairment of hepatic insulin secretion by the liver, and interference with insulin's ability to suppress hepatic glucose production.

Elevated ferritin and diabetes

Oxidative stress and chronic inflammation are being postulated as mechanisms involved in the pathophysiology of diabetes and its complications. Being an inflammatory biomarker and a reflector of iron stores, higher ferritin levels in correspondence to higher iron levels in our study might have been associated with the positive correlation observed in our study with both poor glycemic control and long-term microvascular complications.

Transferrin saturation and diabetes

Based on previous studies, it was speculated that Se transferrin levels may increase as a compensatory mechanism for a reduction in free iron levels that may occur secondary to oxidative stress, and thus, it may serve as a biomarker of some other factor that is causally related to diabetes, and possibly not related to iron load. In the present study, we did not find any association between Se transferrin and diabetes incidence. Interestingly, Se transferrin seemed to be associated with

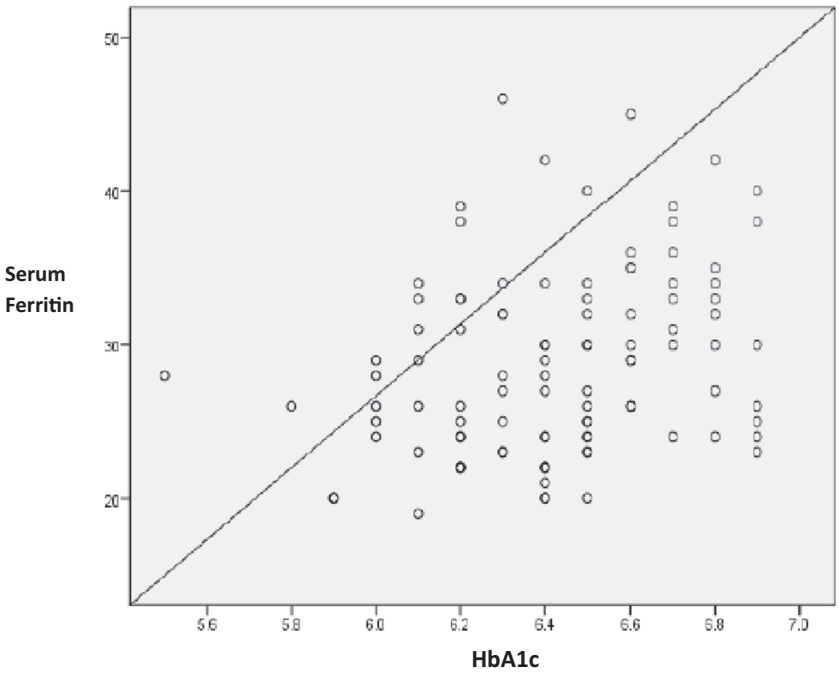

Fig. 1 Scatter graph depicting strength between serum ferritin and HbA1c in control diabetics

Table 4 Glycosylated hemoglobin (HbA1c) and its correlation with microvascular complications in the study group

HbA1c		*N*	Mean	SD	SE	*t* statistic c	*p* value e
Nephropathy	Yes	40	8.96	1.00	0.12	− 5.145	0.001
	No	60	10.19	1.38	0.21		
Retinopathy	Yes	33	9.13	1.18	0.14	− 3.823	0.001
	No	67	10.13	1.32	0.23		
Neuropathy	Yes	31	9.20	1.28	0.15	− 2.986	0.004
	No	69	10.02	1.20	0.21		
Iron	Normal	14	8.59	1.12	0.30	− 2.751	0.007
	Increased	86	9.60	1.29	0.13		

Table 5 Glycosylated hemoglobin (HbA1c) and its correlation with microvascular complications in the study group

Hb1Ac		*N*	Mean	SD	SE	*t* statistic	*p* value
Nephropathy	Yes	12	6.43	0.22	0.06	0.082	0.935
	No	88	6.42	0.29	0.03		
Retinopathy	Yes	11	6.49	0.32	0.09	0.794	0.429
	No	89	6.41	0.27	0.02		
Neuropathy	Yes	12	6.52	0.27	0.07	1.286	0.202
	No	88	6.41	0.28	0.03		
Iron	Normal	81	6.41	0.28	0.03	− 0.709	0.480
	Increased	19	6.46	0.29	0.06		

Table 6 Comparison of nephropathy in the study and control groups

Diabetes type		Patients with nephropathy	Mean	SD	SE	*t* statistic	*p* value
Iron	Study	40	161.98	21.26	3.36	5.828	0.001
	Control	12	107.00	46.15	13.32		
Ferritin	Study	40	304.68	49.11	7.76	7.896	0.001
	Control	12	177.08	49.02	14.15		
Transferrin sat	Study	40	30.83	11.11	1.75	0.885	0.381
	Control	12	27.83	6.46	1.86		

Table 7 Comparison of retinopathy cases in the study and control groups

Diabetes type		No. of patients with retinopathy	Mean	SD	SE	*t* statistic	*p* value
Iron	Study	33	158.18	19.64	3.42	6.335	0.001
	Control	11	99.36	41.85	12.62		
Ferritin	Study	33	308.30	34.06	5.93	6.912	0.001
	Control	11	205.45	62.90	18.96		
Transferrin sat	Study	33	28.67	10.96	1.90	− 0.069	0.945
	Control	11	28.91	6.53	1.97		

Table 8 Comparison of neuropathy cases in the study and control groups

Diabetes type		No. of patients with neuropathy	Mean	SD	SE	*t* statistic	*p* value
Iron	Study	31	165.42	19.15	3.44	6.578	0.001
	Control	12	102.83	43.80	12.64		
Ferritin	Study	31	288.16	53.05	9.52	4.625	0.001
	Control	12	195.58	72.40	20.90		
Transferrin sat	Study	31	29.77	11.21	2.01	1.384	0.174
	Control	12	25.17	3.76	1.08		

Table 9 Correlation values among study diabetes correlations

		Iron	Ferritin	Transferrin sat
Hb1Ac	Pearson Correlation	0.462	0.436	− 0.050
	p value	0.001	0.001	0.620

diabetes risk in individuals with HbA1c values in the control group, but not in those with higher HbA1c values.

Limitations

1. Sample size of the study is small, thereby preventing us from drawing strong conclusions.
2. Diabetes mellitus is a state of chronic inflammation. Though ferritin is a marker of chronic inflammation, the causal relationship between raised ferritin and the study diabetes group is a matter of debate and requires further exploration in establishing the authenticity of causal hypothesis.

Summary and conclusion

In the present cross-sectional, comparative study, we have found significant positive correlation between iron indices (serum iron, serum ferritin) and HbA1c levels in the study group with type 2 diabetes mellitus. We also found that iron indices (i.e., serum iron, serum ferritin, and transferrin saturation) have significant correlation with microvascular complications in them. However, we did not observe any significant correlation between transferrin saturation and HbA1c levels in the present study ($p = 0.62$).

We conclude that serum ferritin and serum iron may be used as surrogate markers of poor glycemic control and microvascular complications in association with HbA1c. Though, the causal association of iron indices is evident by the regression analysis in our study, further larger trials need to be undertaken to establish this causal relationship.

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ORIGINAL ARTICLE

LncRNA Gm4419 promotes the development of cardiac diseases in type 2 diabetic patients with diabetic nephropathy

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Abstract
Diabetic nephropathy has been proved to be correlated with the occurrence of cardiovascular diseases in diabetic patients. However, the mechanism is unclear. LncRNA Gm4419 has been reported to participate in the development of diabetic nephropathy, while its involvement in cardiac diseases is still unknown. Therefore, our study aimed to investigate the correlation between Gm4419 expression and incidence of coronary heart disease (CHD) and stroke. A total of 100 type 2 diabetic patients combined with diabetic nephropathy and 100 type 2 diabetic patients without diabetic nephropathy were included and followed up for 5 years. Renal function and cardiac function indicators as well as serum levels of GM4419 were measured on the day of admission and at the end of follow-up. Occurrence of CHD and stroke was recorded during follow-up and compared between two groups. Renal function of type 2 diabetic patients combined with diabetic nephropathy was worse than that of type 2 diabetic patients without diabetic nephropathy, while no significant differences in cardiac function indicators were found between two groups. Incidences of CHD and stroke were significantly higher in type 2 diabetic patients with diabetic nephropathy than in patients without nephropathy. High serum level of Gm4419 was closely correlated with the occurrence of CHD and stroke as well as poor renal and cardiac functions. LncRNA Gm4419 can promote the development of cardiac diseases in type 2 diabetic patients with diabetic nephropathy.

Keywords Diabetic nephropathy · lncRNA Gm4419 · Coronary heart disease · Stoke

Introduction

With changes in people's lifestyle and diet structure, incidence of diabetes showed an increasing trend [1]. Life quality of diabetic patients was significantly reduced not only by the abnormal physiological conditions that induced by diabetes itself, but also by its severe complications such as diabetic foot ulcer [2], diabetic retinopathy [3], and diabetic nephropathy [4]. Development of diabetes can cause chronic renal failure, leading to unacceptable high mortality and mobility rates worldwide [5]. Diabetic nephropathy is a common complication of diabetes that affects 30% of type 1 diabetic patients and 25% of type 2 diabetic patients, and it is also one of the leading causes of cardiac diseases in diabetic patients [6, 7].

Noncoding RNAs, or ncRNAs, have been proved to play critical roles in both normal biological processes and pathophysiological changes [8]. Long noncoding RNAs, or lncRNAs, is a subgroup of nsRNAs composed of more than 200 nucleotides, which are significantly longer than siRNAs, miRNAs, or other short RNAs [9]. It is well established that the onset and development of diabetic nephropathy require the involvement of different lncRNA to play different functions [10]. A recent study has shown that downregulation of Gm4419 expression inhibits the inflammation in diabetic nephropathy [11], while the functionality of Gm4419 in diabetic nephropathy-mediated cardiac diseases in diabetic patients remains unclear.

Materials and methods

A total of 100 type 2 diabetic patients combined with diabetic nephropathy were selected from January 2015 to January

Qingyan Qiao and Fei Wang contributed equally to this work

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2017 in Shanghai Pudong Hospital. Type 2 diabetes was diagnosed according to the criteria established by the World Health Organization, and diabetic nephropathy was diagnosed according to the existing of persistent macroalbuminuria (300 mg/24 h) in at least two of the three consecutive 24-h urine collections. Those patients included 56 males and 44 females, and age ranged from 33 to 69 years, with an average age of 43 ± 11.2 years. At the same time, 100 type 2 diabetic patients without diabetic nephropathy but with the same age and gender distributions were also enrolled to serve as control group. Patients with other urinary tract diseases, renal diseases, or any cardiac diseases were excluded. All patients were followed up for 5 years (every 3 months) to record the occurrence of stroke and CHD.

Preparation of serum samples

Fasting blood (20 ml) was extracted from each patient on the day of admission and at the end of follow-up for both all laboratory tests (including the tests in this study) and experiments in this study. Blood was kept at room temperature for 2 h, followed by centrifugation at 2000 rpm for 20 min to collect serum samples. Serum samples were kept at − 80 °C before use.

QRT-PCR

Trizol reagent (Invitrogen, USA) was used to extract total RNA from serum samples. Trizol reagent was mixed with serum at a ratio of 10:1. All other operations were performed in strict accordance with instructions of the kit. All RNA samples were tested by NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA), and RNA samples with an A260/A280 ratio between 1.8 and 2.0 were used to synthesize cDNA through reverse transcription. SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA) and cDNA were used to prepare PCR reaction system. PCR reaction conditions were as follows: 95 °C for 55 s, followed by 40 cycles of 95 °C for 12 s and 59 °C for 38 s. $2^{-\Delta\Delta CT}$ method was used to process all data, and relative expression level of Gm4419 was normalized to endogenous control β-actin (see Table 1 for sequences of primers used in PCR reactions).

Detection of laboratory indices

Concentration of urinary albumin in 24-h urine samples was measured by enzyme immunoassay. Serum levels of creatinine were measured by a kinetic Jaffe method. Serum total cholesterol was determined using the Liebermann-Burchard method. Left ventricular end diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), ejection fraction (EF), and fractional shortening (FS) were measured by echocardiography. All those indicators were measured on the day of admission and at the end of follow-up. Estimated glomerular filtration rate (eGFR) was calculated.

Table 1 Sequences of primers used in PCR reactions

	Primer sequences	
	Forward (5′-3′)	Reverse (5′-3′)
Gm4419	GGAACCAAGCAGAC CGAAGAC	CCCCAACCCACAGG AACATAA
β-Actin	GACCTCTATGCCAA CACAGT	AGTACTTGCGCTCA GGAGGA

Statistical analysis

SPSS19.0 (SPSS Inc., USA) was used for all statistical analyses. Measurement data were expressed as mean ± standard deviation, and comparisons of data between two groups were performed using t test, and comparisons among multiple groups were performed using one-way analysis of variance. Count data were analyzed by chi-square test. $p < 0.05$ was considered to be statistically significant.

Results

Comparison of baseline data between two groups

As shown in Table 2, no significant differences in gender, duration of diabetes, BMI, and all four cardiac function indices were found between diabetic patients with and without nephropathy. However, urinary albumin excretion, serum creatinine, and serum cholesterol were significantly higher in diabetic patient with nephropathy than those in diabetic patient without nephropathy ($p < 0.01$ or $p < 0.05$). In addition, eGFR was significantly lower in diabetic patient with nephropathy than those in diabetic patient without nephropathy ($p < 0.01$), indicating the existing of nephropathy.

Comparison of incidence of CHD and stroke between two groups of patients

After 5 years' follow-up, 10 cases of CHD were found in diabetic patients with nephropathy, while only 2 cases of CHD were found in diabetic patients without nephropathy. In addition, 11 cases of stroke were found in diabetic patients with nephropathy, while only 2 causes of CHD were found in diabetic patients without nephropathy. As shown in Fig. 1, cumulative incidences of CHD and stroke were higher in diabetic patients with nephropathy than those in patients without diabetic but with nephropathy. Those data indicate that

Table 2 Comparison of baseline data between two groups

	Nephropathy	Non-nephropathy	*p* value
Gender			
Male	56	53	$p > 0.05$
Female	44	47	
Duration of diabetes (years)	10.9 ± 4.2	10.6 ± 4.9	$p > 0.05$
BMI (kg/m^2)	23.2 ± 2.4	23.1 ± 2.7	$p > 0.05$
Urinary albumin excretion (mg/24 h)	402 ± 244.5**	7.7 ± 5.3	$p < 0.01$
Serum creatinine (μmol/l)	98 ± 34.7*	72 ± 19.6	$p < 0.05$
eGFR			
> 60	33	72	$p < 0.01$
< 60	67	28	
Serum cholesterol (mmol/l)*	5.4 ± 1.2*	4.3 ± 0.89	$p < 0.05$
LVEDD(cm)	4.7 ± 0.8	4.8 ± 0.6	$p > 0.05$
LVESD(cm)	2.8 ± 0.6	2.7 ± 0.6	$p > 0.05$
EF (%)	56.2 ± 4.4	55.7 ± 4.6	$p > 0.05$
FS (%)	29.7 ± 3.6	29.9 ± 4.1	$p > 0.05$

**Compared with diabetic patients without nephropathy, $p < 0.01$; *compared with diabetic patients without nephropathy, $p < 0.05$

nephropathy can significantly increase the incidences of CHD and stroke in type 2 diabetic patients.

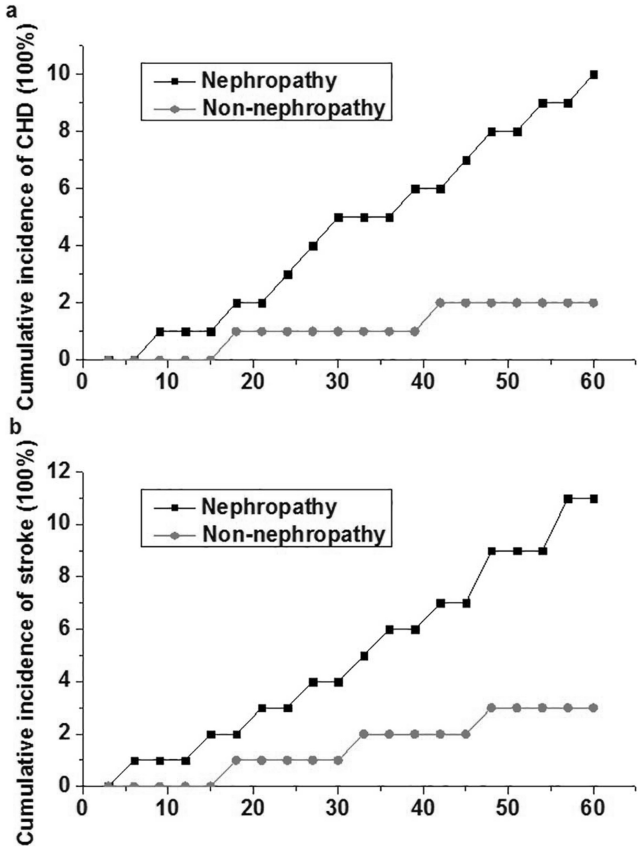

Fig. 1 Comparison of cumulative incidence of CHD and stroke between two groups of patients. **a** Comparison of cumulative incidence of CHD between two groups of patients. **b** Comparison of cumulative incidence of stroke between two groups of patients

Comparison of lncRNA Gm4419 expression levels between different groups of diabetic patients with nephropathy

After 5 years' follow-up, 10 cases of CHD and 11 cases of stroke were found in diabetic patients with nephropathy. In addition, 4 cases of other cardiac diseases and 10 cases of deaths were also observed. Based on this, patients were divided into CHD group ($n = 10$), stroke group ($n = 11$), and diabetes group ($n = 65$). As shown in Fig. 2a, no significant differences in serum levels of Gm4419 were found among three groups on the day of admission ($p > 0.05$). After 5 years' follow-up, serum levels of Gm4419 were significantly higher in CHD and stroke groups than in diabetes group ($p < 0.05$), while no significant differences in serum levels of Gm4419 were found between CHD group and stroke group ($p > 0.05$, Fig. 2b).

Correlation between serum lncRNA Gm4419 levels and renal function as well as cardiac function indices

According to the median serum levels of Gm4419 after follow-up, patients were divided into higher expression group ($n = 45$) and low expression group ($n = 45$). As shown in Table 3, no significant differences in gender, duration of diabetes, and BMI were found between two groups. However, urinary albumin excretion, serum creatinine, and serum cholesterol were significantly higher in high expression group than in low expression group ($p < 0.01$ or $p < 0.05$). In addition, LVEDD and LVESD were significantly bigger, eGFR was significantly lower, while EF (%) and FS (%) were significantly lower in high expression group than in low

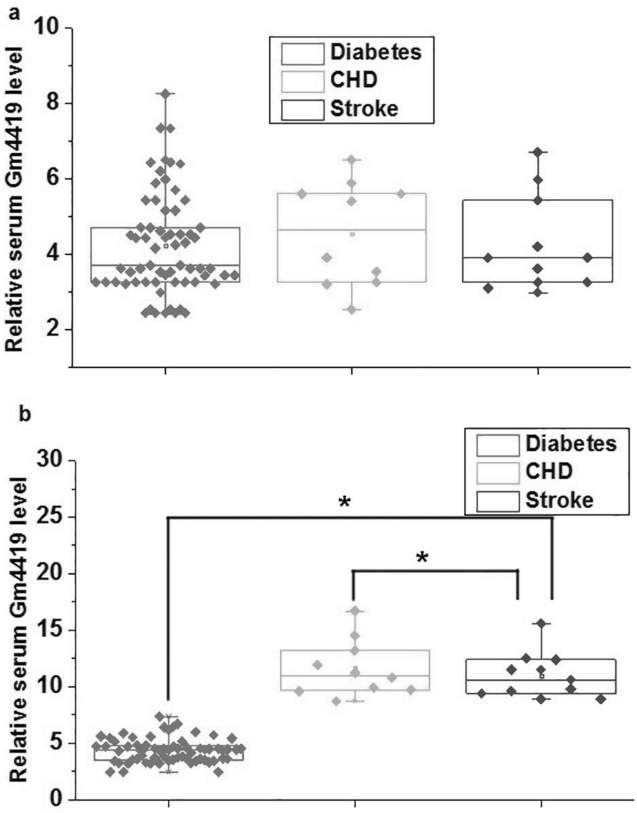

Fig. 2 Comparison of lncRNA Gm4419 expression levels between different groups of diabetic patients with nephropathy. **a** Comparison of lncRNA Gm4419 expression levels between different groups of diabetic patients with nephropathy on the day of admission. **b** Comparison of lncRNA Gm4419 expression levels between different groups of diabetic patients with nephropathy after follow-up. *$p < 0.05$

expression group ($p < 0.05$). Those data suggest that high expression level of Gm4419 in type 2 diabetic patients combined with diabetic nephropathy was significantly correlated with poor renal and cardiac functions.

Discussion

Diabetic nephropathy causes various abnormal structural and physiological changes in diabetic patients, which in turn significantly reduces the life quality of those patients [12]. Clinical symptoms of diabetic nephropathy are frequently ignored at early stages and most patients were diagnosed with severe conditions [13]. Kidney transplantation is considered as a promising treatment strategy for patients with diabetic nephropathy [14]. However, clinical popularization of kidney transplantation is limited by the lack of organ donors and unaffordable costs. Therefore, the aim of the treatment of diabetic nephropathy is to inhibit or control its complications. Renal damage is a leading cause of death in diabetic patients, and renal damage-mediated cardiovascular diseases account for more than two thirds of those deaths [15]. Diabetic nephropathy is also one of the leading causes of cardiac diseases in diabetic patients [6, 7]. A retrospective study carried out by Tuomilehto et al. [16] has shown that the cumulative incidences of CHD and stroke in type 1 diabetic patients with diabetic nephropathy during 20-year follow-up were about 15 and 23%, respectively, while the cumulative incidences of CHD and stroke during the first 5 years were 2 and 1%, respectively. In addition, cumulative incidences of CHD and stroke in type 1 diabetic patients with diabetic nephropathy were higher than those in type 1 diabetic patients without diabetic nephropathy at different time points during follow-up. In our study, the 5 years' cumulative incidences of CHD and stroke in 100 type 2 diabetic patients with diabetic nephropathy were 10 and 11%, respectively, which were significantly higher than the findings in the previous study using type 1 diabetic patients as subjects, which was possibly due to the ethnic differences and the differential pathogenesis of type 1 and type 2 diabetes.

Table 3 Correlation between serum lncRNA Gm4419 levels and renal function as well as cardiac function indices

	High	Low	*p* value
Gender			
Male	26	25	$p > 0.05$
Female	19	20	
Duration of diabetes (years)	15.6 ± 3.8	10.6 ± 4.9	$p > 0.05$
BMI (kg/m^2)	23.1 ± 3.1	23.1 ± 2.7	$p > 0.05$
Urinary albumin excretion (mg/24 h)	707 ± 315.5	307 ± 124.4	$p < 0.01$
Serum creatinine (μmol/l)	127 ± 31.2	88 ± 21.3	$p < 0.05$
eGFR			
> 60	5	16	$p < 0.01$
< 60	40	29	
Serum cholesterol (mmol/l)*	8.6 ± 1.3	5.1 ± 1.5	$p < 0.05$
LVEDD(cm)	6.9 ± 0.9	4.9 ± 0.8	$p < 0.05$
LVESD(cm)	4.1 ± 0.6	3.3 ± 0.7	$p < 0.05$
EF (%)	44.3 ± 3.1	55.3 ± 3.9	$p < 0.05$
FS (%)	19.9 ± 2.6	29.4 ± 4.2	$p < 0.05$

Gm4419 is a novel lncRNA that has been proved to participate in the aggregation of OGD/R injury of cerebral microglial cells [16] and development of trauma-induced astrocyte apoptosis [17]. A recent study also has shown that downregulation of Gm4419 expression inhibits the inflammation in diabetic nephropathy [11]. However, the functionality of lncRNA Gm4419 in other diseases is still unknown. In our study, serum levels of Gm4419 were found to be significantly higher in type 2 diabetic patients with diabetic nephropathy that affected by CHD and stroke than those in patients without any cardiovascular diseases. In addition, high expression level of Gm4419 in type 2 diabetic patients combined with diabetic nephropathy was also found to be significantly correlated with poor renal and cardiac functions. Those data suggest that Gm4419 may participate in diabetic nephropathy-mediated CHD and stroke in type 2 diabetic patients.

Our study provided references for the prevention of CHD and stroke in type 2 diabetic patients combined with diabetic nephropathy, and Gm4419 may serve as a target for this purpose. However, our study is still limited by some shortcomings. Sample size in this study is relatively small and longer follow-up was not performed. In addition, the mechanism of the involvement of Gm4419 in diabetic nephropathy-mediated CHD and stroke in type 2 diabetic patients was not investigated. Our future studies will try to solve those problems.

Conclusions

Incidences of CHD and stroke were significantly higher in type 2 diabetic patients with diabetic nephropathy than in patients without nephropathy. High serum level of Gm4419 was closely correlated with the occurrence of CHD and stroke as well as poor renal and cardiac functions. Therefore, we may conclude that lncRNA Gm4419 can promote the development of cardiac diseases in type 2 diabetic patients with diabetic nephropathy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts 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 The ethics committee of Shanghai Pudong Hospital approved this study, and all patients signed informed consent.

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ORIGINAL ARTICLE

Prevalence and risk factors of diabetes among urban residents in Luzhou City, China

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Abstract

Background Great variation exists in the prevalence rates of diabetes across different geographic regions in China. The purpose of this study was to examine the prevalence of type 2 diabetes among a Chinese population in Western China and to explore associated risk factors.

Methods Adult residents in Luzhou City in Sichuan Province, China, were selected using a multistage area probability design. A validated questionnaire, physical examination, and lab tests were administered to the participants. Bivariate analysis and multivariate analysis using logistic regression were performed to determine factors that may affect the risk of diabetes.

Results The final study sample included 3513 participants with complete questionnaires (response rate was 97.58%). The prevalence rate of diabetes was 17.76%. Multivariate logistic regression analysis demonstrated that residents who fit within the following categories were associated with higher risk for diabetes after controlling for participant demographic factors and other covariates: age groups 50–59, 60–69, and 70 or above; a monthly income of less than ¥1999; family history of diabetes; using mainly animal fat as cooking oil; having a white collar job; BMI above 28; and hypertriglyceridemia. In addition to identifying risk factors previously reported, this study indicates that dietary intake factors may be important to the development of diabetes.

Conclusion This study contributes to the literature of prevalence and risk factors of diabetes in Western China. Future research is needed to further evaluate these risk factors.

Keywords Type 2 diabetes mellitus · Prevalence · Risk factors

Introduction

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia (high blood sugar levels), which may result in serious damage to various body organs [1]. Diabetes has become a global public health issue. It is estimated that the global prevalence of diabetes in 2014 was 9% [2]. Approximately 347 million people around the world are affected by this disease [3]. Diabetes is expected to become the 7th leading cause of death worldwide by the year 2030 [4]. China has a population of over 1.34 billion people and the country is faced with multiple challenges caused by a full-blown epidemic of diabetes [5]. China has the largest number of people with diabetes in the world. The overall prevalence rate of diabetes in mainland China is close to 10% with 92.4 million adults suffer from this condition [5]. A sharp increase in diabetes prevalence has been observed across China over the past few decades. According to diabetes epidemiological surveys conducted by the Chinese Diabetes Society, the prevalence rate of diabetes was 0.67% in 1980, 2.3% in 1994,

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3.2% in 1996, and 9.7% in 2008 [6]. As in other developing countries such as India, the prevalence rate of diabetes in China is expected to increase further in the years to come [6].

There is great variation in the prevalence rates of diabetes across different geographic regions in China. For instance, based on most recent data, the prevalence rate of diabetes in Shanghai was 12.6% whereas Hunan and Guizhou had prevalence rates under 2% (1.5% and 1.9%, respectively), and Hubei, Hainan, Shanxi, and Guangxi had prevalence rates under 3% (2.7%, 2.4%, 2.5%, and 2.5%, respectively) [5]. While genetic, environmental, behavioral, and lifestyle factors may contribute to the development of diabetes, differences in behavior and lifestyle across regions are likely to cause the geographic variation in diabetes prevalence [7, 8]. The prevalence and risk factors of diabetes among Chinese have been reported by several studies [5, 6, 9, 10]. With the exception of one study, few focused on residents of Sichuan Province. Further research is warranted to examine diabetes in this region and investigate risk factors related to diet and lifestyle unique in this region [11].

This study aimed to address this gap in the literature by assessing the prevalence rate of diabetes and associated risk factors among residents of Luzhou City in Sichuan Province, China. Luzhou City is located in Western China and is considered a "medium-sized" city [12]. The western region of China has experienced rapid urbanization and economic development, which results in an elevated level of diabetes risk factors related to diet and lifestyle. Public health concerns caused by overweight and/or obesity among middle-aged adults have become increasingly prominent in Luzhou City. A number of behavioral, lifestyle, environmental, and genetic risk factors associated with diabetes were studied in this study to determine potential factors that may affect the occurrence of diabetes among local residents. Specifically, the following risk factors were considered in this study: gender, age, weight or body mass index, diet and lifestyle, income level, comorbid conditions (i.e., hypertension and hyperglycemia), and family history of diabetes.

Methods

Setting and study population

This study was a cross-sectional study. Study participants were selected using a multistage area probability design. To be included in the study, a participant must be 18 years or older and registered as a permanent resident in Luzhou City. Exclusion criteria included moving to the City within the last 6 months and missing information in the questionnaires, physical examination, or lab tests. At the first stage, a community was randomly drawing from each of the 18 villages, towns, and streets in Luzhou City. At the second stage, a random sample of 200 adult residents was selected based on the resident registration in each community. Consequently, 3600 residents were selected to participate in this study and a survey questionnaire, physical examination, and lab tests were administered. The final study sample consisted of 3513 participants with complete information, among which 624 participants were diabetic and 2889 were not. Diabetes patients were identified based on lab test results and clinical guidelines published by the American Diabetes Association.

Questionnaire, physical examination, and lab tests

A questionnaire "Jiangyang Urban Factors Affecting Adult Health Questionnaire" was used to collect information including demographics such as gender, age, occupation, education level, marital status, family income, disease history, family history of chronic diseases, and behavioral and lifestyle factors such as smoking, drinking, diet, occupation (blue collar/white collar), and physical exercise. The questionnaire adopted questions from national surveys such as China National Health Services Survey when appropriate and was pilot tested in a small sample. Informed consent was obtained from each participant and the survey was carried out by trained investigators. Each participant received the following: (1) investigation via a face-to-face survey; (2) physical examination including measurement of the participant's height, weight, and blood pressure; and (3) lab tests including fasting blood glucose (FBG), triglyceride (TG), and total cholesterol (TC).

Definitions

According to the guidelines enacted by American Diabetes Association in 2012 [13], diabetes is defined as FPG $\geq$ 7.0 mmol/L, or 2-h postprandial blood glucose $\geq$ 11.1 mmol/L, or have been diagnosed as diabetic by a physician/hospital. Hypertension is defined as systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg, or having been diagnosed o have hypertension. According to the guidelines, the *Prevention and Control of Overweight and Obesity of Chinese Adults*, enacted by the Ministry of Health, China, normal weight is defined as BMI 18.5~23.9 kg/m^2, overweight as 24~27.9 kg/m^2, and obesity as $\geq$ 28 kg/m^2. According to the Prevention and Control Proposal of Dyslipidemia in China, enacted in 1997 [14], hypercholesterolemia (HTC) is defined as TC > 5.72 mmol/L and hypertriglyceridemia (HTG) is defined as TG > 1.70 mmol/L.

Statistical analysis

Data was entered and management using EpiData 3.0 software. Data was analyzed using SPSS 19.0 software. The data were described using prevalence rate and composition ratio, and analyzed using chi-square test and logistic regression analysis. The statistical significance level was set at 0.05.

Results

General findings

There were 3513 participants with complete information out of the 3600 selected residents. As such, the effective response rate was 97.58%. There were 1710 men (48.68%) and 1803 women (51.32%), whose ages ranged from 18 to 98 years. The average age was 50.35 ± 16.41 years. There were 624 diabetic participants, which resulted in a prevalence of 17.76%. Table 1 shows that there was no statistically significant difference between different genders and diabetes in terms of the prevalence rates ($p > 0.05$). There was statistically significant difference in the prevalence rates of diabetes across different age groups, education levels, and income levels ($p < 0.05$).

Bivariate analysis

Table 2 shows that the prevalence rate of diabetes was significantly associated with hypertension, family history of diabetes, long-term heavy drinking, mainly using animal fat as cooking oil, low fruit consumption, frequent preserved food consumption, white-collar occupation, BMI, HTC, and HTG ($p < 0.05$).

Table 1 Demographic characteristics of participants with diabetes

Variable	Diabetic subsample ($n = 624$)		χ^2	p value
	N	%		
Gender			0.217	0.641
Men	299	17.45		
Women	325	18.03		
Age group (years)			111.778	< 0.001
18~29	45	9.34		
30~39	51	10.04		
40~49	99	13.04		
50~59	161	22.27		
60~69	155	25.00		
≥ 70	113	26.84		
Education level			74.445	< 0.001
Primary school	411	23.27		
Middle school	185	12.50		
Above college	28	10.49		
Monthly income (RMB)			8.446	0.015
< 1999	483	18.90		
2000~4999	125	14.93		
≥ 5000	16	13.33		

RMB, Ren Min Bi/Chinese Yuan, is the currency used in China

Table 2 Bivariate analysis of risk factors of diabetes

Variable	Diabetic subsample ($n = 624$)		χ^2	p value
	n	%		
Hypertension				
No	388	15.28	38.588	< 0.001
Yes	236	24.23		
Family history of diabetes				
No	509	15.86	92.547	< 0.001
Yes	115	37.95		
Smoking				
No	143	16.86	0.619	0.431
Yes	481	18.05		
Long-term heavy drinking				
No	141	15.31	5.143	0.023
Yes	483	18.63		
Type of cooking oil				
Mainly vegetable oil	350	16.4	6.898	0.009
Mainly animal fat	274	19.87		
Fruit consumption				
Often	5	12.2	35.134	< 0.001
Normal	338	15.03		
Occasionally	281	22.98		
Preserved food consumption				
Never	10	12.05	16.813	0.001
Occasionally	289	16.79		
Normal	234	17.36		
Often	91	25.21		
Occupation				
Blue collar	351	16.59	5.027	0.025
White collar	273	19.54		
BMI				
< 18.5	18	14.63	30.065	< 0.001
18.5~24	372	16.2		
24~27.9	167	19.13		
≥ 28	67	30.45		
High cholesterol (HTC)				
No	505	17.07	6.223	0.013
Yes	119	21.48		
High triglyceride (HTG)				
No	424	16.17	17.931	< 0.001
Yes	200	22.45		

Multivariate logistic regression analysis

Factors with statistical significance in the bivariate analysis were entered as independent variables in the multiple logistic regression analysis with the occurrence of diabetes as the dependent variable. The independent variables included age, monthly income (RMB), family history of diabetes, oil consumption, occupation, BMI, and TG. All included

independent variables were statistically significant with statistically significant categories shown in Table 3. In particular, family history of diabetes had the largest beta coefficient (1.160) which indicates that this variable has the strongest impact on the risk of diabetes. All variables except monthly income had a positive relationship with the occurrence of diabetes, which means the presence of a risk factor was associated with significantly higher risk for diabetes. For example, participants in older age groups were associated with significantly higher risk for diabetes. However, monthly income was negatively associated with the occurrence of diabetes. In other words, participants with higher monthly income were associated with lower risk for diabetes.

Discussion

With the economic reform and urbanization of China, urban residents have faced great health challenges. In a 10-year span from 2003 to 2013, the prevalence of diabetes among middle-older aged adults in China has increased from about 5 to 10–11% [6]. This upward trend in diabetes prevalence suggests a national public health epidemic. The study examined multiple risk factors associated with the prevalence of diabetes in Luzhou City, China. Study results showed that the diabetes prevalence of the residents aged 18 or older was 17.76%. This represents a high prevalence level in comparison to other regions of China [15–17].

This study confirmed that risk factors such as increased age, family history of diabetes, higher BMI (≥ 28), and high triglyceride levels contribute to the occurrence of diabetes. The connection between age and diabetes may be due to the accumulative effect of unhealthy lifestyle. Unhealthy lifestyle habits such as smoking, drinking, and unhealthy diet high in calories, salt, and fat may negatively affect health and manifest at a later age. Additionally, one's physiological function such as blood sugar control declines over time.

According to the regression model (Table 3), the beta coefficient for white-collar occupation is 0.486. This represents a moderate level diabetes risk factor.

In other words, individuals with white-collar occupations were likely to see an increase in diabetes risk compared to individuals engaged in mainly physical work. This is probably due to the fact that white-collar workers have a more sedentary lifestyle, lacking physical exercise and movement while on the job. This finding is consistent with previous studies that a sedentary lifestyle with little to no exercise presents as a risk factor for developing diabetes [18].

Many of the diabetes risk factors, such as obesity or overweight tendencies and high triglyceride levels, are chronic conditions in and of themselves. In an effort to combat diabetes prevalence, patients and physicians alike must address multiple chronic conditions at the same time. Doing so will not only improve one's health status, but also limit their possibility of suffering from diabetes.

Results of this study indicated that as one's education and income increase, the risk of diabetes decreases, suggesting that the more educated and higher income earners are often shielded against developing diabetes possibly due to better access to health care and healthier lifestyle. This study only included three income categories. It is possible that individuals with extremely high incomes may have elevated risk for diabetes if extremely high incomes promote unhealthy lifestyles.

To our knowledge, this study is one of a few studies that evaluated the impact of animal fat and preserved food as risk factors for diabetes. According to the results of the regression model in Table 3, cooking mainly with animal fat (as opposed to vegetable-based cooking oils) was shown to have a positive effect on the risk of developing diabetes. Nevertheless, the beta coefficient for animal cooking fat was relatively low (0.306) compared to other variables. This indicates that although cooking mainly with animal fat can increase one's risk of developing diabetes, it is not necessarily one of the primary risk factors. This is not surprising as animal fat is a source of dietary cholesterol and can result in insulin resistance [18].

Table 3 Multivariate logistic regression analysis of risk factors of diabetes

Variable	Treatment group	Reference group	β	$S_{\bar{x}}$	χ^2	p	OR	95% CI
Age	50~59	18~29	0.843	0.187	20.216	0.000	2.323	1.609~3.354
	60~69		0.919	0.191	23.135	0.000	2.507	1.724~3.647
	≥ 70		0.997	0.202	24.320	0.000	2.709	1.823~4.026
Monthly income (RMB)	2000~4999	≤ 1999	− 0.356	0.118	9.077	0.003	0.701	0.556~0.883
Diabetes family history	Yes	No	1.160	0.136	72.409	0.000	3.189	2.442~4.166
Type of cooking oil	Mainly animal fat	Mainly vegetable oil	0.306	0.119	6.610	0.010	1.359	1.076~1.716
Occupation	With white-collar job	Manual worker	0.486	0.108	20.317	0.000	1.626	1.316~2.009
BMI	≥ 28	< 24	0.528	0.168	9.921	0.002	1.696	1.221~2.355
HTG	> 1.70 mmol/L	≤ 1.70 mmol/L	0.262	0.104	6.310	0.012	1.300	1.059~1.595

The findings of this study highlight the importance of comprehensive intervention measures aimed at changing people's health-related beliefs and lifestyle to prevent diabetes. Based on the factors identified by this and previous studies, we can identify high-risk population groups, disseminate educational knowledge related to diabetes, and promote healthy lifestyle and habits [15, 18]. The Chinese Residents' Dietary Guidelines are one of the valuable information sources that can be used to transform the dietary structure of the general population [15]. Cultivating good living habits, altering unhealthy lifestyle, and encouraging more people to be physically active are important measures to effectively reduce the prevalence of diabetes in Western China and other regions.

This study has several limitations worth noting. First, this study documented the prevalence of type 2 diabetes and associated risk factors among the urban residents in a city in Western China and the findings may not represent other populations in the region. Large-scale survey studies based on data collected from other cities or rural areas in Western China are needed to corroborate the findings of the current study. Second, the current study used the results of fasting blood glucose tests to diagnose diabetes and glycated hemoglobin (HbA1c) test was not used due to limited resources. However, a study based on more than 600 participants suggests that the fasting blood glucose test is more reliable to separate diabetic from non-diabetic participants than HbA1c [19]. Third, we only examined type 2 diabetes in the current study. Even though type 1 diabetes is relatively rare, future research should distinguish type 1 and type 2 diabetes. Fourth, this study did not assess and control for the amount of physical activity of the participants. Physical activity participation is an important moderating factor for diabetes and should be included in future studies.

Conclusion

This study contributes to the literature of prevalence and risk factors of diabetes in Western China. As compared to other regions, this area has an elevated prevalence of diabetes. This increased rate holds multiple health, economic, and behavioral implications for Western China. It is vital for policy makers and health professionals to understand the implications of high diabetes rates and work to reduce these rates. Changing social, behavioral, and lifestyle habits will be necessary to decrease the disease prevalence. Additionally, educating all citizens of risk factors and disease implications will prove useful in the fight against diabetes.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki Ethical Principles for medical research involving human subjects.

Informed consent Written informed consent was obtained from each participant before data collection in the study.

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ORIGINAL ARTICLE

Association between plasma levels of proprotein convertase subtilisin/kexin type 9 (PCSK9) and lipids with rs7903146 polymorphisms of the *TCF7L2* gene in diabetic patients

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Abstract
The rs7903146 polymorphism of *TCF7L2* gene is known as the strongest genetic risk factor for type 2 diabetes mellitus (T2DM). The polymorphism is in association with clinical profile of T2DM patients. PCSK9 is a serine protease that promotes LDLR degradation and regulates circulating levels of lipids. The association of this polymorphism with PCSK9 and metabolic profile of diabetic and healthy subjects was investigated. This cross-sectional study was performed on 132 T2DM patients and the same number of healthy subjects. All the participants were genotyped for the rs7903146 single nucleotide polymorphism by the PCR-RFLP method. Metabolic profile including plasma levels of PCSK9, triglycerides, total cholesterol, non-HDL cholesterol, LDL cholesterol, HDL cholesterol, fasting plasma glucose, and HBA1C was measured. PCSK9, total cholesterol, and LDL-C levels were lower in the diabetic patients as compared to the healthy subjects. There were also direct and significant associations between PCSK9 and TG, TC, LDL-C, and non HDL-C in the subjects. Values of plasma glucose, HbA1c, PCSK9, TC, and LDL-C were higher in patients with TT genotype, but the differences were not statistically significant for all. A positive Spearman correlation was found between PCSK9 levels and the genotypes in all the participants. The results confirm the association of rs7903146 in the *TCF7L2* gene with metabolic parameters and PCSK9. The T allele was associated with higher lipid and PCSK9 levels.

Keywords PCSK9 · rs7903146 polymorphism · *TCF7L2* · Diabetes · Lipid profile

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Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a 692 amino acid serine protease and a circulating protein that is mainly expressed in the liver, intestine, and kidney [1]. PCSK9 acts as a regulator of LDL receptor (LDLR) that binds to the epidermal growth factor-like domain A of LDLR and promotes its degradation through an endosomal and lysosomal pathway, which results in reduced LDL clearance from the circulation [2, 3]. PCSK9 is positively associated with plasma levels of LDL cholesterol [4]. Several demographic and metabolic parameters including plasma LDL cholesterol, HDL cholesterol, triglycerides, apolipoprotein B (apoB), insulin, glucose, smoking, and body mass index appear to correlate with serum PCSK9 levels [5].

Gain-of-function mutations in PCSK9 decrease the number of LDLRs on the cell surface which is a rare cause of familial hypercholesterolemia [6]. Hypercholesterolemia is a major pathogenic risk factor for the incidence of CAD, myocardial

infarction, and death [7, 8]. In African-Americans, loss-of-function mutations in PCSK9 that prevent its secretion are associated with a 30–40% reduction in plasma levels of LDL-C and decrease cardiovascular events [9].

Circulating PCSK9 level was found to be positively associated with both the fasting plasma glucose and insulin concentration, and the HOMA-IR index in human [10]. In contrast, other studies reported that type 2 diabetes mellitus (T2DM) or impaired glucose metabolism did not seem to significantly alter the plasma PCSK9 levels when compared with individuals with a normal glucose metabolism [11].

Effects of diabetes on PCSK9 may be considerably influenced by the racial composition of the population being studied [12–14]. The association of PCSK9 level with insulin and glucose homeostasis remains controversial and different genetic background of the subjects might be involved.

Transcription factor 7-like 2 (TCF7L2) is a member of TCF/lymphoid enhancer factor (LEF) transcription factors which acts downstream of the Wnt signaling pathway. The gene *TCF7L2* located on chromosome 10q25.3 is the most significant and consistent risk conferring gene for diabetes. Two intronic single nucleotide polymorphisms (SNPs), rs7903146 and rs12255372, are significantly associated with the disease. The Wnt signaling pathway is involved in the regulation of pancreatic beta-cell proliferation, differentiation, and insulin secretion. The *TCF7L2* susceptibility variants may impair glucagon-like peptide 1 (GLP1) secretion and beta-cell function and decrease insulin secretion [15, 16]. Different effects of the rs7903146 polymorphisms of *TCF7L2* gene on lipid profile and other metabolic variables were reported. Some studies on diabetic patients reported lower values of LDL-C and lipid factors with minor T allele of this polymorphism, but other studies did not find this association [17–19]. In this study, the authors aimed to evaluate the levels of lipids, lipoproteins, and PCSK9 in diabetic patients with different rs7903146 polymorphisms of *TCF7L2* gene, as compared to matched healthy subjects. Association of the polymorphisms with PCSK9 and the lipid levels was also analyzed.

Patients and methods

Patients

This cross-sectional study was performed on 132 patients with type 2 diabetes and the same number of healthy subjects (age 18–65 years). Type 2 diabetic patients were recruited from the Golestan hospital in Ahvaz, between January 2016 and October 2016. The participants were not related and composed of Arab (65%) and Lor (35%) ethnicities. The diagnosis was done by an endocrinologist based on the American Diabetes Association criteria. Most of the patients were under treatment with hypoglycemic agents or insulin for glycemic control. The patients were not receiving statin or other lipid lowering drugs because we only included diabetic patients with new diagnosis of dyslipidemia, diabetic patients who were not treated for their dyslipidemia, and diabetic patients without lipid abnormalities. Participant's age was 18–65 years. Informed consent was obtained from all the participants.

Genotyping

Genomic DNA was extracted from peripheral blood using a genomic DNA extraction mini kit for the whole blood (YT9040; Yektatajhiz, I.R. IRAN). The rs7903146 single nucleotide polymorphism was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, using the following primers: 5′ACAATTAGAGAGCTAAGCACTTTTTA GGTA′3 (forward) and 5′GTGAAGTGCCCAAG CTTCTC′3 (reverse). The PCR reaction was prepared using 0.1 μg of template DNA, 1 μmol of each primer, 25 μl Ampliqon Taq DNA Polymerase Master Mix, and reaction volume reached 50 μl by distilled water. After amplification, the 188-bp PCR products were digested with RsaI restriction enzyme (1116A; Takara Bio Inc., Japan) at 37 °C for 4 h. The RsaI enzyme recognized the restriction site 5′-GT▼AC-3′. The rs7903146 C allele produced two fragments, 159 and 29 bp, whereas the rs7903146 T allele (mutant) was not cleaved (Fig. 1). The DNA concentration was evaluated by spectrophotometer, and the resulting digestion products were detected following electrophoresis.

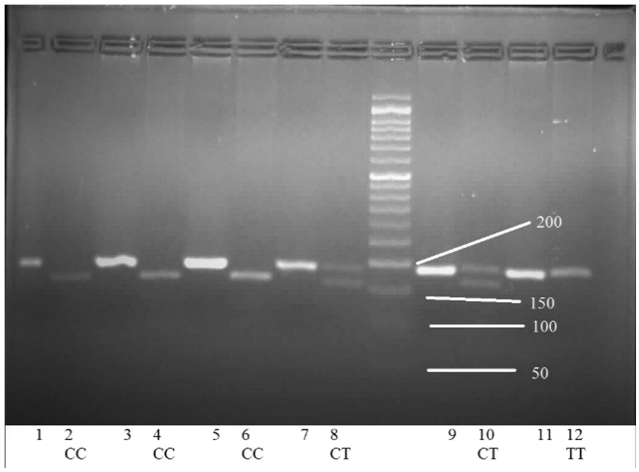

Fig. 1 Electrophoresis gel image of PCR products. Lanes 1, 3, 5, 7, 9, and 11 were PCR products without RsaI digestion. Lanes 2, 4, 6, 8, 10, and 12 were PCR products with RsaI incubation

Laboratory analysis

Blood samples were obtained from all participants after a 12-h overnight fast. The blood was collected into EDTA-containing tubes, and prepared plasma samples were stored at − 80 °C until analysis. Plasma levels of triglycerides (TG), total cholesterol (TC), HDL-C, LDL-C, and glucose were measured by a biochemistry analyzer (Hitachi 7150, Tokyo, Japan) using the measurement kits from Pars-Azmun Co (I.R. IRAN). PCSK9 levels were determined in duplicate using a commercially available quantitative sandwich ELISA kit (ZB-12320-H9648; ZellBio GmbH, Germany), according to the manufacturer's instructions. The intra-assay coefficient of variability (CV) was < 10%. The inter-assay CV was < 12%. HBA1C determination was performed by an enzymatic method provided by Pishtaz Teb Co (I.R. IRAN).

Statistical analysis

The sample size was calculated prior to sampling based on the minor allele frequency of rs7903146 SNP (T allele, 0.39) in Iranian population, test power of 80%, and alpha-level of 0.05. Normality of the variables was assessed by One-Sample Kolmogorov-Smirnov test. Continuous variables are presented as mean ± standard deviation (SD) or as median and interquartile ranges (IQR). Nominal variables are shown as frequency and percentages, and were analyzed by chi-square test. Variables with non-normal distribution were log10 transformed prior to use in the parametric tests. Comparison of the continuous variables was performed using *t* test, ANOVA, and ANCOVA tests. Association between PCSK9 levels and other variables was analyzed by univariate and multivariate linear regression models. Statistical analysis was done using SPSS v16, and $p < 0.05$ was considered significant.

Results

The clinical and biochemical characteristics of the subjects are listed in Table 1. Fasting plasma glucose (FPG) and HBA1C levels in diabetic patients were significantly higher than in the control group ($p < 0.001$), but LDL-C, cholesterol, and PCSK9 levels were lower in the diabetic patients as compared to the healthy subjects ($p < 0.001$ and $p < 0.01$, respectively). Values of PCSK9 were log10 transformed prior to analysis, and the reported *p* value for these parameters was adjusted for sex, age, and BMI.

The results of univariable regression of PCSK9 with the biochemical variables are presented in Table 2. There was no significant correlation between PCSK9 and FPG in both the diabetic and healthy subjects, but values of HBA1C in the diabetic patients were associated with PCSK9 levels ($\beta = 0.244$, $p = 0.008$). There were also direct and significant associations between PCSK9 and TG, TC, LDL-C, and non HDL-C in diabetic patients as well as the control group. In contrast, the correlation of HDL-C with PCSK9 was inverse and significant in the diabetic patients ($\beta = -0.192$, $p < 0.05$). Spearman correlation analysis was also performed and confirmed the significant positive correlations between PCSK9 levels and values of TC, LDL-C, and TG in all the participants ($r = 0.45$, $r = 0.44$, and $r = 0.31$, respectively, $p < 0.001$). Inverse Spearman correlations were also found between PCSK9 and levels of FBS and HBA1C ($r = -0.22$ and $r = -0.16$, $p < 0.05$).

A significant association was found between rs7903146 polymorphism of *TCF7L2* gene and diabetes by chi-square test ($p < 0.05$). Diabetic patients with TT genotypes of rs7903146 polymorphism were at higher risk of T2DM (OR 2.6, CI 1.4–3.4, $p < 0.05$). The genotype distribution among the T2DM and control subjects was in Hardy-Weinberg equilibrium (HWE test: TD2M $\chi^2 = 2.45$, $p = 0.117$, Controls $\chi^2 = 0.11$, $p = 0.734$).

As shown in Table 3, FPG and HBA1C levels in the diabetic patients with TT genotype were higher than those with TC or CC genotypes, and those in TC genotype were higher than in CC genotype ($p < 0.05$). These glycemic parameters increased with higher prevalence of T allele. TG levels were also higher in TT genotype and showed the same profile in diabetic patients ($p < 0.05$ for TT vs. TC). Values of PCSK9, TC, and LDL-C were also higher in patients with TT genotype, but the differences were not statistically significant. In healthy subjects, there were no significant differences in the studied metabolic parameter between the genotypes except for the PCSK9 levels that were significantly higher in TT genotype as compared to TC (256 ± 26 vs. 160 ± 62, $p < 0.05$).

Patients with FPG and HBA1C levels exceeding 140 mg/dl and 7%, respectively, were considered as uncontrolled diabetic patients. As shown in Table 4, patients with uncontrolled glycemic condition showed higher levels of TG, TC, LDL-C, and PCSK9, but the increase was not statistically significant for TG and PCSK9 values.

Spearman correlations between genotypes of rs7903146 polymorphism were also calculated by ranking the genotypes (TT:1, TC:2, CC:3) with PCSK9 levels in all the studied participants. Positive correlations were found between PCSK9 levels and the genotypes in all the patients ($r = 0.123$, $p = 0.058$) and control group ($r = 0.199$, $p = 0.083$), but the correlation was inverse in the diabetic group ($r = -0.04$, $p = 0.335$).

Discussion

The present study aimed to test the hypothesis that circulating PCSK9 levels may be associated with rs7903146 polymorphism of the *TCF7L2* gene, a well-known predisposing locus for T2DM [15, 16]. To the best of the authors' knowledge, this is the first study on the association of plasma PCSK9

Table 1 Comparison of the clinical characteristics of the diabetic and control groups

Variables	T2DM	Control	*p* value
Age (year)	55.15 ± 8.06	52.17 ± 7.85	.014
Weight (kg)	76.62 ± 12.22	76.31 ± 12.45	0.86
Height (cm)	163.8 ± 10.32	168.62 ± 10.591	.002
BMI (kg/m^2)	28.72 ± 4.90	26.9700 ± 4.632	.015
FPG[a] (mg/dl)	206.5 (178.2, 259.0)	88.00 (80.7, 95.0)	.000
HbA1c[a] (%)	7.90% (7.1%,8.4%)	4.90 (4.37, 5.20)	.000
TG (mg/dl)	181.95 ± 71.31	174.11 ± 67.42	.459
Total cholesterol (mg/dl)	189.64 ± 52.46	211.92 ± 63.44	.001[b]
PCSK9[a](ng/ml)	93.25 (74.92,119.62)	141.9 (98.6, 245.5)	.000
LDL-C (mg/dl)	111.53 ± 48.29	128.95 ± 58.49	.003[b]
HDL-C (mg/dl)	41.71 ± 9.46	48.15 ± 10.18	.000[b]
Non-HDL-C (mg/dl)	147.92 ± 55.96	163.77 ± 69.51	.012[b]

[a] Non-normally distributed data, presented as median (interquartile range), were log10 transformed before analysis

[b] *p* values after adjustments for sex, age, and BMI

concentration with this polymorphism. The results confirmed the significant association between this polymorphism and T2DM in subjects from southwest Iran. Interestingly, it was found that the plasma PCSK9 levels in diabetic patients were significantly lower as compared to the healthy subjects. The correlation between diabetes, insulin and glucose metabolism and PCSK9 is the subject of intense debate [20]. In some previous studies, increased PCSK9 levels were reported in T2DM, but in other studies, no associations were found. Studies that found a positive association between PCSK9 and diabetes included significant number of African-Americans [12] or Tunisians [13], whereas studies that did not find an association between PCSK9 and diabetes were performed on populations with mostly Caucasian or Chinese, in which PCSK9 levels were lower in diabetic patients but were not statically significant [11, 14]. The effects of diabetes on PCSK9 may be influenced by the racial background of the participants [20]. In addition, in the current study, no influence of lipid-lowering drugs was considered because the enrolled subjects did not take lipid-lowering drugs prior to the study. However, the patients were under treatment with oral hypoglycemic agents or insulin during the study. Insulin has been shown to be positively associated with PCSK9, and a marked decrease in PCSK9 protein was reported in streptozotocin-induced type 1 diabetic rats [21]. It should be noted that the diabetic participants in the current study were heterogeneous regarding the duration of diabetes. As a result of the deteriorating secretory capacity of insulin during the course of the disease, variable associations of the PCSK9 level with T2DM might be found.

Table 2 Univariate regressions of PCSK9 levels with biochemical variables

Variables	Control		T2DM	
	β	*p* value	β	*p* value
FPG (mg/dl)	.134	.355	.124	.183
HbA1c (%)	.169	.241	.244	.008
TG (mg/dl)	.328	.020	.291	.002
Cholesterol (mg/dl)	.574	.000	.324	.000
LDL-C (mg/dl)	.588	.000	.301	.001
HDL-C (mg/dl)	− .270	.058	− .192	.039
NonHDL-C (mg/dl)	.560	.000	.337	.000

Higher levels of PCSK9 were found in uncontrolled diabetic patients as compared to controlled ones (HbA1c < 7.0%), but it was not statistically significant. Yang et al. [14] also reported higher PCSK9 levels in T2DM with poorly controlled glucose (HbA1c ≥ 7.0%) as compared to well-controlled glucose. Although the PCSK9 level was lower in the diabetic patients as compared to the control group, a poor glycemic control can result in higher PCSK9 levels and its consequences.

FBS and HBA1C in diabetic patients were significantly higher than in the control group. In this study, FBS and HBA1C were higher in the diabetic patients with TT genotype of rs7903146 polymorphism than TC or CC genotypes. This indicates that the T allele is related to FBS and HBA1C levels in diabetic patients and confirms previous studies that reported decreased LDL-C and HOMA-IR and increased glucose in T allele carriers [19, 22]. The results of the present study showed that HBA_{1C} in diabetic patients is associated with PCSK9 which is in accordance with previous reports [14]. The risk T allele of rs7903146 polymorphism affects insulin secretory response to oral glucose and impaired GIP- and GLP-1-induced insulin secretion (30% reduction) [23, 24]. The stimulatory effect of insulin on PCSK9 in mice and cell culture has

Table 3 Comparison of the variables in diabetic patients with different rs7903146 genotypes

Variables	genotypes of rs7903146 polymorphism			*p* value			
	TT	TC	CC	TT vs. TC	TT vs. CC	TC vs. CC	All
FPG[a] (mg/dl)	245.0 (198.5, 309.5)	200.0 (172.0, 276.0)	196.0 (174.5, 230.25)	.025[b]	.028[b]	.025[b]	.004[b]
HbA1c[a] (%)	7.90 (7.3, 8.9)	7.90 (7.1, 8.5)	7.50 (6.87, 8.10)	.013[b]	.014[b]	.013[b]	.003[b]
TG (mg/dl)	198.11 ± 75.30	168.47 ± 66.02	186.42 ± 73.00	.036[b]	.564[b]	.139[b]	.087[b]
Cholesterol (mg/dl)	196.57 ± 56.77	190.25 ± 51.21	181.97 ± 50.31	.570	.232	.454	.484
PCSK9[a] (ng/ml)	100.5 (73.70, 113.50)	92.60 (74.70, 120.60)	89.95 (77.07, 126.32)	.728b	.517b	.708b	.810[b]
LDL-C (mg/dl)	116.54 ± 50.79	113.97 ± 46.14	103.01 ± 49.15	.802	.228	.281	.425
HDL-C (mg/dl)	40.40 ± 11.06	42.57 ± 8.20	41.68 ± 9.659	.280	.561	.654	.557
Non HDL-C (mg/dl)	156.16 ± 60.32	147.67 ± 53.66	140.29 ± 55.32	.474	.223	.531	.473

[a] Non-normally distributed data, presented as median (interquartile range), were log10 transformed before analysis

[b] *p* values after adjustments for sex, age, and BMI

been shown [21]. The transcriptional regulation of PCSK9 and LDLR is in part exerted by the transcription factor sterol regulatory element binding protein 2 (SREBP-2) and SREBP1c, which are regulated by fatty acids and insulin, thereby linking glucose and insulin metabolism to PCSK9 and cholesterol regulation [21, 25, 26].

In the current study, the lipid profiles of the diabetic and control groups were significantly associated with PCSK9 levels. Similarly, Ibarretxe et al. and Yang et al. reported that plasma PCSK9 level was positively associated with TC, LDL-C, non-HDL-C, and ApoB in patients with or without T2DM [27, 28]. In Lakoski et al. [12] as well as Nekaies et al. studies [13], there was a positive association between PCSK9 and TG in diabetic patients. The association can be attributed to the PCSK9 effects on LDL receptor and beyond it by targeting LRP-1, VLDL receptor, ApoE2 receptor, CD36, and ApoB synthesis [1, 5, 27].

The lipid indices were generally higher in TT genotype of the polymorphism, but the increase was only significant for TG and PCSK9 in diabetic and healthy subjects, respectively. Wang et al. in a meta-analysis reported no associations between rs7903146 polymorphism and HDL-C in subjects with metabolic syndrome, non-diabetic subjects and general populations by using four different genetic models [29]. However, in another study, minor allele homozygotes for rs7903146 showed worse postprandial lipemia profile in young males, as seen by a lower HDL-cholesterol and Apo A1 concentration and a trend towards higher triglycerides (TG), than the other genotypes [30]. It has been shown that individuals carrying the CC genotype showed lower risk of developing metabolic syndrome by presenting decreased triglycerides (TG) levels when compared with individuals carrying the allele T [19].

No association has been reported between minor allele (T) and plasma TC and LDL-C in subjects with type 2 diabetes or metabolic syndrome [29]. However, in accordance with the results of the current study, in healthy elderly subjects, carriers of the minor allele showed higher total cholesterol, LDL-cholesterol, Apo B, and TG in the fasting state [30].

Table 4 Comparison of variables between well vs. poorly controlled diabetic patients

Variables	Well controlled (*n* = 29)	Poorly controlled (*N* = 103)	*p* value
FBS[a] (mg/dl)	176.0 (153.0, 199.0)	234.0 (191.0, 273.0)	.013[b]
HbA1c[a] (%)	6.80 (6.15, 6.90)	8.10 (7.60, 8.60)	.000[b]
TG (mg/dl)	164.59 ± 64.95	186.83 ± 72.55	.160[b]
Cholesterol (mg/dl)	165.62 ± 41.09	196.40 ± 53.49	.004[b]
PCSK9[a] (ng/ml)	83.10 (67.0, 105.9)	94.6 (77.25, 123.05)	.107[b]
LDL-C (mg/dl)	90.46 ± 37.032	117.47 ± 49.57	.043[b]
HDL-C (mg/dl)	42.24 ± 9.03	41.56 ± 9.62	.888[b]
Non-HDL-C (mg/dl)	123.38 ± 44.436	154.83 ± 57.10	.043[b]

[a] Non-normally distributed data, presented as median (interquartile range), were log10 transformed before analysis

[b] *p* values after adjustments for sex, age, and BMI

Correlation analysis could not be implemented between PCSK9 levels and lipid factors in the groups of participants with different genotypes in the current study due to the limited number of subjects in each group, especially the people with homozygous minor allele T. However, it is suggested that their correlation can be influenced by the rs7903146 polymorphism. Such influence was previously reported for T2DM [14, 28].

This study has several limitations. The cross-sectional design precludes extrapolating any causality association. Insulin concentrations were not measured; therefore, the association of insulin with PCSK9 in T2DM could not be indicated. The relatively small number of the study population limited the ability to detect weak correlations in both univariable and multivariable analysis, partly because of the difficulty in enrolling T2DM patients with different genotypes who do not take lipid lowering medication.

Conclusion

PCSK9 level was lower in patients with T2DM. Its concentration was associated with HbA1c levels and lipid profile. Patients with TT genotype of the rs7903146 polymorphism in *TCF7L2* gene tend to have higher levels of PCSK9, glycemic, and lipid indices, suggesting that the polymorphism is correlated with PCSK9, thereby modulating its impact on lipid profile.

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Compliance with ethical standards

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

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Human and animal rights The study has been approved by the appropriate local ethics committee at the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.76) 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 Ahvaz Jundishapur 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.

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ORIGINAL ARTICLE

Serum Angiopoietin-2 levels as a marker in type 2 diabetes mellitus complications

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Abstract
Angiopoietin-2 (Ang-2) has been reported to be involved in the development of type 2 diabetes (T2D) and accelerates micro and macro vascular complication. The current study aimed to investigate serum Ang-2 levels in Saudi patients with type 2 diabetes and its complications. In this observational cross-sectional study, serum was collected from 329 T2D subjects aged 34–79 years. All subjects were evaluated for fasting blood glucose (FBG), HbA1c, lipid profile, and kidney profile. Serum concentration of Ang-2 was assayed by ELISA-based method. Mean serum Ang-2 level was significantly higher in female T2D subjects (2881 ± 1550 pg/ml) compared with male T2D subjects (2450 ± 1477 pg/ml). The mean serum Ang-2 levels in female T2D subjects significantly increased with increase in their age and BMI, while mean serum Ang-2 levels did not significantly increase among the male with their age and BMI. A significantly higher level of Ang-2 was found in patients with diabetic complications like neuropathy (3293 ± 1728 pg/ml), retinopathy (2986 ± 1645 pg/ml), and vascular complications (3152 ± 1571 pg/ml). The study has established comprehensive Ang-2 levels in various subsets of adults with diabetes. While interpreting Ang-2 levels, the differences in age and sex need to be taken into account. Diabetic patients with higher Ang-2 levels should be monitored closely and level of serum Ang-2 should be served as a marker to identify patients at high risk of diabetic complications.

Keywords Ang-2 · Angiogenesis · Type 2 diabetes · Microvascular complications · Macrovascular · Cardiovascular diseases

Introduction

Diabetes mellitus (DM) is a growing health concern globally and International Diabetes Federation (IDF) estimated that more than 415 million people were affected with diabetes in 2015. It is also estimated that the number of diabetes cases will increase to 642 million by 2040, putting a huge burden on health costs globally [1]. People with diabetes have an increased risk of developing microvascular complications that lead to blindness, renal failure, nerve damage, and limb amputation [2].

In our body, growth, development, and repair of the tissues are controlled by the process called angiogenesis. For the formation of vascular network in the body, the roles of vascular endothelial growth factor (VEGF) and angiopoietins are well characterized [3]. Any dysfunction in angiogenesis will cause serious health disorders [4]. To stimulate or maintain a healthy vasculature, performance of both VEGF and angiopoietins must be synergistic [5]. The angiopoietin family comprises growth factors—Ang 1–4—that act as ligands for the Tie-2 receptor which is a tyrosine kinase receptor [6]. Among them, Angiopoietin-1 (Ang-1) and Ang-2 have distinctive role in developing and maintaining circulatory vasculature. The action of VEGF is more important during initial stages and Angiopoietin-tie 2 signaling is important towards the later stages. VEGF helps in the initiation of vasculogenesis while Angiopoietin-tie 2 signaling works towards vessel remodeling and maturity processes. The action of Ang-2 is always an antagonist to Tie2/Ang-1 signaling and depends on the tissue conditions and presence of VEGF; it assists vessel growth as well as vessel regression [7].

Several studies reported the role of different markers in diabetes and its complications. Recently, studies on biomarkers (cystatin C, NGAL, transferrin, metalloproteases etc.) in Saudi diabetic subjects have shown good diagnostic accuracy in diabetic complications [8–10]. Even though, these

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studies did not consider sexual dimorphism in the levels of biomarkers and its application in clinical practices is not yet proven because of the study design. A recent study reported the sexual dimorphism in diabetic vascular complications as well as microvascular complications [11]. In view of growing importance for Ang-2 as prospective marker of vascular health status and diabetic complications, we evaluated the gender differences in the level of serum Ang-2 in Saudi diabetic adult and its relevance with the diabetic complications. The study will establish the levels of Ang-2 in Saudi type 2 diabetic adults with different complications.

Materials and methods

This is an observational cross-sectional study conducted in the University Diabetes Center at King Saud University. The study protocol was approved by the Institutional Review Board (IRB) and was conducted in accordance with the guidelines set by Ethics Committee of the College of Medicine, King Saud University.

Study subjects

A total of 370 type 2 diabetic patients, who did not have any other illness, were recruited from general diabetes clinic. We excluded 6 female subjects who were pregnant at the time of recruitment. From the remaining 364 subjects, we excluded 35 subjects with > 30 years of diabetes duration. Finally, a total of 329 subjects were selected; 149 males (45.3%) and 180 females (54.7%), aged between 34 and 79 years.

Diagnosis of type 2 diabetic patients was based on the American Diabetes Association (ADA) criteria or reported to be taking treatment for diabetes [12]. The intact hepatic function was evaluated by aspartate aminotransferase (AST) or alanine aminotransferase (ALT). Patients with microvascular and macrovascular complications were included in this study. Diabetic nephropathy (DN) was evaluated by glomerular filtration rate < 90 mL/min/1.73m^2. The diabetic neuropathy (DN) was evaluated by assessing upper and lower extremities of nerve conduction velocity. The presence of at least one definite microaneurysm in any field photographed was considered as the criterion for the diagnosis of diabetic retinopathy (DR) [13]. Macrovascular complications were confirmed by a history of previous myocardial infarction, angina, and coronary or peripheral revascularization. Selected subjects with different complication were controlled by antihypertensive medications such as Angiotensin II receptor antagonist, Thiazide diuretic, Angiotensin converting enzyme inhibitor (ACEI), Beta blocker, and Calcium Channel blockers (CCB). The diabetes was controlled either by insulin therapy or by oral antidiabetic therapy such as Metformin, Sitagliptin, or Sulfonylurea.

Clinical and demographic data

A research physician interviewed all subjects and clinical data were collected including age, gender, diabetes duration, systolic blood pressure (SBP), diastolic blood pressure (DBP), and smoking. Demographic data such as body mass index (BMI) were calculated as the quotient of weight (kg) divided by height squared (m^2).

Laboratory measurements

After an overnight fast, 5 ml of venous blood sample were collected from each subject in a plain tube. Serum was separated and stored immediately at − 20 °C for further analysis. The biochemical assessments including FBG, HbA1c, lipid profile (triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol (TC)) were analyzed using routine laboratory procedures (RX Daytona clinical chemistry analyzer, Randox, UK). eGFR was estimated using CKD-EPI creatinine equation [14].

Serum Ang-2 was measured using the sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK). The Ang-2 values were expressed in pg/ml. The minimum detectable level of Ang-2 was 46.9 pg/ml, and the intra- and inter-assay coefficients of variation were 5.9 and 8.96%, respectively. The reference values of serum Ang-2 in normal healthy subjects were 800 ± 200 pg/ml and T2D subjects were 2200 ± 700 pg/ml [15].

Statistical analysis

Data entry and analysis were performed using SPSS version 21, IBM, Chicago, Illinois, USA. Results are represented as mean ± SD and percentage. Comparison of means between two groups was carried out using Student's *t* test of normality and equality of variance. For all statistical tests a value of $p < 0.05$ was considered as statistically significant.

Results

The baseline demographic characteristics and biochemical parameters of Saudi type 2 diabetic subjects are shown in Table 1. There were no significant differences between male and female subjects with respect to age, duration of diabetes, SBP, and diabetic complications. Significantly higher BMI, TC, and HDL were observed among female diabetic subjects than males. The glycemic parameters such as mean FBG and HbA1c were also higher in female subjects. Kidney function tests such as serum creatinine were higher in males; while eGFR is higher in females. Diabetic neuropathy was present in 25.5% ($n = 83$) and diabetic retinopathy in 44.4% ($n = 146$) of subjects; while 18.8% ($n = 62$) and 14.6% ($n = 48$) of the

Table 1 Demographic characteristic and biochemical finding of with type 2 diabetic subjects

Variables	Total *n* (329) mean ± SD	Males *n* (149) mean ± SD	Females *n* (180) mean ± SD	*p*
Age (years)	57.7 ± 10.8	58.8 ± 11.7	56.7 ± 9.9	0.090
Duration of diabetes (years)	17.4 ± 8.4	17.6 ± 9.3	17.1 ± 7.6	0.609
Systolic BP (mmHg)	133.7 ± 16.0	135.0 ± 15.4	132.6 ± 16.5	0.193
Diastolic BP (mmHg)	73.4 ± 9.3	74.9 ± 7.9	72.2 ± 10.2	0.008*
BMI (kg/m^2)	32.5 ± 6.0	30.5 ± 5.6	34.2 ± 6.0	0.001*
FBG (mmol/l)	9.0 ± 3.4	8.4 ± 2.9	9.6 ± 3.7	0.002*
HbA1c (%)	8.9 ± 1.8	8.4 ± 1.7	9.2 ± 1.9	0.001*
TC (mmol/l)	4.2 ± 0.9	4.0 ± 0.8	4.3 ± 0.9	0.005*
TG (mmol/l)	1.6 ± 0.8	1.6 ± 0.8	1.6 ± 0.8	0.948
LDL (mmol/l)	2.3 ± 0.7	2.2 ± 0.7	2.4 ± 0.8	0.125
HDL (mmol/l)	1.2 ± 0.3	1.0 ± 0.3	1.2 ± 0.3	0.001*
Creatinine (μmol/l)	71.9 ± 20.9	82.3 ± 18.9	63.6 ± 18.6	0.001*
eGFR (ml/min/1.73 m^2)	88.4 ± 21.1	85.3 ± 22.0	90.9 ± 20.1	0.020*
Hypertensive *n* (%)	245 (74.5%)	111 (78.2%)	134 (77.9%)	0.955
Diabetic neuropathy *n* (%)	83 (25.5%)	33 (24.1%)	50 (30.3%)	0.228
Diabetic retinopathy *n* (%)	146 (44.4%)	66 (45.2%)	80 (46.2%)	0.853
Diabetic macrovascular *n* (%)	62 (18.8%)	26 (18.6%)	36 (22.0%)	0.466
Diabetic nephropathy *n* (%)	48 (14.6%)	28 (19.7%)	20 (12.6%)	0.091
Ang-2 (pg/ml)	2687 ± 1530	2450 ± 1477	2881 ± 1550	0.011*

Results are normally distributed, expressed as mean ± standard deviation or as percentage (%), *DM* diabetes mellitus, *BMI* body mass index, *BP* blood pressure, *FBG* fasting blood glucose, *TC* total cholesterol, *TG* triglycerides, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *eGFR* estimated glomerular filtration rate, *Ang-2* Angiopoietin-2. *p* values were compared by Student's *t* test. *$p < 0.05$ significant

total subjects had macrovascular complications and diabetic nephropathy respectively. Serum Ang-2 level was significantly higher in female subjects compared to male diabetic subjects ($p = 0.011$).

Table 2 presents level of Ang-2 based on different age groups. In type 2 diabetic subjects, the level of serum Ang-2 significantly increases with increase in their age ($p = 0.002$). Similar trend was found in female subjects ($p = 0.018$), while there is no significant increase in Ang-2 level in male subjects. Regardless of gender, when all subjects were considered, Ang-2 was significantly elevated in overweight and obese compared to lean ($p = 0.017$). The differences between the two groups were more marked in female subjects. When individual gender groups were considered, significant differences in Ang-2 levels were seen in female subjects ($p = 0.05$) (Fig. 1).

Levels of Ang-2 did not differ significantly between sexes whose age was < 50 years, while Ang-2 was significantly higher in diabetic females whose age is > 50 years (3046 ± 1574 vs 2583 ± 1561; $p = 0.019$). No statistically significant associations were observed between levels of Ang-2 between sexes whose BMI is < 30 while it was significantly higher among the obese female subjects. (3045 ± 1635 vs 2547 ± 1690; $p = 0.043$).

Table 3 shows the level of Ang-2 according to the diabetic complications. Ang-2 levels were significantly higher among subjects with diabetic neuropathy ($p = 0.001$) and diabetic retinopathy ($p = 0.002$). In addition, Ang-2 remained significantly higher among diabetic subjects with macrovascular complications when compared to those without ($p = 0.008$). However, levels of Ang-2 did not remain significantly different between participants with or without diabetic nephropathy. When compared to males Ang-2 remained higher among female subjects with microvascular complications but in macrovascular complications Ang-2 levels were higher in males.

Discussion

This is a comprehensive report evaluating the levels of Ang-2 in Saudi T2D adults. The diabetic females have comparatively higher Ang-2 level than diabetic males. Diabetic females have significantly increased Ang-2 level with increase in their age

Table 2 Ang-2 levels in type 2 diabetic subjects according to age

Age (years)		< 40	40–50	> 50	*p*
Ang-2 (pg/ml) mean ± SD	Total (*n*)	1881 ± 657 (17)	2200 ± 1285 (53)	2839 ± 1582 (259)	0.002*
	Male (*n*)	1768 ± 568 (10)	2081 ± 1170 (23)	2583 ± 1561 (116)	0.105
	Female (*n*)	2041 ± 825 (7)	2292 ± 1379 (30)	3046 ± 1574 (143)	0.018*

Results are normally distributed, expressed as mean ± standard deviation. Ang-2 (Angiopoietin-2). *p* values were compared by ANOVA . $*p < 0.05$ significant

and BMI. A significantly higher level of Ang-2 was found in obese females with age group more than 50 years. The study also evaluates Ang-2 as a marker for the assessment of vascular health integrity and addresses DM patients at higher risk of diabetic complications. Since no previous data on reference levels of Ang-2 in Saudi adult are available, results of the present study cannot be compared with the other studies from the region.

Cardiovascular diseases are considered as one of the major complications of diabetes. There are only few studies reported on factors affecting cardiovascular diseases in Saudi population. In a previous study, diabetes, hypertension, obesity, dyslipidemia, smoking habits, and physical inactivity were mentioned as the major factors affecting the development of cardiovascular diseases in Saudi population. In addition, poor lifestyle habits such as lack of physical inactivity and high intake of total and saturated fat, and cholesterol are also related to the development of vascular disease [16]. Ang-2 is a glycoprotein exclusively expressed by the endothelial cells. It can act as an antagonist for Tie-2 and stimulate endothelial cell proliferation to induce vascular inflammation, accelerating the generation and development of vascular disease in patients with diabetes [17, 18].

In this study, we found that the Saudi diabetic females have comparatively higher Ang-2 level than diabetic males and it increases with increase in their BMI. To demonstrate the sexual dimorphism in serum concentrations of Ang-2 levels, a significantly higher level of serum concentrations of Ang-2 levels were observed in obese females compared to obese male subjects [19]. A previous study also demonstrated a gender dimorphism for Ang-2 levels, with women showing higher Ang-2 than men [20]. Differences in the gender on vascular functions and impact of feminine sex hormones on the expression of vascular growth factor have also been reported [21–23]. The exact mechanism behind the sexual dimorphism of Ang-2 has not been elucidated yet, so a detailed future study is suggested. In previous studies, it was reported that the Ang-2 expression may elevate by the inhibitory action of estrogen hormone on angiopoietin-1–Tie-2 receptor [24].

In the current study, we found that serum Ang-2 significantly increases with the increase in age for T2D and diabetic females but not in males and Ang-2 is significantly higher in diabetic females whose age is > 50 years. The angiogenic factors like VEGF, angiopoietins, fibroblast growth factor (bFGF) etc. are released by inflammatory cells having mitogenic and migratory effects on endothelium [25]. Increased transcription of angiogenic genes, such as VEGF and Ang-2, was triggered by a common stimulus for both chronic inflammation and angiogenesis [26]. Interleukin-6 (IL-6) is a proinflammatory cytokine, considered as mediator of chronic inflammation. The production of IL-6 is controlled by the macrophages produced by adipose tissue and adipocytes. In a previous study, it was found that postmenopausal women with the metabolic syndrome showed higher IL-6 as well as Ang-2 [27]. In our study, females above > 50 years were obese, and they were diabetic also. Hyperglycemia is considered as a major cause which exerts toxic effects on the endothelium and leads to inflammation which in turns increases the production of Ang-2 [28]. From the above possible reasons, we believe that females above 50 years are more likely to have

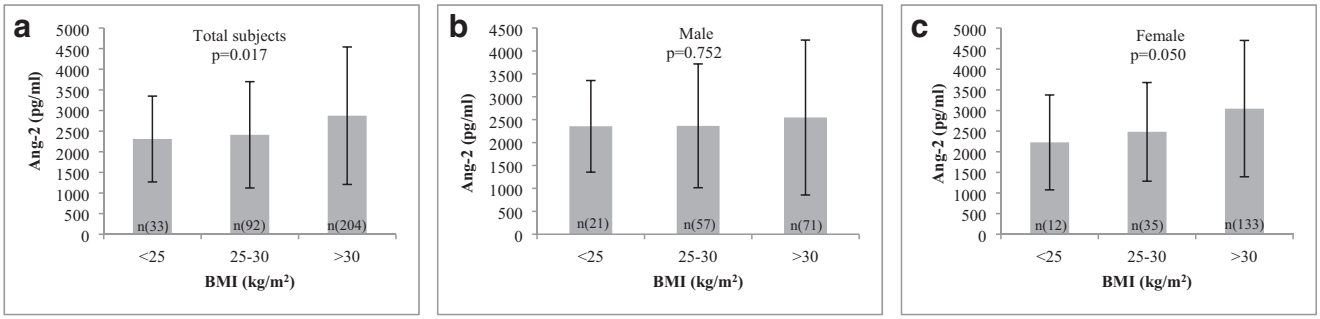

Fig. 1 Ang-2 levels in type 2 diabetic subjects according to BMI. **a** Total, **b** male, and **c** female. BMI body mass index, Ang-2 Angiopoietin-2. *p* values were compared by ANOVA . $*p < 0.05$ significant

Table 3 Levels of Ang-2 in type 2 diabetic subjects with and without diabetic complications ($n = 329$)

Diabetic complications		Ang-2 (pg/ml) levels according to the state of diabetic complications			
		Total Ang-2 (pg/ml) mean ± SD (*n* (%))	*p*	Male Ang-2 (pg/ml) mean ± SD (*n* (%))	Female Ang-2 (pg/ml) mean ± SD (*n* (%))
Neuropathy	Yes	3293 ± 1728 (83 (25.2%))	0.001*	3006 ± 1620 (33 (22.1%))	3482 ± 1782 (50 (27.8%))
	No	2481 ± 1403 (246 (74.8%))		2290 ± 1400 (116 (77.9%))	2650 ± 1389 (130 (72.2%))
Retinopathy	Yes	2986 ± 1645 (146 (44.4%))	0.002*	2980 ± 1762 (66 (44.3%))	2991 ± 1554 (80 (44.4%))
	No	2449 ± 1393 (183 (55.6%))		2034 ± 1043 (83 (55.7%))	2794 ± 1550 (100 (55.6%))
Nephropathy	Yes	3059 ± 2005 (48 (14.6%))	0.072	2969 ± 2121 (28 (18.8%))	3181 ± 1884 (20 (11.1%))
	No	2624 ± 1431 (281 (85.4%))		2334 ± 1274 (121 (81.2%))	2844 ± 1506 (160 (88.9%))
Macrovascular	Yes	3152 ± 1571 (62 (18.8%))	0.008*	3192 ± 1650 (26 (17.5%))	3124 ± 1534 (36 (20%))
	No	2578 ± 1503 (267 (81.2%))		2291 ± 1394 (123 (82.5%))	2821 ± 1554 (144 (80%))

Results are normally distributed, expressed as mean ± standard deviation. Ang-2 (Angiopoietin-2). *p* values were compared by Student's *t* test. *$p < 0.05$ significant

chronic inflammation than males and the combined action of both hyperglycemia and inflammation leads to more circulating serum Ang-2 level.

In Middle Eastern countries, prevalence of obesity is more likely to be among women than in men. Like Saudi Arabia, the high-income countries such as Kuwait, UAE, Bahrain, and Qatar also show > 25 kg/m^2 of mean BMI for both males and females [29]. A pervious study in Saudi Arabia reported that prevalence of obesity was higher among Saudi women than men (33.5 vs 24.1%) [30]. In Saudi females, the average BMI over age 30 years was reported to be > 30 kg/m^2; the prevalence of overweight and obesity increased with age [31]. In our study, we found that Ang-2 level significantly increases with BMI in all T2D and diabetic females. Different evidences suggest that angiogenesis may be enhanced in human obesity. The evidence from the experimental study with both human and mice revealed that increased obesity is associated with Ang-2 mRNA expression in visceral adipose tissue and insulin resistance is linked with adipose tissue inflammation [32]. The above findings speculate that increased levels of angiogenic factors lead to adiposity and are involved in the pathogenesis of obesity. It is also demonstrated that Ang-2 is elevated in overweight and obese compared to lean control subjects, although Ang-2 was significantly elevated only in female obese subjects compared to lean female subjects. The sexual dimorphism was observed in serum concentrations of Ang-2 levels with significantly higher levels in obese females compared to obese male subjects. Although several studies reported the association between Ang-2 in the regulation of angiogenesis, the exact mechanism of angiogenic factors in both serum and adipose tissues is not expounded yet [19].

Hyperglycemia promotes toxic effect on vascular endothelium by the production and activation of advanced glycation end products (AGE) and reactive oxygen species (ROS) which leads to the development of micro and macrovascular complications in diabetes mellitus [33, 34]. In our study, we found that serum Ang-2 was significantly higher in DR subjects than without retinopathy. Previous studies also confirmed that Ang-2 levels show a significant difference among retinopathy group and are more prominent in proliferative group [35].

Various growth factors and cytokines play crucial role in the development of proliferative diabetic retinopathy (PDR). The main process involved in the development of retinal angiogenesis in diabetic retinopathy is angiogenic switch and proteolytic processing of the extracellular matrix (ECM). In PDR, involvement of proteases in ECM remodeling and Ang–Tie system in vessel remodeling has been reported [36–38]. In earlier studies, it has been reported that the increased level of Ang-2 indicates the severity or progression of diabetic retinopathy especially in PDR [39]. A previous clinical study indicated that the increased level of Ang-2 in diabetic patients is not because of leakage of blood from ischemic retinal capillaries but due to excessive production of Ang-2 from endothelial cells. This confirms the above statement; upregulation of stored Ang-2 is found in Weibel-Palade bodies of retinal endothelial cells [40].

When compared to healthy controls, serum Ang-2 is significantly higher in non-diabetic patients with acute and chronic congestive heart failures, while the level of Ang-1

level remains same in both groups and comparison with chronic and acute cases reported that Ang-2 level was found to be elevated in non-diabetic patients with acute coronary syndrome [5, 41]. Without considering the presence and absence of CVD, Ang-2 level seems to be elevated in diabetic patients; while no differences were found in the level of Ang-2 in diabetic patients with and without CVD. A reduction of Ang-2 levels only in diabetics without CVD reveals the altered relationship of Ang-2 with glycemic control in the presence of CVD [42, 43]. In the present study, the Ang-2 levels were elevated in DM patients with vascular complications. These findings are in agreement with a previous cross-sectional study, among diabetic patients showing the elevated level of circulating Ang-2 and were also associated with various cardio-metabolic parameters [44].

Moreover, in our study, we found a significant increase in Ang-2 level in diabetic patients with diabetic neuropathy. A previous study also reported that the polyneuropathy and insulin therapy were associated with higher Ang-2 levels. But even after controlling the presence of macrovascular complications, no differences were found between the levels of Ang-2 in subjects with and without diabetic polyneuropathy [44]. To our knowledge, the role of Ang-2 level in diabetic neuropathy is not yet reported anywhere. To clarify our results, future well-designed prospective studies on pathophysiological role of Ang-2 on diabetic neuropathy are needed. A well-defined role of Ang-2 in kidney diseases is already described in previous studies. The increased level of Ang-2 may lead to kidney damage [45]. A positive correlation with Ang-2 and proteinuria in kidney diseases was reported earlier [46]. In comparison with healthy controls, the diabetic patients with normoalbuminuria have significantly higher levels of both serum and urine Ang-2 and increased level of Ang-2 is related to the severity of diabetic nephropathy [47]. In our study, serum Ang-2 level was higher in DN group even though there is no significant difference among the patients with and without DN. This may be due to fact that majority of subjects in this study population were having eGFR > 90 ml/min/1.73 m^2 and the sample size for DN group was very small. Another possible reason may be glycemic control which is considered as a primary determinant of reductions in serum Ang-2 [42].

In conclusion, present study establishes the levels of serum Ang-2 level in Saudi adult population with T2D in different subsets of age, gender, BMI, and diabetic complications. Our study shows that levels of Ang-2 are higher among female diabetic subjects. It also reveals that in female T2D subjects, Ang-2 levels rise with their age and BMI. A significantly higher level of Ang-2 was found in obese females with age group more than 50 years. Ang-2 levels are significantly higher in patients with diabetic neuropathy, retinopathy and diabetic vascular complications. Higher Ang-2 levels may serve as a marker to identify patients at high risk of vascular complications.

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Authors' contribution KS conceived and designed the study. KS, SSJ, and SSN conducted the research, provided the research materials, and collected and organized the data. SSN analyzed and interpreted the data. KS wrote the initial and final draft of the article. All of the authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Compliance with ethical standards

Conflicts 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 College of Medicine, King Saud University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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REVIEW ARTICLE

HbA1c reduction and weight-loss outcomes: a systematic review and meta-analysis of community-based intervention trials among patients with type 2 diabetes mellitus

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Abstract
Managing blood glucose and maintaining weight could prevent the risk of diabetes complications and maintain of T2DM. The study aimed to provide the systematic review and meta-analysis to test the effect of community-based interventions on HbA1c reduction and weight loss after receiving the community-based intervention and to determine any gaps in the literature and set up the recommendations for future intervention. Two databases PubMed and Medline were included to extract the relevant articles. "Type 2 diabetes (T2D)," "Community-Based Intervention," "glycemic control," and "weight reduction" were used as the keywords. Appraisal of systematic review was based on PRISMA format. Out of 475 publications identified, 12 studies that fulfilled inclusion criteria which characterized by predominantly measure HbA1c and weight were included in the meta-analysis. Overall, the community-based intervention decreased the HbA1c levels by − 0.25% (− 0.33, 0.16) and ($Z = -582$, $p = 0.00$) and weight loss ($Z = -5.110$, $p = 0.00$). The community-based intervention positively decreased the HbA1c level. Our findings could guide the significance of community-based interventions for T2DM patients in the future.

Keywords Diabetes mellitus community-based intervention · HbA1c reduction · Weight loss

Introduction

The estimated global prevalence of diabetes mellitus has risen more rapidly in the past two decades. International Diabetes Federation (IDF) estimated 415 million people have lived with DM [1]. This number had been predicted as 642 million in 2040.

Type 2 diabetes mellitus (T2DM) is often associated with adverse consequences, including nephropathy, retinopathy, and cardiovascular problem [2]. Emerging complications linked with unhealthy diabetes mellitus self-management (DMSM) practice include unhealthy eating habits, physical inactivity, non-medication adherence, lack of regular blood glucose monitoring, and uncontrolled weight [3–6].

DMSM practice is a key strategy to prevent complications and to maintain health-related behaviors. T2DM patients are required to perform multiple tasks such as to attend medical appointment regularly, to adhere medication regimens, and to engage in self-care behaviors including home blood glucose monitoring, healthy eating, and increasing physical activity [7]. However, it is difficult for T2DM to engage in healthy behaviors continuously [8]. The problem is due to some common barriers including low self-commitment, low self-efficacy to maintain an activity, and insufficient support from family and community [9, 10].

Community members are important aspect of the DMSM practice since most of self-management activities for many T2DM patients occur within the community environment. Communities are the ultimate coordinators and key stakeholders for supportive system of long-term care for people with T2DM including their health-related behaviors.

A community-based approach may improve the DMSM practices by addressing barriers related to facility-based approaches and individual-based approaches [11]. Engaging

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community in facilitating DMSM practice is fundamental strategy for prevention of diabetes among risk groups as well as prevention of complications among T2DM patients [12, 13]. The Global Partnership for Effective Diabetes Management recommended that the diabetes healthcare team should be multi and interdisciplinary teamwork [14].

Several community-based interventions obtained positive impacts on weight loss [15], HbA1c reduction, raising physical activity and decreasing of waist circumference [16], increasing of self-efficacy and quality of life [17]. In contrary, some studies showed negative effects of the community-based intervention in HbA1c and weight loss [9, 18]. A meta-analysis reported that the interventions decreased HbA1c by 1.6% (95% CI = 0.1–3.1%) compared to the control group over 12 months follow-up [9].

Even though, those studies reported a tendency toward positive effects of community-based intervention in health outcomes [9, 15, 16]. However, there was still being a gap of analysis of the study on a controversy of health outcomes from community-based interventions for DMSM practice that need to be explored by both systematic review and meta-analysis. The result of this study was to ensure congruent findings of community-based interventions as one approach for effective management of DMSM practice to improve health outcomes and to elevate quality of life among T2DM patients.

Methods

Data sources

Databases through PubMed and Medline were used to extract relevant articles. More than 400 articles were initially obtained using the inclusion criteria and critical appraisal using systematic review articles followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) framework. Community-based intervention was applied as the primary search term and entered as the medical subject heading (MeSH) in an abstract and title of an article. This initial review includes 12 articles that almost fit to the systematic review.

Search strategy

A term used to find the relevant articles in this review, including "type 2 diabetes (T2D)," "Community Based Intervention," "HbA1c," "blood glucose control," and "weight reduction." Available title and abstract related to the community-based intervention were reviewed in systematic way to find out the most suitable articles. The searching articles were limited to those articles, which had been published between 2012 and 2017 to make sure that the articles were up-to-date and relevant to the current situation.

Eligibility criteria of study

The PICO (Participant-Intervention-Comparison-Outcomes) format was used to design the inclusion criteria for reviewing the articles.

P Type 2 diabetes mellitus (T2DM)
I Community-based intervention
C Control group
O HbA1c reduction, A1c, weight loss

The inclusion criteria compromised of (1) English language articles published between 2012 and 2017, (2) full articles of randomized control trial (RCT), (3) a community-based intervention was applied to improve health outcomes, and (4) the outcomes measured were HbA1c or body weight. Description of what an appropriate program (e.g., articles in which not include community as a part of diabetes prevention) would be listed as reasons to exclude from this study. Types of study designs include a single design, descriptive, qualitative design, one group quasi-experimental, and quasi-experimental study with two groups pretest-posttest design with non-equivalent control group. While unpublished dissertation, and inappropriate populations such as type 1 diabetes, gestational diabetes as well as other metabolic diseases were also excluded from this study.

Literature search

Figure 1 described the flow chart of screening and selection of articles based on the PRISMA flow chart. Two databases provided 485 documents during the years of 2012–2017. The process was conducted on December 21, 2018. After duplication of study has been verified, 475 articles were recruited. The researchers screened the relevant studies based on the abstracts and excluded 325 articles out.

After selecting articles based on abstracts and research titles, there were 123 articles that fulfilled the requirements with full texts of publication. Referred of inclusion criteria, only 12 articles can be examined in the meta-analysis stage. In contrary, 111 articles have to be excluded for several reasons including the following: 25 articles designed the program by using other approaches rather than community-based approach. Seventeen articles measured outcomes other than HbA1c and weight loss were not included as outcomes of this meta-analysis. Twelve studies focused on populations such as type 1 diabetes mellitus, gestational diabetes, hypertension or other population who are not related to T2DM. Since this review was focused on RCT studies, 48 studies were excluded due to inappropriate study designs that did not fit with our purpose, such as single design, descriptive study, qualitative study, one group quasi-experimental study, and two groups quasi-experimental, pretest-posttest design with non-equivalent control group. Other reasons for being excluded in this study are no full texts available (18

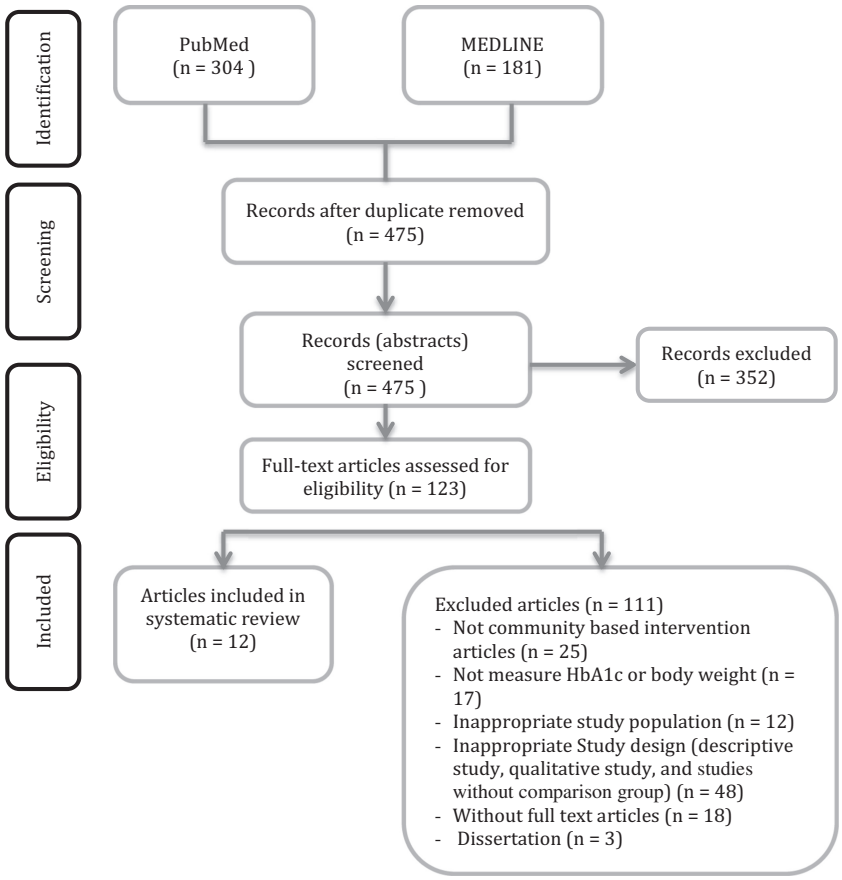

Fig. 1 Summary of evidence search and selection criteria

articles) and the others were published in dissertation formats (3 articles).

Data extraction procedure and quality assessment

A single reviewer was tasked to assess the eligible articles based on the study title. Then, two authors assessed abstracts independently based un-blinded standardized manner. We extracted the articles published between 2012 and 2017 to make sure up-to-date information. Special characteristics of each study have been identified and extracted by two authors. We extracted the following information from each study as follows: authors and year of research publication, study design which is a community-based approach, sample size, details of the community-based program, duration of interventions, and health outcomes measured in terms of HbA1c reduction as well as weight loss (Table 1).

Quality assessment was performed according to the Consolidated Standards of Reporting Trials (CONSORT), which is a validated scale for intervention studies in meta-analysis. This scale awards a maximum of 9 points of each study comprised of 2 for assessment of sample size and sample allocation, 1 for assessor blinding, 4 for selection of outcomes and adequate data, and 2 for addressing biases of study. We assigned scores of 0–3, 4–6, and 7–9 as a low, moderate, and high quality of studies. When the study has several adjustment models, we extract all information that reflects the maximum adjustment level for controlling potential bias.

To verify the degree of acceptance for articles to pool in meta-analysis, we assigned the mean difference and standard deviation in each study. When the mean difference was not reported in that study, a midpoint of the upper and lower boundaries in each study was assigned.

Controlling of potential bias

A nine-item checklist tool adapted from Consolidated Standards of Reporting Trials (CONSORT) was used to assess the risk of bias by [19]. The following items included are (1) adequate sequence generation, (2) allocation adequately concealed, (3) assessor blinding, (4) incomplete outcome data adequately addressed, (5) selective reporting, (6) free of other bias, and (7) free of bias X.

Each item scored using "yes" (✓ = score 1), "no" (✗ = score 0), and unclear (? = score 0). The total scores were calculated for

Table 1 MeSH, PubMed, and Scopus headings and free text (keyword) for community-based intervention, HbA1c, blood glucose control, and weight reduction

Term	PubMed	MEDLINE
Subject heading	MeSH (controlled vocabulary of PubMed)	MeSH (controlled vocabulary of MEDLINE)
Community-based intervention	Method/Intervention/Community based/	Exp[a] Community-based intervention/Consumer/ Community health systems/Population-based planning/Intervention study/Early medical intervention/
Blood glucose	Blood glucose/Control group/Prevention/Dextrose/ Relion glucose/	Blood sugar/Self-monitoring/Blood glucose/Glucose dehydrogenases/Glucose monohydrate/L-Glucose
Weight loss	Weight reduction/Reduction/	Weight reduction/Weight loss program/Weight loss diet/Anti-obesity agents/
Free text		
Community-based intervention	[b]Ti (community-based intervention) OR Ab (Community-based intervention)	[b]Community based intervention. ab, kw, ti
Blood glucose	Blood glucose*. ab, kw, ti (Blood Glucose OR HbA1c* OR blood Sugar* OR Fasting blood sugar) ab, kw, ti	Blood glucose*. ab, kw, ti (Blood Glucose OR HbA1c* OR blood Sugar* OR Fasting blood sugar) ab, kw, ti
Weight loss	Weight loss * ab, kw, ti (Weight loss OR weight reduction OR body weight * OR Weight) ab, kw, ti	Weight loss * ab, kw, ti (Weight loss OR weight reduction OR body weight * OR Weight) ab, kw, ti

[a] Explode a subject heading in PubMed and MEDLINE (retrieved results using the selected descriptor and its more specific descriptor)

[b] Runs a search through these field: abstract (ab), keywords (kw), and title (ti) in PubMed and Medline

each study, in which a score of low-risk bias was 7–9; a moderate risk of bias presented scores of 4–6 and a high risk of bias scored was 0–3.

Statistical analysis

The meta-analysis was conducted using Revman version 5.1 software [The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark; Review Manager (RevMan), 2011]. Effectiveness of community-based intervention on HbA1c reduction and weight loss between experimental group and control group was described by using mean difference. All data were considered continuous and thereby the mean difference with 95% confidence intervals was used to determine effect measures. The heterogeneity of variance was described via Chi-square and I^2-index test. Fixed effect model was conducted when the Chi-square for the heterogeneity was not significant. The statistical analysis with the standard of $p < 0.05$ performed a review manager. Funnel plots as well as Egger's strategy were conducted to discuss the publication bias.

Results

Population and setting

The mean average age of study participants was 56 years, the percentage of female participants (67.76%) is more likely than male (32.24%). The median duration of illness was 7 years (5 to 9). The percentage of type 2 diabetes mellitus was more than twice in the adult population compared to the young population or the older population. This group was more likely to develop glycemic uncontrolled and diabetes complications.

This study was restricted to intervention in which a community-based dimension was included. Among 12 studies recruited participants were recruited from various groups of population including two studies were American-Indian [20, 21], two studies were African-American [15, 22], one study was Latino [22], one study was Asian [16], and one study was Korean-American [17]. Three studies did not mention race or ethnicity of the study population [18, 23–26]. Studies also described the different regions of research conducted including the western countries [15–22, 26], Australia [23, 24], and Asian countries [9, 25].

Controlling of potential bias

Table 1 showed an assessment of potential bias from each study. Seven studies were identified as low risk of bias, five studies were moderate risk of bias. Four studies reported assessor blinding to avoid the performance bias during implementing the program. Twelve studies employed an objective measured of HbA1c and/or body weight loss.

Community-based intervention

Twelve studies examined the effectiveness of the community-based intervention on health outcomes among type 2 diabetes

mellitus. All studies were randomized control trials (RCT), pretest and posttest design with non-equivalent control group. Therefore, it is considered as a high valid and reliable research design.

Intervention approach

The intervention was led by several disciplines including community health workers and village health volunteer [16–18, 21–25], community leaders [22], nurse practitioners [9, 15, 17, 22, 23], pharmacists [15], physicians [9, 15, 24, 25], dietitians [21, 23], certified diabetes educators [9, 18, 20, 23], nutritionists [22], psychologists [22], fitness trainers [26], general practitioners [26], and local community experts [21]. Multidisciplinary approaches to diabetes self-management may particularly be necessary for patients with T2DM. Increasing the role-play by allied health professionals in diabetes care may represent a more effective diabetes self-management.

Duration of program

Duration of the intervention ranged from 2 to 18 months. Eight studies offered the program within 12 months [9, 15, 18–21, 23, 25, 26], one study spent duration on 18 months [24], and two studies conducted the community-based intervention within 6 months [16]. One pilot study examined the feasibility of the community-based intervention program within 2 months [22]. With regard to the duration of the intervention, lengths of the program also varied from weekly to monthly sessions.

Intervention strategies

In general, intervention strategies are compromised of an individual education and group-based education toward diabetes self-management followed by encouraging the patients' active involvement in the learning process. Moreover, group discussion sessions, goal setting, problem-solving, decision-making, and communication skills are also included in the strategies.

Twelve studies can be classified as combined didactic learning with participatory learning strategies including skill building and goal setting [15, 16, 20–23], problem-solving [17, 20, 21, 23, 24], rewarding system [15], effective communication [17, 23], and emotional support [18, 23, 24]. In addition, health literacy skills in food preparation, food choice, and read food label were included in this review [15–17, 20–22, 25, 26]. Five studies mentioned engaging participants in exercise behaviors as an initial planning phase of the intervention in managing the blood glucose and body weight [15, 16, 22, 25, 26].

Community-based intervention

Commonly, the community-based intervention incorporated the program into the community and involved several stakeholders in the program to facilitate on health behaviors, blood glucose control, body weight control, and promote effective day-to-day coping with stress. Some studies applied the weight loss strategies by controlling food intakes such as increasing fruits and vegetable consumption, decreasing calorie intake and saturated fat as well as promoting in actively physical activity [15, 16, 21, 25].

In Patel et al study [16], the researcher encouraged to increase physical activity in daily life about 150 min/week. A group-based lifestyle program followed by goal setting, problem-solving, group discussion, and rewarding strategy are also implemented in this study to promote behavioral change. A pedometer is used to record the physical activity change every week. In this study, the roles of the community to facilitate the orally translated information, adopting health behaviors, were evaluated. Using the community-based approach concept, the facilitators also encourage the fruits and vegetables intake to help the weight loss. The myPlate plastic plate model measures trans fat intake and fruits and vegetables intake minimum of 5 servings per day. Engaging community in the program was also used to demonstrate the exercise and cooking of healthy foods, a grocery store tour, and a recipe makeover potluck party. Findings demonstrated that participation in a culturally tailored, lifestyle intervention program in a community setting can effectively improve the health outcomes.

Another study conducted in China has introduced the "Zhiji management." This Zhiji management is a 3-month lifestyle program designed to calculate and balance the nutrition intake with energy consumption from physical activity. Health education followed by weekly consultation, encouragement, and community involving of physical activity and dietary restriction were conducted in this program. The role of a community physician is to provide dietary and physical activity information from the computer software into charts and tables format in order to make it more understandable. The physician also illustrated ways to balance energy intake and consumption based on the prescription. The findings of this study found positive effects on community participation in physical activity, increasing energy consumption up to 54.6 kcal per day and total reducing the dietary intake by 328.5 kcal [25].

In addition, a study involved community members and university advisory board to create the faith-based adaptation of the Group Lifestyle Balance (GLB) programs and health education (HE) programs. The HE program consisted of strategies for reducing calorie intake, dietary fat consumption, and behavioral style followed by stimulus control, goal setting, and problem-solving. Involving community members in the weight loss program provided 12 weeks following six-booster

session to perform at least 150 MET-minutes/week of physical activity. The church health advisors (CHAs) who are compromised on nurses, pharmacists, and physicians were invited to deliver a faith-based adaptation of the GLB program. The program consisted of the weekly sessions followed by six monthly 1-h post-cores "booster" sessions. This program was positively affected on decreasing the body weight at least 3, 5, or 7% weight loss at either 12 weeks or 12 months post-baseline and maintaining the lifestyle change among diabetes patients [15].

Effectiveness of community-based intervention on health outcomes

We found that 12 studies were included in this review based on inclusion criteria. Summary findings of effects of the community-based intervention on health outcomes between the intervention and the control groups are presented as follows:

HbA1c

HbA1c level associated with the community-based intervention was also examined in seven studies, while two studies measured only fasting blood glucose to monitor the blood glucose level. Of those nine studies which measured the HbA1c/FBG, six studies showed the improvement of HbA1c after implementing the community-based intervention [16, 17, 20, 21, 24, 25]. Three studies reported non-significant difference in HbA1c level between the intervention group and the control group [9, 15, 18].

Weight

This review examined an association of the community-based intervention with weight reduction. Regarding the 12 studies, 60% of the studies described positive impacts of the community-based intervention on BMI as the primary outcomes of the intervention [15, 16, 20–22, 25]. Two studies confirmed non-significant in decreasing body weight after receiving the program between baseline and follow-up period [18, 24].

Other outcomes

More physical activity has been confirmed with HbA1c reduction and maintaining the body weight. In this systematic review, three studies examined the physical activity as a primary outcome [15, 16, 25]. Other health outcomes included improvement of quality of life [9, 17], controlling levels of both systolic and diastolic blood pressure [22, 25], reduction of waist circumference [16, 21], and improving the insulin resistance among patients with T2DM.

Summary of effects analysis of interventions

HbA1c reduction

Eleven randomized control trials contributed to this meta-analysis and were pooled to establish the effects of interventions in HbA1c levels (see Fig. 2 and Table 2). Community-based intervention has a positive effect on HbA1c reduction. There was considerable heterogeneity among interventions [$\chi^2 = 92.16$, df = 9, ($p < 0.05$); $I^2 = 99\%$]; the random effect models were used in this study. The impact of community-based intervention has significantly effect on HbA1c levels (0.25%), (− 0.33, 0.16) and ($Z = -0.581$, $p = 0.00$).

Weight loss

Figure 3 summarized the effect of the community-based intervention in weight loss. Body weight of the intervention group was slightly reduced than in the control group ($Z = -5.110$, $p = 0.00$) (Table 3). The data analysis showed heterogeneity among all interventions [$Tau^2 = 0.538$, df = 7 ($p < 0.05$), $I^2 = 97.343\%$].

Discussion

This study is aimed at conducting a systematic review and meta-analysis to investigate the effectiveness of the community-based interventions in weight reduction and HbA1c control among T2DM patients. Managing blood

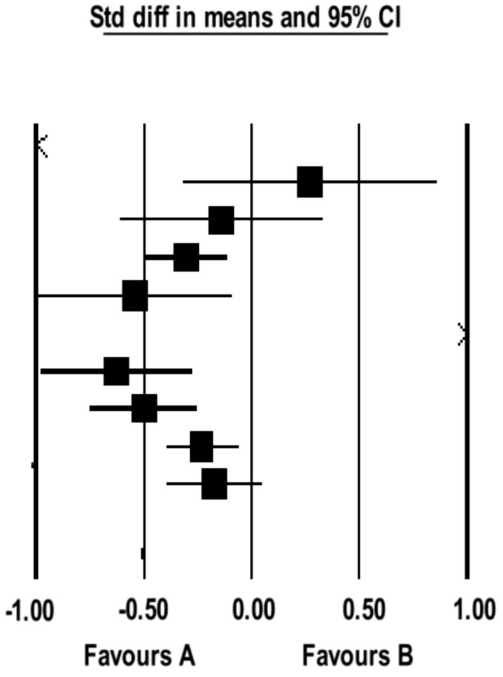

Fig. 2 Forest plot for HbA1c

Table 2 Meta-analysis results of HbA1c

Studies	Intervention		Total	Control		Total	Std diff in mean	95% CI (%)	Z-value	*p* value
	Mean (%)	SD (%)		Mean (%)	SD (%)					
Sattin et al. (2016) [15]	5.8	0.48	317	6.8	0.48	287	− 2.083	− 2.282, − 1.885	− 20.596	0.000
Patel et al. (2017) [16]	5.7	0.37	26	5.6	0.37	20	0.27	− 0.315, 0.856	0.905	0.366
Riddell et al. (2016) [23]	0.06	1.1	35	0.22	1.2	35	−0.139	− 0.608, 0.330	− 0.581	0.561
Sugiyama et al. (2015) [9]	− 1.0	1.6	224	− 0.5	1.7	217	0.303	− 0.491, − 0.115	− 3.163	0.002
Kim et al. (2009) [17]	1.2	1.3	40	1.7	0.1	39	− 0.539	− 0.988, − 0.090	− 2.352	0.019
McDermott et al. (2015) [24]	− 0.2	0.2	84	− 0.7	0.2	107	2.5	2.120, 2.880	12.891	0.000
Tucker et al. (2014) [22]	2.1	0.01	64	2.11	0.02	66	− 0.63	− 0.982, − 0.277	− 3.50	0.000
Yu et al. (2014) [25]	− 0.3	1	175	0.2	1	98	− 0.5	− 0.751, − 0.249	3.907	0.000
Simmons et al. (2014)	− 0.1	1.3	272	0.2	1.3	283	− 0.231	− 0.398, − 0.064	− 2.709	0.007
Ockene et al. (2012) [20]	− 0.10	0.3	162	− 0.04	0.4	150	− 0.171	− 0.393, 0.052	− 1.503	0.133
Miyong et al.(2015)	− 1.3	0.1	105	− 0.7	0.1	104	− 6.00	− 6.636, − 5.364	− 18.493	0.000

Heterogeneity: Tau2 = 1.554, df = 10 ($p < 0.05$), 12 = 98.753%. Test for overall effect Z = −13.573 ($p = 0.00$)

glucose and maintaining weight could prevent the risk of diabetes complications and manage the condition of T2DM.

Obesity is one of an issue concerning among T2DM patients. It has a negative impact on uncontrolled glycemic level and associated with severity of diabetes and risk of cardiovascular problem. Since there is an increasing number of obese patients with T2DM, managing and preventing of complications among this population becomes a public health priority.

Twelve studies were conducted in various countries and represented across ethnic groups comparison. An RCT design was considered as a high visibility design to verify health outcomes achievement with minimum of measurement bias (Table 4). The primary outcomes indicated from those studies include HbA1c, weight loss, and other health outcomes. The other health outcomes were waist circumference, systolic and diastolic blood pressure, and quality of life. In conclusion, the present study confirmed that the community-based intervention affects on increasing of physical activity level that can induce the reduction of body weight.

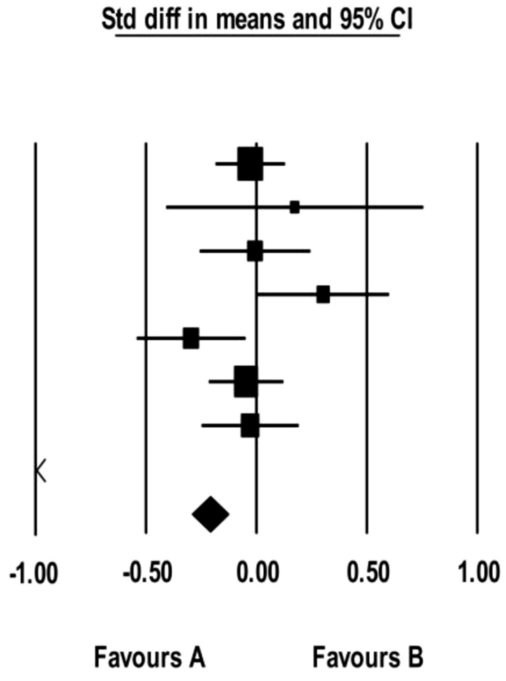

Fig. 3 Forest plot for weight loss

Eleven from 12 of the studies reported HbA1c as an outcome that has been pooled in the meta-analysis. Meta-analysis showed that community-based interventions had contributed significantly in decreasing of HbA1c by − 0.25% ($p < 0.001$). It was consistent with the previous meta-analysis which revealed that community-based PA program has a positive effect on lowering HbA1c by − 0.32 respectively [30]. However, it is interesting to note that meta-analysis of clinically based exercise intention by promoting physical among T2DM patients can decrease HbA1c by − 0.66% (− 30.71 mmol/mol) [31]. Another study by Plotnikoff et al. also reported that stronger intervention had contributed to lower of HbA1c [32]. The findings noticed that a more significant intervention doses had been significantly impacting on increasing the physical activity and healthy food intake. As the result, the improvement on HbA1c was identified (Table 5).

Eight studies revealed that community-based interventions also decreased body weight in this meta-analysis. The intervention had contributed to reduce body weight index by Z = − 5.110, $p < 0.001$. The high weight loss changed among target population does not necessarily increase even among the group which fully participated. Therefore, scalability of diabetes intervention program is an essential element to gain high participation at the population-level. For this reason, besides the importance of intensive intervention that plays an important role in losing weight, participation in the program is also important as a fundamental factor that contributed on decreasing blood sugar levels and reducing diabetes complications.

Table 3 Meta-analysis results of weight loss

Studies	Intervention		Total	Control		Total	Std diff in mean	95% CI (%)	Z-value	*p* value
	Mean (%)	SD (%)		Mean (%)	SD (%)					
Sattin et al. (2016) [15]	98.4	21	317	99	22.1	287	− 0.028	− 0.188, − 0.132	− 0.342	0.732
Patel et al. (2017) [16]	67.4	11.6	26	65.5	10.1	20	0.173	− 0.411, 0.757	0.581	0.561
Riddell et al. (2016) [23]	87.3	18.4	120	87.7	8.7	120	− 0.006	− 0.259, 0.247	− 0.049	0.961
McDermott et al. (2015) [24]	91	23.1	81	84.7	18.6	92	− 0.302	0.002, 0.603	1.974	0.048
Yu et al. (2014) [25]	68.7	10.7	175	72	11.9	98	− 0.296	− 0.545, − 0.048	− 2.335	0.020
Simmons et al. (2014)	89.2	17.0	245	90	17.2	283	− 0047	− 0.218, 0.124	− 0.536	0.592
Ockene et al. (2012) [20]	190.19	31.9	162	191.16	36.3	150	0.028	− 0.251, 0.194	0.251	0.802
Katula et al. (2011) [21]	87.44	1.28	151	90.93	1.37	150	− 2.633	− 2.941, − 2.324	− 16.717	0.000

Heterogeneity: Tau2 = 0.538, df = 7 ($p < 0.05$), 12 = 97.343%. Test for overall effect Z = − 5.110 ($p = 0.00$)

It is essential that the weight loss appears consistent as plateau at 6 months. The findings demonstrated that weight loss program not only focused on how to reduce the weight but also how to maintain it within an acceptable range [34]. Therefore, emphasis on the lower-energy intake and regular exercise behavior is the cornerstone to reduce the weight. This finding supported by the previous study reported that the mean weight loss of participants that underwent the interventions-diet alone, diet and exercise, and meal replacements are accounted as 5 to 9% more after 6 months follow-up [35]. Another systematic review also confirmed that regular exercise with low-energy intake has positive impact to reduce

Table 4 Assessment scores of potential risk of bias in community-based interventions among T2DM patients

Authors	Adequate sequence generation	Adequate allocation concealment	Adequate blinding	Incomplete outcomes data addressed	Free of selective reporting	Free of other bias	Free of bias X	Objective measures HbA1c	Objective measures weight	Score
Sattin et al. (2016) [15]	✓	✓	✓	✓	?	✗	✓	✓	✓	7/9
Patel et al. (2017) [16]	✓	✓	✓	✓	✗	✗	✓	✓	✓	7/9
Riddell et al. (2016) [23]	✓	✓	?	✓	✓	?	?	✓	✓	6/9
Sugiyama et al. (2015) [9]	✓	✓	✓	✓	✓	?	✓	✓	✗	7/9
Kim et al. (2009) [17]	✓	✓	✗	✓	✓	?	✓	✓	✗	6/9
McDermott et al. (2015) [24]	✓	✓	✗	✓	✓	✓	✓	✓	✓	8/9
Tucker et al. (2015) [27]	✓	✓	?	✓	?	✓	✓	✓	✗	6/9
Yu et al. (2014) [25]	✓	✓	✗	?	✓	?	✓	✓	✓	6/9
Simmons et al. (2014) [28]	✓	✓	✓	✓	✓	?	✓	✓	✓	8/9
Ockene et al. (2012) [20]	✓	✓	?	✓	✓	✓	✓	✓	✗	7/9
Kim et al. (2015) [29]	✓	✓	✗	✓	✓	?	✓	✓	✓	7/9
Katula et al. (2011) [21]	✓	✓	?	✓	✓	?	✓	✗	✓	6/9

The quality of articles was described based on the score of articles assessment as following; score of 0–3 was low quality article, score of 4–6 was moderate quality article, and score of 7–9 was high quality article

Table 5 Community-based intervention and health outcomes

References	Design	Community stakeholder	Sample size	Community-based program	Program duration	Result
Sattin et al. (2016) [15]	Cluster—randomized trial (RCT)	• Church health advisors (CHAs) • Church's health ministry (nurses, pharmacists, and physicians)	• Intervention group ($n = 317$) • Control group ($n = 287$)	• Weight loss strategies by reducing calories and dietary fat consumption, • Facilitated to stimulus control, goal setting, and problem-solving • Providing health education about information and risk improvement strategies about mental health and stress	12 months	• The intervention group showed a significant on weight reduction • Non-significant on fasting blood glucose (FBG) and physical activity
Patel et al. (2017) [16]	Randomized control trial (RCT)	• Community • Mandir's medical officer • Executive committee • Volunteer	• Intervention group ($n = 26$) • Control group ($n = 20$)	• Weight loss strategies by increasing the physical activity 150 min per week, increase the fruit and vegetable intake minimum of 5 servings per day, and decreasing the saturated and trans fat intake • Group-based lifestyle intervention session about 75 min • Set the goals • Rewarding strategies for success achievement • Reinforcement and follow-up strategies	6 months	• The intervention group showed a significant on weight reduction and decreasing HbA1c level • The intervention group showed a significant on increasing of physical activity and reducing waist circumference
References	Design	Community involving	Sample size	Community-based program	Program duration	Result
Riddell et al. (2016) [23]	Randomized cluster design	• Volunteer • Dietitians Diabetes educator	• Intervention group ($n = 35$) Control group ($n = 35$)	• Assisting how to self-management in daily living • Promotion and support of regular linkage to clinical care • Providing the emotional support • Provision of ongoing and sustained support to assist in the lifelong needs of diabetes self-management • Briefly the phone call for remaining and seeking their intentions to attend the program • Skill in goal setting, problem-solving and effective communication	12 months	• The intervention group showed a significant on decreasing HbA1 and improving the medication adherence • The intervention group showed a significant on fruit and vegetables intake per day, and participating in exercise • The intervention group showed the improvement of peer support and satisfaction
Sugiyama et al. (2015) [9]	Randomized control trial (RCT)	• Diabetes educator • Doctor • Community	• Intervention group ($n = 224$) • Control group ($n = 217$)	• Training on self-monitoring blood glucose • Six weekly 2-h groups self-care session • The 1-year training program and 8 h of education program related to diabetes and its clinical presentations and complication • 12 h of training and implementation of the empowerment sessions	12 months	• The intervention group showed a significant on mental health related to the quality of life • The community-based intervention showed that there is no significance of the HbA1c level and social support

Table 5 (continued)

				• A one-on-one discussion session with the health educator to review his or her baseline and follow-up laboratory and biometric data		
Tucker et al. (2014) [22]	Randomized control trial (RCT)	• Researcher team • Community leader • Nutritionist • Nurses • Psychologist	• Intervention group ($n = 64$) • Control group ($n = 66$)	• Didactic presentation regarding healthy eating and physical activity behavior • Demonstration of how to read and understand the nutrition label • Demonstration of how to prepare a healthy meal • A small discussion on sharing strategies and overcoming barriers • Training on assertiveness anger and depression management, and stress/anxiety management • Individualized personal goals	2 months	• The intervention group showed a significant on lower levels of BMI, diastolic blood pressure, and physical stress
Yu et al. (2014) [25]	Randomized control trial (RCT)	• Volunteer • Doctor • Community	• Intervention group ($n = 175$) • Control group ($n = 98$)	• The 3-month lifestyle intervention program designed to quantify and balance dietary energy intake and energy consumption • Monitor the energy consumption of physical activity, by using an electronic accelerometer-like (Zhiji Energy Monitor) • Encouraging to keep the dietary diary for 2 days a week and a weekend day • 3-month of health-related individualized consultations • Trained community physicians in each the clinic, aided with customized computer software designed for the Zhiji intervention for addressing the improvement	12 months	• The intervention group showed a significant on increasing the physical activity and decreasing the total dietary intake • The intervention group showed a significant on lower of body weight, waist circumference, as well as systolic and diastolic blood pressure
Simmons et al. (2014) [28]	Cluster—randomized control trial (RCT)	• Peer support facilitator (PSF) • Diabetes educator	• Intervention group ($n = 272$) • Control group ($n = 283$)	• Group education workshop for diabetes overview of approximately 3.5 h • 2-day training and PSF support • 4–6 months discussion on how to address barriers to care for DM patients, social, and emotional aspect of diabetes and health care received • A meeting of PSFs and nurse to share positive and challenging experiences, generate solutions, discuss clinical issues related to the intervention • PSFs kept the record their peers improvement and	12 months	• The community-based program was not positive impact on HbA1c, diastolic BP, weight, total cholesterol, diabetes knowledge, depression, quality of life, medical adherence, and self-efficacy

Table 5 (continued)

				reflect the experience of delivering the intervention		
Ockene et al. (2012) [20]	Randomized control trial (RCT)	• Diabetes educator • Community	• Interventi-on group ($n = 162$) • Control group ($n = 150$)	• Providing basic information on diabetes prevention • Promoting positive attitudes to behavior changes • Building skills in goal setting, self-monitoring, problem-solving challenges, healthy cooking skills, and grocery shopping skills • Demonstrations of healthy cooking methods, demonstration of portion sizes with real foods, and practice walking with pedometers during the sessions • Training in motivational counseling and group management skills	12 months	• The intervention group showed a significant on weight reduction, HbA1c level, and improving the insulin resistance
Katula et al. (2011) [21]	Randomized control trial (RCT)	• Community health worker • Register dietitians • Research team • Local community expert	• Interventi-on group ($n = 151$) • Control group ($n = 150$)	• Six months program of limiting the caloric intake • Weekly for CHW led group session and three personalized consultations • One group session and telephone contact • Facilitating on healthy eating, goal setting, and problem-solving	12 months	• The intervention group showed a significant on blood glucose control, body weight, BMI, and waist circumferences
Miyong, Kim et al. (2015) [33]	Randomized control trial (RCT)	• Team of RNs • Community health workers	• Interventi-on group ($n = 105$) • Control group ($n = 104$)	• A series of structured behavioral education programs including knowledge of DM and its treatment • Advancing the problem-solving skill, cognitive reframing, and belief in self--monitoring, health literacy skill (reading the food label, medical terminology, etc.) and encour-aging to actively engage including multimedia presentations, teach-back, role-play, and group discus-sions • Ongoing self-monitoring of glucose • Individualized counseling using a motivational interviewing method that was conducted by nurses/ community health workers (CHWs) who had an extensive training in DM management	12 months	• The intervention group demonstrated 1.0–1.3% reductions in HbA1c • The intervention group showed a significant improvement in diabetes-related self-efficacy and quality of life when compared with the control group.
Kim et al. (2009) [17]	Randomized control trial (RCT)	• Nurses	• Interventi-on group ($n = 41$)	• Weekly education session (overview self-care, healthy eating, reading food label, exercise,	30 weeks	• The intervention was effective in significantly lowering HbA1c and fasting blood glucose

Table 5 (continued)

		• Nutritionist • Physician	• Control group ($n = 42$)	medication, and food-drug interactions • Home glucose monitoring • Monthly telephone counseling • Problem-solving and communication skill		• The intervention also improved knowledge, self-care activities, self-efficacy, attitudes, depressive score, and quality of life among the experimental group
Tucker et al. (2014) [22]	Randomized control trial (RCT)	• Research team members • Team of RNs • Community health workers • Psychologist	• Intervention group ($n = 65$) • Control group ($n = 65$)	• Health promotion workshop consisted of didactic presentations about healthy eating and physical activity behaviors • Cognitive-behavior skills and strategies to facilitate the health promoting behaviors • Demonstrations by a nutritionist on how to read and understand nutrition labels • Demonstrations on how to shop for and prepare desired culture-linked meals in a healthier way • Small group discussion to share strategies for engaging in health promoting behaviors and overcoming barriers	12 months	• The intervention group showed significantly lower levels of BMI, diastolic blood pressure, and physical stress

the mean weight by approximately 6.7 kg during the last 1 year of intervention [36]. In addition, types of exercise such as resistance training (RT), aerobic training (AT), and high-intensity interval training (HIIT) are able to avoid and fight insulin resistance as well as maintain the weight among patients with T2DM [37].

This study highlights the significant results that focused on community-based diabetes prevention program. The program has a high degree of participation and is highly effective against outcomes mainly for weight loss. These programs have varieties of basic protocols for maximizing the programs fidelity. However, some barriers to the program approach related to participation rate tend to be low. As the findings, no studies had been counted on diabetes risk reduction.

Strength and limitation

This systematic review was focused on RCTs to test the effects of the community-based interventions on health outcomes. This design was considered as the rigorous methodology to ensure validity of outcome measures. Another strength was concerned on representativeness of the studies with variety of cultural groups among different countries. Whereas, limitation of this study was concerned on the articles published between 2012 and 2017 to ensure that the articles were up-to-date and relevant to the current situation. Therefore, only 12 studies related to community-based intervention on HbA1c reduction and weight reduction were included in this study. The total number of studies might not cover all target groups in different setting. However, some limitations that are still found in this review include the difficulty of generalization of the contribution to multi-component interventions and the relatively small number of studies that were included in the meta-analysis. We analyze the potential sources of heterogeneity including types of community-based interventions, different ethnicity, sex, and how the researchers provide the intervention. Some studies combined the intervention of low-energy intake with regular exercise that showed more impact than a single intervention. Another limitation related to the interpretation of study results that should be interpreted with caution due to outcome measured in this study was focused only the reduction of HbA1c and body weight.

Conclusions

This review confirms that community-based intervention significantly affected on HbA1c and body weight reduction.

Even a low level of small changed effect was found, but it could be meaningful for T2DM patients. These results provide a valuable evidence to support development of the community-based intervention to improve self-management behaviors in order to reduce and to maintain the HbA1c and body weight of T2DM patients. The community-based intervention studies have shown positive results, but further studies should be strengthened on long-term community trials with other health outcomes measured need to be investigated.

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Compliance with ethical standard

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REVIEW ARTICLE

Gestational diabetes is associated to the development of brain insulin resistance in the offspring

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Abstract
Gestational diabetes mellitus (GDM) is defined as a form of glucose intolerance. Evidences for late metabolic and behavioral consequences to offspring born from GDM are emerging. More recent and concerning evidences point to detrimental effects of GDM on the behavior and cognitive capacity of the offspring. We aimed to review what is known about the consequences of GDM to brain and behavior of the offspring. A research was made in PubMed using the words gestational diabetes, insulin resistance, memory, cognition, brain, and offspring. The most relevant papers according to citations and with results suggesting that hyperglycemia in pregnancy is associated with inflammation and some mechanisms which are potentially involved with the late brain insulin resistance and its consequences in the GDM's offspring brain were selected. An increased risk of developing neuropsychiatric disorders to offspring from GDM mothers has been suggested. Transient intra-uterine exposure to high glucose levels can interfere with neuronal integrity, survival and connectivity to offspring's brain. Fighting neuroinflammation during gestational period may avoid susceptibility for neurodegenerative disorders in the offspring.

Keywords Gestational diabetes mellitus · Insulin resistance · Brain · Inflammation · Memory · Offspring

Introduction

Gestational diabetes mellitus (GDM) is defined by the American Diabetic Association [1] as a glucose intolerance triggered during the second and third trimesters of pregnancy in previously normoglycemic women. Thus, hyperglycemia during pregnancy is a hallmark of GDM [2]. Diabetes in pregnancy is the most important metabolic condition occurring in approximately 10% of all pregnancies in developing countries like Brazil and India, and reaching greater incidence in developed countries such as USA [3, 4]. Family history of obesity or diabetes, non-white race, and maternal age are risk factors for developing GDM [5, 6].

GDM is related to several detrimental effects on the offspring, including an increased risk of congenital malformations and inflammation that affect several organs and tissues, including the central nervous system (CNS) [7–9]. The immediate consequences of GDM to infants include macrosomia, neonatal hypoglycemia, hypocalcemia, and respiratory distress syndrome at birth [10]. The most common morbidity is macrosomia occurring in 30% of infants who were exposed to fetal hyperglycemia [11]. Maternal factors that contribute to fetal macrosomia include obesity and elevated concentrations of lipids and amino acids [12].

There is also an increase of inflammation markers in GDM mothers such as higher plasma levels of tumor necrosis factor alpha (TNF-α) [13]. GDM contributes to offspring neuroinflammation and influences neuronal distribution in the brain, as well as neural stem cell (NSC) proliferation and apoptosis during embryogenesis, possibly contributing to cognitive deficit, behavioral changes, and memory loss in the adult life [9, 14, 15].

Learning and memory deficits are related to inflammation and changes in insulin signaling in the brain. Brain insulin resistance (IR) is present in diseases related to cognitive impairment, including neurodegenerative disorders, such as Alzheimer's disease, and sepsis [16–19]. However, while the effect of brain IR is well documented in neurodegenerative diseases, the effect of insulin and its receptors on molecular mechanisms in inflammation, memory, and cognition in the

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offspring of GDM is poorly understood, and very few studies in literature are underlying the theme [7–9, 14, 15, 20–26].

This review aims to provide an overview about how intrauterine exposure to hyperglycemia during GDM influences the development of brain IR in the offspring, analyzing putative mechanisms or inflammation that could contribute to behavioral alterations and cognitive decline seen in adult offspring. A research was made in PubMed using the words gestational diabetes, insulin resistance, memory, cognition, brain, and offspring. The most relevant papers according to citations about GDM suggest hyperglycemia in pregnancy is associated with inflammation and putative mechanisms, which are potentially related to consequences to offspring brain.

Shared mechanisms between insulin resistance in type 2 diabetes and GDM

Insulin is a peptide hormone produced by β cells of the pancreas. It controls glucose metabolism and it is mainly secreted in response to the rise of circulating nutrients that occur immediately after food ingestion [27]. Thus, the elevation of the insulin level does not avoid the development of insulin resistance in muscle and adipose tissues [28, 29].

Physiological intracellular insulin signaling begins with the binding of insulin to its receptor in the surface of cells. Insulin receptors are formed by two alpha (α) and two beta (β) subunits. Insulin binding to its cell surface transmembrane receptor stimulates protein kinase activity of the β subunit. This leads to a conformational alteration of the molecule and to the insulin receptor phosphorylation in tyrosine residues [30]. Insulin receptors activate many intracellular substrates of which the most important are part of the insulin receptor substrate (IRS) family [31, 32]. IRS-1 and IRS-2 are directly related to glucose control in brain and peripheral tissues.

When insulin is bound to its receptor, the receptor phosphorylates IRS in tyrosine residues. Impaired kinase activity of insulin receptors appears to play an important role in insulin resistance in T2DM [32, 33]. The phosphatidylinositol-3-kinase (PI3-K) is an important compound in insulin signaling pathway. PI3-K is activated by IRS and consists of two subunits: p110 which has a catalytic subunit and the p85 regulatory subunit. The binding of IRS to the SH2 domains of the p85 subunit of PI3-K activates the associated catalytic domain [34].A number of receptors for growth factors and many oncoproteins work as intracellular binding sites for proteins containing SH2 domains such as PI3-K [35–37].

IR occurs when there is a failure in the activation or inhibition of IRS-1 and IRS-2 or if there is a reduced activation of other proteins such as activated kinase tyrosine (AKT) [36]. In peripheral tissues, inhibition of IRS-1 does not cause hyperglycemia but it leads to insulin resistance and growth retardation [32]. Reduced sensitivity to insulin induces β cells to increase the production of this hormone in order to maintain glycemia in the normal range. Besides the increasing of insulin, the tissue gets more resistant to it, favoring the return of the hyperglycemia [1, 38]. IRS-2 inhibition leads to hyperglycemia and hyperinsulinemia in the peripheral tissues.

There are differences in the IR mechanisms described in T2DM and GDM. During gestation, there are compensatory mechanisms such as hypertrophy and hyperplasia of β pancreatic cells to keep normoglycemic levels for the mother and the offspring [39, 40]. In GDM, these compensatory mechanisms tend to fail, leading to hyperglycemia, IR, and DNA damage to β pancreatic cells of the mother, rendering them more susceptible to develop obesity, diabetes, and neurodegenerative disorders later in life. Although, these events are well established in rodents, the compensatory alterations that happen in human β pancreatic cell during pregnancy remain controversial [39, 40].

Hyperglycemia in GDM is responsible for causing modifications in genes involved in proliferation and differentiation of NSC of the offspring, and these changes may favor in adult life oxidative stress and metabolic disturbance [14, 41].

Long-term consequences of GDM

For many years, GDM was considered to be a transient condition, which ruled out investigation of whether it was related to long-lasting consequences to either the mother or the fetus [7, 9]. It is known that mothers who develop diabetes during gestation are at increased risk of developing T2DM later in life due to the induction of metabolic changes and DNA damage to β pancreatic cells [42]. Risk of adverse maternal, fetal, and neonatal outcomes is increased as a function of maternal hyperglycemia [2, 42]. A study made by Cheung and Byte [42] with females who developed diabetes during pregnancy reviewed earlier studies examining the prevalence of GDM and risk of subsequent diabetes through an epidemiological tool known as population etiological fraction. They found over 31% of women who had GDM developed T2DM later in life and concluded that effective measures to prevent this situation are necessary and could have a significant impact over population health.

Infants born from diabetic mothers also show an increased risk of becoming obese and developing T2DM later in life [2]. The magnitude of fetal-neonatal risks and the reduction on neuronal activity and neurogenesis are proportional to the severity of maternal hyperglycemia [10, 43]. Besides changes in metabolism in the offspring of diabetic mothers, there are also morphological and functional alterations in hypothalamic structures caused by abnormal hormone levels [44]. Catecholaminergic system contributes to changes in the hypothalamus of the offspring affecting the neurodevelopment and promoting metabolic alterations such as an impaired leptin sensitivity [45, 46]. These changes can happen during pregnancy or lactation and are associated with

obesity in adulthood, and also to brain changes [44, 45, 47]. More recent and concerning evidences point to detrimental effects of GDM on the behavior and cognitive capacity of the offspring [9, 20]. An increased risk of developing neuropsychiatric disorders in children from GDM mothers has also been suggested [9]. Due to the need of new researches in this field and the difficulty of doing human biochemical research in children's brain, studies using experimental models are necessary to indicate possible ways to avoid or inhibit offspring brain damage or metabolic alterations in the offspring.

GDM impairs the development of CNS and causes cognitive and behavioral abnormalities in the offspring [48]. Studies using animal models have shown that GDM influences the behavior and fetal phenotype [9, 11]. An original research made with rats induced to diabetic pregnancy by a single intraperitoneal streptozotocin (STZ) injection (30–35 mg/Kg) showed that GDM's offspring at 60 days of age presented increased anxiety levels when exposed to challenging situations, including the social interaction test and the elevated plus maze, and also revealed hyperactivity in the open-field test [49]. Identical results were seen when evaluating the offspring of a transgenic model for metabolic syndrome and correlating these behavior changes with hippocampal inflammation [50].

Changes in brain structure can occur in fetuses from GDM mothers resulting in increased susceptibility to autism and schizophrenia later in life [51]. More recently, a study made with humans analyzed children born from multiethnic mothers diagnosed with maternal diabetes at 26 weeks of gestation revealed a strong association between GDM and increased risks of developing autism in the offspring [23].

A review also pointed to an increased risk of schizophrenia in GDM offspring due to the classic mechanisms of maternal diabetes such as hyperglycemia, oxidative stress, alterations in lipid metabolism, and mitochondrial structure affecting memory and cognition [52].

There are a few studies about how hyperglycemia and hyperinsulinemia effect the developing brain in humans [31, 53, 54]. A research conducted by Linder and colleagues (2015) assessed the influence of maternal metabolism in the development of the fetal brain through fetal magneto encephalography, a modern technique to evaluate fetal brain activity. They determined fetal brain postprandial activity to be slower in the offspring of women with GDM. Their results indicated that maternal diabetes affects brain development and leads to CNS IR in the fetuses [53–55].

Evidence for interference of GDM in insulin signaling pathways in the offspring's brain

Insulin has an important neuroprotective role and also modulates synapse plasticity mechanisms [56]. Insulin signaling also controls synapse density with consequent regulation of circuit function and plasticity [57–59]. Insulin receptors have been described in several brain structures, but those with the highest density include the hippocampus and cerebral cortex, where they were found to regulate memory and cognition [60].

Interestingly, for a long period of time, the brain was considered insensitive to insulin, and the CNS would not be insulin-dependent within this old concept [61]. However, it has been demonstrated that systemic metabolic expenditure and energetic homeostasis in the CNS are insulin-dependent and that neurons in the hippocampus, hypothalamus, and other brain regions that are sensitive to the presence of insulin are directly related to activities such as ingested food control and the energy expended for example [61]. Subjects with diabetes have a pre-disposition to develop cognitive deficits and show a greater chance of dementia, Alzheimer's disease, and Schizophrenia [17, 18, 61].

The reduced activation of insulin pathway is proposed to have negative effects on GDM's offspring cognition and memory [62]. Insulin and insulin-like growth factor-1 (IGF-1) are important in the development of these functions, and diabetes during pregnancy strongly influences the regulation of the receptors of insulin and IGF-1 [20, 22]. Alterations in IGF-1 gene expression and epigenetic regulation in hippocampus increase the risk for dementia to the offspring of maternal diabetes [22]. Recent studies in rodents revealed that retardation of fetal dendritic development in GDM is associated with IGF-1 signals [63].

Evidences suggest that the inhibition or malfunctioning of the IRS-1 is the main cause of memory loss and cognition failure due to brain IR [11, 22]. The reduction of the expression of the insulin receptors is related to memory loss and cognitive deficits at the brain [64]. Unfortunately, these molecular mechanisms of brain IR are not described in the GDM offspring, and most of what is known about brain IR comes from researches involving chronic neurological disorders such as Alzheimer's or Parkinson's diseases, among others [18, 48, 65].

Hyperinsulinemia and hyperglycemia in several brain areas could not be associated with changes in glucose transporter (GLUT) expression in the past decade [66]. Most of the past studies analyzing glucose transporters evaluated GLUT4 expressions which are present in peripheral tissues, but not in the brain [66] which expresses mostly GLUT1 transporting glucose across the blood-brain barrier and into the astrocytes and GLUT3 that carries glucose into neurons [67, 68]. However, a recent study evaluating middle-aged rats under a high-fat diet revealed that an increase in GLUT1 is associated with tissue damage in the hippocampus [69]. Expression levels of GLUT1 in cell membranes are normally increased by diminished glucose levels in hypoxia and reduced by increased glucose levels being widely distributed in fetal tissues [70]. It has been hypothesized that higher levels of GLUT1 expression are correlated with hypoxia, blood-brain barrier disruption, and may actually be a compensatory

response to energy demands by the cells [71]. GLUT1 expression has also been described as a measure of how glucose enters endothelial cells and in neural stem cells when in a hypoxia situation [69]. Although these studies were not done under GDM conditions, they provide molecular evidences of the role of hyperglycemia, hypoxia, and inflammation in the brain, and there are a few studies showing evidence of their effect over maternal diabetes [69].

A recent meta-analysis [54] evaluated maternal diabetes and the cognitive performance in the offspring. Exclusion criteria were no control group population, the presence of any pathology in the offspring, and pre-clinical researches. The studies analyzed were made in humans and involved 6140 infants, and they found that children born from GDM and between 1 and 2 years old had lower scores in psychomotor and cognitive development.

Putative mechanisms involved in long-term consequences to brain function in GDM offspring

Morphological changes in the brain of the GDM offspring occur, most of the times, in the embryonic state and may have long-term consequences [51]. Hyperglycemia present in GDM pregnant is thought to generate a choric hypoxia state in the fetus [9]. These metabolic maternal changes affect neurodevelopment in many different ways such as a reduced myelination [72], neuron distribution [73] and connectivity at cortex [9], neurogenesis in hippocampus and cortex [74], and neuron apoptosis [56].

In the presence of hypoxia, the fetus needs to maintain the iron plasma level, and its supply does not attend its demand. Human babies born from mothers with GDM possess iron content estimated in just 40% of normal [25, 56]. Iron plays an important role in neurotransmitter synthesis, neurogenesis, and myelination [75, 76]. Iron deficiency leads to hippocampal changes which compromise its normal functions and result in behavioral alterations similar to those seen in schizophrenic patients [77]. Other neurodegenerative disorders that developed in fetuses originated from maternal diabetes which may be related to iron deficiency and hypoxia as results from the hyperglycemia and hyperinsulinemia present in this condition [77]. It is also common in fetal iron deficiency to the offspring manifesting higher levels of irritability and a depressive behavior [78].

Hypoxia and iron deficiency can also contribute to an increase in the inflammatory burden incurred by the fetus [79]. Iron deficiency, hypoxia, and higher TNF-α levels are factors which can be triggered for the changes in glycemia leading to possible neurocognitive sequelae in offspring of diabetic mothers [25].

Metabolic alterations occur in GDM [25] such as higher plasma TNF-α levels [13] and increase in total blood glucose in the dams [20] and can influence neuron development of the offspring [80].Recently, a study showed that alterations in glyoxalase 1, a detoxifying enzyme and its pathway, lead to increased levels of circulating methylglyoxal, which causes a reduction in embryonic mice cortical neural precursor cells causing long-lasting alterations in adult neurons, such as a diminished number of mature neurons and the reduction of neurogenesis, affecting behavior and cognition during the animal's whole life [9]. The inflammation of the CNS may begin due to infection, toxic metabolites, or brain injury, and the immune response depends mainly on glial cells [9].

A maternal pro-inflammatory state can influence the brain development in the offspring

Microglia and astrocytes are glial cells directly related to neuroinflammation influencing memory and cognition [81, 82]. Usually, when having an exacerbated activation of microglia and astrocytes, a diminished activity of PI3-K insulin pathway and consequently a higher inhibition of insulin receptors and substrates are observed [81, 83, 84]. Even knowing the importance of microglia and astrocytes in inflammation, there are no studies evaluating their roles during all brain stages of development, starting from the embrionary period until adult life in GDM offspring. Hyperglycemia during pregnancy can contribute to a maternal pro-inflammatory state that can influence the brain development in the offspring [20, 24].

Microglia and astrocytes are responsible for producing and increasing the TNF-α levels having a harmful impact in the developing brain [82, 85].It has been shown that increased brain levels of TNF-α lead to peripheral and brain IR [64]. Higher plasma levels of TNF-α might cross placenta and could cause morphological alterations in the offspring's brain [64]. A study revealed that circulating TNF-α concentrations may inhibit the insulin production [13, 86].

It is already known that inflammation plays an important role in generating morphological changes in offspring and/or worsening the IR consequences in offspring from GDM leading to severe brain alterations such as an abnormal distribution of neurons in the cortical areas and a reduced neurogenesis [7, 9]. A study published recently by Vuong and colleagues [87] provides evidence that in rats, maternal obesity associated with GDM influences microglial activation and neuroinflammation in newborn offspring. GDM was induced in female Sprague-Dawley rats using a high-fat and sucrose diet for 6 weeks prior mating, throughout gestation and lactation, while lean control dams were fed a low-fat diet. The dams experienced increased weight gain during pregnancy and starting mid-gestation developed hyperinsulinemia and moderate hyperglycemia. Offspring of

GDM dams presented impaired recognition memory in the object recognition test, associated with reduced synaptophysin expression in CA1 and dentate gyrus (hippocampus areas). Increased levels of pro-inflammatory cytokines interferon gamma, IL-1α, IL-4, and TNF-α were also observed in the brain of the neonatal offspring of GDM dams. Finally, the microglial morphological transformation and astrogliosis observed in the GDM offspring persisted into young adulthood. GDM appears to condition microglia to a neuroinflammatory environment such that resulted in derangement of the hippocampal CA1 pyramidal neurons and cognitive impairments in young adulthood [87].

Serum maternal oxidative stress canals can induce or contribute to the development of a pro-inflammatory state and is associated with increased rates of perinatal problems [88–91]. Oxidative stress happens when the levels of reactive oxygen species (ROS) present within a tissue are greater than can be counteracted by the antioxidants present at the same tissue [88]. Pregnant women with GDM have increased level of DNA damage. A recent study showed that markers of lipid peroxidation and DNA oxidation are significantly increased in GDM women compared to healthy group of patients which indicates increased oxidative stress in GDM patients [89]. The human placenta is susceptible to oxidative stress and oxidative damage but methods of predicting pregnancy complications are limited [88]. The usage of biomarkers of oxidative stress in pregnancy for clinical applications must be detectable in biological fluids and be highly stable [92]. Some multiple markers of oxidative stress are known such as protein carbonyls and superoxide; ROS production provokes oxidation of lipids such as polyunsaturated fatty acids (PUFAs) and advanced glycation end-products (AGEs) [88].

Changes in the antioxidant levels of enzymes contribute to the development of oxidative stress in maternal diabetes [93]. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that produces free radicals and leads to chain reactions that might damage cells [94]. Diet [95], medicine intake [96], and physical exercise influence the antioxidant levels, and cells are protected from oxidative stress by an interacting network between antioxidant enzymes [94]. A study made by Aziz and colleagues [93] with rats induced to maternal diabetes through a combination of different methods such as being fed with high-fat sucrose diet, and receiving a single intraperitoneal injection of streptozotocin (35 mg/Kg) and nicotinamide (120 mg/Kg) on gestational day 0 showed reduced activity of some antioxidant enzymes. The authors analyzed the superoxide dismutase, which catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide [97]; catalase, which catalyzes the conversion of hydrogen peroxide to water and oxygen [98]; and glutathione peroxidase, whose main role is to prevent the organism from oxidative damage [99], and their levels were significantly reduced in the dams induced to maternal diabetes [93]. This study highlight that impaired antioxidant condition can be linked with maternal diabetes stress.

Another marker of oxidative stress can be the receptor for AGEs (RAGE), which can trigger a pro-inflammatory pathway, and is associated with the risk of developing psychiatric disorders [13]. RAGE alterations have been described as important for GDM-induced offspring changes in the CNS [20]. Maternal diabetes was induced in rats during mid-pregnancy by STZ creating a pro-inflammatory state, which was triggered by RAGE signaling. Primary hippocampal neurons in culture obtained from the offspring showed an altered excitability and a hyperpolarized membrane-resting potential. As NF-kB, a pro-inflammatory transcription factor, is increased by RAGE, Western blots were performed with hippocampus samples, and its levels were found to be significantly higher in the offspring of STZ-treated dams. In early adulthood, offspring of GDM animals had reduced anxiety-like

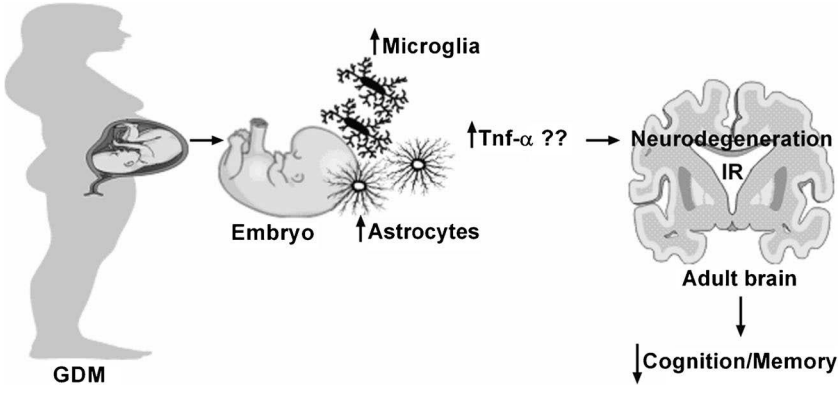

Fig. 1 GDM consequences in the offspring's brain. GDM is caused by hyperglycemia during pregnancy that leads to maternal serum inflammation and embrionary inflammation, which can influence the offspring's brain development. One of the possible mechanisms that could be responsible for these changes in the embryo, and our hypothesis is that the microgliosis and astrogliosis during embrionary period would lead to an elevation of the TNF-α expression. In adult life, offspring born from diabetic mothers would be more susceptible to develop brain IR and consequently neurodegeneration to be revealed by the early cognition impairment and memory loss

behavior, and altered object place preference revealed in behavior tests. These results suggest a disturbance in hippocampal development and alteration in behavior mediated by increasing levels of RAGE in fetal brain caused by hyperglycemia during pregnancy [20].

A recent study investigated another possibility of influencing the CNS development of the offspring through changes in the expression of genes responsible for regulating apoptosis in the hippocampus of neonate Wistar rats born to mothers who were induced to diabetes with a single intraperitoneal injection of STZ [56]. The study evaluated male offspring at P0, P7, and P14 and revealed that maternal hyperglycemia may cause disturbances in the expression of Bcl-2 and Bax genes, two extremely important genes in apoptosis regulation. The authors suggest that these disturbances may be the reason for the anomalies in cognition and behavior observed in offspring born to diabetic mothers.

Conclusions

The embryo exposure to GDM induces neuroinflammation [87], derangement of hippocampal neurons [9], and cognitive changes [20]. The offspring brains can suffer the consequences of inflammatory mechanisms during gestation leading to brain IR [87]. Failure in brain insulin signaling in the GDM offspring might explain in part the delay in cognitive development, and this hypothesis has never been evaluated properly (Fig. 1). Cognitive decline and memory loss in adults, especially at mild age or third age, might be a consequence of an embryo exposition to inflammation during a gestational period.

Lifestyle intervention is necessary and requires a scientific basis for applying these possible interventions in order to achieve and deliver results from an economic and effective perspective in the fight against IR [54].An effective strategy to prevent and combat hyperglycemia and IR during GDM is the adoption of a balanced diet associated with physical exercises, which will favor the better functioning of insulin-dependent and non-insulin-dependent pathways, contributing to increased insulin sensitivity and glucose uptake [21]. Lifestyle intervention in pregnant women who were obese has shown to reduce plasma levels of inflammation markers [100].

Hyperglycemia that occurs in GDM stimulates an inflammatory state that can lead the fetus to brain IR driving them to develop memory loss and cognition impairment [101]. The data reviewed suggests therapies using inhibitors of inflammation pathways could be used in order to reduce inflammation avoiding these embryo malformations during GDM. This would contribute to the inhibition of memory loss and cognitive deficit in the offspring diminishing the long-lasting effects of the GDM. Finally, we suggest that blocking TNF-α signaling at different stages of embryo development could be an effective approach, as this pathway seems to mediate several alterations that affect cognition [9, 20]. The development of effective anti-inflammatory therapies is of great relevance to prevent the long-lasting effects of GDM over the offspring's brain.

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Compliance with ethical standards

All procedures did not involve any human participants and/or animals, because it is a review article. This study is in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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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

RSSDI Research Grants

- 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's 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 18 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.	Aditya Diagnostics & Hospital	Dibrugarh, Assam
11.	Sunil's Diabetes Care N' Research Centre Pvt Ltd.	Nagpur, Maharashtra
12.	Marwari Hospital and Research Centre	Guwahati, Assam
13.	Down Town Hospital	Guwahati, Assam
14.	St.Theresa's Hospital	Hyderabad, Telangana
15.	Aegle Clinic	Pune, Maharashtra
16.	Tulip Hospital	Sonipat, Haryana
17.	Lilavati Hospital & Research Centre	Bandra West, Mumbai
18.	Srajan Hospital	Udaipur, Rajasthan

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.

Announcements

Dear Member,

Please update your Membership details like Complete Postal Address, Email Id, Pan No. & Mobile no. after log in your membership area on our website www.rssdi.in under sub heading Membership corner, so that we can send you RSSDI Newsletter & Journals.

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