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EDITORIAL

Diabetic retinopathy: An often missed window of opportunity

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Type 2 diabetes mellitus (T2DM) is a global pandemic with South-east Asia as its epicentre [1]. Uncontrolled diabetes damages the entire vascular tree, leading to microvascular complications primarily affecting the small vessels of the retina, nerves and kidneys and macrovascular complications that involve the larger vessels of the brain or the heart. Not only do they contribute to increased morbidity and mortality, but they also hugely reduce the quality of life of people with diabetes. The prevalence of these complications varies across different ethnicities. Diabetic retinopathy is the leading preventable cause of blindness amongst working age individuals [2] that affects small vessels of the eye and has a linear relationship with the duration of diabetes and glycemic control. The prevalence of diabetic retinopathy (DR) is lower in Asians compared to their western counterparts [3].

Diabetic retinopathy is a neurovascular complication where neuronal injury as a result of inflammation precedes clinical microvasculopathy. Pathophysiologic mechanisms like inflammation, epigenetic changes and insulin resistance that damage the pancreatic beta-cell also cause organ dysfunction, increasing the risk of diabetic retinopathy and other vascular complications. The quest to detect and predict early damage to the neurovascular tree prior to the development of full blown microangiopathy is ongoing [4]. There is a need to evolve strategies for earlier detection and treatment of diabetic retinopathy with an attempt to not only preserve good vision, but also prevent simultaneous inflammatory and destructive process in other organs [4].

There is proven evidence today that inflammation and retinal neurodegeneration contribute to diabetic retinal damage in the early stages of DR. Numerous recently identified molecular mechanisms may provide direction for the development of new early interventions [5].

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An interesting study by Tamer Ibrahiem Salem et al. [6] published in the present issue evaluated the "Expression profile of microRNAs as promising biomarkers in early detection as well as potential targets for management of proliferative diabetic retinopathy". Whole blood samples from 180 diabetic patients (60 without DR, 60 with non-proliferative diabetic retinopathy (NPDR), 60 with proliferative diabetic retinopathy (PDR)) and 60 normal individuals as control were tested for gene expressions of miR-21, miR-181c and miR-1179 using two-step reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). PDR group had much higher levels of microRNA 181c and miRNA 1179 compared to NPDR and control groups, which can be used to anticipate and follow-up the progression. However, miRNA 21 was similar in PDR or controls. Combination of miRNA 1179 and miRNA 21 improved the accuracy rate to 90%. Combination of miR-181c and miR-1179 increased the accuracy to 100% in discriminating between PDR and NPDR. This study proved that microRNAs may play a role in pathogenesis of diabetic retinopathy. It's likely that in the near future, microRNA antagonists or mimics could be used to modify DR by reducing its progression and subsequent blindness.

Another study by Sincer Abide featured in the current issue has highlighted the role of epicardial fat thickness for prediction of proliferative diabetic retinopathy [7]. Epicardial fat thickness (EFT) and MHR (monocyte to HDL ratio) were analysed in three groups of patients with diabetes namely, 36 without DR (NDR), 35 with proliferative DR (PDR) and 41 with non-proliferative DR (non-PDR). Monocyte counts, HDL, mean MHR and EFT values of NDR, non-PDR and PDR groups were significantly different. Study concluded that MHR and EFT were significantly increased in proliferative DR and negatively correlated with NDR. Authors recommend that increased EFT may be used to predict the presence of PDR in type 2 DM.

A recent large real-world study by Chawla et al. published in Prim Care Diab Europe [8] established relationship between diabetic retinopathy, microalbuminuria and other modifiable risk factors. A significant association between

presence of microalbuminuria, BMI, glycemic control, diabetes duration, peripheral neuropathy and the degree of retinopathy was highlighted. The study suggested that the presence of microalbuminuria be used as a simple clinical biomarker for the development of proliferative diabetic retinopathy.

Artificial intelligence has come a long way today, aiding healthcare professionals on many fronts. An interesting study by Sangeethaa and Jothimani [9] published in the present issue highlights the importance of artificial intelligence in the detection of exudates from clinical fundus images using machine learning algorithms in diabetic maculopathy. The main aim of this study is to assist in diagnosing DR using a computer-aided technique (AI) by detection of hard exudates. Methodology of the study focuses on the identification of the yellow lipids that include hard exudates. The classifier is provided with images of diseased retina as input, and it produces the output as exudates or non-exudates. Support vector machine (SVM) and multilayer perceptron (MLP) interpret the images to accurately predict the presence of exudates and non-exudates. Fundus images are pre-processed to get the filtered, contrast enhanced image. Then, for the detection of hard exudates, features such as blood vessel segmentation implement morphological operation by measuring the size of the lesion, and optic disc (OD) are measured and eliminated by comparing the parameters with the size of the lesions. Subsequently, segmented images are used as an input to the classifier such as SVM and MLP, which classifies and gives output about the presence or absence of the exudates. SVM and MLP classifiers collected 140 images from real-time databases from Aravind Eye Hospital, Coimbatore, with an accuracy of 88% and 95% respectively.

In the recent times, digital innovations include 5th generation (5G) telecommunication networks, Internet of Things (IoT), and artificial intelligence (AI), with immense potential for creating an inter-dependent ecosystem. These digital innovations have revolutionized the model of eye care [10].

A recently published study by Chawla et al. [11] "Trained nurse–operated tele ophthalmology screening approach as a cost-effective tool for diabetic retinopathy" highlighted the role of teleophthalmology for economical screening for diabetic retinopathy in developing countries. The photographs taken on a dilated fundus using an approved imaging camera by a trained health care provider and streaming them through the Internet to a specialty eye centre or ophthalmologist for reporting enabled basic screening for the presence of diabetic retinopathy on a large scale. Telescreening for diabetic retinopathy is fast emerging as a cost-effective, accurate, and reliable method for diabetic retinopathy screening and could be the way forward in developing countries like India where the delivery of cost-effective eye care to patients with diabetes in a practical and viable mode is a huge challenge. The study proposed that large scale adoption of tele ophthalmology should be encouraged as a means towards providing low-cost access to DR screening for timely detection as well as management of DR to reduce the menace of this devastating, vision-threatening condition.

Diabetic retinopathy is an often-overlooked microvascular complication of diabetes, essentially because of its silent asymptomatic course in early disease. Regular screening and early detection is a key factor that can attempt to change the trajectory of its progression to prevent eventual blindness. Advancements in technology and biosciences have opened a wide vista of diagnostic as well as management approaches. Molecular biology has thrown up new biomarkers that can serve as not only effective screening tools but also treatment targets for future strategies aimed at preventing progression from NPDR to PDR and vitreous haemorrhage, diabetic maculopathy and vision loss. Digital innovations incorporating artificial intelligence have brought forward teleophthalmology as an important means for screening and diagnosis of diabetic retinopathy aiding in its optimal management. It's time that we take proactive steps in incorporating newer advancements into our clinical practice and more research needs to be encouraged in this rapidly emerging arena.

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REVIEW ARTICLE

A systematic review and meta-analysis of randomized control trials of vitamin D supplementation in diabetic nephropathy

Sun Xuan[1] · Zhang Jin[1] · Wang Zhe[1] · Bu Huai-en[1] · Tian Chun-ying[1] · Wang Dong-jun[1] · Guan Yuan-yuan[1] · Wang Hong-wu[1]

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Abstract

Objective The aim of this study is to explore the correlation between vitamin D and diabetic nephropathy.

Methods Relevant evidences were searched from PubMed, Embase, Web of Science, Ovid and China Knowledge Resource Integrated (CNKI), Wanfang Data Knowledge Service Platform databases (WANFANG), and VIP dating from inception to December 2019 to obtain the randomized controlled trials (RCTs) of vitamin D in the treatment of diabetic nephropathy. According to inclusion and exclusion criteria, two researchers independently screened the literature, extracted data, and evaluated the quality of included studies. Rev Man 5.3 software was used to conduct statistical analysis.

Results A total of 10 studies involving 651 patients were identified. These studies were finally included into the meta-analysis. A meta-analysis results showed that vitamin D is the protection factor in diabetic nephropathy, the group treated with vitamin D did better than the traditional drug and the placebo group. After taking vitamin D, the level of vitamin D in the patient's body increased significantly. Pooled results showed that there was a significant difference for vitamin D (MD = 38.24, 95%CI = 32.69–43.79, $p < 0.001$.) The patient had a significant decrease in urinary protein; the difference was statistically significant (MD = − 180.92, 95%CI = − 212.67 to − 149.16, $p < 0.001$). The blood creatinine content decreased obviously (MD = − 17.13, 95%CI = − 27.88 to − 6.37, $p < 0.01$). However, most of the included studies did not report the quality of life and adverse reactions of patients, making it impossible to analyze these measures.

Conclusion This study showed that vitamin D played an active role in the treatment of diabetic nephropathy and can be used in future clinical applications. However, there are still some studies of low quality in the included studies, so it is suggested that clinical and scientific researchers carry out more high-quality, large sample, multi-center randomized controlled trials (RCTS) to provide more evidence-based medical evidence for future studies on vitamin D treatment of diabetic nephropathy.

Keywords Vitamin D · Diabetic nephropathy · Randomized controlled trials · Meta-analysis

Introduction

According to the World Health Organization (WHO), there are currently at least 415 million people with diabetes and 318 million people with impaired glucose tolerance [1]. Diabetic nephropathy is one of the most concerned chronic microvascular complications in diabetic patients, and is an important cause of death in diabetic patients, as well as the main cause of end-stage nephropathy [2, 3]. Diabetic nephropathy is managed by controlling blood sugar, blood pressure, lipids, urine protein, and improving the way of life to delay the process of end-stage nephropathy [4].

Vitamin D is a steroid hormone which is converted by the liver and kidneys into bioactive 1,25 (OH) 2D3 and acts on the body. Studies showed that vitamin D was associated with pathogenesis of inflammation, immunity, cancer, musculoskeletal system, metabolic disease, cardiovascular system, and psychiatric nervous system, including diabetes and its complications [5]. A growing body of research has linked vitamin D to diabetic kidney disease, but there is no detailed and reliable evidence for evidence-based medicine. Therefore, this paper searched and screened the data of randomized controlled trials of vitamin D and diabetic nephropathy. Meta-analysis was used to study the correlation between

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vitamin D and diabetic nephropathy, so as to provide guidance for the subsequent clinical prevention and even treatment of diabetic nephropathy.

Materials and methods

Literature retrieval

Two researchers independently searched databases including PubMed, Web of Science, Embase, Ovid databases, China Knowledge Resource Integrated (CNKI), Wanfang Data Knowledge Service Platform databases (WANGFANG), and VIP from inception to December 2019. Using the search terms "Vitamin D," "25(OH)D," "diabetic nephropathy," and "DN." Publications were limited to the English and Chinese.

Inclusion criteria

Studies were included if they met the following inclusion criteria: (1) The literature type is randomized controlled trials (RCTs). (2) The subjects were diabetic nephropathy patients. (3) The study focused on the relationship between vitamin D and diabetic nephropathy. (4) The research report provided the required data.

Exclusion criteria

Studies were excluded if they met the following inclusion criteria: (1) Unclear statistical methods and data description. (2) Full text unavailable. (3) Repetitive literature. (4) Case reports, literature, and review articles. (5) Experiments on animals.

Study selection and data extraction

Firstly, two researchers independently complete the preliminary screening of the article by reading the title and abstract, and the third researcher makes the decision if there are different opinions. Then, according to the inclusion and exclusion criteria, the two researchers screened the articles by reading the full text, if there were differences between the two researchers, the third researcher would make a judgment. Data were also extracted independently by two investigators to ensure that the precise targeted data were collected. The following data were extracted from each of the included studies: general information of patients, the number of cases and intervention measures in treatment group and control group, effective rate, adverse reaction, and other outcome indicators, author information, year of publication, etc.

Qualitative evaluation

The qualitative evaluation of the studies was based on Cochrane Handbook for Systematic Reviews of Interventions (version 5.0) [6]: The main evaluation content includes the following several aspects: random sequence, allocation concealment, blinding of participants and personal, blinding of outcome assessment, incomplete outcome data, selective reporting, other sources of bias. In all cases, the answer "yes" indicated a low risk of bias, the answer "no" indicated a high risk of bias, and the answer "unclear" indicated an uncertain risk of bias. The quality evaluation shall be conducted independently by two professional researchers and cross-checked. In case of differences, the third party shall make a decision.

Statistical method

The RevMan 5.3 software provided by the Cochrane collaboration was used for meta-analysis of the included RCTs. Combined OR ratio (OR) was used for counting data, and weighted standard deviation (MD) was used for measurement data, with 95% confidence interval (CI) as the effect size for both. The heterogeneity of the included data was analyzed by chi-square test. If $I^2 \leq 50\%$, there was no statistical heterogeneity among the studies, and the fixed effect model was selected for analysis. If $I^2 > 50\%$, it indicated statistical heterogeneity between studies. Random effect model was selected for meta-analysis, and the causes of heterogeneity were analyzed.

Results

Literature search results

Through NoteExpress document management software combined with manual re-checking, repetitive documents were eliminated, and irrelevant documents were excluded by reading the titles and summaries. Download the full text of relevant literature and to read it carefully before further screening and completing subsequent data extraction. A total of 1273 studies were retrieved through the retrieval strategy, including 299 PubMed, 4 Web of Science, 105 Ovid databases, 0 EmBase, 366 CNKI, 410 Wanfang database, and 89 VIP database. According to the inclusion and exclusion criteria, 269 studies were checked and excluded. Finally, 10 studies were included, with a total of 651 patients. The screening processing is summarized in Fig. 1.

Study characteristics

Ten studies met the inclusion criteria, with a total of 651 patients, including 335 in the treatment group and 316 in the

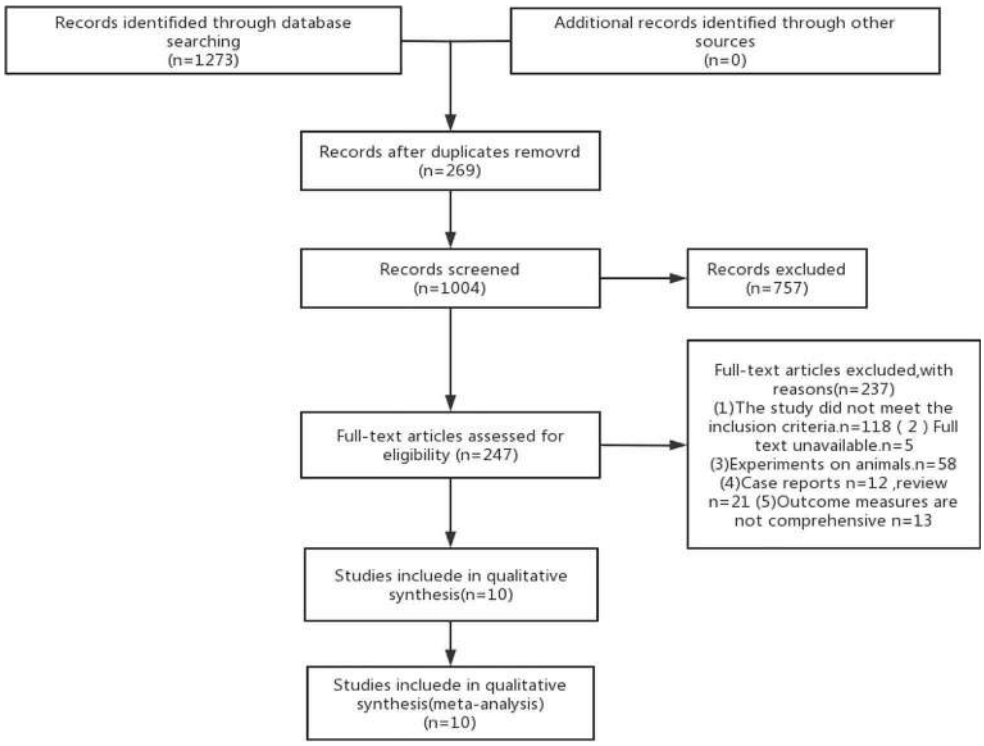

Fig. 1 Flow chart of literature selection

control group, which were involved in both domestic and foreign studies. The study period of 4 studies [9–11, 16] was 12 weeks, that of 3 studies [8, 12, 14] was 24 weeks, and 3 studies [7, 13, 15] was 8 weeks. General information of the included studies was shown in Table 1, including the general information of patients, the first author and year of publication, country, the number of cases and intervention measures in treatment group and control group, intervention, the duration of treatment, and outcomes. For more details, see Table 1.

Quality assessment

Risk bias graphs were drawn from 10 included studies. Only 4 [9, 13, 15, 16] referred to specific randomized methods, 3 [13–15] to allocation concealment, 4 [13–16] to double-blind methods, and only 1 to adverse reactions, all referring to changes in patients' quality of life. The methodological quality evaluation of the included studies was shown in Table 2.

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

Author	Year	Sample size		Intervention		Time (week)	Outcomes
		Treatment	Control	Treatment	Control		
Zhang 2017 [7]	2017	30	30	①	②	8	A+B+C+D+E
Long 2014 [8]	2014	37	17	①+④	④	24	A+J+K+L+M+N
Li 2019 [9]	2019	26	28	①+⑤	⑤	12	B+C+D+O+P+Q+R+U
Shi 2016 [10]	2016	62	62	①+⑤	⑤	12	D+G+P+Q+S+T+U+V+W
Wang 2014 [11]	2014	21	24	①+⑤	⑤	12	D+O+U+Q
Li 2013 [12]	2013	39	39	①+③	③	24	F+W
A. Esfandiari 2018 [13]	2018	25	25	①+③	③	8	A+B+E+J+X+Y+Z
Gayani C.Liyanage 2017 [14]	2017	42	43	①	⑤	24	A+C+G+H+T+U+Y+A1
Maliheh Barzegari 2019 [15]	2019	25	25	①	②	8	A+A1+A2+G+H+J+T
Ahmadi, N 2013 [16]	2013	28	23	①	②	12	A+A3+B

① Vitamin D; ② placebo; ③ basic hypoglycemic measures; ④ hemodialysis; ⑤ Irbesartan

A: Vitamin D levels; B: HbA1c; C: FBG; D: Urine protein; E; FBS; F: UAER; G: TC; H: HDL; I: UA; J:Ca; K: P; L: HOMA-IR; M: BNP; N: QTd time; O: NAG; P: hs-CRP; Q: TGFβ1; R: 2hPPG; S:RR; T: TG; U: Scr; V: BUN; W: HCY; X: TNF-a AND IL-6; Y: albumin ; Z: GFR; A1: LDL; A2: MDA; A3: UACR

Table 2 Methodological quality evaluation of the included studies

Studies	Random method	Allocation concealment	Blind method	Incomplete outcome date	Selective reporting	Other bias
Zhang 2017 [7]	Unclear	Unclear	No	Unclear	Yes	Unclear
Long 2014 [8]	Unclear	Unclear	No	Unclear	Yes	Unclear
Li 2019 [9]	YES	Unclear	No	Unclear	No	Unclear
Shi 2016 [10]	Unclear	Unclear	No	Unclear	Yes	Unclear
Wang 2014 [11]	Unclear	Unclear	No	Unclear	Yes	Unclear
Li 2013 [12]	Unclear	Unclear	No	Unclear	Yes	Unclear
A. Esfandiari 2018 [13]	Yes	Yes	Yes	Unclear	Yes	Unclear
Gayani C. Liyanage 2017 [14]	Unclear	Yes	Yes	Unclear	Yes	Unclear
Maliheh Barzegari 2019 [15]	Yes	Yes	Yes	Unclear	Yes	Unclear
Ahmadi, N 2013 [16]	Yes	Unclear	Yes	Unclear	Yes	Unclear

In the table, "yes" means low risk, "no" means high risk, and "unclear" means not aware of risk bias

Meta-analysis results

Vitamin D levels

There were 6 studies [7, 8, 13–16] mentioned vitamin D after the intervention patients before and after the change of the vitamin D levels in the body. There were statistical heterogeneity ($p < 0.001, I^2 = 95\%$) between the treatment group and control group. Thus, the random effect model was used for analysis. The pooled results indicated that there was a significant difference in the two groups (MD = 32.87, 95%CI: 20.59 to 45.16, $p < 0.001$). The detailed results were shown in Fig. 2A below. The studies were transformed into fixed-effect models, and the included studies were statistically analyzed again. Each study was removed one by one, and a new meta-analysis was conducted. Sensitivity analysis showed that there was no statistical heterogeneity ($p = 0.18$, $I^2 = 42\%$) after removing the studies of Esfandiari et al [13], Barzegari et al [15] and Ahmadi et al [16]. The pooled results indicated that there was a significant difference in the two groups (MD = 38.24, 95%CI: 32.69 to 43.79, $p < 0.001$), suggesting that Esfandiari et al [13], Barzegari et al [15], and Ahmadi et al [16] were the source of heterogeneity. The detailed results are shown in Fig. 2B.

HbA1c

HbA1c reflected a patient's glycemic control over the past 3 months, so HbA1c provided a patient's long-term glycemic trend. Three studies [7, 13, 16] mentioned changes in HbA1c before and after intervention, including 83 patients in the treatment group and 78 patients in the control group. There was no statistical heterogeneity ($p = 0.88$, $I^2 = 0\%$) between the treatment group and control group. Thus, the fixed effect model was used for analysis. The pooled results indicated that there was no significant difference in the two groups (MD = 0.02, 95%CI: − 0.37 to 0.41, $p = 0.92$). The meta-analysis of HbA1c between the treatment group and control group was shown in Fig. 3.

Urine protein analysis

Three of the included studies [7, 10, 11] involved changes in urinary protein. Proteinuria is one of the major markers of renal disease, and accurate measurement of clinically significant proteinuria is important for the diagnosis and management of renal disease [17]. There was statistical heterogeneity between groups in the included studies ($p < 0.001$, $I^2 = 93\%$). The randomized model was used for meta-analysis. The pooled results indicated that there was no significant difference in the two groups (MD = − 564.42, 95%CI: − 1153.85 to 25.02, $P = 0.06$). The result was shown in Fig. 4A. The studies were transformed into fixed-effect models, and the included studies were statistically analyzed again and a new meta-analysis was conducted. After removing Shi et al [10], the heterogeneity test of the remaining two studies showed no significant statistical heterogeneity ($p = 0.21$, $I^2 = 35\%$), indicating that Shi et al [10] was the source of heterogeneity. The pooled results indicated that there was a significant difference in the two groups (MD = − 180.92, 95%CI: − 212.67 to − 149.16, $p < 0.001$). Moreover, it can be seen that the treatment group with vitamin D has a statistically significant effect on the reduction of urinary protein, as shown in Fig. 4B.

Creatinine analysis

As can be seen from Fig. 5A, there was statistical heterogeneity between groups ($p = 0.02$, $I^2 = 75\%$), and meta-analysis using a random model showed no statistical significance (MD = − 10.23, 95%CI: − 24.02 to 3.55, $p = 0.15$). The included studies were transformed into fixed effect models, and the included studies were statistically analyzed again. Each study was removed one by one and a new meta-analysis was conducted. After removing Shi et al [10], there was no statistical heterogeneity between the

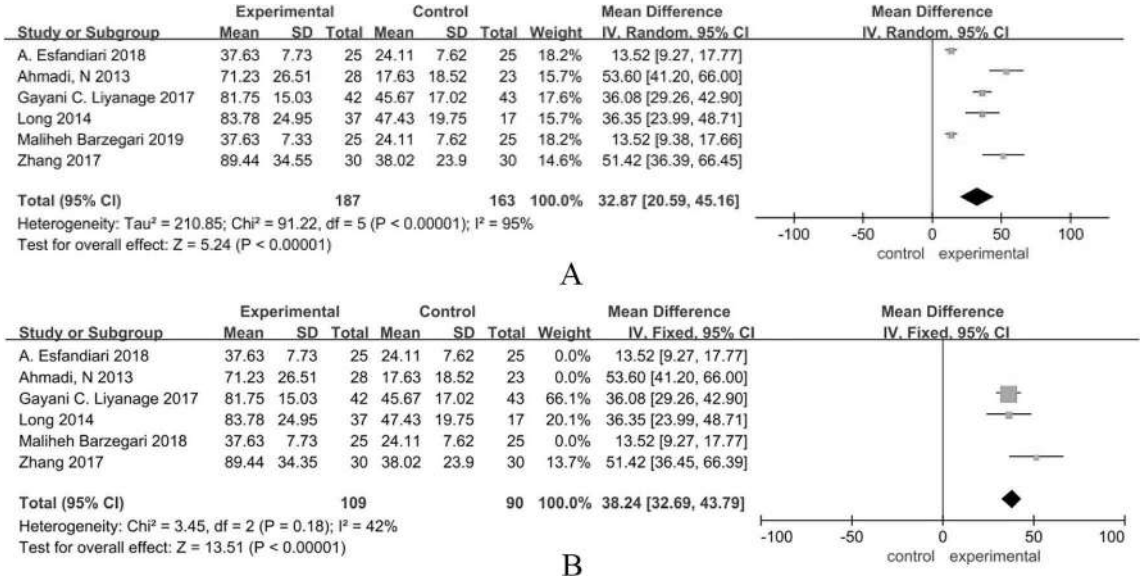

Fig. 2 **A** Meta-analysis of vitamin D levels in treatment group vs. control group. **B** Meta-analysis of vitamin D levels in treatment group vs. control group

remaining two groups ($p = 0.88$, $I^2 = 0\%$), which indicated that Shi et al [10] was the source of heterogeneity. After removal, meta-analysis was conducted again, and the results were shown in Fig. 5B. The results were statistically significant (MD = − 17.13, 95%CI: − 27.88 to − 6.37, $p = 0.002$) indicating that vitamin D was a protective factor for patients with diabetic nephropathy.

Adverse reactions and quality of life

None of the included studies mentioned changes in quality of life, and only Mei et al [12] mentioned adverse reactions, so it cannot be analyzed here.

Discussion

Diabetes is a chronic infectious disease that is currently prevalent. Like other epidemics, it is characterized by the fact that it is difficult to cure within a short period of time, and patients with diabetes often undergo long-term treatment. Diabetic nephropathy (DN) is a serious diabetic microvascular complication that reference for statistic quarter of diabetics are affected and is one of the leading causes of end-stage nephropathy worldwide. Diabetic nephropathy is caused by the changes in the structure of glomerular capillaries and renal tubules as well as the disorder of glucose homeostasis. However, the research on the treatment and prevention of diabetic nephropathy is still an ongoing task.

At present, Mogensen staging is widely used in clinical staging of diabetic nephropathy [18]. Diabetic nephropathy was divided into 5 stages, including acute glomerular hyperfiltration, normal albuminuria, early stage diabetic nephropathy, clinical diabetic nephropathy stage, and renal failure. The treatment of diabetic nephropathy is mainly in the first three stages; at this time, the further deterioration of diabetic nephropathy could be prevented by strict control of blood sugar and good control of blood pressure and lipids, and the application of ACEI or ARB drugs to reduce urinary protein. Once the disease progresses to stage 4 or 5, the use of these drugs can only delay the rate of deterioration of kidney function, which will eventually progress to the stage of uremia. Diaz et al. [19] found that diabetic patients with low vitamin D levels had a higher risk of kidney disease. Tiryaki et al. [20] found that proteinuria was significantly reduced in patients with early diabetic nephropathy treated with vitamin D. Another analysis found that diabetic nephropathy patients

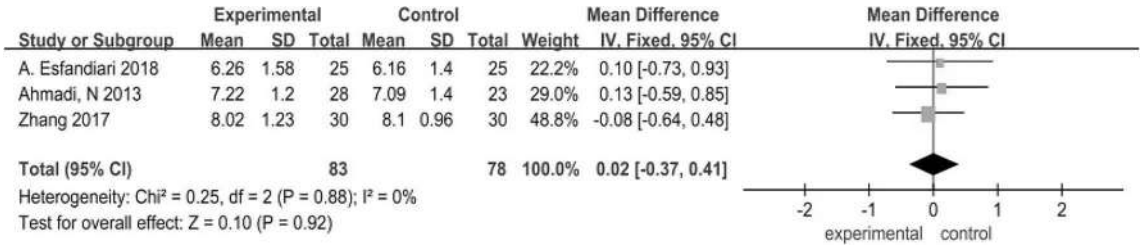

Fig. 3 Meta-analysis of HbA1c in treatment group vs. control group

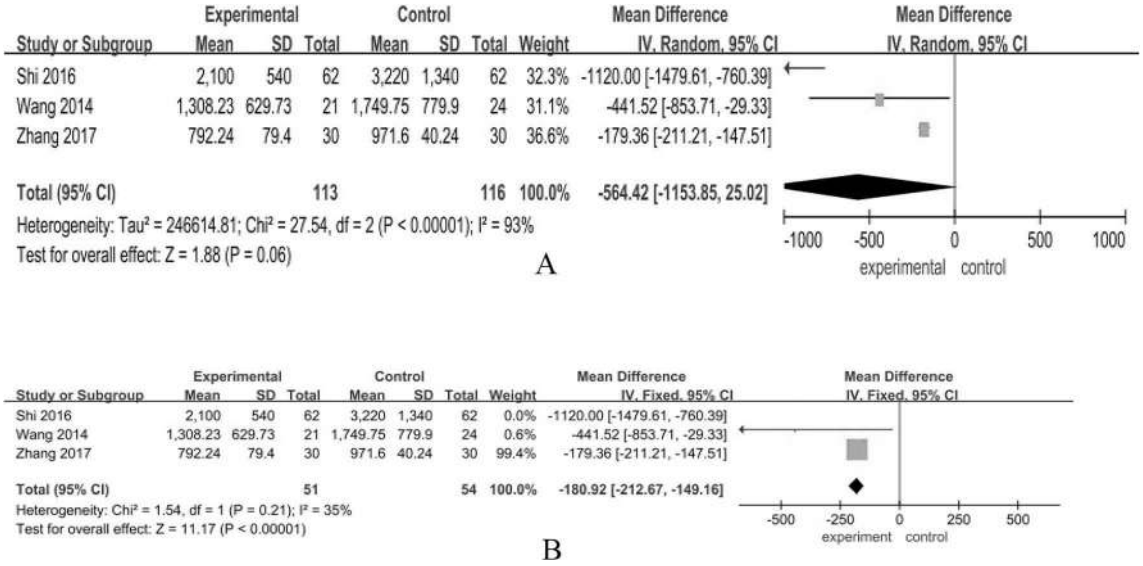

Fig. 4 **A** Meta-analysis of urine protein forest in treatment group vs. control group. **B** Meta-analysis of urine protein forest in treatment group vs. control group

with urine protein significantly decreased, and not affected by GFR, blood pressure, and ACEI, after 23 weeks of oral pericalcinol [21]. Therefore, early detection of 25-OH-VD level and timely supplementation of vitamin D in diabetic nephropathy is of great significance to protect the kidney and delay the deterioration of diabetic nephropathy. This is also consistent with the results of this study.

The main mechanism of vitamin D in diabetic nephropathy is reflected in the following aspects. First and foremost, vitamin D had the effect of inhibiting inflammatory factors such as interleukin (IL)-1, IL-6, and tumor factors [22]. Manion et al. [23] believed that patients with vitamin D deficiency had a 23% higher level of IL-6 than those with normal vitamin D. An open, prospective, single-center clinical study showed that oral administration of pericalcitol for 12 weeks significantly reduced serum and peripheral blood monocytes TNF-α and IL-6 levels in patients [24]. Secondly, vitamin D can improve insulin sensitivity and reduce the risk of diabetes. Type 2 diabetes accounts for up to 90% of diabetes patients. The study found that the main pathogenesis of type 2 diabetes mellitus is decreased function and number of pancreatic beta cells and insulin resistance [25]. Wang and Chen [26] found that islet β-cell function was significantly positively correlated with 25 (OH) D level in patients with type 2 diabetes. Several studies showed that taking vitamin D not only increased insulin sensitivity, but also positively regulated insulin signaling pathways [27, 28]. Thirdly, by adjusting the renin-angiotensin system (RAS), to reduce the formation of Angiotensin II, kidney damage caused by high blood sugar has a protective effect [29]. Studies showed that patients with diabetes renal interstitial angiotensin II 1000 times higher than that of healthy people [30]. Compared with RAS inhibitors alone, the combination of RAS inhibitors with

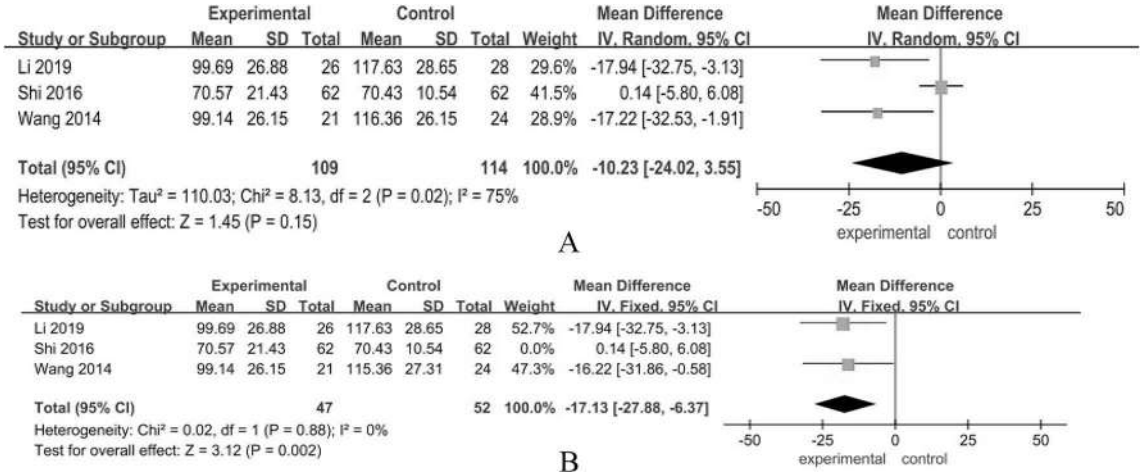

Fig. 5 **A** Meta-analysis of creatinine analysis in treatment group vs. control group. **B** Meta-analysis of creatinine analysis in treatment group vs. control group

VDRA palicalcinol was more effective in reducing proteinuria and renal damage in patients with diabetic nephropathy, a randomized controlled clinical study showed [31]. Therefore, vitamin D and RAS inhibitors have a synergistic effect in the treatment of diabetic nephropathy. Finally, vitamin D reduces podocyte hypertrophy and loss in the kidney, thereby reducing proteinuria and glomerulosclerosis. A number of recent randomized clinical trials have confirmed the antiproteinuric activity of vitamin D analogs in diabetic patients with CKD. Potent antiproteinuric activity of vitamin D has also been demonstrated in a variety of animal models of kidney disease. Treatment with 1,25-dihydroxyvitamin D (1,25(OH)2D3) or activated vitamin D analogs reduced albuminuria and prevented podocyte injury in 5/6 nephrectomized rats [32].

Vitamin D has important biological functions, such as regulating the immune system, affecting insulin secretion, improving insulin resistance, etc. For patients with diabetic nephropathy, vitamin D can well promote the absorption of calcium in glomerular filtrate. Given the prevalence of vitamin D deficiency in many populations and the potential link between vitamin D deficiency and adverse health outcomes, vitamin D deficiency is listed as a major public health problem [33]. Since complications of diabetes are closely related to microvascular disease, we wanted to study the correlation between vitamin D and diabetic nephropathy. However, in view of the small sample size of some included studies, we conducted a systematic review of meta-analysis to provide guidance for the clinical prevention and treatment of diabetic nephropathy in the future.

Our research has several advantages. The methodology was systematic and detailed, since the study's efficacy was small, which may lead to a greater therapeutic effect than a large study. In addition, we made a meta-analysis of the researchable outcome indicators as much as possible. Although the results were uneven to some extent, we also used the random effects model, which took into account the changes at the research level. Furthermore, most of the RCTS included in this study were multi-center randomized controlled trials, and both Chinese and English studies were involved. The overall quality of the study was relatively high, and the results were of certain reference value. The results of the meta-analysis showed that, from the point of view of HbA1c in patients after taking vitamin D, the significance of taking vitamin D in lowering blood glucose was not obvious in the data obtained from this study. But compared with the control group, in the implementation of the vitamin D after the intervention, the patient's body vitamin D levels rise obviously, urine protein and creatinine levels significantly lower for the patients with diabetic nephropathy, and renal protection has a positive meaning, thus the clinical treatment of diabetic nephropathy patients with vitamin D is also feasible.

However, this study also has some limitations. First, although randomized controlled trials at home and abroad are included, due to the small sample size and other problems, publication bias also exists to a certain extent. Second, although all of the studies included were randomized controlled trials, the blindness of several of the studies to evaluate the results was not clear, so there could be testing bias or confusion. Moreover, the existence of heterogeneity is inevitable due to the different duration of treatment. Finally, as diabetic nephropathy is a chronic disease, We think the quality of life of patients should be evaluated in the research. There is no research on the quality of life of patients. Therefore, the results should also be drawn with caution.

Conclusion

In conclusion, many patients with diabetic nephropathy are deficient in vitamin D. Vitamin D deficiency may play an important role in the pathogenesis of diabetic nephropathy, and timely supplementation of vitamin D may play an important role in the prevention and treatment of diabetic nephropathy. However, research on vitamin D intervention in diabetic nephropathy is limited, and more clinical trials or further evidence are needed to determine the effectiveness of vitamin D and provide additional evidence to guide vitamin D supplementation depending on the patient's circumstances.

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Author contribution Xuan Sun and Hong-wu Wang participated in the conception and design of this article. Xuan Sun and Zhe Wang completed the data retrieval. Xuan Sun and Chun-ying Tian completed the preliminary article screening according to the title and abstract. If they have different opinions, Yuan-yuan Guan makes the decision. Then Xuan Sun and Dong-jun Wang completed the article screening according to the existing standards, if there were differences between the two researches, Huai-en Bu would make a judgment. Xuan Sun, Zhe Wang, and Hong-wu Wang completed the data extraction. Xuan Sun and Huai-en Bu completed the data evaluation. Xuan Sun completed the statistical analysis.

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Declarations

Ethics approval and consent to participate Not applicable.

Conflict of interest The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Moderating effect of coping on the relationship between depression and chronic DM complications among patients with diabetes mellitus

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Abstract

Background The connection between depression and medical complication of DM is well established; however, the role of coping and how effective patients are able to manage DM complications are still not adequately tested. The purpose of this study was to assess the moderation effect of coping patterns on the relationship between depression and chronic DM complications of DM.

Methods Using a cross-sectional design, 384 patients diagnosed with DM type II have been recruited conveniently from a specialized national center. Data were collected in relation to depression, ways of coping, and medical complication using self-administered questionnaire and medical records.

Results The analysis showed no significant moderation effect of coping, or domain of coping, on the relationship between depression and chronic DM complications ($p > .05$). Depression was a significant predictor of chronic DM complications, while coping was not. Gender differences were found in depression with higher mean score of females compared to males, while no significant difference was found between male and females in relation to coping.

Conclusion Patients with DM type II need psychological counseling and enhancement of ways of coping to enable them manage their psychological disturbances including depression to prevent and/or minimize long-term effect of DM on their physiological functions.

Keywords DM type II · Depression · Coping · Chronic DM complications

Introduction

Diabetes mellitus (DM) is a common chronic condition affecting approximately 10.0% of the population worldwide [1]. International records are showing a rapid increase of number of people diagnosed with DM in the world from 171 million in 2000 to 366 million in 2030 [2, 3]. It has been noted that such an increment was more obvious in low-income countries compared to high incomes ones [2, 3]. Managing diabetes depends primarily on the personal management style and self-care and individuals' capabilities and resources available to them [1]. Previous reports showed that psychological status has been linked to DM and that managing DM is considered a stressful experience [4]. For example, depression was found to be common in patients with diabetes and was associated with worse treatment outcomes [4]. The estimated prevalence of depression among patients with diabetes mellitus ranged from 28 to 52% and has been associated with hyperglycemia in both types I and II [4, 5]. Depression has also been linked to greater morbidity and mortality rates in patients with type II DM; thus, it is considered one significant factor of poor health care outcomes [6]. Therefore, the ways of coping and management that patients with DM use are one significant component to overcome depression and prevent consequences on the bio-psycho-social well-being. Effective coping has been linked to effective management of psychological disturbances that lead to biological and psychological problems and DM complications [7].

Patients with diabetes mellitus are suffering from a number of stressful situations and depressive symptoms which

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makes them vulnerable to a psychosocial and physiological problems. Several studies have suggested that patients with diabetes, both adults and youths, can benefit significantly by learning coping patterns that they can apply to dealing with diabetes [8]. Although coping has been defined as one significant component to manage DM, coping strategies used by patients with DM are still not well defined by the literature. Therefore, addressing coping patterns is one important step towards understanding the connection between development of depressive symptoms among patients with DM and the development of chronic DM complications. Depression has been connected to chronic DM complications among patients with DM; however, factors that may interfere with occurrence of these complications vary [9, 10]. To improve coping patterns, we need to assess and explore the role of coping patterns and its effect on depressive symptoms. Studies showed that a number of factors can be employed to improve patients' responses to DM including social and psychological one [11, 12]. Nevertheless, the ability of patients to manage and how effective their coping strategies are might affect and be affected by their psychological status; among is depression [13]. Therefore, examining the psychosocial factors that contribute to development of chronic DM complications is needed in the context of their coping abilities. This will enable health care providers to better understand their patient's experience of diabetes mellitus and better plan for their care. The literature is scarce in studies that address connection and role of types of coping patterns in development of depression and chronic DM complications among patients with DM. The *purpose* of this study was to investigate the degree to which coping pattern can act as a moderator of the relationship between depression and chronic DM complications among Jordanian patients with type- II diabetes mellitus. Secondary question:

Are there any differences in coping patterns and depressive symptoms related to sociodemographic characteristics of patients diagnosed with type II diabetes mellitus?

Hypothesis

Coping patterns will have a positive moderation effect on the relationship between depressive symptoms and chronic DM complications among patients diagnosed with type II diabetes mellitus.

Methods

Design

Correlational design was utilized to answer the research questions. Data was collected using self-administered questionnaire from Jordanian patients diagnosed with DM type-II referred to outpatient unit. Information collected regarding depression, coping patterns, and DM medical complications.

Sample and setting

A convenience sample of 343 patients was diagnosed with type II diabetes mellitus receiving care at one national center specialized in diabetes and hormonal and genetic disorders. A total of 400 patients were approached, and 343 agreed to participate and completed and returned the survey data with response rate of 86%. Inclusion criteria were (1) at age of 18 years or above and (2) able to read and write Arabic. Exclusion criteria included (1) no history of mental or cognitive disorders.

Data collection

Prior data collection, ethical approval was obtained from the XYZ center; a national center specialized in treating individuals with hormonal and genetic problems and disorders. Anonymity of the respondents was ensured during and after study completion, and data secured and saved to provide anonymity. Moreover, the questionnaires were coded by numbers to maintain confidentiality of the data. Participants were informed that their participation is voluntary, and they have the right to withdraw at any time during the study. Furthermore, the participants instructed that their completion of the questionnaire will be considered as a written consent for their participation. The cover letter included information about the purpose of the study, its significance, and a statement that they can withdraw at any time without any consequences and their responses will be treated confidentially. The whole package was all in Arabic.

Measurement

The data was collected using the Arabic version of the instruments. The instruments were:

1. Depressive symptoms were measured using the Beck Depression Inventory-II [14]. The scale is formed of 21 items with respondents on a four-point Likert scale in which 0 represents the absence of symptoms and 3 represents an extreme problem. The total score ranges from 0 to 63, and the standard cutoff points are as follows: 0–13 indicate no or minimal symptom, 14–19 indicate mild symptoms, 20–28 indicate moderate symptoms, and 29–63 indicate severe symptoms [14]. A score of 13 is the cut-off point indicating depression. The test–retest *r* was 0.88, and Cronbach's alpha is 0.87 [14]. The Arabic version has been used in this study [9] where Cronbach's alpha is 0.88.

2. The Ways of Coping Scale (WCQ) Questionnaire [15]. The WCQ is a self-report instrument that is used for measuring coping patterns. It asks participants to recall a recent stressor and then rate how often they have used 66 different behaviors to cope with that particular stressor. Scale scores are additively derived from individual items and divided by a total score to provide relative scores for a total of eight subscales: confrontive coping, accepting responsibility, distancing, escape-avoidance, planful problem solving, positive reappraisal, self-controlling, and seeking social support. In a factor analytic study [15], the internal consistency ranged from 0.66 to 0.79 for the eight subscales [15]. The reliability of the Arabic version of WOC [16] was good where Cronbach's alpha was .88.
3. Chronic DM complications have been measured based on the medical files of patients confirmed by their primary clinician of the most reported complications utilizing the national, international guidelines, and medical literature, and those are hypertension, retinopathy, neuropathy, and foot ulcer.

Anthropometric measurements: Anthropometric measurements include recent weight and height using a detect scale with accuracy of + 100 g. Standing height was measured without shoes to the nearest centimeter using a stadiometer with the shoulders in a relaxed position and arms hanging freely. Blood pressure was measured using standardized sphygmomanometers EN 1060 (RIESTER) with cuff circumference of 24–32 cm to cover 80% of the upper arm (for obese patients, larger cuffs were used, 42–50 cm). The other sociodemographics such as gender, age, marital status, type of DM, duration of DM, smoking status, education level, and work status have been obtained from an investigator-developed subject profile.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (IBM-SPSS, 25). Descriptives were obtained to describe depression and coping patterns and using central tendency measures (means and medians) and the dispersion measures (standard deviation and ranges). The estimated descriptive statistics was compared to normative samples in the literature. Associations between variables were examined using Pearson *r* coefficient. *t* test was used for two independent samples, and analysis of variance (ANOVA) was used to examine differences in variables of interest in relation to demographic and health remark variables. To examine the moderation effect of coping on the relationship between depression and chronic DM complications, two-model multiple hierarchical regression analysis was used. Alpha was set to 0.05.

Results

Demographic characteristics

The sample consisted of 343 patients with diabetes mellitus type II. As shown in Table 1, the mean age of the patient was 56.8 (SD = 11.6). The patients' age ranged from 22 to 96 years with 50% ($n = 172$) of the patients below the age of 57. Males represented 47.8% ($n = 164$), 28.3% ($n = 97$) have baccalaureate level of education, 37.9% ($n = 130$) are currently working, and 85.1% ($n = 292$) of patients were married. With regard to smoking, 72.3% ($n = 248$) never smoked, while 14% ($n = 48$) and 13.4% ($n = 46$) were current and former smokers, respectively.

Health measurement

Among the patients who participated in this study, the mean of DM duration was 8.2 years (SD = 6.44), and about 50% ($n = 171$) of the patients had DM duration of more than 6.5 years. More than half of the patients (53.6%, $n = 184$) were on oral hypoglycemic agent only, 29.2% ($n = 100$) were on combination of oral hypoglycemic agent and insulin, 14.9% ($n = 51$) were just on insulin, and very few of the patients are on diet (2%, $n = 7$). The mean of insulin duration was 37.6 months (SD = 48.3), and also 50% of the patients

Table 1 Demographic characteristics of patients with type II diabetes mellitus attending National Center for Diabetes Endocrinology and Genetics (NCDEG) ($N = 343$)

Variable		*n*	%
Gender	Male	179	52.2
	Female	164	47.8
Educational level	Illiterate	40	11.7
	Elementary	56	16.3
	Secondary	70	20.5
	Diploma	52	15.2
	Baccalaureate	97	28.4
	Graduate	26	7.6
Job status	Worker	130	37.9
	Not worker	115	33.5
	Retired	94	27.4
	Disable	3	9
Social status	Married	292	85.1
	Single	7	2.0
	Widow	35	10.2
	Divorce	6	1.7
Smoking status	Current	48	14.0
	Former	46	13.4
	Never	249	72.6

insulin duration is less than 18 months. Of the patients, 52% ($n=127$) have hypertension, 25.4% ($n=67$) of the patients had retinopathy, and 4.1% (10) have retinopathy and foot ulcer. The mean of HBA1c was 8.1 (SD = 4.2), and 63.3% ($n=216$) of the patients had HBA1c > 7 indicating uncontrolled DM (see Table 2).

Psychosocial health indicators

Depression

The mean score for depression was 12.3 (SD = 8.2) ranging from zero to 48, and 50% ($n=172$) had a score of 11 or above. About 25% of the sample had a score of less than six, and 25% of them had score above 18. The analysis indicates that 43.4% ($n=149$) were categorized as not depressed, 23.3% ($n=80$) as mild depressed, 21.3% ($n=73$) as moderate depressed, and those with severe depression were 12.0% ($n=41$) (see Table 3).

Coping patterns

The mean score for coping was 74.8 (SD = 16.0). With regard to subscales of the ways of coping, the highest mean scores were observed in positive reappraisal ($M=11.95$, SD = 3.58), planful problem solving ($M=10.91$, SD = 2.95), and self-controlling with mean of 10.24 (SD = 3.65). While the lowest mean scores were observed in accepting responsibility ($M=5.05$, SD = 2.46), confrontive coping ($M=7.0$, SD = 2.72), and distancing coping ($M=9.62$, SD = 3.55). Regarding the two major coping domains, the mean score for problem focused coping was 40.2 (SD = 9.0), while the mean score for emotional focused coping was 34.7 (SD = 9.6), indicating that patients tend to use more of problem-focused coping rather than using emotional-focused coping (see Table 3).

Model testing: moderation effect of coping on the relationship between depression and chronic DM complications

Two-model hierarchical regression analysis was used to examine the moderation effect of coping on the relationship between depression and chronic DM complications (see Table 4). In block 1, depression as independent (predictor) and chronic DM complication as dependent (outcome) were entered, and in block 2, coping patterns were entered to test the add-up as independent (predictor) to test the effect of moderation of coping on the relationship between depression and chronic DM complication.. In model 1, where depression entered, the analysis showed that the model was statistically significant ($F=16.1$, $p<0.001$). The model was able to explain only 6.3% ($R^2=0.063$; adjusted $R^2=0.059$) of variation in occurrence of chronic DM complications in relation to depression. In block 2, in which coping was added to test its moderation effect, the analysis showed (see Table 3) that

Table 2 Health measurement of patients with type II diabetes mellitus attending National Center for Diabetes Endocrinology and Genetics (NCDEG) ($N=343$)

Health remarks	Mean	Median	SD	P_{75}	P_{25}	Min	Max
DM duration	8.2	6.5	6.44	12	3	0.5	30
Last HbA1c	8.1	7.9	4.22	8.9	7	5.2	16
Insulin duration	11.0	10	8.2	14	6	0	36

Table 3 Description of Psychological Indicators – depression, stress, coping and coping sub scales of patients with type II diabetes mellitus attending National Center for Diabetes Endocrinology and Genetics (NCDEG) (N = 343)

Variable	M	Md	SD	P_{75}	P_{25}	Min	Max	Range
Depression	12.3	11	8.2	18	6	0	48	48
Coping total	74.8	77	16.0	123	30	65	83	93
Emotional-focused coping	34.7	36	9.6	64	13	30	41	51
Confrontive	7.0	7.00	2.7	9	5	0	15	15
Distancing	9.6	10	3.6	11	8	2	32	30
Self-control	10.2	10	3.7	12	9	2	32	30
Seeking support	10.3	11	3.3	12	8	3	29	26
Problem-focused coping	40.2	40	9.0	62	14	34	45	53
Accepting responsibilities	5.1	5	2.5	6	4	0	28	28
Escape avoidance	9.8	10	4.4	13	6	0	19	19
Painful problem solving	10.9	11	3.0	13	9	4	19	15
Positive appraisal	12.0	12	3.6	15	10	4	35	31

Table 4 Regression examining moderation effect of coping on the relationship between depression and medical complications among patients with DM ($N = 343$)

Variables	Block 1		Block 2	
	β	p value	β	p value
Depression	.047	< .001	.047	< .001
Coping			− .003	.583
R^2	.063		.064	
R^2_{adj}	.059		.056	
R^2 change	.001		$p = .582$	

the model was statistically significant ($F = 8.6$, $p < 0.001$) with $R^2 = 0.064$ and adjusted $R^2 = 0.056$. The R^2 value of 0.064 indicates that 6.4% of the variation in the relationship between depression and chronic DM complications is related to the moderation effect of coping. This could be explained in terms of the R^2 changes that seem very minimal and non-significant ($R^{2\,=}$ 0.001, $p > 0.05$). The analysis indicates that adding coping to the relationship between depression and medical complication has not influenced significantly the relationship, and the added R^2 value was non-significant and very minimal (> 1.0%). The analysis also showed that depression is a significant positive predictor of medical complication ($\beta = 0.047$, $p < 0.001$), while coping was not ($\beta = -0.003$, $p = 0.582 < 0.001$).

Using the error of variance to compare the two models in which model one includes depression and chronic DM complication, the error was ($e = \sqrt{1 - R^2} = \sqrt{1 - .063} = \sqrt{.94} =$ 0.96.7), while the error in variance in model two in which coping was added was ($e = \sqrt{1 - R^2} = \sqrt{1 - .64} = \sqrt{.937} =$ 0.97).Therefore, it is obvious that adding coping to the model has not changed the error of variance which indicates that coping is not a significant moderator on the relationship between depression and chronic DM complication.

Differences related to demographic and personal characteristics

Pearson r has been used to examine the relationship between the depression and coping in relation to age, DM duration, insulin duration, and HBA1c. The analysis showed that age and insulin duration have no significant correlation with depression and coping. DM duration has significant and negative correlation with depression ($r = -0.14$, $p < 0.05$). Using t test to examine the difference in the depression and coping in relation to gender, the analysis (see Table 5) showed that there is a significant difference between male and female patients in relation to depressive symptoms ($t = -3.93$, df = 1, $p < 0.001$) with mean of females' score of higher ($M = 14.1$, SD = 8.6) than male patients ($M = 10.7$, SD = 7.4), while coping total and all subscale and domains of coping had no significant differences. Moreover, ANOVA test (see Table 5) has been used to examine the relationship between the depression and coping in relation to working status, DM treatment, and smoking status. The analysis showed that there was only significant difference in depression in relation to the working status of patient ($F_{4,\,341} = 6.48$, $p > 0.001$), while no other significant differences were detected ($p > 0.05$).

Discussion

The psychological status of patients with diabetes mellitus is a crucial issue that might interfere in effective treatment plans. Therefore, chronic DM complications are more likely to occur with lower level of psychological well-being among patients with DM. This has indicated that adaptation and coping with the disease are key factors that need to be considered while managing care for patients with DM. In this study, we tested the moderation effect of coping on the relationship between depression and development of chronic DM complications. We found that coping has no moderating effect on the relationship between depression and development of chronic DM complications. This infers that patients diagnosed with DM type II and suffering from depressive symptoms, regardless of their ways of coping, have developed chronic DM complications. One explanation is related to the high level of depressive symptoms found among patients in this study where about more than a half suffered various levels of depressive symptoms and about one-third suffered moderate to severe form of depressive symptoms. Another explanation is related to the effect of depressive symptoms that might have disabled patients from being able to use the appropriate ways of coping or results using more emotional-focused coping rather than problem-focused ones. Depression has both behavioral and cognitive components that results in lack of concentration and indecisiveness [4, 7]. Therefore, patients with higher levels of depression had lower ability to manage effectively their illness-related issues. Chronic DM complications, thus, are developed due to psychosomatic symptoms of depression that exacerbates the physical and physiological well-being of patients with DM type II [5]. The results provide a novel perspective on how effective coping should be employed to prevent the DM complications. It is assumed that patients with DM need to use effective coping strategies than focuses, in addition to daily requirement and demands of patients with DM, on how to manage psychological factors such as depression to lower the risk to develop DM complications. Here, we emphasize the type of coping used by the patients and its role in improving the psychological status of patients reaching better self-care and quality care outcomes. Interestingly, and contrary to our findings, a recent study found

Table 5 Differences in depression and coping related to selected demographic and health related ($N=343$)

Variable		*n*	*M*	SD	*t* test	*p* value
Coping × gender	**Male**	179	75.2	15.0	.384	.701
	Female	164	74.5	17.0		
Depression × gender	**Male**	179	10.7	7.4	3.93	< .001
	Female	164	14.1	8.6		
					F	
Coping × treatment of DM	**Diet**	7	65.4	14.7	2.06	.105
	Oral	184	74.9	15.5		
	Insulin	51	71.6	16.5		
	Oral and insulin	100	76.9	16.2		
Depression × treatment of DM	**Diet**	7	13.1	5.3	2.27	.080
	Oral	184	11.5	7.8		
	Insulin	51	14.8	9.4		
	Oral and insulin	100	12.6	8.0		
Coping × working status	**Worker**	130	75.9	15.8	.827	.482
	Not worker	115	73.2	16.6		
	Retired	94	75.6	15.7		
	Disable	3	68.7	8.4		
Depression × working status	**Worker**	130	10.8	7.5	6.48	< .001
	not worker	115	14.9	9.0		
	Retired	94	11.0	7.2		
	Disable	3	13.7	11.9		
Coping × smoking status	**Current**	48	80.0	2.2	2.99	.056
	Former	46	74.7	2.5		
	Never	249	73.8	1.0		
Depression × smoking status	**Current**	48	12.6	1.2	.165	.898
	Former	46	12.8	1.1		
	Never	249	12.1	.5		

that patients diagnosed with DM and using more frequently the emotion-focused coping strategies are more likely to use diabetes' self-care activities than other forms of coping [17]. However, such positive link might be affected also by other factors such as having or suffering from other medical disorders [18]. Therefore, coping, which could be adaptive or maladaptive, affects and are affected by patients' psychological well-being leading to deterioration or improvement in quality of self-care.

The findings of this study also showed that more than half of the patients reported depressive symptoms, and they were using problem focused coping patterns more frequently than emotional focused coping. To our knowledge, this is the first study that estimated the level of depression and coping patterns among type II diabetics among Jordanian population and in the Arab countries. Although the culture and health care systems vary across countries, patients with DM are now able to retrieve information and seek consultation through various forms of electronic resources. While age might be an issue, however, most of older persons in Arab world are taken care of by their adult children who are able to retrieve needed information. This could have contributed to enhance their problem-focused coping over the emotional one. Nevertheless, using problem-focused coping did not predict chronic DM complications. The results of this study do partially agree with international reports [10, 19] which reported depression among 28 to 58%. Others [20] have also found that about 17% of their sample of patients with type 2 DM have scored higher than the cutoff point indicating a high prevalence of depression. Such variations could be related to many factors such as age of patients, having comorbidity, and way of measurement of depression. Using self-reported format seems to lead to higher rates of depression compared to more robust and objective measures such as interviews. The clinical evaluation and using observational and longitudinal approach for detecting depression among patients with DM could be significant factors that might reveal robust and consistent rates of depression [20].

On the other hand, the results do not correspond with previous international studies that reported a greater proportion of patients with diabetes using avoidance coping styles than using problem-focused ones [21]. The results infer that depression is considered a significant factor that health professionals need to consider when investigating and

promoting mental and psychological well-being of patients with type II diabetes mellitus. Controlling and lowering depressive symptoms among patient with type II diabetes mellitus are required to enable long-term glycemic control [22]. Such positive influence might give courage and sense of mastery for the patients to use more problem coping strategies and, consequently, prevent development or delay complications.

The study also examined the relationship of depression and coping with demographics and health-related factors. The findings showed that, and contrary to previous studies, no significant association was found between age and depression [22]. In addition, this study suggested no correlation between insulin duration and depression and coping which support what Clark and colleagues [23] previously found. However, depression was found to associate negatively with DM duration, while no significant correlation with HbA1c was found. Such finding does not agree with previous studies that connected depression to deterioration of HBA1c and diabetic control [24]. On the other hand, we found that males and females are different in their depressive symptoms with a mean score of females higher than males. This result corresponds with other studies that linked higher depressive levels among females with DM than males [25].

One limitation of this study is related to using self-report format. Although self-administered format seems more feasible, depression should be clinically evaluated to ensure depressive symptoms and their effects on the daily life of patients. Another limitation is related to sample selection where all patients were seeking treatment from the targeted national center, while other places might have different level of care, resources, and services.

Conclusion and implication

We found that a high proportion of patients with DM type II suffer from depression although using more frequently problem-focused coping. Depression was associated with chronic DM complications, while coping was not. Such findings have implications to mental health counselors and DM clinicians caring for patients with DM type II regardless of their age and gender. There is a need to assess psychological factors such as depression and test individuals' ability to cope effectively with their health conditions and needs. While, addressing medical and physiological problems are considered priority, psychological ones need also to be prioritized to prevent and minimize the risk to develop chronic DM complications. There is a need to indicate early detection and psychological counseling at DM outpatient's clinics. Research in DM should focus on the effectiveness of psychological intervention on depression and other psychological problems among patients with DM and the long-term effect on the development, or prevention, of DM complications, comorbidity, and mortality.

Declarations

Ethical consideration Prior to data collection, ethical approval was obtained from the Scientific Research Committee from the XYZ institution. Patients were assured of confidentiality, anonymity, and that their participation is voluntary. Patients signed informed consent after having all their questions answered and assured of their rights to withdraw from the study without any direct or indirect influence of their received care. Patients were also informed that the data will be used for research purposes only.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Role of epicardial fat thickness for prediction of proliferative diabetic retinopathy

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Abstract

Purpose Plenty of factors including inflammation are responsible for development of diabetic retinopathy (DR). Epicardial fat produces adipokines, cytokines and inflammatory products. Monocyte count to high density lipoprotein (HDL) ratio (MHR) has been recently suggested as an inflammatory marker.

Methods Epicardial fat thickness (EFT) and MHR were analyzed in 36 diabetics without DR (NDR), 35 diabetics with proliferative DR (PDR) and 41 diabetics with nonproliferative DR patients (nonPDR).

Results Monocyte counts, HDL, mean MHR and EFT values of NDR, nonPDR and PDR groups were significantly different. One-way analysis of variance test with post hoc Tukey test revealed that the significance of differences in MHR and EFT were dependent on the differences between NDR and PDR ($p<0.001$ for both) and nonPDR and PDR groups ($p<0.001$ and $p=0.001$). The differences between NDR and nonPDR ($p=0.81$ and $p=0.06$) were not significant. MHR and EFT were significantly positively correlated with PDR ($r=0.453$, $p<0.001$ and $r=0.394$, $p<0.001$) and negatively correlated with NDR ($r=-0.256$, $p=0.006$ and $r=-0.380$, $p<0.001$). Only EFT was found to be independently associated with PDR ($p=0.002$, 95% CI: OR: 1.643 (1.206–2.237)). An EFT value of >5.90 mm classified the presence of PDR with a sensitivity 74% and specificity of 61% (AUC = 0.750, 95% CI, 0.658–0.843), and a MHR value of >12.8 ratio classified the presence of PDR with a sensitivity of 83% and a specificity of 79% (AUC = 0.811, 95% CI, 0.728–0.893).

Conclusion We suggest that MHR and EFT were significantly increased in proliferative DR, and increased EFT may predict the presence of PDR in type 2 DM.

Keywords Epicardial fat thickness · Diabetic retinopathy · Monocyte to HDL ratio

Introduction

Diabetes mellitus (DM) has been associated with increased risk for coronary artery disease, and it is accepted as a major risk factor for atherosclerosis [1]. It has been associated with both micro- and macrovascular disease. Early detection of microangiopathy for vascular risk assessment in DM is an important target [2]. One of the earliest finding of diabetes-related microvascular involvement is retinopathy. A serious complication of DM, diabetic retinopathy (DR), is an important cause of blindness [3]. Diabetic retinopathy (DR) is grouped either as nonproliferative or proliferative, according to vascular structural changes or development of new aberrant vessels. Multifactorial etiologies contribute to the development of DR [4]. Inflammatory cytokines and chemokines were found to be increased in plasma and/or vitreous and aqueous humor samples of DR patients. Although some inflammatory markers like pigment epithelium-derived factor (PEDF), insulin-like growth factor (IGF-1), placental growth factor (PGF) and interleukin-10 (IL-10) were not increased in plasma and/or vitreous and aqueous humor samples of NDR and nonPDR patients they were increased in PDR patients [4].

Epicardial fat thickness (EFT) may act as an endocrinologically active tissue via paracrine and vasocrine

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mechanisms and regulates the functions of the heart and blood vessels. Moreover, it has been shown that epicardial fat produces several adipokines, cytokines and inflammatory products [5]. Macrophages and monocytes are the most important cell types that secrete proinflammatory and prooxidant cytokines in the inflammatory process [6]. High-density lipoprotein (HDL) had been demonstrated to protect endothelial cells from negative effects of low density lipoprotein (LDL) and inhibit oxidation of LDL molecules [7, 8]. Recently, ratio of monocyte counts to HDL cholesterol (MHR) has been used as an inflammatory marker in various conditions [9, 10].

As far as we know, there has been no study searching association between DR and EFT and MHR. Therefore, we aimed to investigate the relations between DR and EFT and MHR in patients with type 2 DM.

Methods

Sample size was calculated by power analysis. For a 95% power, sample size was calculated as 35 participants in each group. Ten percent more subjects were enrolled for possible loss during the study. General characteristics such as gender, age, systolic and diastolic blood pressure of the subjects and laboratory data were obtained from database of the institution. Exclusion criteria were as follows: inadequate transthoracic echocardiographic imaging, <18 years of age, acute diabetic complications, accompanying diseases such as anemia, moderate to severe renal or liver disease, thyroid disorders, electrolyte imbalances, the patients with established systemic inflammatory disorders or infectious diseases. The diagnosis of DM was established according to the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) guidelines in 2020 [11]. International Clinical Diabetic Retinopathy Disease Severity Scale was used in determination of the DR [12].

Laboratory parameters, including, serum glucose, creatinine, total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were recorded from the laboratory database (Architect C8000, USA). Simultaneous optical and impedance measurements (Cell Dyn 3700; Abbott Diagnostics, Lake Forest, IL, USA) were used in determination of hemogram parameters. The monocyte to HDL ratio (MHR) was measured by ratio of monocyte count by HDL levels. Body mass index (BMI) was calculated by ratio of weight in kilograms by the square of height in meters.

Echocardiographic examination was performed with Vivid S6 (GE Vingmed, N-3191 Horten-Norvay) machine using 4-Mhz transducer. Examinations were performed by a cardiologist blinded to the study. One-lead electrocardiography (ECG) was recorded continuously, and three consecutive cycles were averaged for every measured parameter during echocardiographic examination. Patients were in the left lateral position. Left ventricular end-diastolic diameter (LVEDD, mm), left ventricular end-systolic diameter (LVESD, mm), interventricular septum thickness (IVSD), left ventricular posterior wall thickness (PW), left ventricular ejection fraction (LVEF %) and EFT were measured. The echo-free space between the outer wall of the myocardium and the visceral layer of the pericardium was identified as epicardial fat, and EFT was measured on the free wall of the right ventricle at the end diastole from the parasternal long- and short-axis views in three cardiac cycles [13].

Statistical analysis

For the analyses, Statistical Package for Social Sciences for Windows (SPSS) version 18.0 (SPSS Inc. Chicago, IL, USA) was used. Quantitative variables were normally distributed and were expressed as mean ± standard deviation (SD). Qualitative variables were presented as numbers and percentages. A one-way analysis of variance (ANOVA) test was used, and post hoc analyses were performed with Tukey's HSD to assess the dependence of differences between groups. Pearson correlation analysis was used to assess the correlations between EFT, MHR, HDL and monocyte count. Multiple logistic regression analysis was used to analyze the value of EFT, MHR, HDL and monocyte count as independent predictors of DR. A receiver operating curve (ROC) analysis was used to find sensitivity and specificity of MHR and EFT to classify the presence of DR. A p value of <0.05 was considered as significant.

Results

A total of 112 patients (69 males, 43 females) were included in the study: 36 diabetics without DR (NDR), 35 subjects with proliferative DR (PDR) and 41 nonproliferative DR patients (nonPDR). Baseline demographic characteristics were not significant between the study groups (Table 1).

Echocardiographic parameters including LVEF, LVDD, LVSD, PW and IVSD were similar in all groups. However, EFT values were significantly different in NDR, nonPDR and PDR groups (4.49±1.59;5.47±1.81 and 6.57±1.50 mm, respectively p <0.001). HDL values of NDR, nonPDR and PDR groups were significantly different (54±15 mg/dL, 52±17 mg/dL, 41±8, mg/dL, respectively, p<0.001). Monocyte counts of NDR, nonPDR and PDR groups were also significantly different (0.48±0.15 ($\times 10^9$/L), 0.50±0.14 ($\times 10^9$/L) and 0.6±0.12 ($\times 10^9$/L), respectively, p= 0.001). Mean MHR values were also

Table 1 General characteristics, clinic and laboratory findings of the study population

	Negative DR (NDR) (n=36)	Nonproliferative DR (nonPDR) (n=41)	Proliferative DR (PDR) (n=35)	p
Male/female	21/15	22/19	16/19	0.56
Hypertension (%)	19 (53%)	26 (63%)	27 (77%)	0.10
Smoking	5 (14%)	1 (2%)	4 (11%)	0.17
Family history	16 (44%)	12 (29%)	12 (34%)	0.37
Age (years)	62±10	64±10	64±9	0.19
Body mass index (kg/m^2)	27 ±4	26±3	26±3	0.29
Duration of DM (years)	7.7±3.9	9.2±4.2	9.6±3.7	0.11
Creatinine (mg/dL)	0.88±0.19	0.99± 0.47	0.94± 0.30	0.38
LDL cholesterol (mg/dL)	118±33	103± 35	101±30	0.06
HDL cholesterol (mg/dL)	54±15	52± 17	41±8	<0.001
Triglyceride (mg/dL)	164±109	125±57	139± 57	0.09
Total cholesterol (mg/dL)	177±40	177± 36	171±31	0.11
Hemoglobin (gr/dl)	14.1±1.6	13± 1.0	13.0± 2.0	0.12
Platelets (×10^9/L)	252±58	238±54	233±63	0.34
Neutrophils (×10^9/L)	4.5±1.5	4.8±1.6	4.2±1.5	0.22
Monocytes (×10^9/L)	0.48±0.15	0.50±0.14	0.6±0.12	0.001
MHR	9.9±5.2	10.6±4.0	15.2±4.3	<0.001
EFT (mm)	4.49±1.59	5.47±1.81	6.57±1.50	<0.001

SD standard deviation, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *EFT* epicardial fat thickness, *MHR* monocyte to HDL-C ratio

significantly different in NDR, nonPDR and PDR groups (99.7±52.2; 106.1±40.5; 152.4±43.6, respectively p <0.001). One-way ANOVA test with post hoc Tukey test revealed that the significance of differences in MHR and EFT was dependent on the differences between NDR and PDR (p<0.001 for both) and nonPDR and PDR groups (p<0.001 and p=0.001, respectively). The differences between NDR and nonPDR (p=0.81 and p=0.06) were not significant. Pearson correlation analysis revealed that MHR was significantly positively correlated with PDR (r=0.453, p<0.001) and negatively correlated with nonPDR (r= −0.187, p=0.048) and NDR (r=−0.256, p=0.006). EFT was significantly positively associated with PDR (r=0.394, p<0.001) and negatively correlated with NDR (r= −0.380, p<0.001), and correlation with nonPDR was not significant.

Multiple logistic regression analysis enter stepwise model was also performed to define predictive value of variables for the presence of PDR. Monocyte, EFT, HDL and MHR values were included in this model. Only EFT was found to be independently associated with PDR (p=0.002, 95% CI: OR:1.643 (1.206–2.237). Receiver operating curve (ROC) analysis revealed that an EFT value of >5.90 mm classified the presence of PDR with a sensitivity 74% and specificity of 61% (AUC = 0.750, 95% CI, 0.658–0.843), and a MHR value of >12.8 ratio classified the presence of PDR with a sensitivity 83% and specificity of 79% (AUC = 0.811, 95% CI, 0.728–0.893) (Figure 1).

Discussion

The main finding of the present study is that MHR and EFT were significantly increased in PDR compared to both NDR and nonPDR, and MHR and EFT were not significantly different between NDR and nonPDR, and increased EFT is an independent significant predictor of the development of PDR in type 2 DM.

Microvascular complications of DM have been associated with inflammation and immune response [14, 15]. DR is also a complex microvascular complication of DM and involves inflammation and multiple events [16, 17]. Inflammatory cytokines like tumor necrosis factor (TNF)-a, interleukin (IL)-6, IL-1β) and intracellular adhesion molecule (ICAM) have been demonstrated in both serum and vitreal fluid of the subjects with DR [18]. Tumor necrosis factor (TNF-α), IL-6, resistin, visfatin, omentin, leptin, plasminogen activator inhibitor-1 (PAI-1) and angiotensinogen are produced by epicardial fat, since it is an active endocrine tissue [19, 20]. Elevated EFT is considered as a marker of inflammation and related with cardiovascular events [21]. Other studies in literature revealed association between DR and EFT [22]. Similarly, EFT was also associated with diabetic nephropathy which is another microvascular complication of type 2 DM [23]. Accordingly, we have found increased EFT in patients with PDR compared to those nonPDR and NDR. Only EFT (not monocyte, HDL or MHR) was an independent predictor

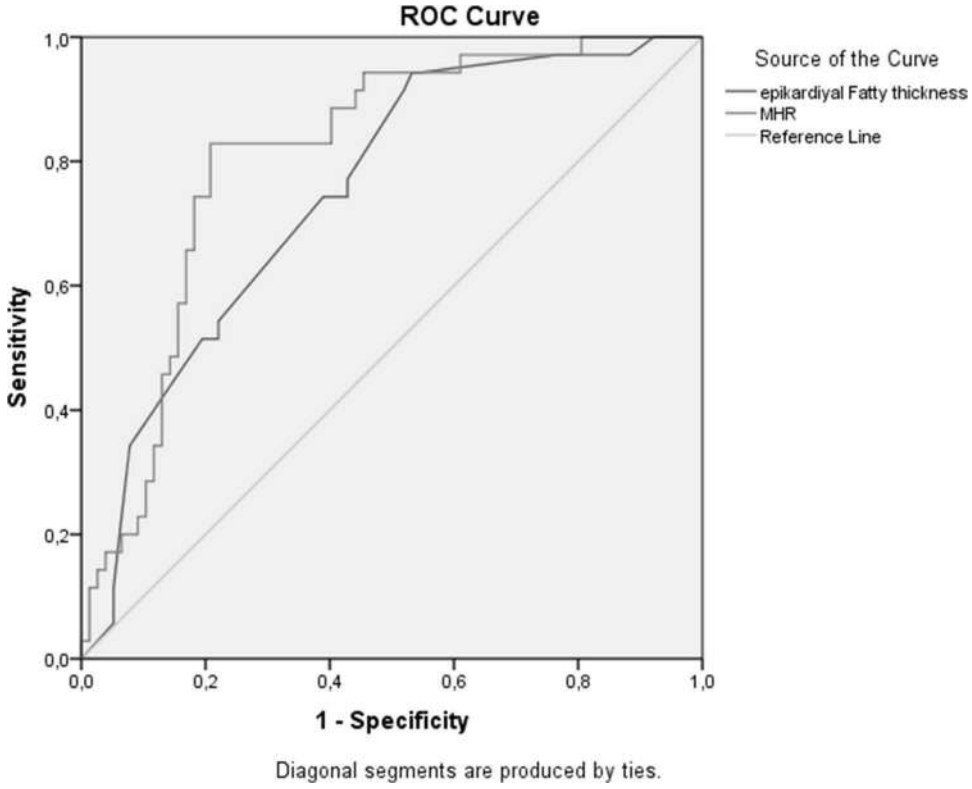

Fig. 1 Receiver operating curve (ROC) analysis was revealed that an EFT value of >5.90 mm predicted presence of PDR with a sensitivity 74% and specificity of 61% (AUC = 0.750, 95% CI, 0.658–0.843) and a MHR value of >128.8 ratio classified presence of PDR with a sensitivity 83% and specificity of 79% (AUC = 0.811, 95% CI, 0.728–0.893). AUC area under the curve, CI confidence interval

of PDR. Increase in thickness of epicardial fat might be associated with PDR through increased inflammation.

MHR has been suggested to be a better indicator of inflammation than leukocyte count [24, 25]. Recent studies showed that, MHR could be an inflammatory predictor [26, 27]. Indeed, monocytes are responsible of cytokine release in inflammation [28]. Interestingly, monocytes have been suggested to have a role in diabetic microvascular complications. [29]. HDL particles have been reported to be associated with anti-inflammatory effects by decreasing monocyte migration and accumulation, in the endothelium [30]. Accordingly, we found that MHR values greater than 12.8 were associated with PDR with a sensitivity and specificity (83% vs 79%, respectively) in patients with diabetes. Furthermore, we have found MHR to be significantly increased in PDR group compared to nonPDR and NDR groups. Since inflammatory burden of the PDR is higher than the other two groups, it may induce greater MHR values in subjects with PDR. Oxidative stress and inflammatory process are associated with increased monocyte count and decreased HDL levels [31]. Accordingly, our finding of increased EFT and MHR in PDR may be associated with inflammation. Increased EFT in PDR group could be a consequence of increased pro-inflammatory markers that are secreted from that tissue, which could trigger the development of PDR. However, prospective cohorts are needed to confirm our results.

Limitations of the present study include lack of measurement of serum levels of the inflammatory markers, such as, C-reactive protein, TNF, IL-6, IL-8, IL-1β, monocyte chemoattractant protein-1. Secondly, relatively small study population may make our results controversial. Thirdly, despite cardiac magnetic resonance is the gold standard for visualization of EFT, we just used echocardiographic imaging. There are limited data and limited study about association of MHR with EFT in diabetic retinopathy. Finally, we did not follow up the patients prospectively for adverse cardiovascular outcomes.

Conclusions

We suggest that MHR and EFT were significantly increased in proliferative DR and increased EFT may predict the presence of PDR in type 2 DM.

Author contribution Concept—A.S., I.S.,T.D.,M.C.; design—A.S.,I.S.,Y.G.,Y.A.; supervision—F.U.,U.D.,Y.G.; fundings—A.S.,T.D.,M.C.; materials—T.D.,Y.A.,F.U.; data collection and/or processing—U.D.,I.S.,M.C.,Y.A.; analysis and/or interpretation—A.S.,I.S.,Y.G.,M.C.; literature review—A.S.,I.S.,U.D.,M.C.; writing—Y.A.,I.S.,F.U.,U.D.; critical review—A.S.,İ.S.,Y.G.,F.U.

Declarations

Ethical consent After obtaining institutional consent and ethical approval (07.03.2019; application number: 2019/59), diabetic patients diagnosed to have DR were examined by transthoracic echocardiography in Cardiology clinic of our institution between April 2019 and August 2020 in this prospective cohort study.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Detection of exudates from clinical fundus images using machine learning algorithms in diabetic maculopathy

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Abstract

Purpose of the study As per the estimation of the World Health Organization, around 400,000,000 people may be affected by diabetes in the year 2014. It is rapidly increasing double by the upcoming year 2030. Diabetic mellitus, also called diabetes, can cause critical issues of retinal disease such as diabetic retinopathy (DR) which causes blindness. Exudates are considered as the major sign of diabetic maculopathy (DM) which may lead to visual disturbances.

Methods The main purpose is to develop a computer-aided technique for the detection of hard exudates to assist in the diagnosis of DR. It mainly focuses on the identification on the yellow lipids that include hard exudates. The diseased retinal image is given as an input to the classifier; it produces the output as exudates or non-exudates. In the proposed work, the classifier such as support vector machine and multilayer perceptron are used to find out the accurate prediction of exudates and non-exudates. First, the fundus images are allowed to preprocessing technique to get the filtered, contrast enhanced image. Second, the features such as blood vessel segmentation implements morphological operation for the detection of hard exudates by measuring the size of the lesion, and optic disc (OD) is measured and eliminated by comparing the parameters with the size of the lesions. Third, the segmented images are given as an input to the classifier such as SVM and MLP; it classifies and given as an output about the presence or absence of the exudates. In the proposed work, the experimental tests are carried out, and the results are verified on a different set of images.

Results The average accuracy values obtained using SVM and MLP classifiers for 140 images from real-time databases collected from Aravind Eye Hospital, Coimbatore are 88% and 95% respectively.

Keywords Diabetic maculopathy · Yellow lipids · Morphological operation · Hard exudates

Introduction

Diabetes causes severe damages in the main organs of the body such as the heart, liver, kidney, and eyes. The retinal eye disease caused by diabetes such as diabetic maculopathy (DM) and its advanced stage diabetic macular edema (DME) are the main cause of blindness for the people having diabetes more than 10 years. DR affects the retina by blocking or leakage of tiny blood vessels. The lesions are created by the outflow of lipids from the abnormal vessels [1]. The lesions in the retina are differentiated as hard exudates and soft exudates. The hard exudates (HE) are mostly located in outer retinal layer by lipid-filled macrophages and lipoprotein. The HE is developed by the cause of lipids segregated from the nearby capillaries and microaneurysms. It appears as clusters of spots which are in yellow color with different shapes and boundaries. Soft exudates appear as cotton wool spots with uncertain and blurred boundaries by blocking the retinal precapillary arterioles. These lesions are found in the nerve fiber layer of the retina. Drusen occur under the retina and are more often than not, a symptom of age-related macular degeneration (break of tissues in the rear of the eye). Exudates typically are inside, or immediately under the retina and usually associated with the retinal blood vessel problems. Drusen are small yellow deposits of fatty proteins (lipids) that accumulate under the retina. Drusen are likely similar to the exudates, but the drusen more often occur beneath the retina. Imaging tests such as fundus photographs and optical coherence tomography will distinguish between

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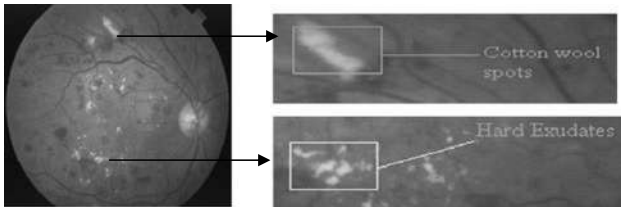

Fig. 1 Color retinal image with HE-cotton wool spots

the two conditions. The exudates such as hard and soft are shown clearly in Fig. 1.

The DR is categorized into retina in normal, diabetic retinopathy, non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR), and/or macular edema (ME). The severity stage of DR is categorized by the presence of microaneurysms (MS), hemorrhages (HR), and exudates (Ex). The regular screening of DR can reduce the risk of vision loss of patients. The presence of hard exudates (HE) is the major cause for blindness when they take place near to fovea or present on the fovea [2]. The DR can be diagnosed and analyzed by using the retinal image captured by the fundus camera. In many countries, for clinical practice, the fundus images which are all taken from the various parts of the countries are sent to examine the disease by a human expert or the ophthalmologists. For screening a mass population, it would be essential to buy more efficient fundus cameras and have to give more training for humans to become qualified experts. There is a strong demand for well-trained machine and human experts. To reduce the number of experts or the ophthalmologists' burden and to enumerate automatic detection of exudates in the fundus images becomes rising. In the fundus images, the lesions are in white color/yellow color soft structures of different pixel sizes. For instance, in the various dataset [3], the small lesions found only in a small quantity of pixels while the large one found to be in many of the pixels. It may have different shapes, different structures, and different intensities. Optic nerve fibers and the light reflections bounded inside the vessels may display the same appearance and it can bar the extraction of exudates regions.

As the disease progresses, continuous monitoring is essential to diagnose and to take the timely effective treatment. Manual segmentation of HE is quite difficult. The recognition of hard exudates in color retinal images takes part in an important role in diabetic maculopathy analysis and monitoring the next process of treatment. As a result, the proposed work mainly focuses on HE detection. HE detection is a prolonged process and vulnerable to viewer errors. The automatic finding of HE assists in achieving fast and precise analysis. This method of screening systems can diminish the workload of the experts [4].

The proposed work is more efficient and less expensive compared to the earlier stage of methods. The proposed method uses SVM and MLP classifiers for exudates recognition.

The main contribution of the paper is to identify the presence of exudates by preprocessing the fundus image; the blood vessels and optic disc are isolated. It is attempted to quantify retinal vessel damage and the cause of optic disc through various image processing techniques. Machine learning algorithms are introduced to classify the image as exudates or non-exudates. The preprocessed image is processed for segmenting the blood vessels, optic disc (OD), and exudates. After performing the above segmentation, machine learning techniques such as SVM and MLP are used to classify the detection of exudates. The overall workflow of the proposed work is shown in Fig. 2.

This paper is categorized as follows. "Related work" section explains the literature review on the existing works that engage segmentation of exudates and diagnoses using machine learning algorithms. In "Material and methods" section, it specifies the contribution of the proposed work to detect the exudates and explains the training methods proposed in this work. "Experimental evaluation" section gives the evaluation of experiments and results from the classifier. The conclusion and future enhancement are explained in "Conclusion" and "Future enhancement" sections.

Related work

The main aim is to develop the computational tools which aid in quantification and visualization of the anatomical structures of lesions. It includes analysis of blood vessels, analysis of optic disc, analysis of macula, and analysis of microaneurysms detection and exudates detection.

The dark and light structures are removed such as blood vessels and nerve fibers [3, 5], [6]. Texture feature is characterized to the local variance, and uses local binary pattern in the refining stage, to place the exudate region. The features of the exudates are extracted to classify the extreme level of the disease by using SVM classifier [7, 8]; the presence of

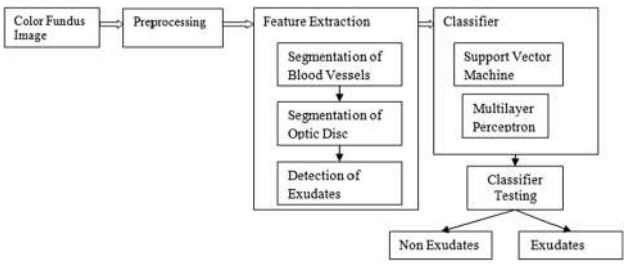

Fig. 2 Overall workflow of the proposed work

any abnormality for diagnosis of DR has been identified; and the global attributes of the image have been recorded [9]. A new model for the detection of exudates by using fuzzy logic techniques to diagnose DR by using morphological operation and fuzzy logic is proposed [10, 11].

MLP and radial basis function (RBF) for the final segmentation of exudates are used for extracting their features and separating hard exudates from background by using logistic regression [12].

The threshold is calculated by intensity based parameters of the image for extracting and eliminating OD effectively [13]. A database of 30 images of size 1504×1000 collected from the ophthalmology department and developed an automated system for the detection of cotton wool spots, and achieved sensitivity of 82.16% was implemented [14].

Deep CNN with high accuracy is implemented in [15]; it is flexible and works efficiently on retinal image data. It does not require feature removal and pre-training. It has some disadvantages such as it uses black box approach, and it slows down the training speed if the GPU is not good.

Some of the related works use CAD-based technique [5, 16] for classification and detection of DR. It identifies the differences in normal and affected eye images to frame feature space. These features specify the exact recognition of diabetic maculopathy.

The computer-aided DR method is evaluated by various methods, such as neural network [17], pattern recognition [18], and Gabor filter [19], mainly focused on blood vessel segmentation. The detection process becomes complex due to various tools and approaches. Annunziata et al. [20] segmented the vessel detection and exudates using a neighborhood estimator. A voxel classification [21] is introduced which is based approach using OCT image for layer-dependent sampling strategy. The grayscale morphology [22] is used for automatic segmentation of exudates by candidate extraction. By using the Markovian segmentation model, the appropriate contour of candidate pixels is resolved.

The main contribution of the proposed technique is preprocessing; blood vessel, optic disc, and exudates segmentation; and classification using SVM and MLP classifier. Image resize, median filtering, and CLAHE are used in pre-processing. The image is thresholded using binary thresholding in the segmentation phase. Feature extraction is done by circular Hough transform for the segmentation of optic disc, which is later subjected to neural network for exudate detection.

Material and methods

Retinal image databases

The retinal images are more essential for the development of computer-aided models for the detection of retinal-related disease. The proposed method was implemented and tested on real-time datasets of retinal images collected at Aravind Eye Hospital, Coimbatore, Tamil Nadu. The database contains 140 color fundus retinal images with 90° field of view (FOV). Each image has the resolution of 96 dpi, 3388×2588 pixels in JPG format. These images are captured by using Topcon trc-50dx retinal camera, and the proposed work uses the images with the ages from 20 to 70 years. It contains 95 positive images with soft exudates and/or hard exudates, for which the optic disc is also available. The other 50 images are normal and with other lesions.

Preprocessing

Preprocessing is very essential to enhance the quality and feature of the image. Basic preprocessing includes image resize, filtering, and enhancing the image. All the collected retinal images were resized into 128×128 pixels. In the proposed work, median filter is used to smoothen and remove the unnecessary pixels in the image. Here, the image is split into small regions. This filter recognizes the noisy pixels within every small region and replaces the noisy pixels with the median of the nearest pixels within the regions. To enhance the filtered image, the output of the filtered image is given as the input to the CLAHE, to get the enhanced images by transforming the pixel values into its intensity of images. Figure 3 shows the preprocessing technique which consists of image resize, median filter, and CLAHE.

Retinal blood vessels extraction

Retinal blood vessels act as significant role in the identification of exudates. After the preprocessing step, the enhanced image is applied to morphological operation to extract the blood vessel from the CLAHE; image is shown in Fig. 4.

Morphological operation is accomplished by combining the kernel and the pixel values of the enhanced image. The morphological opening and closing operation is processed for three times in different kernel sizes. First, the kernel size and shape are chosen as 5×5 and elliptical, respectively. Here, the

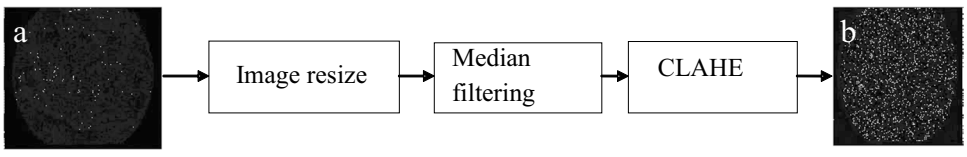

Fig. 3 Workflow of the preprocessing method

kernel slider moves in and around the image to perform opening and closing operation. Again, kernel size is set to 11×11 and 23×23 for the second and third time, respectively, and the shape of the kernel is again chosen as elliptical. Backgrounds are eliminated by performing the subtraction of the morphological image from the contrast image enhancement. The resultant image is then given to CLAHE to improve the contrast of the image. This resultant image is given as the input for binary thresholding. Here the contours are detected by find contour and draw contour functions. Inverse binary thresholding is performed to get the clear view of the segmented blood vessels. The unnecessary chunks has been removed by appluing the bitwise_NOT to the eroded image, such that the contour can be detected. Again, it is processed to bitwise and bitwise_NOT to extract the blood vessel. Figures 5 and 6 show the step-by-step process of how the blood vessels are extracted from color fundus image.

Optic disc localization

It is considered as a main stage in the aspect of computer-aided recognition in retinal screening, for the reason that OD has as much as the same properties of exudates such as color and brightness. The optic disc position helps to identify whether the retinal images are left side or right side of the eye. The OD has the characteristics of a bright yellow disc in the retinal image. OD is vertical oval in shape with mean dimension of 1.76 mm horizontally by 1.92 mm vertically. The OD disc is masked from the fundus image before the exudates' detection. The false responses are avoided by detecting the localization of the disc such that if the optic disc is not segmented, the probability of the optic disc is wrongly identified as the lesion. OD localization is quite simple in normal retinal images, and it is the challenging task in the exudates' affected images, because it has the large area of the bright lesions [22]. In the proposed work, the OD localization phase includes the evaluation of the retinal blood vessels segmentation, center of the optic disc localization, and OD segmentation. Figure 7 shows the proposed work of the localization of OD.

Optic disc segmentation

Optic disc segmentation plays a significant role in the analysis of the extraction of exudates. Extraction of the OD is the first step for computer-aided diagnosis which helps to assist the ophthalmologist in detecting DR. OD methods are considered by identifying the OD properties such as intensity, shape, or size. To identify the OD boundary, the size, $m \times n$ was calculated based on the center of the OD

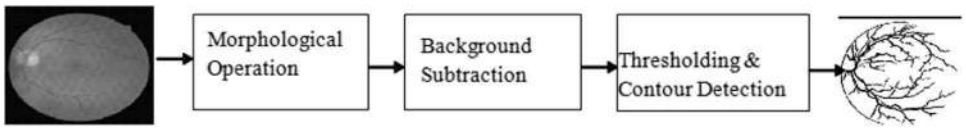

Fig. 4 Proposed workflow for the segmentation of blood vessels

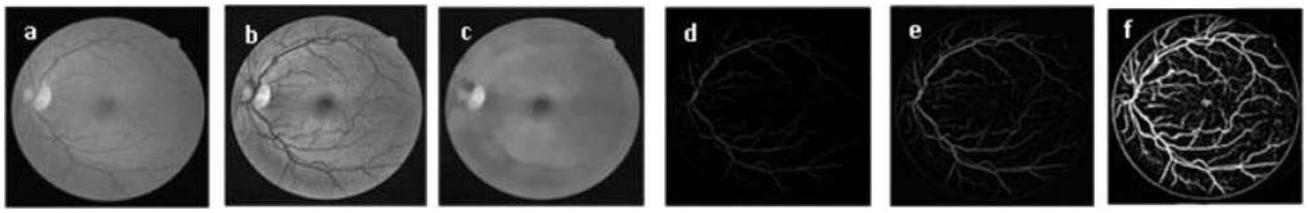

Fig. 5 **a** Color fundus image. **b** Contrast image enhancement. **c** After performing morphological operation. **d** Background subtraction. **e** Image enhancement. **f** Thresholding binarization

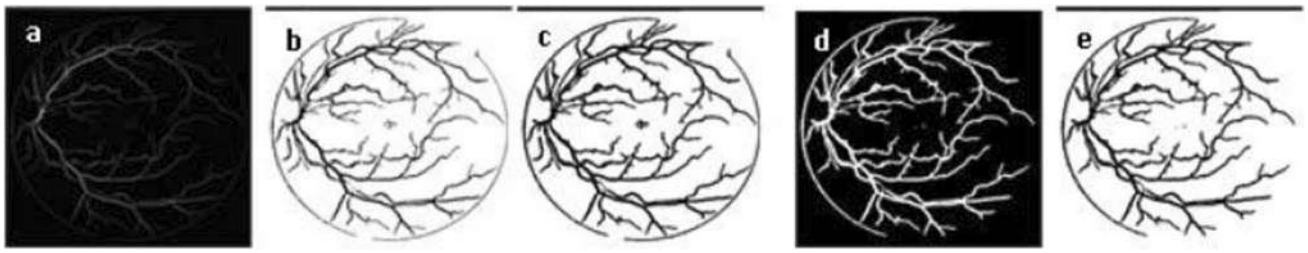

Fig. 6 **a** After masking. **b** After binarization. **c** After erosion. **d** Contour identification. **e** After bitwise operation

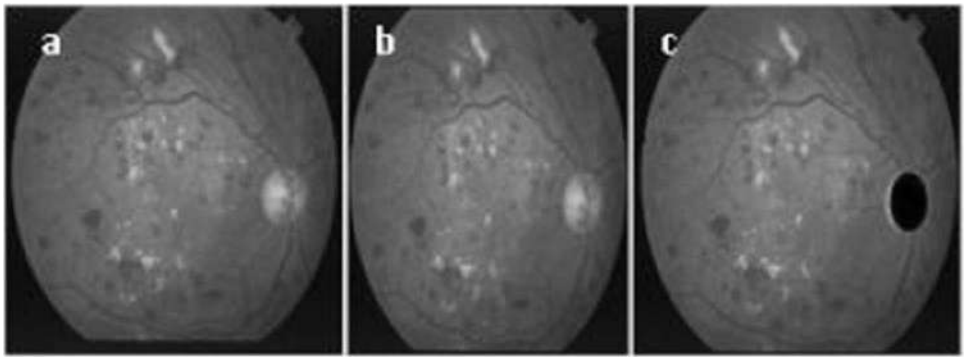

Fig. 7 OD localization. **a** OD center localization. **b** Segmentation of OD. **c** Masking of OD

localization. Circular Hough transform was performed to extract the boundary of the OD due to its circular boundary shape [23].

Detection of hard exudates

The injured blood vessels may leak yellowish fluid and a few amounts of blood into the eye in the advanced stage of NPDR [24]. The result of vascular damage leads to leakage of yellowish lipid identifies the presence of exudates. The count of exudates increases when the disease progresses.

Preprocessing for retinal images is performed by splitting the RGB channel into red, green, and blue channels and applying CLAHE for contrast enhancement. Binary threshold is performed for the red and green channels of the color fundus image, such as minimum threshold value as 127 and maximum threshold value as 255. Exudates are yellow lipid, so red and green channels are used to identify the yellow pixel value. Bitwise Bitwise_AND operation is performed for both the thresholds of the red and green channels to get the yellow color. The morphological dilation and erosion operation is performed by structuring elements or kernels. The kernel size is chosen as 7×7 with all ones. Dilation is performed for the output of bitwise_and operation. Now, the kernel slider moves around the image and repeats for four times. The output of the dilation process is given as the input to the erosion operation; it repeats the loop up to ten times to get the clear image of the exudates. The output from the erosion is given as the source image of the canny edge detection method; the upper and lower threshold values chosen here as 200 and 30 respectively.

Find and sort contours are based on their sizes by using sorted function. While sorting, it is possible to find the maximum area that may be an optic disc. Optic disc is found out by the specific radius and eliminates it by using mask operation. The contour is drawn for the sorted array in which the green channel is specified with the thickness 3. Thus, the exudates are identified. Figure 8 shows the original fundus image, OD with exudates, and the elimination of OD.

SVM classifier

A support vector machine (SVM) is a powerful technique, which performs linear or non-linear classification, regression, and outlier detection. It is the best for the classification of small or medium datasets. It is based on supervised learning methods, which is used to analyze the training data to find an exact solution to classify the images into normal or the presence of the exudates. After the extraction of blood vessels and OD, the normal and abnormal images are separated by SVM classifier. This classifier minimizes the risk and prevents the over-fitting problem. Based on the result, a better performance may be achieved. After extracting the regions of the blood vessel and optic disc in the color fundus images, the segmented images are ready to process using the SVM classifier. It is used for analyzing the training data and also determining the various stages of exudates.

The segmented features of the normal and abnormal images are combined and saved in a matrix form. This matrix is given as the input to the SVM classifier, and the training output is also saved as in the form of the matrix such that it is used for the testing. For testing, the test image features are segregated and combined to form a matrix. Before giving the input of the test image, the training mode is loaded to the SVM classifier to find the severity of the disease. This SVM classifier architecture consists of three layers which include input, hidden, and output layers. Figure 9 represents the architecture of the SVM classifier.

SVM classifier classifies the normal and abnormal images by using the kernel function. The output of the abnormal images is the presence of exudates.

The SVM classification is performed by python language and is executed in TensorFlow. The proposed work is based on non-linear classification and works on mathematical function defined as kernel. In general, the kernel takes the input as data and transforms it in the required form. There are different types of kernel available, but every kernel carries the non-linear kernel function. They are as follows:

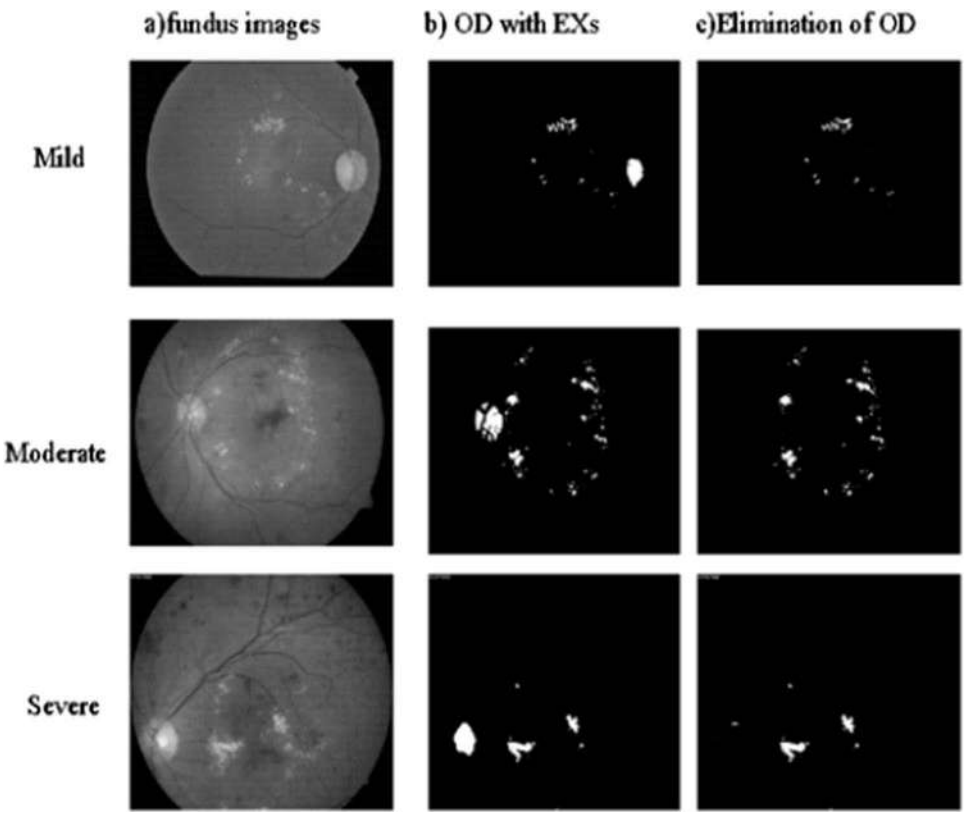

Fig. 8 Removal of OD and the segmentation of exudates

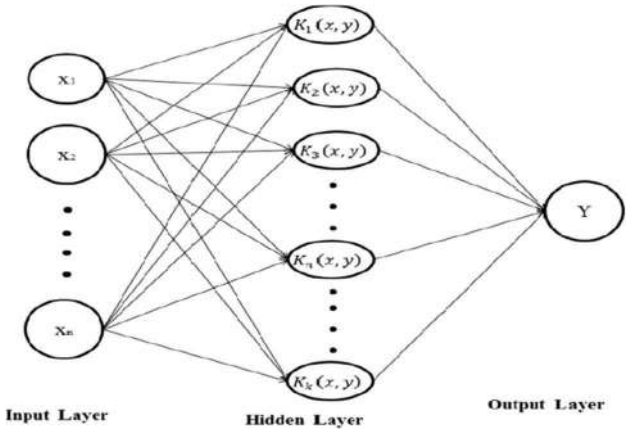

Fig. 9 Architecture of the SVM classifier

Polynomial kernel

It is a homogeneous kernel and is represented by the following:

$$k(\vec{x_i}, \vec{x_j}) = (\vec{x_i}.\vec{x_j})^d \tag{1}$$

where $k(x_i, x_j)$ represents the kernel function, x_i and x_j are the feature space vectors, and d is the degree of the polynomial function.

Radial basis function kernel (RBF kernel)

Here, square of Euclidean distance is used and it is only for non-linear hyperplanes.

$$k\left(x, x^{'}\right) = \exp(-\frac{\left|\left|x - x^{'}\right|\right|^2}{2\sigma^2}) \tag{2}$$

where x and x' represents feature space vectors and σ is a parameter for free. The typical parameter value may direct to overfitting of the data.

Polynomial kernel

It is the non-homogeneous kernel and is represented as follows:

$$k(x, y) = (x^T y + c)^d \tag{3}$$

where "c" is a constant term, represented for a free parameter. x and y are the feature space vectors. D represents the degree of the polynomial function.

In this research, a polynomial kernel is used, which is popular for image processing. The degree of the polynomial chosen here is 3. Some of the properties of SVM classifier are complexity of training, model selection, classification accuracy, and storage and execution complexity.

MLP classifier

A feed forward artificial neural network (FFNN) class is normally referred as multilayer perceptron (MLP). It consists of three or more layers such as input, output, and one or more hidden layers, where each layer gives the input to the other layer. Except the input layer, the activation function for the nodes in all the layers is the nonlinear function. Every hidden layer consists of many perceptrons which are called hidden units. A single hidden layer consists of array of perceptrons. The output from the hidden layer is formulated as follows:

$$f(x) = G(W^T x + b) \tag{4}$$

Each node is termed as a neuron, which utilizes a nonlinear activation function, except the input nodes. The activation of softmax can be expressed by using the following formula:

$$\mathrm{Softmax}\left(x_i\right) = \frac{\exp(x_i)}{\sum_j \exp(x_j)} \tag{5}$$

where x_i represents the ith element given as an input to softmax, which corresponds to the class i, and j represents the number of classes specified. The cross entropy of the function of a softmax is calculated as follows:

$$\mathrm{loss} = -\log \frac{e^a \mathrm{Correct}}{\sum_i e^{a_i}} \tag{6}$$

It uses a supervised learning technique called backpropagation for training. It is differentiated from linear perceptron by consisting of multiple layers and non-linear activation. Figure 10 shows the architecture of a multilayer perceptron.

Neural networks mostly preferred a training algorithm called stochastic gradient descent. In this algorithm, the input is taken as the row of data reveals to the network at a time. The network processes exposed input by activating the neurons upward as it repeats until it produces an output value. This is referred to as a forward pass. The network is trained to predict the new data, the forward pass used.

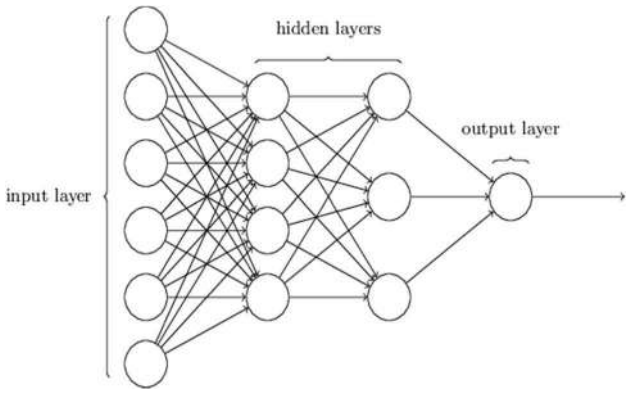

Fig. 10 Architecture of the MLP classifier

An error is calculated by comparing the network output with the expected output. If the error occurs, it propagates back through the network, exactly by one layer, and its weights are adjusted by maintaining how much amount they contributed to the error. This is called a backpropagation algorithm. This process is repeated until all of the images in the training data. Epoch is used for updating the network once for the entire training. The network can be trained for ones, tens, hundreds, or many thousands of epochs. The weight of the output gradients is calculated by the following:

$$\frac{\partial E}{\partial W_{kj}} = \sum\nolimits_{n=1}^{n} \partial_k^{(h)} h_j^{(n)} \tag{7}$$

where ∂_k is the error with respect to the net input for the unit k. After hidden gradient weights are computed through backpropagation by using the following formula:

$$\frac{\partial E}{\partial h_j^{(n)}} = \sum\nolimits_k \partial_k^{(h)} W_{kj} \tag{8}$$

$$\frac{\partial E}{\partial V_{ji}} = \sum\nolimits_{n=1}^{N} \bar{\partial}_j^{(n)} x_i^{(n)} \tag{9}$$

The weights can be updated from the errors calculated for each training is called as online learning. The errors can be saved for all the training images and at the end, the network can be updated. This is called batch learning. Predictions can be performed on test data or validation data, to estimate model on unseen data. It can be deployed operationally and make predictions continuously.

Experimental evaluation

In the emerging medical field, ophthalmologists have to handle large amounts of retinal fundus images for the detection of exudates. An automated segmentation technique makes it easy for the experts to analyze the retinal disease faster. The proposed method automatically segments the exudates in retinal fundus images in the progression of retinal disease.

A total of 140 images were collected from a real-time dataset collected from Aravind Eye Hospital, Coimbatore. To train SVM and MLP classifiers, 13 normal retinal images, 17 abnormal images, 68 exudates-affected retinal images, and 42 images were used. For testing, 8 normal images, 12 abnormal images, and 22 exudates-affected retinal images were used. The images which are separated for training and testing are shown in Table 1.

Table 1 Number of images for training and testing

Images	Normal	Abnormal lesions	Exudates	Total
Training	13	17	68	98
Testing	8	12	22	42
Total	21	29	90	140

Segmentation may vary naturally based on quality of the retinal image and methodological view of the experts. In the proposed technique, there is certain statistical performance measures used to obtain the effectiveness of automated identification of the exudates used.

Sensitivity finds the probability of retinal images with exudates. It is determined by the ratio of number of correctly detected exudate images to the total number of retinal fundus images.

True positive (TP) represents the number of correctly identified exudate images and false negative (FN) represents the number of exudate images identified wrongly as non-exudate images.

$$\text{sens} = \frac{TP}{TP + FN} \tag{10}$$

Specificity finds the retinal image is not affected by the exudates. It is determined by the ratio of the number of correctly classified non-exudate images identified wrongly as non-exudate images.

True negative (TN) refers to the number of non-exudate images identified correctly and false positive (FP) refers to the number of normal images that are identified as DR images.

$$\text{spec} = \frac{TN}{TN + FP} \tag{11}$$

Accuracy is determined by the ratio of calculating the total number of retinal images determined correctly to the total number of retinal images taken for the classification.

$$\text{Acc} = \frac{TP + TN}{TP + TN + FP + FN} \tag{12}$$

Precision is determined by the ratio of correctly classified exudate images to the number of non-exudate images identified wrongly as the exudate images.

$$\text{Precision} = \frac{TP}{TP + FP} \tag{13}$$

F-score is used to measure the trade-off between precise prediction and avoiding false negatives. It is based on the arbitrary, which depends on the classifier. It is calculated by the ratio of the average of precision and the true positive rate.

$$F - \text{score} = \frac{(2 * TP)}{(2 * TP + FP + FN)} \tag{14}$$

Table 2 Computation of statistical parameters

Parameters specified	Total no. of images	
	SVM	MLP
TP	19	21
TN	18	19
FP	3	1
FN	2	1

Table 3 Computation of evaluation of metrics

Evaluation metrics	Values	
	SVM	MLP
Sensitivity	0.86	0.95
Specificity	0.90	0.95
Accuracy	0.88	0.95
Precision	0.90	0.95
F-score	0.88	0.95

Table 4 Comparison of result identified

Image no	Result by experts (annotated)	Result by SVM	Result by MLP
DR009	Normal	Normal	Normal
DR042	Abnormal	Abnormal	Abnormal
DR055	Normal	Abnormal	Normal
DR056	Exudates	Exudates	Exudates
DR069	Exudates	Exudates	Exudates
DR078	Abnormal	Exudates	Exudates
DR076	Exudates	Exudates	Exudates
DR080	Exudates	Abnormal	Abnormal
DR101	Exudates	Exudates	Exudates
DR142	Abnormal	Normal	Abnormal

The test performances of SVM and MLP classifiers are calculated by the computation of the statistical parameters shown in Table 2.

In Table 3, the metrics of the proposed classifier are evaluated and shown.

Table 4 represents the results given by the experts for every image, and the result achieved by the proposed system such as SVM and MLP is noted for the respective image. Here, the image DR055 is actually a normal image, but SVM predicts that the image DR055 as abnormal. The image

Table 5 Confusion matrix

	SVM			MLP		
	Normal	Abnormal lesions	Exudates	Normal	Abnormal lesions	Exudates
Normal	7	1	0	8	0	0
Abnormal lesions	1	9	2	0	10	2
Exudates	0	2	20	0	2	20

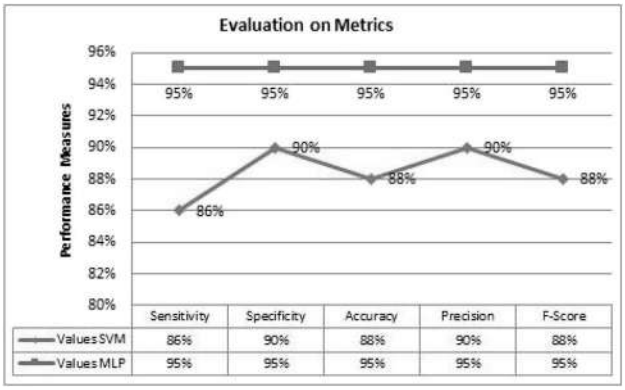

Fig. 11 Graphical representation of performance measure

DR078 is actually an abnormal lesion, but SVM and MLP predict that the image DR078 as exudate image.

A confusion matrix is for describing the performance measures of test data for which the true values are known, as shown in Table 5.

In SVM, 12.5% of normal images were screened wrongly as abnormal lesions, whereas 8% and 16% of abnormal lesions are wrongly detected as normal and exudate images respectively. Finally, 9% of exudate images are wrongly screened as abnormal lesions.

In MLP, 100% of normal images were screened correctly as normal images, whereas 16% of abnormal lesions are wrongly detected as exudate images. Finally, 9% of exudate images are wrongly screened as abnormal lesions.

The average accuracy values obtained using SVM and MLP classifiers for the abovementioned database are 88% and 95% respectively. The precision and *F*-score values obtained for SVM are 90% and 88% respectively. Sensitivity and specificity values are obtained for SVM as 86% and 90%, respectively. The precision and *F*-score values obtained for MLP are 95% and 95% respectively. Sensitivity and specificity values obtained for MLP as 95% and 95% respectively. The graphical representation of the performance measure is shown in Fig. 11.

In the existing work [25], the achieved accuracy is about 90% by using FCM classifier of 142 images for detecting the exudates. Nayak et al. achieved sensitivity is about 90%, specificity is about 100%, and accuracy is about 93% with 140 images by using neural network. Table 6 shows the comparison of related work with the proposed work. Figure 12 shows the graphical representation of comparison of related existing work with the proposed work.

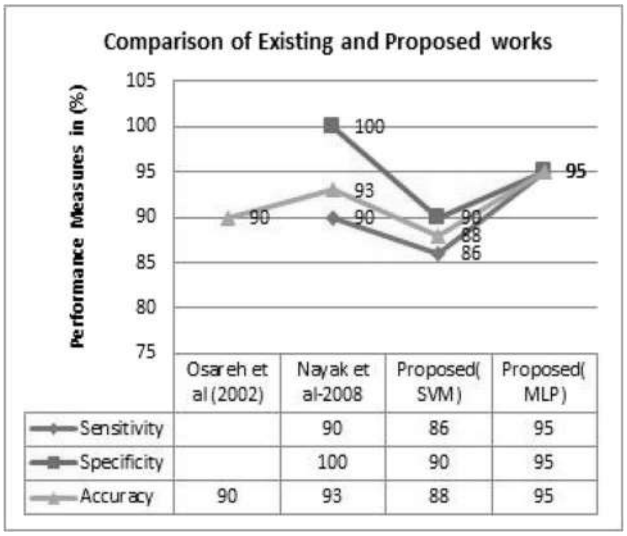

Fig. 12 Comparison of existing and proposed works

Table 6 Comparison with existing methods

Related works	No. of images	Feature detection	Classifier used	Sensitivity	Specificity	Accuracy
Osareh et al. (2002)	142	MAs, HAs, exudates	FCM	-	-	90.10%
Nayak et al. (2008)	140	EXs, BV, MAs	NN	90%	100%	93%
Proposed	140	MAs, HAs, exudates	SVM	86%	90%	88%
			MLP	95%	95%	95%

Conclusion

The retinal fundus image is preprocessed to remove the noises. The given image is identified to be exudate or non-exudate images by features of exudates mentioned. The optical disc is extracted using Hough transform, and the blood vessels were extracted by morphological operations. If the resultant image is affected by the exudates, then the exudates were segregated from the images by segmentation methods. The performances are measured using statistical parameters such as specificity, sensitivity, and accuracy. The proposed SVM and MLP classifier results in 88% and 95% of accuracy.

Future enhancement

This method is quite simple, it detects disease faster, and it works efficiently even on a poor illumination system. The proposed work can be further enhanced by using the large training dataset. It can be further analyzed with different classifiers with different diseases by different segmentation methods. Drusen and exudates are appeared to be similar; the detection and differentiation of the exudates, drusen, and cotton wool spots are to be performed in future. Diabetes may cause hypoxia and ischemia of the retina due to blockage of the microvasculature. This induced the formation of new vessels which can be termed as neovascularization. This is more detrimental to the vision on a long term. Our machine does not have the ability to detect the new blood vessels. In future, it can be analyzed to predict the new blood vessels earlier to prevent from the vision loss.

Abbreviations DR: Diabetic retinopathy; DM: Diabetic maculopathy; OD: Optic disc; DME: Diabetic macular edema; HE: Hard exudates; OCT: Optical coherence tomography; NPDR : Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; ME: Macular edema; MS: Microaneurysms; HR: Hemorrhages; Ex: Exudates; HE: Hard exudates; FOV: Field of view; RBF: Radial basis function; CLAHE: Contrast limited adaptive histogram Equalization; SVM: Support vector machine; MLP: Multilayer perceptron; FFNN: Feed forward neural network

Author contribution NIL.

Data availability and material NIL

Code availability NIL.

Declarations

Competing interests The authors declare no competing interests.

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ORIGINAL ARTICLE

Expression profile of microRNAs may be promising in diagnosis of proliferative diabetic retinopathy: an Egyptian study

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Abstract

Purpose Diabetic retinopathy (DR) is still a leading cause of blindness. The role of miRNAs in diabetic retinopathy still needs more research. We aimed at validating the role of circulating miRNAs: 21,181C and 1179 in early detection, dynamic monitoring, and management of DR.

Methods Whole blood samples were collected from 180 diabetic patients and 60 normal individuals as control. The diabetic patients were subdivided into 60 subjects without retinopathy, 60 with non-proliferative diabetic retinopathy (NPDR), and 60 with proliferative diabetic retinopathy (PDR). Gene expression of miR-21, miR-181c, and miR-1179 were estimated in each sample using two-step reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

Results MicroRNA 181c and miRNA 1179 were significantly higher among PDR group compared to NPDR,W DM and control groups, while no significant difference was detected regarding miRNA 21. The areas under the receiver operating characteristic (ROC) curves of the validated two-serum miRNAs were 0.983 and 0.927 respectively. Combination of miRNA 1179 and miRNA 21 improved the accuracy rate to 90%. Combination of miR-181c and miR-1179 possessed high ability to discriminate between PDR and NPDR with an accuracy rate of 100%.

Conclusion MicroRNAs play a role in pathogenesis of diabetic retinopathy. In our study, miR-181c and miR-1179 were significantly high in PDR patients compared to NPDR and controls hence can be used to anticipate and follow up the progression. MicroRNA antagonists or mimics can be tried as new medications to modify DR by reducing the rate of progression, and subsequent blindness.

Keywords MicroRNAs · Proliferative diabetic retinopathy · miRNA181c · miRNA1179 · miRNA21

Introduction

Diabetic retinopathy (DR) is a common complication of DM and it may lead to complete blindness. It has long been known as a microvascular disease [1]. Clinically, DR has two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is the early stage and characterized by increased vascular permeability and the patient may be asymptomatic. PDR, more advanced stage, is characterized by neovascularization. The patient may complain from severe vision impairment because of vitreous hemorrhage or tractional retinal detachment [2]. Vascular endothelial growth factor (VEGF) and hypoxia-induced factor 1 (HIF-1α) are important factors for angiogenesis condition [3, 4].

MicroRNAs (miRNAs) are a class of highly conserved 19-25 nucleotide noncoding RNAs that regulate gene expression at the posttranscriptional level [5]. Recently, miRNAs have been demonstrated to play an important role in diabetes and its complications [6]. Serum miR-21, miR-181c, and miR-1179 levels could be sensitive and cost-effective biomarkers for the early detection of proliferative DR (PDR). Thus, investigation into the circulating levels of miRNAs in samples obtained from patients with different stage disease is guaranteed [7].

miR-181c targets many signaling pathways and cellular processes that are critical in diabetes-impaired angiogenic responses to ischemia. This includes HIF-1α stabilization and VEGFA expression in the early phase. In the late phase, it

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promotes (phosphatase and tensin homolog) PTEN inhibition and collagen dysregulation [8]. miR-21 was reported to target PTEN, leading to activation of HIF-1α and VEGF expression [9]. Also, miR 1179 has been listed as candidate miRNA being over expressed in cases of diabetic retinopathy [10]. Although recent studies have revealed a link between the expression of miRNAs and the development of DR, these studies mainly focused on miRNAs expressed in tissues of animal model. Few studies have specifically examined the role of miRNAs in DR patients [11]. It is likely that deepening our understanding in this field, and hopefully, more experiments to prove evidence for the correlation between miRNAs and DR are needed in the future [12].

In this study, we aimed to investigate the role of plasma (miR181c,miR1179 miR-21) in the pathogenic process and in diagnosing the severity of DR. Our study might provide a valuable reference for the clinical diagnosis of patients with DR.

Patients and methods

This prospective comparative study was conducted at Departments of Ophthalmology and Medical Biochemistry & Molecular Biology, Faculty of Medicine, Benha University, in the period between May 2015 and April 2019. This study protocol was approved by the Local Ethical Committee at Benha University. The study included 240 Egyptian adults (180 diabetic cases, 60 control) and the sample size was calculated using EPI-Info (Epidemiological information package) software version 7.2.4.

- 180 diabetic cases were further subdivided into three groups:
- **Group I**: DM group without retinopathy (WDM) included 60 patients diagnosed according to World Health Organization (WHO) guidelines [13] and the diagnosis validated at recruitment by patient's history and review of the medical records. The exclusion criteria were gestational diabetes and severe advanced diabetic complications defined as being registered blind.
- **Groups II and III**: diabetic retinopathy groups included 60 patients with non-proliferative diabetic retinopathy and 60 patients with proliferative diabetic retinopathy.

Retinopathy diagnosis was made by clinical examination done by a retina specialist, fluorescein angiography (FA), and macular optical coherence tomography (OCT) of both eyes. The worst eye defined the level of retinopathy [14]. The exclusion criteria were as follows: history of previous laser photocoagulation, presence of cataract, glaucoma, history of eye trauma or previous ocular surgery. None of these patients was on anti-vascular endothelial growth factor (anti-VEGF) or laser therapy.

- **Group IV**: for comparative purposes, the study also included 60 normal individuals of cross matched age attending the diabetic clinic to have blood glucose testing and proved to be non-diabetic. They gave blood samples as control group.

All study individuals signed written fully informed consent including the investigations to be performed.

Measurements

Measurements

The following data were recorded and coded: age (years), sex, height, weight, body mass index (kg/m^2), duration of DM, and type of DM (IDDM &NIDDM). Additionally, HbA1c (%) was measured at recruitment.

Blood sample collection

Peripheral venous blood samples were collected in vacutainer tubes containing EDTA under complete aseptic conditions then immediately frozen at −80°C till molecular assay of microRNA was done according to manufacturer's instructions.

Total RNA extraction

Total RNA was extracted from whole blood samples using RNeasy kit; Qiagen, Valencia, CA, according to manufacturer's instructions.

Molecular assay of microRNAs

Two-step RT-PCR for hsa-miR-21, hsa-miR-181c, and has-miR-1179 by real-time PCR was performed using Qiagen miScript preAMP RT-PCR kit (Qiagen GmbH Hilden, Germany) for conversion of microRNA to cDNA in a G-storm thermocycler (UK). Then, amplification and quantification of RNAs was done by real-time PCR in ABI7900 (Applied Biosystem, USA) using SuperReal Premix Plus Quanti Tect. Kit, SYBR Green (Tiagen, Shanghai) according to manufacturer's instructions and using the specific primers for each. Real-time cycler conditions were 95°C for 15 min for initial denaturation, followed by 40 cycles of 95°C within 30 s for

denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for extension step. The target sequences were hsa-miR-21, hsa-miR-181c, and has-miR-1179, and the calibrator sample is normal lens. The identification of suitable housekeeping genes is a crucial step for deriving reproducible results when investigating the differential expression of miRNAs [15]. The reference gene (housekeeping gene) was RNU6B (U6) as it is the most commonly used gene to normalize miRNA RT-qPCR data [16].

Sequence name	Forward primer	Reverse primer
miR-21 were	5′- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TC -3	5′- ACA CTC CAG CTG GGT AGC TTA TCA GAC TGA -3′
miR-181c	5′- ACA CTC CAG CTG GGA ACA TTC AAC CTG TCG -3′	5′- CTC AAC TGG TGT CGT GGA GTC GGC AA T TCA GTT GAG ACT CAC CG -3′
miR-1179	5′-ACA CTC CAG CTG GGA AGC ATT CTT TCA TT-3′	5′-AAC GCT TCA CGA ATT TGC GT -3′
RNU6B	5′- CTC GCT TCG GCA GCA CA -3′ [15]	5′-AAC GCT TCA CGA ATT TGC GT -3′ [17]

The threshold cycle (CT) served as a tool for the calculation of the starting template amount in each sample, and gene fold expression changes were calculated using the equation $2^{-\Delta\Delta ct}$ and because of the relative nature of quantification using the $2^{-\Delta\Delta ct}$ method, adjustment was required for each sample. Briefly, cDNA was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}-fold prior to amplification by real-time PCR and a standard curve was derived in order to obtain optimal amplification conditions [18].

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 16 software (Spss Inc, Chicago, ILL Company). Categorical data were presented as number and percentages, using Fisher's exact test (FET) for their analysis. Continuous data were expressed as mean ± standard deviation, median, IQR, and range. Data were tested for normality using Shapiro-Wilks test, assuming normality at $p>0.05$. Differences between groups were tested using ANOVA (F test) for normally distributed variables or Kruskal-Wallis test for non-parametric ones. Significant ANOVA or Kruskal-Wallis was followed by post hoc multiple comparisons using Bonferroni test to detect the significant pairs. ROC curve analysis was used to detect cut-off values for the studied miRNAs with optimum sensitivity and specificity in prediction of PDR. The accepted level of significance in this work was stated at 0.05 (p <0.05 was considered significant).

Results

This study included 240 cases; 60 patients had PDR, 60 patients had NPDR, 60 diabetic patients without diabetic retinopathy, and 60 non-diabetic individuals as a control group. Details of the socio-demographic characters of enrolled patients and controls are shown in Tables 1, 2, and 3, which showed statistically significant increase in the duration of DM (13.5 ±5.4 years) in PDR group compared to diabetic patients without retinopathy (WDM group) (8.5±3.02 years). There were non-significant differences between them regarding age, weight, height, BMI, HbA1c, sex, and type of DM.

Comparing the groups regarding the studied markers showed that the mean values expressed in FRU of miRNA181C and miRNA1179 were significantly higher among PDR group (26654.4 ± 12857.8 and 22202.4 ±

Table 1 Comparison of the studied groups regarding socio-demographic characters

Variable	Controls (n=60)			DM (n=60)			NPDR (n=60)			PDR (n=60)			ANOVA (F test)	p
	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range		
Age (ys)	47.1	10.3	29–65	50.9	9.6	34–65	48.9	8.2	41–65	44.9	8.2	33–57	**0.78**	**0.51 (NS)**
Weight (kg)	82.0	4.92	75–89	88.7	10.31	75–105	90.0	9.88	74–102	89.1	7.18	77–100	**1.93**	**0.14 (NS)**
Height (m)	1.66	0.06	1.57–1.75	1.66	0.10	1.54–1.83	1.67	0.08	1.55–1.8	1.67	0.07	1.55–1.8	**0.028**	**0.99 (NS)**
BMI (kg/m^2)	29.6	3.16	25.3–34.4	32.4	5.88	23.3–44.3	32.5	5.71	22.8–42.4	32.0	3.78	26.3–39.5	**0.78**	**0.51 (NS)**
Duration of DM (Ys)	----------			8.5	3.02	4–13	12.2	2.09	9–15	13.5†	5.40	7–25	**4.73**	**0.017 (S)**
HbA1c	----------			7.79	2.78	3.9–12.1	7.30	2.80	4.1–12.2	8.70	2.56	5.2–12.2	**0.68**	**0.51 (NS)**

†Significant in comparison with the DM

Table 3 Comparison of the studied groups regarding type of DM

			Groups			Total
			DM	NPDR	PDR	
Type of DM	IDDM	Count	24	36	18	78
		% within groups	40.0%	60.0%	30.0%	43.3%
	NIDDM	Count	36	24	42	102
		% within groups	60.0%	40.0%	70.0%	56.7%
Total		Count	60	60	60	180
		% within groups	100.0%	100.0%	100.0%	100.0%

FET=1.86; p=0.53 (NS)

10606.4 respectively) in comparison with NPDR (3756.7 ±1785.9 and 6736.4 ±3271.1 respectively), WDM (4346.0 ±2110.4 and 7523.5±3564.6 respectively), and control groups (4958.8± 2407.9 and 5454.9 ±2648.8 respectively) (p <0.001), while no statistically significant difference was detected regarding miRNA21 whose mean values (FRU) were 9354.9 ±4542.5 for PDR group, 4811.0 ±2336.2 for NPDR group, 5773.3 ±2803.2 for DM without retinopathy group, and 5345.6 ± 2595.6 for the control group (Table 4 and Figures 1, 2, and 3). Relative quantitation of the studied markers shows that miRNA 181c level was 5.39 folds in PDR, 0.76 folds in NPDR, and 0.8 folds in DM without retinopathy, respectively, compared to the control group.

While miRNA 1179 level was 3.97 folds in PDR, 1.3 folds, in NPDR, and 1.52 folds in DM without retinopathy, respectively, compared to the control group.

Finally, miRNA 21 level was 1.69 folds in PDR, 0.9 folds in NPDR, and 1.09 folds in DM without retinopathy respectively compared to the control group. Because samples of control group are used as calibrators, the expression levels are set to one. Gene expression levels were plotted as log10 values (log10 of 1 is 0), so the expression level of the calibrator samples appears as 0 in the graph [12] (Figures 4 and 5).

The receiver operating characteristic (ROC) curve analysis shows that miRNA181C ≥ miRNA1179 significantly predicts PDR. For miRNA 181C: (AUC=0.983, 95%CI =0.94–1.0), sensitivity was 90% and specificity was 100%. For miRNA1179: (AUC=0.927, 95%CI =0.82–1.0), sensitivity was 90% and specificity was 80%. In combining markers, the sensitivity improved to 100% for (miRNA 181+ miRNA 1179), 90% for both (miRNA 181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100% (Figure 6).

Discussion

DR is a highly specific vascular complication and a sight-threatening problem related to diabetes. PDR is characterized by gradually progressive alterations in the retinal microvasculature, leading to retinal hypo perfusion and increased vascular permeability [19]. Thus, detecting a novel factor to monitor the progression of the proliferation of vascular endothelial is very important. Few studies had listed three PDR-associated miRNAs, including miR-21, miR-181c, and miR-1179. miR-181c inhibition promoted tubule formation and VEGFA expression [12, 20].

Table 2 Comparison of the studied groups regarding sex

			Groups				Total
			Controls	DM	NPDR	PDR	
Sex	Male	Count	30	18	36	42	126
		% within groups	50.0%	30.0%	60.0%	70.0%	52.5%
	Female	Count	30	42	24	18	114
		% within groups	50.0%	70.0%	40.0%	30.0%	47.5%
Total		Count	60	60	60	60	240
		% within Groups	100.0%	100.0%	100.0%	100.0%	100.0%

FET=3.4; p=0.39 (NS)

Table 4 Comparison of the studied groups regarding the studied markers

Variable	Controls (n=60)			DM (n=60)			NPDR (n=60)			PDR (n=60)			Kruskal-Wallis test	p
	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range		
miRNA181C (FRU)	4958.8	2407.9	1234–7895	4346.0	2110.4	1074–7027	3756.7	1785.9	975–6071	26654.4	12857.8*†‡	6713–42080	**21.8**	**<0.001 (HS)**
miRNA1179 (FRU)	5454.9	2648.8	1357–8685	7523.5	3564.6	1968–12258	6736.4	3271.1	1676–10725	22202.4	10606.4*†‡	5701–35505	**17.4**	**<0.001 (HS)**
miRNA21 (FRU)	5345.6	2595.6	1330–8511	5773.3	2803.2	1437–9192	4811.0	2336.2	1197–7660	9354.9	4542.5	2328–14894	**6.85**	**0.077 (NS)**

*Significant in comparison with the controls
†Significant in comparison with the DM
‡Significant in comparison with the NPDR

We have studied the three miRNAs including miR-21, miR-181c, and miR-1179 in predicting PDR out of NPDR which is very important for detecting the progression of patients with NPD developing to PDR.

Our results indicated the mean values expressed in FRU of miRNA181C and miRNA1179 were significantly higher among PDR group (26654.4 ± 12857.8 and 22202.4 ± 10606.4 respectively) in comparison with NPDR (3756.7±1785.9 and 6736.4 ± 3271.1 respectively), DM (4346.0±2110.4 and 7523.5±3564.6 respectively), and control groups (4958.8± 2407.9 and 5454.9 ±2648.8 respectively) (p <0.001) while no statistically significant difference was detected regarding miRNA21 whose mean values (FRU) were 9354.9 ±4542.5 for PDR group, 4811.0 ±2336.2 for NPDR group, 5773.3 ±2803.2 for DM group, and 5345.6 ±2595.6 for the control group (Table 4 and Figures 1, 2, and 3). In combining markers, the sensitivity improved to 100% for (miRNA 181c+ miRNA 1179), and 90% for both (miRNA181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100% (Figure 6).

Consistent with our results, Qing et al. [7] reported an accuracy rate of the three-miRNA (mir-181c and miR-1179 and miR-21) profile was 82.6%.

Previous studies had revealed the overexpression of mir RNA 181c in the pathogenesis of retinopathy [8 and 12, 21].

Unlike the results obtained by us, studies revealed a significant increase in miR-21 expression in the vitreous humor in proliferative diabetic retinopathy [22].

Demographic data revealed that there were not any significant differences between the three groups' (DMC, NPDR, and PDR) HbA1c levels. These observations agree with that reported by Ma et al. [23]. On the other hand, Raum et al. [24] reported that elevated levels of HbA1C were associated with increased risk of DR.

Conclusion

Since DR may continue to progress even in patients with controlled diabetes, there was a need to search for new biomarkers that can help identify high-risk DR patients and start therapy. MicroRNAs play a role in pathogenesis of diabetic retinopathy specially angiogenesis. In our study, miR-181c and miR-1179 were found to be significantly high in PDR patients compared to NPDR patients and controls so they could be used effectively to anticipate and follow this disease. MicroRNA antagonists or mimics can also be tried as new medications to modify the occurrence and progression of DR. This can reduce the rate of progression of DR, and the development of blindness.

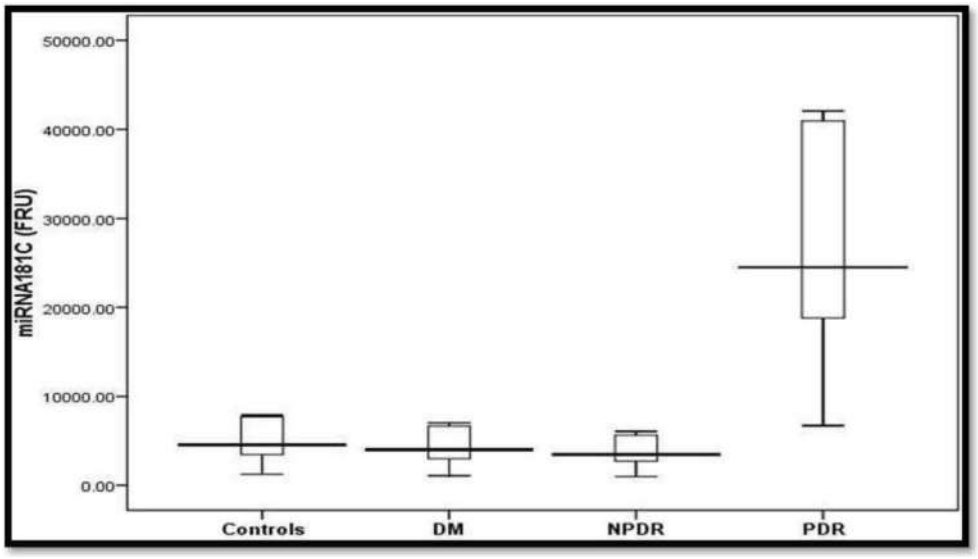

Fig. 1 Box plot showing the median and interquartile range (IQR) of miRNA181C among the studied groups. The median values were (4550.5, 4004.0, 3469.5, 24503.5) and the IOR values were (3178.3–7685.0, 2765.3–6686.0, 2478.5–5686.8, 17225.5–41172.3) among control, diabetes mellitus without retinopathy, non-proliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

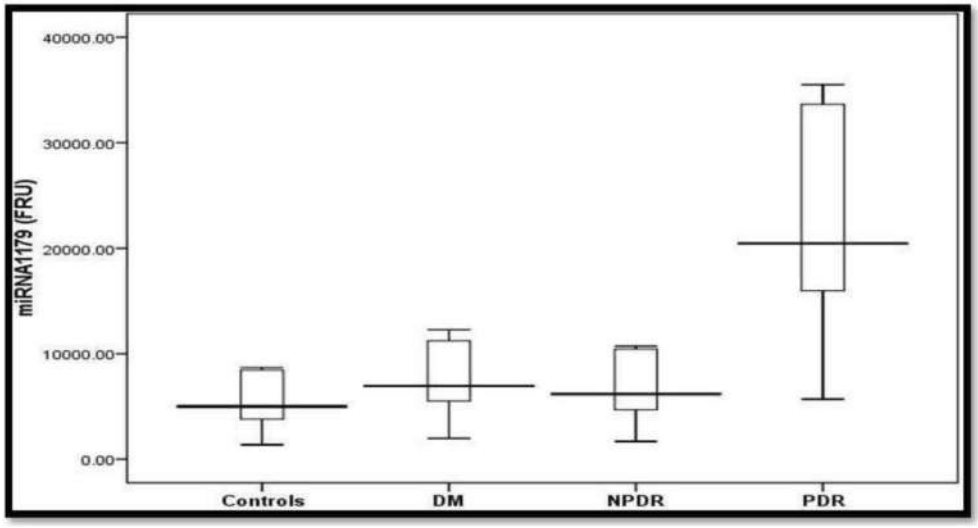

Fig. 2 Box plot showing the median and interquartile range (IQR) of miRNA1179 among the studied groups. The median values were (5005.5, 6957.0, 6181.5, 20470.5) and the IOR values were (3496.5–8454.0, 4991.8–11319.8, 4317.8–10440.0, 14541.8–33874.8) among control, diabetes mellitus without retinopathy, nonproliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

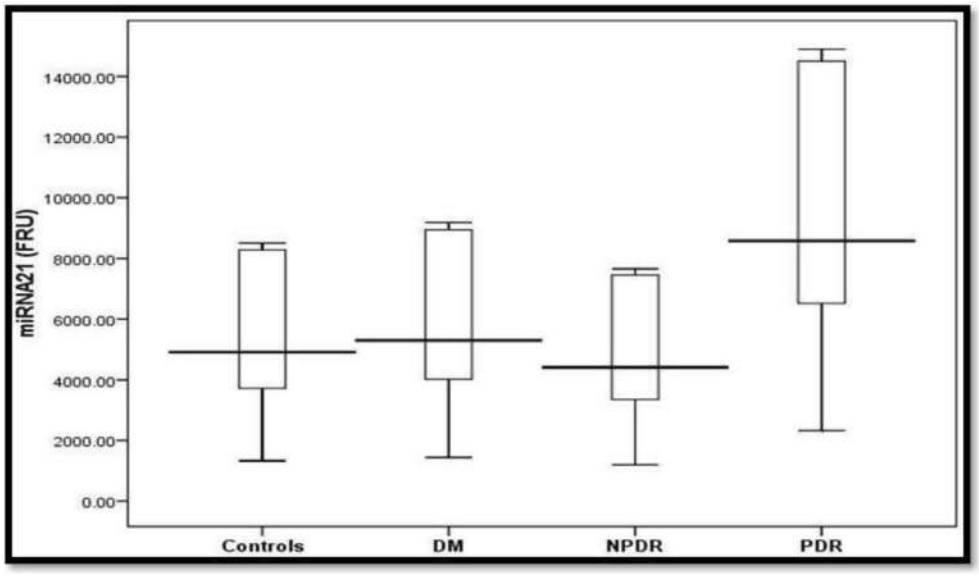

Fig. 3 Box plot showing the median and interquartile range (IQR) of miRNA21 among the studied groups. The median values were (4905.5, 5298.0, 4415.0, 8584.5) and the IOR values were (3426.5–8284.0, 3700.5–8947.0, 3083.5–7456.0, 5996.0–14498.0) among control, diabetes mellitus without retinopathy, non-proliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

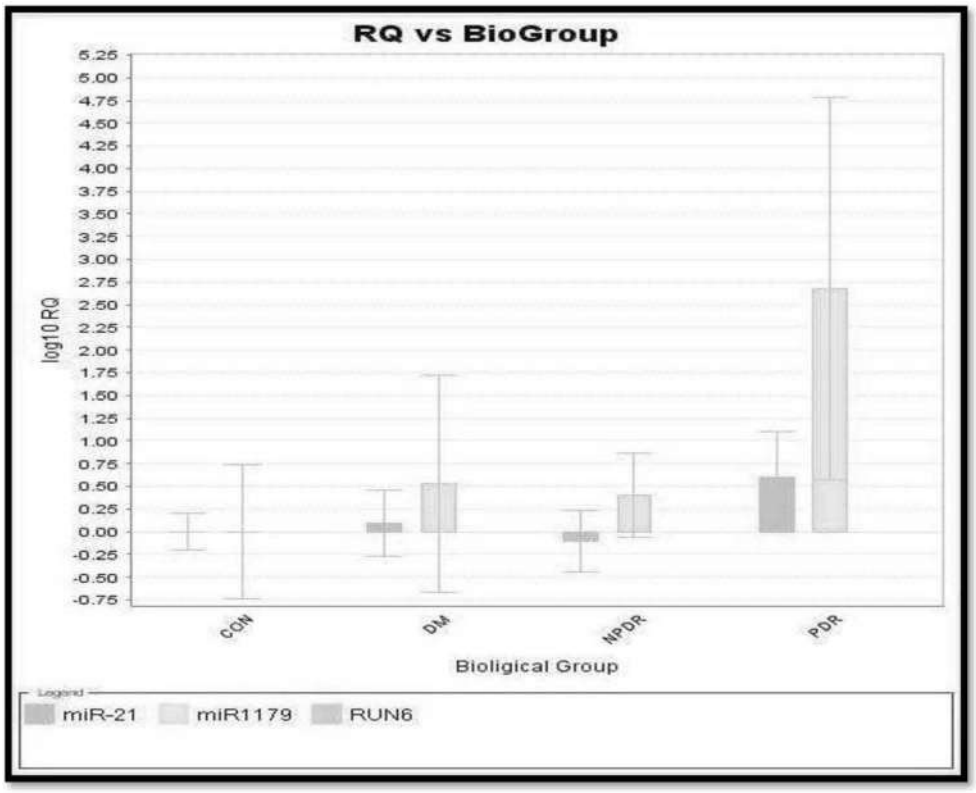

Fig. 4 Expression pattern of miR-21 and miR-1179 (RQ) in the studied groups compared to control group plotted as log 10 value. miRNA 1179 level was 3.97 folds in PDR, 1.3 folds in NPDR, and 1.52 folds in W DM, respectively, compared to the control group. While miRNA 21 level was 1.69 folds in PDR, 0.9 folds in NPDR, and 1.09 folds in W DM respectively compared to the control group. The control group is used as a calibrator so the expression levels of other groups are set to 1. As log 10 of 1 is 0, the expression level of the calibrator appears as 0 in the graph [12]

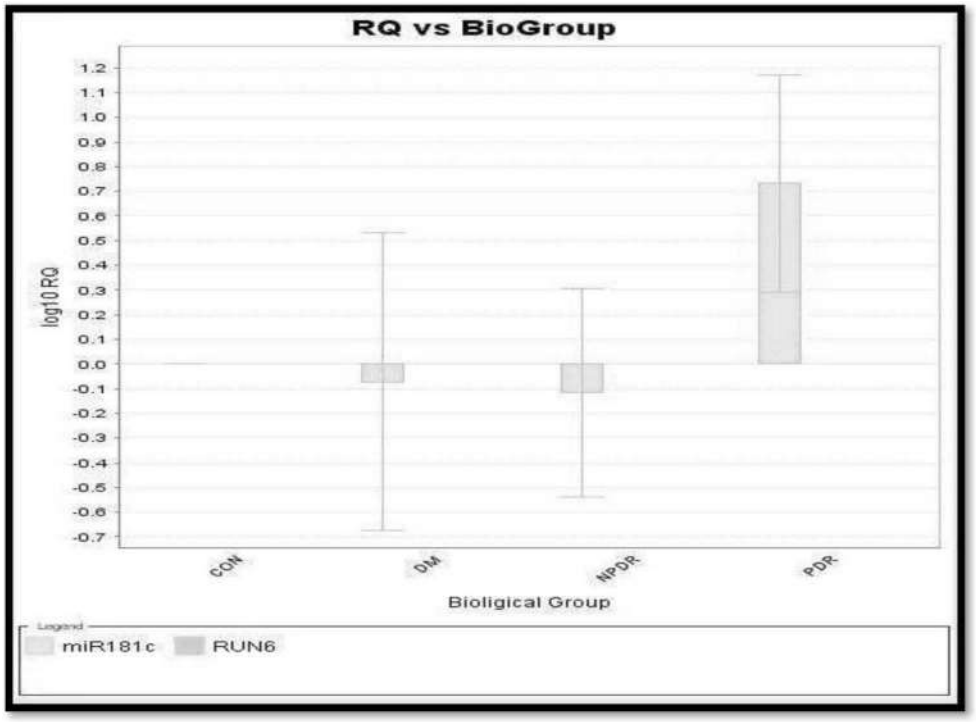

Fig. 5 Expression pattern of miR-181c (RQ) in the studied groups compared to control group plotted as log 10 values. Relative quantitation of the studied markers shows that miRNA 181c level was5.39 folds in PDR, 0.76 folds in NPDR, and 0.8 folds in WDM, respectively, compared to the control group

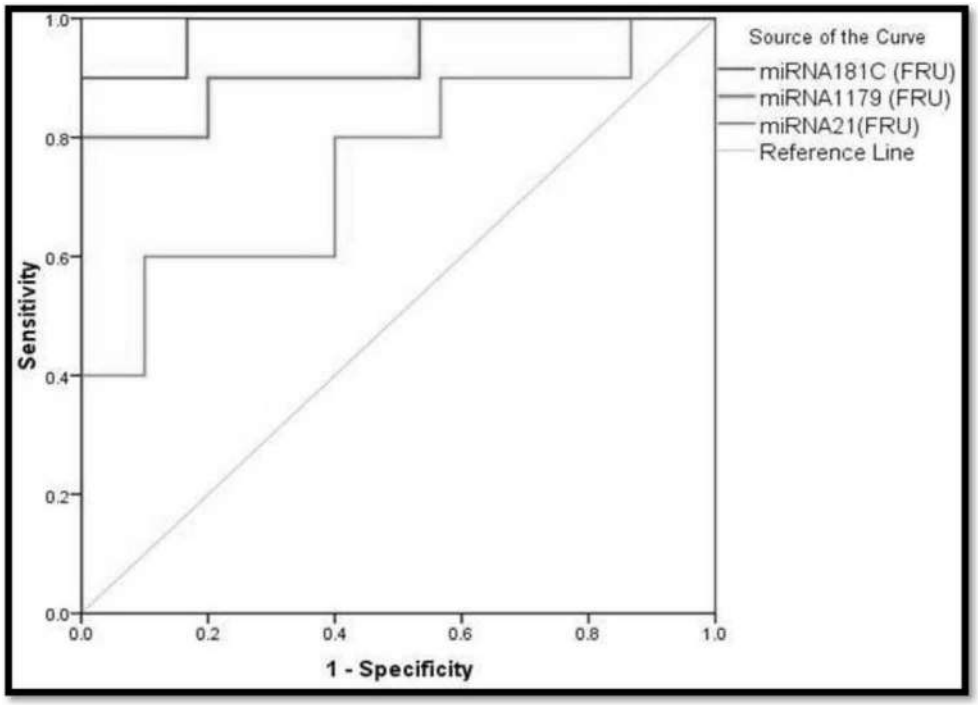

Fig. 6 ROC curve for the performance of the studied markers in the prediction of PDR. The receiver operating characteristic (ROC) curve analysis shows that miRNA181C ≥ miRNA1179 significantly predicts PDR. For miRNA 181C: (AUC=0.983, 95%CI =0.94–1.0), sensitivity was 90% and specificity was 100%. For miRNA1179: (AUC=0.927, 95%CI=0.82–1.0), sensitivity was 90% and specificity was 80%. In combining markers, the sensitivity improved to 100% for (miRNA 181c+ miRNA 1179), and 90% for both (miRNA181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100%

Availability of data and materials All relevant raw data will be freely available to any scientist wishing to use them for non-commercial purposes without breaching participant confidentiality.

Author contribution Tamer Ibrahiem Salem: collecting samples and clinical data, drafted the manuscript and revision of the manuscript

Nashwa Badr Eldin: collecting samples and clinical data, drafted the manuscript and revision of the manuscript

Naglaa Fathy Alhusseini: molecular biology technique

Omnia Alsaied Abdullah: conceived and designed the research, molecular biology technique, analyzed and interpreted the data, critical revision of the manuscript and corresponding author

Nashwa Elsayed Ahmed: rewrote the parts previously been commented on by the reviewers and needed to be corrected in the manuscript

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Declarations

Competing interests The authors declare no competing interest.

Animal research This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publications 85-23, revised 2011). All protocols were approved by the institutional review board for animal experiments of the Faculty of Medicine, Benha University, Egypt.

Consent to participate and consent to publish The research protocol was accepted by the Medical Faculty Ethical Committee, Benha University. Before participation, written informed consents were received from all patients at the study initiation, both consent to participate in the study and consent to have the data published.

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ORIGINAL ARTICLE

Regional inequalities in type 2 diabetes epidemiologic indices in Brazil

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Abstract

Introduction Type 2 diabetes mellitus is one of the main non-transmissible chronic diseases in the world and mainly affects developing countries such as Brazil. Current data on the burden of this disease on the health of the population are needed for the formulation of more effective public policies.

Objective The aim of this study was to analyze the regional inequalities of epidemiological indicators of type 2 diabetes mellitus in Brazil, its administrative regions, and federative units in 2017.

Methods This is a secondary analysis of Brazilian rates in 2017 extracted from the Global Burden of Disease. The epidemiological indicators for type 2 diabetes mellitus studied were as follows: incidence, prevalence, and mortality per 100,000 inhabitants; disability-adjusted life years; years living with disability; mortality by incidence ratio; years of life lost. To describe the epidemiological indicators of type 2 diabetes mellitus, averages and respective confidence intervals of 95% were used.

Results In 2017, there was an important regional disparity of type 2 diabetes mellitus according to the Brazilian regions. The Northeast region was the most impacted in all indicators analyzed and the Midwest region was the region less impacted.

Conclusion There is an important disparity of type 2 diabetes mellitus rates in Brazilian regions, representing an important public health challenge in coping with the burden of the disease and reducing regional disparities.

Keywords Diabetes mellitus type II · Public health · Epidemiology · Brazil · Regional disparities

Introduction

Socioeconomic, contextual, and individual characteristics, such as income inequality, were related to the production of health problems, and the performance of the Brazilian health system should be analyzed under political, social, and economic contexts that show its history and its current conformation, and its objectives and priorities. In this scenario, the determinants associated with health problems should be identified and the assessment of which should be made considering their impact on different social groups and specifically, in Brazil, according to the administrative regions and federative units [1].

According to the World Health Organization (WHO), in 2000, approximately 2.8% of the world population had diabetes and by 2030 this rate will be estimated at 4.4%. This increase is seen especially in developing countries and linked to population aging and obesity. As a result of these data, the WHO itself already classifies diabetes mellitus (DM) as an epidemic [2, 3].

Thus, DM may be related to the growing economic impact on health systems due to patient costs, which occur due to the high incidence of acute and chronic complications, hospitalizations, physical disability, years of life lost due to disability and mortality, and premature mortality [4]. Researches indicate a higher prevalence of DM in elderly with lower socioeconomic status, and it is known that people with lower socioeconomic conditions have less access or difficult access to health services [5–7].

The increase in the cost of living of the inhabitants and the high demand for care of the public services, together with the insufficient supply of health resources offered by the State, and regional differences in socioeconomic size and development make health care unequal among Brazilian administrative regions [8, 9]. With less access to health services, lower education, and unhealthy lifestyle habits, in addition to delays in the provision of these services, people are more likely to develop chronic diseases in Brazil, due to

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the regional disparities that were related to the worst socioeconomic and development indicators [10].

A study [11] published in 2017 with data from 2008 observed a regional disparity as a factor related to the burden of epidemiological indicators of type II diabetes mellitus (T2DM) in Brazil. However, after almost a decade of changes in the socioeconomic and development profile of Brazilian regions, what is the burden of T2DM in Brazil in 2017? Thus, the aim of this study was to analyze the regional inequalities of epidemiological indicators of T2DM in Brazil, its administrative regions, and federative units in 2017.

Materials and methods

Study design, geographical and temporal delimitation

A secondary analysis was conducted using data from the Global Burden of Disease database for 2017 to estimate the findings for the five Brazilian administrative regions (North, Northeast, Midwest, Southeast, and South) and for the country.

Data sources

The Global Burden of Disease (GBD) is a database that quantifies health loss due to hundreds of diseases, injuries, and risk factors so that health systems can be improved and disparities thus eliminated. Through this tool, it is possible to measure the morbidity and mortality of various diseases worldwide, which has grown exponentially in recent decades and has become an international consortium of more than 3600 researchers, where their estimates are updated annually. GBD is managed by the Institute for Health Metrics and Evaluation (IHME) which feeds the database with a set of interactive data visualizations [12]. The GBD was the database to obtain these data, which were classified according to the tenth revision of the International Classification of Diseases (ICD-10) [13].

Study variables

The epidemiological indicators for T2DM studied in this study were as follows: incidence, prevalence, and mortality per 100,000 inhabitants; DALY (disability-adjusted life years); YLD (years living with disability); mortality by incidence ratio; YLL (years of life lost). The mortality-by-incidence ratio was estimated from the data extracted from the GBD by the relationship between the estimated mortality rate and the incidence rate for every 100,000 people [14].

Data analysis

Only a descriptive analysis of the data was performed for Brazil and stratified to administrative regions and federative units. The Shapiro–Wilk test was used to classify the distribution of data and a p-value higher than 0.05 was classified as a normal distribution. To describe the epidemiological indicators of T2DM, means and respective confidence intervals of 95% were used. The program used was Stata® (StataCorp, IC) version 11.0.

Ethical aspects

In this study, a secondary data extracted from public databases where it is not possible to identify the study participants were used. According to resolution no. 510 of April 7, 2010 [15], this type of research should not be submitted to ethical consideration by the National Health Council.

Results

Epidemiological indicators of type 2 diabetes mellitus were presented in Brazil. On an average, the prevalence (per 100,000 inhabitants) was 3834.3 cases, with 195.8 new cases diagnosed per 100,000 people, with a mortality rate of 20.8 deaths per 100,000 people. Regarding the burden of T2DM in the life of the Brazilian population, the relationship between incidence and mortality was 9.4 people, which represents 9 deaths per 100 sick people and impacted on 717.3 years of disability-adjusted life related to T2DM, as a result of 363.3 years of life lost and 353.4 years living with disability (Table 1).

Regarding the regional variability of epidemiological indicators of T2DM, the Northeast region was the most impacted in all indicators analyzed (prevalence = 4142.7, incidence = 214, mortality = 26.3, MRI = 12.1, DALY = 843.1, YLL = 460.4, and YLD = 382.7). Additionally, some indicators in the Southeast region are similar,

Table 1 The burden of type 2 diabetes mellitus in Brazil in 2017

Epidemiological indicators (per 100,000 inhabitants)	Brazil Average (95% CI)
Prevalence	3932.2 (3733.2; 4131.3)
Incidence	203.0 (192.22; 213.81)
Mortality	23.69 (21.0; 26.4)
Mortality-to-incidence ratio	11.4 (10.7; 12.2)
Disability-adjusted life year	775.7 (710.7; 840.9)
Years of life lost	413.1 (365.3; 461.0)
Years lived with disability	362.6 (343.9; 381.3)

such as prevalence, incidence, and mortality, although the indicators related to the burden of the disease are lower than those observed in the Northeast region.

On the other hand, the Midwest region presented the lowest epidemiological indicators among the regions (prevalence = 3482.5, incidence = 179.7, mortality = 18.0, MRI = 10.0, DALY = 631.8, YLL = 311.7, and YLD = 320.1) (Table 2).

When epidemiological indicators were analyzed by Brazilian federative units and federal district, it was observed that there are disparities between the rates of T2DM of the federative units of the same region, with emphasis on the inequalities observed in the Northeast and Southeast regions of the country. In the States, Maranhão in relation to prevalence, incidence, and YLD, and of São Paulo, with emphasis on the low indicator of disease lethality (MRI) (Table 3).

Discussion

When analyzing the regional disparities of epidemiological indicators of T2DM in Brazil, its administrative regions and federative units in 2017, it was observed that the disparity of epidemiological indicators is present in both when the focus is on the regions and when the focus is on the federative units and the Brazilian Federal District, focusing on the Northeast region and with higher lethality in the Southeast region and alerting to heterogeneity related to the burden of type 2 diabetes mellitus in Brazil in 2017.

T2DM is also associated with chronic complications such as renal failure, lower limb amputation, blindness, and cardiovascular diseases, in addition to others, which may compromise the functional capacity, autonomy, and quality of life of the population to different degrees of severity [16].

The most part of the rates related to diabetes mellitus was attributed to individual and avoidable risk factors, which can be modified with the effectiveness of public policies aimed at promoting healthy lifestyle habits, such as balanced diet and physical activity [17]. These actions can have a significant impact on reducing the burden of diabetes mellitus in Brazil, being the important tools to manage resources and define priorities in the most diverse health interventions at all levels of care.

The epidemiological, nutritional, and demographic transitions observed in recent decades have resulted in increased morbidity and mortality from chronic non-communicable diseases (NCDs), considered as indicators of the cause of morbidity and mortality worldwide [18]. Data from the Global Burden of Disease Study shows that NCDs accounted for 43% of disability-adjusted life years (DALYs) in 1990, increasing to 54% in 2010 [19] and variated according to the local socioeconomic development.

The World Health Organization proposes that the human and socioeconomic impact of NCDs can directly affect the progress of the Millennium Development Goals, and the consequences will be diagnosed in most countries, especially those with low and middle incomes and with the most vulnerable populations [20].

Although access to prevention methods is the most effective in detecting any disease, the Brazilian health system does not have preventive interventions that function equally throughout the national territory, mainly due to the high

Table 2 Regional disparities in epidemiological indicators of type 2 diabetes mellitus in 2017

Epidemiological indicators*	Administrative regions Mean (95% CI)				
	Midwest	Northeast	North	Southeast	South
Prevalence	3482.5 (3252.9; 3712.1)	4142.7 (3693.9; 4591.5)	3837.0 (3457.4; 4216.7)	4142.4 (3377.5; 4907.4)	3842.4 (2462.1; 5222.8)
Incidence	179.7 (168.1; 191.2)	214.0 (189.5; 238.5)	199.6 (176.4; 222.9)	211.0 (166.4; 255.7)	198.4 (136.6; 260.3)
Mortality	18.0 (13.9; 22.0)	26.3 (19.9; 32.8)	23.7 (18.7; 28.8)	24.3 (9.4; 39.2)	22.5 (13.2; 31.7)
MRI	10.0 (8.2; 11.7)	12.1 (10.3; 13.8)	11.8 (10.7; 12.9)	11.3 (6.3; 16.2)	11.3 (8.9; 13.7)
DALY	631.8 (520.8; 742.8)	843.1 (685.8; 1000.4)	764.5 (656.0; 869.0)	807.9 (50.8; 1112.0)	749.3 (409.6; 1089.0)
YLL	311.7 (220.8; 402.6)	460.4 (343.1; 577.7)	410.6 (340.4; 480.7)	426.3 (183.4; 669.2)	395.0 (173.4; 616.6)
YLD	320.1 (298.4; 341.8)	382.7 (340.5; 424.9)	353.9 (318.4; 389.4)	381.6 (309.0; 454.2)	354.2 (225.4; 483.0)

MRI, mortality-to-incidence ratio; *DALY*, disability-adjusted life year; *YLL*, years of life lost; *YLD*, years lived with disability

*Per 100,000 inhabitants

Table 3 Distribution of epidemiological indicators by federative units in Brazil in 2017

Federative units	Prevalence	Incidence	Mortality	MRI	DALY	YLL	YLD
Acre	3551.9	182.4	20.2	9.0	686.1	359.6	326.6
Alagoas	4815	251.9	40.2	6.3	1169.5	722.6	446.9
Amapá	3587.1	183.8	18.9	9.7	675.5	344.6	330.9
Amazonas	3640.4	189.1	23.7	8.0	759.2	422.4	336.8
Bahia	4179	215.9	27.4	7.9	882.3	495.2	387
Ceará	3318.8	172.8	18.4	9.4	605.6	300.2	305.4
Distrito Federal	3361.2	177.9	15.8	11.3	553.1	244.1	244
Espírito Santo	4250.2	209.3	17.4	12.0	698.4	309.3	389
Goiás	3356.4	170.3	16.3	10.4	602	293.9	308.1
Maranhão	4515.3	235.8	28.1	8.4	937.4	518.3	419.1
Mato Grosso	3631.5	187.3	21.4	8.8	714.8	379.6	335.2
Mato Grosso do Sul	3580.8	183.1	18.5	9.9	657.2	329.1	328.1
Minas Gerais	3442.3	172.5	15.3	11.3	598.4	282.7	315.7
Paraná	3801.5	198.2	24.4	8.1	792.6	441.3	351.3
Paraíba	4436.8	232.6	31.8	7.3	948	537	411
Pará	3676.8	191.2	21	9.1	701.2	362.4	338.8
Pernambuco	4969.5	255.9	34.3	7.5	1036.8	579	457.8
Piauí	3871	198.8	24.2	8.2	788.7	431.2	357.5
Rio Grande do Norte	3577.8	182.5	17.8	10.3	634.2	306.8	327.5
Rio Grande do Sul	4417.4	223.5	24.8	9.0	859.1	451.6	407.5
Rio de Janeiro	4349.1	225.6	30.3	7.4	930.2	527.9	402.3
Rondônia	3818	198.5	24.4	8.1	769.9	418.4	351.4
Roraima	4732.7	255.1	35.4	7.2	1006.2	568.4	437.8
Santa Catarina	3308.3	173.7	18.2	9.5	596.1	292.2	303.9
Sergipe	3601.2	179.6	15.1	11.9	585.4	253.3	332.1
São Paulo	4528.1	236.7	34.2	6.9	1004.7	585.3	419.4
Tocantins	3852.4	197.4	22.6	8.7	753.4	398.1	355.3

Per 100,000 inhabitants; *DALY*, disability-adjusted life year; *YLL*, years lived with disability; *YLD*, years lived with disability; *MRI*, mortality-to-incidence ratio; *DALY*, disability-adjusted life year; *YLL*, years of life lost; *YLD*, years lived with disability

territorial extension and the difficulties recognized for the implementation of public health policies through the needs and barriers encountered in each region [20].

In addition, the main investments related to T2DM in Brazil were directed to treatment, which demands government strategies to reduce costs and improve health care [21] and other factors such as changes in eating habits and lifestyle may also be related to regional and intraregional disparities in the burden of T2DM observed in the present study.

The interior of Brazil and the less economically developed regions have faced problems in the implementation of the Unified Health System (SUS) since its creation, due to difficulties in the supply of medicines and tests and referrals for specialized care, in addition to the long waiting time in primary care units, generating dissatisfaction of users and health professionals with the system [19], and, like most of these remote cities, they have small populations, which suffer from inadequate human and financial resources and infrastructure.

A study on the burden of T2DM in Brazil showed that the highest rates of years lost (YLL) and years of life lived with the disease (YLD) were identified in the Northeast and South regions of the country, respectively, as well as the chronic complications that represented 80% of the YLDs, which is considered one of the main health problems in Brazil, due to relevant mortality and morbidity rates [19].

When establishing a correlation with developed countries, the highest fraction of the resulting burden from T2DM in Brazil was the result of YLD. In this context, the Northeast region presented a significant portion of the YLL, reflecting the differences in compliance with the health conditions and economic development of this region in relation to the other administrative regions [22].

The Northeast of Brazil is a region in which the profile of T2DM has longer years of life lost by the disease, which may be a reflection of an epidemiological profile with a lower rate of early diagnosis due to difficulties in accessing health services, as well as barriers to ongoing treatment, which

also corroborates the increase in mortality from diabetes. Other relevant aspects include issues of health education with diabetics for health professionals and patient adherence to treatment in an already chronic condition [19, 23].

Despite the fact the Brazilian human development index has increased over the years, the municipalities of the Northeast region are still classified as low human development. This situation is expected to be alarming, since access to health services in Brazil is strongly influenced by the social conditions of individuals and by the acquisition of adequate housing [24].

The South and Southeast regions, considered the most developed, have higher percentages of T2DM attributable to obesity, while in the North, the increase in the attributable population fraction is due to overweight itself, which may be related to a late nutritional transition in the region, due to economic advances leading to a more recent exposure to risk factors for DM [25].

Risk factors such as population aging, obesity prevalence, sedentary lifestyle, and urbanization commonly observed in more developed regions, such as the Southeast, may contribute to the increase in the mortality rate [26]. When addressing T2DM and its various chronic complications, the morbidity burden (YLDs) is generally composed mainly of diabetic retinopathy; however, the regional variation shows a higher concentration of YLDs in the Southeast region, in relation to the North region, for example.

Another important factor that may also be related to the worse epidemiological rates in the Southeast is the fact that in this region the population density is higher and there is migration of residents from other Brazilian regions to the Southeast, which occurs due to the fact that it is in the Southeast that the main technological parks related to health care are concentrated. This is the reason many people leave other regions to seek more specific treatment in this region [27].

It is observed that the non-transmissible diseases (NTD) represented the majority of the burden of diseases in Brazil in 2008, corroborating previous studies conducted in the country, when NTDs represented 66.3% of the burden of diseases in 1998, reaching the rate of 77.2% in 2008. This percentage is similar to current findings in other Latin American countries such as Mexico (73.0%) and Colombia (74.0%), the latter being higher than Peru (58.5%) and Costa Rica (62.0%) and lower than the percentage of Chile (84.0%) [28, 29].

About the burden of T2DM, this disease is usually among the main causes of disability-adjusted lost years of life (DALY). In Australia, T2DM accounted for 5.5% of DALY in 2004, resulting similarly to the Brazilian percentage, despite methodological differences in the DALY estimate between the 1998 and 2008 studies. Also, according to data from these studies, 39.0% of cases of T2DM could have been avoided in Canada, reducing exposure to the risk factor overweight. In Switzerland, 42.5% of cases of T2DM were also attributed to obesity in 2002; until in 2010, overweight was considered the sixth most important risk factor for DALY worldwide [19].

There was a remarkable increase in the spread of T2DM due to obesity and overweight in Brazil between 2002–2003 and 2008–2009, indicating that the importance of other factors in the definition of the T2DM load in the country decreased, proportionally increasing the prevalence of physical inactivity and unhealthy diet over the years [30].

Among the strengths of the study, it is possible to observe the predominance of some limitations present both in the development of this research and in most studies on the global burden of diseases in the world. The scarcity of national studies with relevant representativeness of the prevalence and incidence rates of T2DM, in addition to effective diagnosis and exhaustive list of chronic complications, still needs to be investigated, not counting acute complications, the duration and proportion of treatments performed, and underreporting and incorrect classification of the number of deaths.

In addition to these limitations mentioned, there are still those related to the complexity of DALY estimates, which require several studies to calculate parameters, but have weak data systems in terms of quality and amount of data available, without considering the specificities of the different health systems.

Policies aimed at promoting healthy lifestyle habits can reduce the burden of T2DM in the country, given the high prevalence of overweight in younger people, and thus are important actions aimed at school-aged children, for example. In this context, population-based actions, those that are not focused on risk groups, should be prioritized [30], since the research shows that a significant fraction of the T2DM load is attributed to moderately high values of body mass index (BMI).

Finally, it is worth mentioning that the weights used in the calculation of YLD are standardized worldwide, so they may not consider the specificities of each health system, limitations that are mainly related to the use of parameters in the international literature.

Epidemiological, nutritional, and demographic transitions make it difficult to establish preventive forms of intervention, given the difficulties in implementing public health policies. Therefore, promotion actions aimed at investing in raising awareness of the population in maintaining healthy life habits through motivational lectures in basic health units, holding sports events, allied to healthy cooking classes, are actions that can impact cost reduction and improvement of health care in communities.

In view of the results of the present study, Brazil has an important regional disparity in relation to the burden of

T2DM and that the control plan for this disease should be directed to the needs and impact on Brazilian regions.

Author contribution MAM was responsible for the conception and design or acquisition of data or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published. FMFS was responsible for the drafting the article or revising it critically for important intellectual content and final approval of the version to be published. FWSF was responsible for the conception and design or acquisition of data or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published.

Availability of data and material The data will be available upon request to the authors.

Code availability The code will be available upon request to the authors.

Declarations

Ethics approval This study was performed with secondary data and according to the Brazilian Law no. 510 of 7th April, and ethical assessment is not necessary by the ethics committee.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Regular hospital visits and treatment outcomes among people living with type 1 diabetes: a 7-year study from South India

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Abstract

Aim Literature on how people with type 1 diabetes (TIDM) manage their condition in India is sparse. The aim was to explore the profile, practice of regular visits to the hospital, regular monitoring of glucose and treatment outcomes among people with T1DM.

Methods A retrospective study was conducted among 239 people with T1DM using electronic medical records at a diabetes specialty care centre in Chennai from 2012 to 2018. Parameters such as socio-demographic, anthropometric and clinical data were collected and categorised into two groups-group 1 (n=141, Regular visit) and group 2 (n=98, Irregular visit).

Results The median age and duration of diabetes for group 1 and group 2 (age; 19(8,60) and 25(12,50) years, (p<0.001) and duration; 10(2,35) and 8(3,25) years, (p=0.002)) showed significant difference. The median hospital visits were (group 1 vs. group 2; 10.0(7,14) vs. 3(1,9.0) times; p<0.001). No significant difference was seen in HbA1c among the groups. Self-monitoring of blood glucose (SMBG) was high among group 1 (16.3%) compared to group 2 (2%), (p<0.001). The presence of chronic kidney disease (CKD) was found to be high in group 2 (8.2%) than group 1 (0.7%), (p=0.004) and diabetic retinopathy (DR) was also higher among group 2, but did not reach statistical significance (15.3% vs. 9.9%, p=0.147). No gender difference was observed in the presence of complications. None of the individuals who had presence of complications was practicing SMBG regularly.

Conclusion The individuals who made a minimum of one or more than one hospital visit per year were found to have nil or lesser complications. Individuals who practiced SMBG had less complications compared to those not practicing SMBG.

Keywords Type 1 diabetes · Regular hospital visit · Self-monitoring of glucose · South India

Introduction

Type 1 diabetes (T1DM) is caused by auto-immune destruction of beta-cells in the pancreas and results in very minimal secretion or complete failure of insulin secretion. T1DM is the major form of diabetes prevalent among children and adolescents, but it may occur at any age. This cannot be prevented unlike type 2 diabetes (DM) but persons with T1DM can lead a healthy life by taking regular insulin and following appropriate healthy behaviors. Around 128,900 children and adolescents in the age group of 0–19 years are affected with T1DM every year globally. Currently, a total of 184,100 children are living with T1DM in India [1]. Registry of People with Diabetes with Young Age at Onset (YDR), an observational multi-centric clinic-based registry revealed that 63.9% of the individuals under 25 were found to have T1DM [2]. People with T1DM need intensive diabetes education and counselling for self-monitoring of glucose (SMBG) in order to prevent development of acute and chronic complications of diabetes. SMBG allows to take necessary interventions and also it helps for adjustment of insulin dosage and also improves health outcomes [3]. Thus, frequent glucose monitoring will allow patients to identify, prevent or manage episodes of hypoglycemia and hyperglycemia [4]. SMBG is recommended at least four times per day, but many patients need to increase the frequency to achieve good metabolic control.

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As T1DM is chronic in nature and requires life-long insulin, intensive care and continuous glucose monitoring, it is an expensive and also an exhaustive disorder for the individuals affected and also the care takers [5]. Achievement of good glycemic control among people with T1DM is crucial. In addition, regular visits to the hospital and screening for complications always remain as challenging tasks. It also imposes challenges and burden to the country's health care system. Literature on how people with T1DM manage their condition with these demanding tasks in low-income countries is limited. Hence, our study aimed to explore the profile, regular visits to the hospital, practice of regular SMBG and treatment outcomes among people with type 1 diabetes from South India.

Methods

This is a retrospective study conducted among people with T1DM attending the outpatient department of a tertiary care centre for diabetes in Chennai, South India, from 2012 to 2018. A total of 530 individuals had attended the outpatient department during this study period. Individuals with DM and secondary DM and those with duration of diabetes of less than 5 years were excluded from the final analysis. Those who had definite diagnosis of T1DM according to WHO criteria [6] and currently who were on insulin treatment were selected and a total of 239 individuals with duration of diabetes more than 5 years and who had undergone screening for complications such as chronic kidney disease (CKD) and diabetic retinopathy (DR) were included for the final analysis. Sociodemographic details, anthropometric measurements, clinical and biochemical data and details on the presence of complications of diabetes, follow-up visits and practice of SMBG of all the individuals were collected from electronic medical records. Glycosylated hemoglobin (HbA1c) was measured by high-performance liquid chromatography (HPLC) method using Bio RAD turbo variant equipment. Average HbA1c was calculated using HbA1c measurements of all the visits during the study period of 7 years (2012–2018). CKD was assessed using KDIGO classification [7]. Retinal examination was done by an ophthalmologist and fundus photography was taken for all the participants and the presence of microaneurysm in the macular area was classified as DR. The diagnosis of DR was done based on ETDRS classification [8]. Latest visit information on body mass index (BMI) and clinical and other biochemical parameters during the study period were included. The detail on pattern of SMBG performed by the participants was recorded from individual medical records. It was educated to monitor the blood glucose levels for a minimum of at least 2 times every day at different time intervals based on mealtime in a week. Individual informed consent was not obtained since the study was done using routinely maintained medical records. Institutional Ethics Committee EC was obtained for this study (IEC/N-006/03/2019). They were categorised into two groups—group 1 and group 2—based on their annual hospital visits for health check-up. All the individuals who made a minimum of one or more than one visit to the hospital for their health check-up every year were categorised into group 1 (n = 141) (Regular visit) and the individuals who have not made at least one visit every subsequent year or who visited alternate years were categorised into group 2 (n=98) (Irregular visit).

Statistical analysis

The normality of the data was checked and median (min, max) were presented for continuous variables and percentages for categorical variables. Mann Whitney U test and chi-square tests were performed to analyse statistical significance between the study groups respectively. The statistical analysis was performed using SPSS version 20.0. A p value of < 0.05 was considered as statistically significant.

Results

The baseline characteristics of the study groups are presented in Table 1. The individuals who had regular hospital visits for follow-up were found to be younger than individuals who had irregular hospital visits and the difference was statistically significant (19(8,60) vs. 25(12,50); $p<0.001$). The duration of diabetes for group 1 (10(2,35)) was observed to be longer compared to group 2 8(3,25); $p = 0.002$. The age at diagnosis of T1DM was significantly higher in group 2 than group 1 ($p<0.001$). The median hospital visits during 2012 to 2018 by group 1 (10(7,14)) was considerably greater than group 2 (3(1, 9); $p<0.001$). There was no significant difference found in BMI, levels of HbA1c, creatinine, estimated glomerular filtration rate (eGFR) and albumin-to-creatinine ratio (ACR) between the study groups.

Table 2 shows the presence of complications and details on practice of SMBG among the study groups. The prevalence of CKD showed a significant difference between the groups (group 2 vs. group 1: 8.2% vs. 0.7% ($p = 0.004$)). The proportion of individuals who practiced SMBG was found to be higher among group 1 (16.3%) compared to group 2 (2%) and it was statistically significant ($p<0.001$). The presence of DR was higher among the individuals who made irregular hospital visits compared to the individuals who made regular hospital visits (15.3% vs. 9.9%).

Table 3 depicts the comparison of the study participants by their practice of SMBG. The individuals who performed SMBG were found to be younger than those who did not perform SMBG (16(9,37) vs. 23(8,60); $p<0.001$). BMI was higher among those who did not perform SMBG compared to

Table 1 Baseline characteristics of the study participants

Variables	Group 1 (n = 141) Regular visit	Group 2 (n = 98) Irregular visit
Age (years)	19 (8,60)	25 (12,50)*
Duration of diabetes (years)	10. (2,35)**	8. (3,25)
Age at diagnosis of DM (years)	9. (1.,28)	16 (0.,29)*
BMI (kg/m^2)	22. (13,42)	22.6 (14,40)
No. of visits to the hospital (2012 to 2018)	10. (7,14)*	3 (1,9.)
HbA1c%	8.9 (6.9,13.4)	9.2 (6.3,15.)
Serum creatinine (mg/dl)	0.8 (0.6,1.4)	0.8 (0.5,5.8)
eGFR (ml/min/1.73m^2)	108 (69,148)	102 (9,219)
Albumin/creatinine (A/C) ratio (μg/mg creatinine)	7. (3,108)	6. (2,109)

Values are median (min, max), *p<0.001, ** p <0.01

the individuals who performed SMBG (22.3(13,42) vs. 20.8(14,27); p =0.049). The age at diagnosis of DM was less among individuals who performed SMBG (6(0,22)) compared to those who did not perform SMBG (13(0.,29)) and the difference was statistically significant (p<0.001). The number of hospital visits was significantly higher among individuals who performed SMBG (11(2,14)) compared to individuals who did not perform SMBG (7(1,14); p = 0.001).

The age-wise comparison of SMBG and presence of complications is presented in Table 4. The median ages of the adults (aged ≥ 20 years) and the children and adolescents (< 20 years) were 28 years and 17 years respectively. The age at diagnosis of DM was significantly high in the adults (17(0,29)) compared to the children and adolescents (8(0,14); p<0.001). The median BMI was observed to be high in the adults (23.5(15,40)) compared to the children and adolescents (20.4(13,42); p=0.002). The children and adolescents had more hospital visits compared to the adults (10(1,14) vs. 5(1,14)) and the difference was statistically significant (p=0.001). The practice of SMBG was high among children and adolescents compared to adults (22% vs. 3.4%; p <0.0001). The adults had higher prevalence of complications compared to the children and adolescents. About 6.1% of the adults were found to have CKD compared to no CKD in children and adolescents. A significantly higher proportion of adults had the presence of DR compared to children and adolescents (18.9% vs.1.1%; p<0.001).

Table 5 depicts the gender differences in hospital visits, glycemic control and presence of complications. Male participants were found to be older than the females (p = 0.020). The number of hospital visits made by females was more than the male participants (8(1,14) vs. 6(1,14); p=0.012). There was a significant gender difference in BMI among those aged ≤ 19 years with female participants having higher BMI (21.2(13,42)) than their male counterparts (18.6(14,28); p<0.001). The proportion of practice of SMBG was observed to be more among females (16%) than males (4.4%); p=0.003.

The comparison of SMBG and glycemic control based on the presence of complications is given in Table 6. The individuals with complications were found to be older than the individuals without complications (35(19,60) vs. 21(8,50); p<0.001). The number of hospital visits was high among individuals without complications (7(1,14)) compared to those with complications (4(1,14)) and the difference between the groups was statistically significant (p=0.031). None of the individuals who had presence of complications was practicing SMBG regularly compared to 12.2% who practiced SMBG regularly.

Table 2 Comparison of presence of complications and details on practice of SMBG among the study groups

Variables	Group 1 (n = 141) Regular visit	Group 2 (n = 98) Irregular visit
Chronic kidney disease (CKD)	1 (0.7%)	8 (8.2%) **
Diabetic retinopathy (DR)	14 (9.9%)	15 (15.3%)
Practice of SMBG	23 (16.3%)*	2 (2%)

Values are n (%), *p<0.001, **p<0.01

Discussion

Our study among individuals with T1DM diabetes highlights that all those who made a minimum of one or more than one hospital visits per year had lesser prevalence of complications of diabetes. They had nearly ten hospital visits during the study period as compared to three visits by the irregular group of individuals. This reflects that at least 1 visit to the health care team per year (including not only the physician but also the dietician, educator, psychologist) would have a positive effect on their condition. The age at diagnosis for regular visit group (group 1) was closer to Indian Council of

Table 3 Comparison of the study participants by practice of SMBG

Variables	Practice of SMBG (n = 25)	No practice of SMBG (n = 204)
Age (years)	16 (9,37)*	23 (8,60)
BMI (kg/m^2)	20.8 (14,27)	22.3 (13,42)ǂ
Age at diagnosis of DM (years)	6 (0,22)*	13 (0,29)
Duration of diabetes (years)	10. (5,21)	8.5 (2,35)
HbA1c%	9.3 (6.9,13.8)	9.0 (6.3,15)
No. of hospital visits	11(2,14)*	7 (1,14)

Values are median (min, max), *p<0.001, ǂp<0.05

Medical Research cohort [2]. A review of childhood and adolescent on-set T1DM in India [9] reported the prevalence of diabetic retinopathy varies from 5 to 31.1% and nephropathy 4 to 21.6%. Our study findings are also comparable with their findings. Another study from North India reported that in individuals with age at onset of diabetes less than 18 years with mean HbA1c% of 8 and with disease duration of 10.2 years, DR occurred in 22% and nephropathy in 18% [10]. Individuals who did not visit hospital regularly were older, had late onset of T1DM and high prevalence of complications even with lesser duration of diabetes compared to the individuals who made at least one or more than one visit per year in this study.

The study conducted among type 1 diabetes in Northern California reported that 34% were performing SMBG 3 times or more per day [11] and another study done in Sweden by Mostrom et al [12] showed 43.9% of subjects were doing SMBG ≥ 4 times/day. Our study reported 16% of the subjects who visited hospital regularly doing SMBG, which was lesser proportion than the above studies. The reasons could be expenses towards SMBG, fear of needle prick, lack of knowledge and skills to handle glucose monitors, perceived/unperceived stigma and discrimination and so on, while the reported reasons for not performing more SMBG in the Sweden study were lack of time, non-remembering and self-consciousness. Older age and female sex were associated with more frequent SMBG than younger age and male sex [11]. In our study, children and adolescents < 20 years practiced SMBG more than adults aged ≥ 20 years (22% vs. 3.4%). Gender difference was clearly observed as practice of SMBG was more among females (16%) compared to male participants (4.4%), their visits to hospital were more compared to males and hence they have lesser complications compared to males.

Our study showed similar HbA1c level between the individuals who practiced and not practiced SMBG regularly. This result was consistent with another retrospective study conducted in Western Kenya that showed no association between adherence to SMBG and glycemic control [13]. However, a study conducted in the USA showed a strong association of increased number of SMBG with lower level of HbA1c [14]. Adherence to interventions remains key to

Table 4 Age-wise comparison of practice of SMBG and presence of complications

Variables	Age < 20 years (n = 91)	Age ≥ 20 years (n = 148)
Age (years)	17(8,19)	28 (20,60)*
Age at diagnosis of DM (years)	8 (0,14)	17 (0,29)*
Duration of diabetes (years)	7 (4,15)	10 (2,35)
BMI (kg/m^2)	20.4(13,42)	23.5 (15,40)**
No. of hospital visits	10 (1,14)*	5 (1,14)
HbA1c%	9.4 (6.9,15)	8.5 (6.3,14.8)
Practice of SMBG#	20 (22%)*	5 (3.4%)
Presence of CKD#	0.0%	9 (6.1%)
Presence of DR#	1 (1.1%)	28 (18.9%)*

Values are median (min, max); #values are n (%),*p<0.001, **p<0.01

Table 5 Gender differences in hospital visits, glycemic control and presence of complications

Variables	Male (n = 114)	Female (n = 125)
Age (years)	23 (9,60)ǂ	21(8,49)
Age at diagnosis	13 (1,29)	11(0.,28)
Duration of DM (years)	9 (2,35)	8 (3,21)
HbA1c%	9 (6.9,15)	8.8 (6.3,14.8)
No. of. visits to hospital	6 (1., 14)	8 (1,14)ǂ
BMI (in age categories)		
≤ 19 years	18.6 (14,28)	21.2 (13,42)**
20–39 years	23.6 (15,40)	23 (18,31)
≥ 40 years	24.6 (21,29)	24.1 (20,32)
Practice of SMBG #	5 (4.4%)	20 (16%)**
Presence of complications#		
Diabetic retinopathy	18 (15.8%)	11 (8.8%)
Chronic kidney disease	5 (4.4%)	4 (3.2%)

Values are median (min, max); #values are n (%),**p<0.01, ǂp<0.05

Table 6 Comparison of SMBG and glycemic control among individuals with and without complications

Variables	Individuals with complications (n = 34)	Individuals without complications (n = 205)
Gender (M:F)	20:14	94:111
Age (years)	35 (19,60)*	21(8,50)
Duration of diabetes (years)	7 (2,35)	9 (2,28)
Age at diagnosis of DM	21 (1, 29)*	11 (1,27)
No. of hospital visits	4 (1,14)	7 (1,14)*
HbA1c%	8.6(6.9,14.8)	9 (6.3,15)
Practice of SMBG#	-	25 (12.2%)

Values are median (min, max); #values are n (%), *p<0.001

achieve good outcomes [15]. Medication adherence, adherence to lifestyle modification, type of medication and family support also play an important role other than adherence to SMBG alone in maintaining good glycemic control. Better glycemic control was associated with higher education and regular follow-up in people with type 1 diabetes from North India [16]. Age of the patients and duration of disease were most significant predictors of poor glycemic control in another study from Egypt [17]. Poor attendance to outpatient visits adds to the poor glycemic control.

In a large cohort of type 1 diabetes from Sweden showed a slow improvement in glycemic and risk factor control from 1997 to 2004 indicating additional measures to be taken to improve risk factor control [18]. None of the individuals who had presence of complications was practicing SMBG in our study. No significant difference was found in the duration of disease and glycemic control among individuals with and without complications in our study. There was also no difference in HbA1c among those who practiced or not practiced SMBG. We could not rule out the reasons for the above findings since data was not available for compliance to treatment.

From our study, we observe that age of the participants and less number of hospital visits may be the important determinants of the presence of complications. We also note that there was a clear demarcation between the individuals for whom age at diagnosis was comparatively late among the irregular group than that of the regular group.

Moreover, the proportion of those practicing SMBG was less in our study and that itself can be noted as an important limitation to derive a strong conclusion. The practice of SMBG was observed to be high among children and adolescents compared to adults in our study. But our study did not capture the details on frequency of SMBG performed by the study participants. The number of hospital visits and median HbA1c was found to be high among children compared to the adults. The role of caregiver, dietary pattern, care at school/college may also play a role and exploring these factors may give us an in-depth understanding on the achievement of good glycemic control. In addition, the role of diabetes self-management and education support (DSMES) is also crucial in this regard and hence a qualitative in-depth study would illustrate the interaction of good glycemic control and SMBG from behavioral aspect.

There is a strong need for a structured diabetes education at individual level and also to improve the knowledge on T1DM care and management at family level and school level which may create a conducive environment to maintain the good glycemic control. A systematic review of diabetes self-management education and support by Rohilla et al showed that there is heterogeneity of DSMES implementation and evaluation [19] and hence called for long-term assessment of psycho-social and behavioral outcomes among people with T1DM.

The main strength of our paper is the illustration of the 7-year information on overall profile of treatment seeking behavior and treatment outcomes among people with type 1 diabetes. A limited number of participants were practicing SMBG and we could not assess the frequency of SMBG performed by the participants throughout the study period as we recorded only whether SMBG was performed regularly or not. Few limitations of the study were data was not available for compliance to prescribed medication, hospital admission, episodes of hypoglycemia and which could throw some light on the risk of developing complications. We cannot generalize the findings of this study since the data was collected in a tertiary care centre for diabetes.

Conclusion

The individuals who made a minimum of one visit or more than one visit per year were found to have nil or lesser complications. Similarly, the subjects those who had practiced SMBG have lesser complications compared to those who were not self-monitoring their glucose.

There is a need for in-depth qualitative study and further prospective cohort to understand the process of management of type 1 diabetes including the individual's interaction within

home, interaction with school or college (students) and other social context where they were situated in and importantly interaction with health services system.

Declarations

Conflict of interest The authors declare no competing interests.

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CORRECTION

Correction to: Regular hospital visits and treatment outcomes among people living with type 1 diabetes: a 7-year study from South India

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In Table 4 3rd column heading, it should be Age ≥ 20 years (n = 148).

The Original article has been corrected.

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ORIGINAL ARTICLE

Evaluation of drug utilization pattern of antidiabetic drugs and 10-year cardiovascular risk in new and recently diagnosed type 2 diabetes mellitus patients: a prospective, longitudinal, observational, hospital-based study

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Abstract

Purpose To evaluate changes in drug utilization pattern of antidiabetic drugs on prospective follow-up of new and recently diagnosed type 2 diabetes mellitus patients along with their 10-year cardiovascular risk assessment.

Methods A prospective observational hospital-based study was conducted among new and recently diagnosed (≤ 6 months) type 2 diabetes patients attending the medicine outpatient department (OPD) at tertiary care teaching hospital, Raipur, after taking written informed consent. Antidiabetic drug prescription and socio-demographic characteristics were noted in the Case Record Form. Recruited patients were followed up at 3, 6, and 9 months. Ten-year cardiovascular risk was determined using the QRISK®3 risk calculator.

Result A total of 90 patients, 48 (53.33%) new and 42 (46.67%) recently diagnosed type 2 diabetes patients, were recruited. An average number of antidiabetic drugs prescribed in patients was 2.1 at the final visit. The average daily dose of antidiabetic drugs was assessed against the WHO/ATC-defined daily dose (DDD). It was decreased for metformin and other antidiabetic drugs, except for glimepiride. More than one-third of patients had a QRISK3 score >10% at each visit.

Conclusion This study highlighted that patient education and periodic review of prescription of new and recently diagnosed diabetic patients play a vital role in managing type 2 diabetes mellitus by tailoring treatment regimen. The present study revealed overtreatment of low-risk groups and under-treatment of high-risk groups based on QRISK3 score and statins. This observation signifies that risk stratification is essential for preventing CVD risks in new diabetic patients.

Keywords Drug utilization pattern · Type 2 diabetes patients · QRISK3 score · Cardio-vascular risk

Introduction

Diabetes mellitus is a spectrum of metabolic disorders arising from various pathogenic mechanisms, resulting in hyperglycemia that may lead to acute symptoms and metabolic abnormalities. Chronic complications like retinopathy, neuropathy, nephropathy, and cardiovascular disease arise from prolonged hyperglycemia [1, 2]. In the twenty-first century, type 2 diabetes mellitus (DM) is the primary driver of the epidemic, accounting for more than 90% of all diabetes cases [3]. Many diabetes patients also suffer from obesity, hyperlipidemia, and cardiovascular diseases [4]. QRISK is a statistical model used to predict a patient's risk over 10 years of developing cardiovascular disease (CVD) (including coronary heart disease, stroke, or transient ischemic attack). The current guidelines state that for the prevention and management of atherosclerotic cardiovascular disease (ASCVD) and heart failure, cardiovascular risk factors should be systematically assessed, at least, annually in all patients with diabetes [5]. Moreover, most of the drug utilization studies on antidiabetic medication use are cross-sectional studies without focusing on cardiovascular risk assessment. Hence, this study was

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designed to evaluate the changes in the drug utilization pattern of antidiabetic drugs in new and recently diagnosed type 2 DM patients. Also, our objective was to assess drug therapy's rationality and follow them prospectively along with their 10-year cardiovascular risk assessment.

Materials and methods

The total duration of this study was 15 months. The patients were recruited from Internal Medicine OPD and Diabetic Clinic over six months (May 2018 to October 2018). The inclusion criteria were adult ≥ 18 years of either gender, newly diagnosed type 2 DM as per diagnostic criteria of American Diabetes Association (ADA) guidelines 2018, type 2 DM patients diagnosed in preceding 6 months and on continuous antidiabetic treatment and patients willing to participate in the study giving "written informed consent." The patients with type 1 DM, gestational DM, comorbid severe conditions (heart failure, previous history of stroke, myocardial infarction, pancreatitis, cystic fibrosis, drug-induced DM, substance and drug abuse, pregnant women, females in the postnatal period (less than 6 weeks)) were excluded from the study.

Each patient was followed up at every 3-month interval, for 9 months, from the date of recruitment. The participants were given a schedule for three subsequent visits, and they were informed telephonically of their scheduled visit one week prior.

Data collection methods

Data of the selected patients were collected using the case record form (CRF). Socio-demographic characteristics were obtained from the patients at the time of enrolment into the study. Recruited patients were classified into different socioeconomic classes according to modified Kuppuswamy Classification [6]. The antidiabetic drugs prescribed to recruited patients were recorded in the semi-structured CRF from patients OPD card. The QRISK3 predicted risks were calculated using the QRISK3 software-open access algorithm [7] at the baseline as well as subsequent follow-ups and were stratified according to statin use (statin users and non-users). According to new statin therapy criteria (NICE Guidelines 2014), patients using statin were categorized into statin under-users (QRISK3 score >10% but not using statin) and statin over-users (QRISK3 score <10% and still using statin) [8].

Statistical analysis

The data were entered into an excel sheet. These data were codified and analyzed in SPSS version 21.0. Socio- demographic variables like age, height, weight, *BMI* were expressed in mean ± SD. Average doses of individual antidiabetic drugs were calculated using a master chart/excel sheet and compared with their defined daily dose (DDD) obtained from the updated ATC/DDD Index [9].

Observation and results

Total 90 patients {48 (53.33%) new and 42 (46.67%) recently diagnosed type 2 DM} were recruited in this study. The mean age of patients was 50 ± 11.18 years. Eighty four patients completed the final follow-up at nine month (Fig. 1).The highest number of patients 31 (34.44%) were in the age group of 40–49 years. Forty patients (44.44%) had family history of diabetes. A higher preponderance of 57.78% (*N*=52) was observed in females. The average weight (in kg) was 64.18 ± 15.19 at the baseline which remained unchanged during subsequent follow-ups. From the baseline data, patients were categorized into underweight, normal weight, and overweight using Body Mass Index (*BMI*) according to WHO criteria. Majority of patients (58.9%) in this study had *BMI* < 25 kg/m^2 (47.78% of normal weight and 11.11% underweight). *BMI* also remained unchanged during subsequent follow-ups.

Among the patients, 55.55% of patients had completed their primary education compared to 23.33%, who were illiterate. 21.1% patients were graduate or post-graduate. According to the modified Kuppuswamy Socioeconomic Scale, more than half of patients recruited in the study belong to lower socioeconomic status 51.11% (*N*=46) of class IV and 14.44% of class V (*N*=13).

Antidiabetic drugs prescribed

More than 2/3rd of patients—73.33% (*N*=66) were taking combination therapy at the time of recruitment which

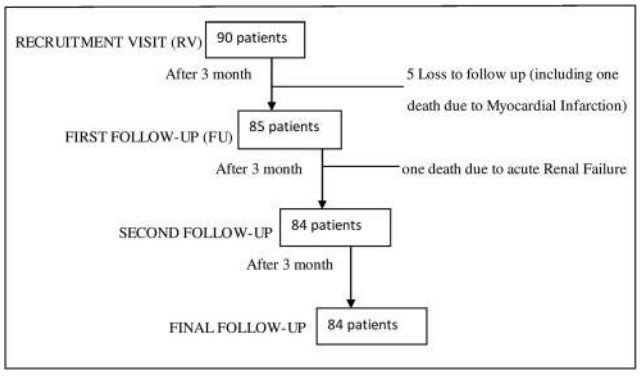

Figure 1 Flowchart of patients observed at each follow-up

Table 1 Antidiabetic drug therapy

Drug therapy	RV (*N*=90)	1 FU (*N*=85)	2 FU (*N*=84)	3 FU (*N*=84)
Monotherapy	24(26.67)	16(18.82)	11(13.10)	22(26.20)
Dual drug	36(40.00)	33(38.82)	38(45.24)	32(38.10)
Triple drug	21(23.33)	30(35.29)	28(33.33)	21(25.00)
≥ Four drug	09(10.00)	06 (07.06)	03(03.57)	05(05.95)
On diabetic diet and exercise	00	00	04(04.76)	04(04.76)

(Value given in the parenthesis indicate percentages)

{*RV* recruitment visit, *FU* follow-up}

decreased to 69.05% (*N*=58) in final follow-up (Table 1). 4.76% (*N* = 4) people stopped antidiabetic drugs and switched to diabetic diet and exercise. The metformin remained to be most commonly prescribed drug from baseline (97.85%, *N*=88) until final follow-up (90.5%, *N*=76). The second most common drug prescribed was glimepiride. Teneligliptin was the most commonly prescribed drug among the DPP-IV inhibitors. Voglibose was prescribed to 17 patients (18.9%) at the baseline; however, more patients received this drug in subsequent follow-up. Ten (11.1%) patients were also receiving insulin at the time of recruitment. In the subsequent follow-ups, insulin was not prescribed to any patient.

Three patients (3.5%) switched to Ayurvedic medications in the first follow-up and two patients continued to take until final follow-up. After second visit, one patient (1.2%) started taking Homeopathic medicine. Metformin + glimepiride was the most commonly prescribed fixed-dose combination (FDC) at the baseline (42.2%) as well as in all three follow-ups (Table 2). The WHO core prescribing indicators are illustrated in Table 3. The utilization of drugs from the National List of Essential Medicines was 75% while 93.33% of antidiabetic drugs were prescribed by generic name at the baseline. Average daily doses of most of the drugs were less than that of their defined daily dose (DDD) except glimepiride (Table 4).

Evaluation of 10-year cardiovascular risk using QRISK3 score

The individual QRISK3 score did not change significantly from baseline to the final follow-up visit and we observed that more than half of patients had a QRISK3 score <10%. More than 75% patients were statin non-users at the baseline and subsequent follow-ups (Table 5). On considering 2014 NICE

Table 2 Distribution of prescribed fixed-dose combination of antidiabetic drugs

	RV *N* (%)	1 FU *N* (%)	2 FU *N* (%)	3 FU *N* (%)
1)Metformin + glimepiride	38(42.2)	21 (24.7)	21(25.00)	27 (32.1)
500 mg + 1 mg	20(22.2)	13 (15.3)	14 (16.7)	20 (23.8)
1000 mg + 1 mg	09(10.0)	01 (01.2)	03 (03.6)	03 (03.6)
1000 mg + 2 mg	07(07.8)	04 (04.7)	02 (02.4)	02 (02.4)
500 mg + 2 mg	02(02.2)	03 (03.5)	02 (02.4)	02 (02.4)
2) Metformin + Teneligliptin	2(02.2)	02 (02.4)	06(07.1)	06 (07.1)
500 mg + 20 mg	1(01.1)	01 (01.2)	04(04.8)	02 (02.4)
1000 mg + 20 mg	1(01.1)	01 (01.2)	02 (02.4)	04 (04.8)
3) Metformin + Sitagliptin	2 (02.2)	02 (02.4)	01 (01.2)	-
500 mg + 50 mg	1(01.1)	01 (01.2)	-	-
1000 mg +100 mg	1(01.1)	01 (01.2)	01 (01.2)	-
4) Metformin + voglibose	-	-	06(07.1)	06(07.1)
500 mg + 0.2 mg	-	-	06(07.1)	05 (05.9)
500 mg + 0.3 mg	-	-	-	01 (01.2)
5) Metformin + glimepiride + voglibose	02 (02.2)	06 (07.1)	12(14.3)	17 (20.2)
(500 mg + 1 mg + 0.2 mg)	01(01.1)	05 (05.9)	11 (13.1)	16 (19.1)
(500 mg + 1 mg + 0.3 mg)	01(01.1)	01 (01.2)	01 (01.2)	-
(1000 mg + 1 mg + 0.2 mg)	-	-	-	01 (01.2)

(*N* number of patients, *RV* recruitment visit, *FU* follow-up)

Table 3 WHO core prescribing Indicators

	Baseline	1 FU	2 FU	3 FU
Average no. of drugs per prescription	4.18	3.89	3.69	3.62
Average no. of antidiabetic drugs per prescription	2.17	2.30	2.25	2.11
Average no. of nondiabetic drugs per prescription	2.62	1.90	1.86	2.08
Percentage of antidiabetic drugs prescribed by generic name	93.33%	89.52%	84.31%	79.79%
Percentage of antidiabetic drugs prescribed from Essential drug list India(NLEM 2015)	75%	69.19%	71.04%	72.62%

{*FU* follow-up}

guidelines, we observed that statin under-users (QRISK3 score >10% but not using statin) were more compared to over-users (QRISK3 score <10% and still using statin).

Adverse drug reactions

During the study period, adverse drug reactions were noted in the case record form at the baseline and all three follow-ups. Total 28 adverse drug reactions (ADRs) were reported during the entire study period. Two serious adverse events in the form of mortality due to myocardial infarction and acute renal failure were also reported. Both these events were unrelated to antidiabetic therapy. The most common ADR reported was GI upset. Abdominal discomfort was observed in 7 patients (25%), diarrhea in 4 patients (14.3%), and anorexia in 2 patients (7.14%) receiving metformin. Flatulence was observed in 5 patients (17.8%), abdominal bloating in 3 patients (10.7%), and diarrhea in 1 patient (3.6%) receiving voglibose. Hypoglycemia was reported by three patients (10.7%) receiving glimepiride and urinary tract infection was reported by one patient (3.6%) receiving empagliflozin.

Discussion

In our study, the average age of recruited patients was 50 years. An observational study conducted by Chaudhary et al. (2019) [10] had the highest number of patients (31.2%) in the 51- to 60-year age group while in our study, the highest number of patients (34.44%) were in age group 40–49 years and between 50 and 59 years were second highest (27.78%). 58.9% of recruited patients had *BMI* < 25 kg/m^2, which indicates that even underweight or normal weight persons are being affected by diabetes nowadays. The reason underlying this could be their sedentary lifestyle and altered food habits [11].

About 2/3rd of recruited patients were from lower socioeconomic status (class IV and V). Diabetes was prevalent in higher socioeconomic class 50 years ago, but now, the epidemiological pattern is changing. This gradient shift can be attributed to rapid sedentary lifestyle changes in the lower socioeconomic class [11]. Therefore, the procurement of antidiabetic medication in this class is a matter of concern, which may lead to increased morbidity and financial burden on the health care delivery system.

Table 4 Comparison of average dose v/s defined daily dose

Drugs (ATC code)	Defined daily dose	Average dose (/day in mg)			
		RV	1 FU	2 FU	3 FU
Metformin (A10BA02)	2000	1119.32	1036.58	967.53	967.10
Glimepiride (A10BB12)	2	02.24	02.27	01.92	01.93
Voglibose(A10BF03)	0.6	00.52	00.54	00.47	00.39
Teneligliptin (A10BH)	--	22.00	21.90	21.18	23.64
Sitagliptin(A10BH01)	100	100.00	100.00	100.00	-
Acarbose(A10BF01)	300	50.00	75.00	75.00	75.00
Insulin (A10A)	40	18.4	-	-	-
Empagliflozin(A10BK03)	17.5	-	25	-	-
Repaglinide(A10BX02)	4	-	3.00	3.00	2.00
Pioglitazone(A10BG03)	30	18.75	15.00	-	15.00
Linagliptin(A10BH05)	5	-	5.00	-	-

(Note: Unit of all the drugs are in mg; unit of insulin is in IU)

{*RV* recruitment visit, *FU* follow-up}

Table 5 Distribution of diabetic patients by statin users and non-users

Visit	Statin	No. of patients	< 10%	> 10%
Baseline (*N*= 90)	Users	21(23.3)	13 (14.4)*	08(08.9)
	Non-users	69(76.7)	40(44.5)	29 (32.2)#
1 follow-up (*N*= 85)	Users	19(22.4)	14 (16.5)*	05(05.9)
	Non-users	66(77.6)	38(44.7)	28(32.9)#
2 follow-up (*N*= 84)	Users	19(22.6)	12 (14.3)*	07(08.3)
	Non-users	65(77.4)	40(47.6)	25(29.8)#
3 follow-up (*N*= 84)	Users	19(22.6)	13 (15.5)*	06(07.1)
	Non-users	65(77.4)	41(48.8)	24(28.6)#

(Value given in the parenthesis indicate percentages) [* = over-users; # = under-users]

The drug utilization pattern of the antidiabetic drugs was similar to other studies conducted in India, e.g. Mandal et al. (2016) [12] Geetha et al. (2017) [13]. In these studies, the average number of antidiabetic drugs per prescription was 2.18 and 2.56 respectively while in our study, it was near about two or more antidiabetic drugs per prescription. This indicates that polypharmacy is an observed trend in the management of type 2 diabetes mellitus. Antidiabetic drugs prescribed by generic name (baseline) were 93.33%. However, the prescription by generic name was very less in other studies 4.66% [14] and 11% [15]. Similarly, the utilization of drugs from the National List of Essential Medicines reported was less in other studies 53.79% [14] and 51.2% [15] while it was 75% in our study.

As per recommendations ADA 2021, treatment should be started with monotherapy. In our study, 26.67% patients were on monotherapy at the start of study whereas more than 2/3rd of patients (73.33%) were prescribed two or more antidiabetic drugs at the time of recruitment. The studies conducted by Kapur et al. (2019) [16] and Joshi et al. (2018) [17] also reported that 70% and 85% of patients, respectively, were receiving polypharmacy.

ADA guidelines also recommend that dual therapy in patients with newly diagnosed type 2 diabetes can be considered who have A1C ≥ 1.5% above their glycemic target [18]. However, in our study, among the newly diagnosed, 10.4% of patients were started with dual drug therapy despite having A1C below the glycemic target to start dual therapy. In these patients, dose of metformin used was 500–1000 mg per day. However, maximum recommended daily dose of metformin is 2 gm but doses more than 1 gm per day are often associated with side effects and intolerance. Initially at the time of recruitment, 21 patients were prescribed metformin > 1000 mg/day, out of which 7 patients experienced ADR due to metformin and the dose was decreased. Blood sugar level of 8 patients was controlled, so their daily doses of metformin were reduced to < 1000 mg/day on further follow-ups. On the other hand, 6 patients were continued to receive metformin with same dosage of > 1000 mg/day without any ADR. An average daily dose of metformin was decreased from 1119.32 mg (baseline value) to 967.10 mg (third follow-up visit). In the study conducted by Mandal et al. (2016) [12], average daily doses of metformin were 990.97 mg/day which was greater than average daily dose of metformin (967.10 mg/day) in our study. The doses of other drugs like glimepiride (1.50 mg/day) and Sitagliptin (50 mg/day) were observed to be less compared to average daily doses of these drugs in our study (Table 4).

Metformin was the most commonly prescribed antidiabetic drug. It is an euglycemic drug, causes weight loss, improves lipid profile, reduces the production of advanced glycosylated end products, and prevents both microvascular and macrovascular complications in type 2 diabetes patients [19]**.** The second most common drug prescribed was glimepiride throughout the study. A similar finding was observed in previous studies conducted by Mandal et al. (2016) [12] and Patel et al. (2013) [20]. Among the DPP-IV inhibitors, Teneligliptin was the most common drug, prescribed to 22.2% of patients at the time of recruitment. In a similar study conducted by Chaudhary et al. (2019) [10], DPP-4 inhibitors were prescribed to 29.78% of patients comprising of Sitagliptin, Vildagliptin, and Linagliptin. Gliptins are mainly used as adjuvant because they are weight neutral, safe, well-tolerated, have convenient dosing schedule and also negligible risk of hypoglycemia. Alpha-glucosidase inhibitors are added to control post-prandial hyperglycemia. Voglibose has better GI tolerance and more potent in this class. Hence, this could be the reason for prescription of this drug in a greater number of patients in subsequent follow-ups to control post-prandial hyperglycemia.

Despite the known CV benefits of SGLT-2 inhibitors and GLP-1 agonists [21], only few patients were prescribed these drugs as these drugs are very costly and GLP-1 agonists are injectable too. India is a developing country and such costly injectable drug was not affordable to them as most of the recruited patients were belonging to lower socioeconomic class IV and V. Compared to these drugs, metformin which shows significant reduction of cardiovascular events [21] was most economical and acceptable to recruited patients.

Metformin (500 mg) + glimepiride (1 mg) was the most commonly prescribed FDC. A similar finding was observed in the studies conducted by Patel et al. [20] and Acharya et al. [22]. FDC of antidiabetic drugs in DM management is considered irrational as all the drugs are not needed in every patient with diabetes and the time of administration of individual drugs may not match leading to pharmacokinetic incompatibility. The FDC of metformin with 2nd Generation Sulfonylurea and α-glucosidase inhibitor is considered irrational as metformin is given with food or after food to prevent gastric irritation. In contrast, sulfonylurea is given before the

meal and voglibose is given with the meal's first bite. Therefore, different antidiabetic drugs when needed can be given separately instead of FDCs. Ten (11.1%) patients (baseline) had poorly controlled blood sugar level, so they were prescribed insulin as per ADA recommendation [18].

Four patients stopped allopathic medicines and completely switched to Ayurvedic/ Homeopathic medication. Of these, two patients had got reasonable glycemic control. However, planned scientific studies are needed to establish the safety and efficacy of these medicines of complementary system. The most common ADR reported in our study was GI upset in the form of abdominal discomfort and diarrhea. In a similar observational study on type 2 DM, patients carried out by Deb et al. [23] found that metformin contributed 51% of total ADR reported, in the form of dyspepsia and diarrhea, followed by glimepiride-induced hypoglycemia.

Recent guidelines do not anymore consider diabetes as a CAD risk equivalent and recommend cardiovascular risk stratification for primary prevention. Stratification of diabetic patients improves accuracy in prediction of subclinical CAD, silent ischemia, and future cardiovascular events. Stratification also discriminates higher from lower risk patients who may need intensive statin or aspirin prevention, while avoiding overtreatment in lower risk cases [24]. Recruited patients with QRISK3 score of < 10% were categorized as low-risk group and QRISK3 score of > 10% as high-risk group as per NICE guidelines [8]. This study revealed overtreatment of low-risk groups (i.e., QRISK3 score is < 10% but still taking statin) and under-treatment of high-risk groups (i.e., QRISK3 score is > 10% but not taking statin). In fact, statin under-users were more compared to over-users. Moreover, recent guidelines state that diabetic patients ≥ 40 years of age having no ASCVD risk factors should be offered moderate-intensity statin [19], and coincidently, all statin under-users of our study were > 40 years. In the present study, around 15% of patients were statin over-users throughout the follow-up, which is relatively higher than other studies, 2.7% [25] and 5.0% [26]. These findings indicate that factors other than risk score were being considered while initiating statins. Therefore, for judicious use of statins, the QRISK3 score should also be considered. Estimates of CVD risk can be helpful for both clinicians and patients: for clinicians, it gives prognostic information that can support them in the choice of therapeutic and preventive strategies such as use of aspirin, statins, and SGLT-2 inhibitors and GLP-1 receptor agonists; for patients, it can be a motivation tool to adopt healthy lifestyle measures [27]. Even the American College of Cardiology/American Heart Association recommends the use of ASCVD risk calculator tool (Risk Estimator Plus) to estimate 10-year risk of first ASCVD event in newly diagnosed diabetic patient [5]. This Risk Estimator Plus tool is available online which can also be downloaded as mobile app to estimate the ASCVD risk [28].

Strength of the study

This study was a prospective follow-up study of new and recently diagnosed type 2 DM patients at a 3-month interval for 9 months; however, the study designs of most of the drug utilization studies in diabetes mellitus are cross-sectional. We observed the changes in the drug utilization pattern of antidiabetic drugs on prospective follow-ups of these patients through this study.

Limitation of the study

Diabetes patients were selected from only one hospital in Raipur, which is located in an urban area, so the representation of the rural population was relatively low. The study had a limited sample size (90 patients) and was conducted for a limited duration of time, i.e., 15 months and recruited patients were followed only for 9 months.

Conclusion

Patient education and periodic review of diabetic patients play a vital role in managing type 2 DM. The prevalence of type 2 DM was more in the middle-age group (40–49 years), having low or normal *BMI* and patients belonging to lower socioeconomic class. More extensive and elaborative studies are needed to explore the factors responsible for paradoxical observations of our study.

Polypharmacy was observed trend in prescription in this prospective follow-up study. Instead of using maximum recommended dose of most commonly used drug metformin, other drugs were added in prescription. The prescription analysis also revealed that irrational FDCs were prescribed e.g., metformin + glimepiride; metformin + glimepiride + voglibose. Metformin plus glimepiride was the most commonly prescribed FDC. The prescription of antidiabetic drugs by generic name and adherence to national list of essential medicine (NLEM) were significant findings in our study. This study emphasizes assessment of cardiovascular risk using QRISK3 score. This will also be helpful for judicious use of statin in diabetes.

Abbreviations *ADA,* American Diabetes Association; *A1C,* hemoglobin A1C; *AHA/ACC,* American Heart Association/American College of Cardiology; *ASCVD,* atherosclerotic cardiovascular disease; *CAD,* coronary artery disease; *CV,* cardiovascular; *DM,* diabetes mellitus; *FDC,* fixed dose combination; *NLEM,* National List of Essential Medicine

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Declarations

Ethics approval The study was a prospective, longitudinal, observational hospital-based study initiated after obtaining approval from Institute Ethics Committee. Written informed consent was obtained from all the patients.

Consent for publication All patients provided written informed consent before study entry.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Automation of insulin bolus dose calculation in type 1 diabetes: a feasibility study

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Abstract

Background Diabetes care in type 1 diabetes has remained a challenge over time. Ability to count carbohydrates in every meal and varying insulin dose according to individual insulin/carbohydrate ratio allows people with type 1 DM to have wider food choices with better glycemic control. Difficulties in carbohydrate counting may largely be solved by use of technology.

Methods This work was done at the endocrine unit of a superspeciality centre involved in care of people with type 1 diabetes. The process of development of software and its preliminary application and results from its use in clinical care in a small group of interested patients is presented in this manuscript. Carbohydrate counting tool for Indian foods was developed, and subsequently, bolus dose calculation was automated by using the reinforcement algorithm in an android app platform named "T1-Life". Data on app usability and acceptability is documented in this pilot study report.

Results Five patients completed 3 months of this app usage. Among five people with combined usage over 115 patient weeks, a total of 2661 insulin dose predictions were made. This translates to 3.31 patient-initiated bolus dose predictions per day. Of the total bolus dose predictions made, 82% were accepted by the participants. With usage of app, time in range (70–180 mg/dl) increased by an average of 16.67% in children who used CGMS in their first week as well as last week of observation.

Conclusion T1-life, integrated carbohydrate counting and reinforcement-based insulin bolus dose prediction system, has good patient usability and acceptability.

Keywords Type 1 diabetes · Expert system · Bolus insulin dose prediction · Carbohydrate counting

Introduction

Incidence of type 1 DM is increasing worldwide. Incidence of type 1 diabetes in India has been reported to be 3.7/100,000 in boys and 4.0/100,000 in girls [1]. Prevalence has been estimated to be 26.6/100,000 in urban and 4.27/100,000 in rural areas [2]. Type 1 DM contributes to only 5–10% of the total population affected by diabetes [3]. But its impact on the affected child and his/her family is tremendous. There have been significant improvements in armamentarium for type 1 diabetes care with inventions of insulins, recombinant human insulins, painless pen devices for injection, continuous glucose monitoring systems, insulin pumps, and so on. But glycemic control in people with type 1 diabetes still remains poor worldwide [3–6]. Similar trend is also seen in studies from India [7].

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Factors responsible for poor control might be related to patient factors like poor adherence to treatment recommendations like self-monitoring of blood glucose, compliance to insulin therapy, diet recommendations, and poor participation in treatment decision-making. Glycemic control may also be poor due to healthcare deficiencies like poor access to expert care, poor access to insulin, and inability to impart diabetes education. Moreover, it is being identified that a vision beyond Hba1c control may be warranted in care of people with type 1 diabetes [8]. A consensus statement suggests use of various other clinically meaningful outcome measures like hypoglycemia, hyperglycemia, diabetic ketoacidosis, and time in range besides HbA1c measurements [8].

Carbohydrate counting (CC) is one of the ways to improve glycemic control and quality of life in people with type 1 diabetes [9, 10]. Carbohydrate counting has been shown to consistently reduce HbA1c in different meta-analyses [9, 10]. It is considered to be one of the best options among insulin dosing strategies [11]. Carbohydrate counting gives flexibility in food choice by tailoring insulin dose according to carbohydrate quantity and quality. However, it requires knowledge of carbohydrate content of different food products, frequent blood glucose measurements, and complex arithmetic skills to calculate individual insulin sensitivity factor (ISF) and carbohydrate insulin ratios (CIR). There are few basic formulas available as a good starting point to calculate ISF and CIR, but these need to be individualised according to the activities and daily routine of each person. Few tools have been reported for this purpose in literature, but these tools have only automated calculation part of the entire process and are very mechanistic in nature [12]. They need manual calculation of carbohydrate amount in a meal, and individuals need to calculate their ISF as well as CIR themselves [12]. Even with these inherent limitations, these calculators are still preferred by people than conventional ways of manual insulin dose calculation. None of these applications is available outside research domains though.

Use of technology and machine learning can help in developing an expert system to solve all the steps needed to automate insulin bolus dose prediction. There is very limited work done so far in this field. We intend to develop a mobile application, which could help in automation of CC and bolus insulin dose prediction. This manuscript presents preliminary results from use of the prototype of this mobile application in clinical care of a small group of interested patients.

Methods

Setting This work was done at the endocrine unit of a superspeciality centre involved in care of people with type 1 diabetes.

Food database A food database of commonly used Indian foods with their nutrient contents was created with the help of local registered dietitians. Using this information, an Android platform mobile application (app) "T1-Life" was created to automate carbohydrate counting. Patients could enter their actual meal content, and the app would let them know its macronutrient composition as well as total calories.

Instructions to participants Participants (and their families, in cases of children) were imparted skills for carbohydrate counting skills in a one-to-one setting. They were also explained about the usage of the app to record their food intake, exercise duration/type, blood glucose values, and insulin dose taken. Patients were also initiated on Abbott libre pro continuous glucose monitoring system and were instructed to follow every week.

Approach to insulin prediction CGMS (continuous glucose monitoring system) data, food data, exercise data, and insulin dose records over 3 months were analysed for initial two patients to refine machine learning algorithms for automation of suggestions for doses of insulin.

An expert system has been created where initial insulin prediction is rule-based and subsequent insulin dose adjustment is done based on reinforcement principle. Reinforcement algorithms are applied in an individual-specific as well as meal-specific manner. In the expert system, starting bolus doses are decided as per 500 and 1500 rule. People are asked to enter their pre-meal blood glucose values along with actual food about to be taken. Insulin dose is calculated automatically at the back-end by extracting carbohydrate content of food, ISF and CIR. Algorithm also includes modifications for already present insulin on-board from previous insulin doses as well as any correction dose needed based on pre-meal blood glucose values and ISF. ICR of each patient was modified weekly as per their CGMS/self-monitoring of blood glucose (SMBG) data entered in mobile app. The flow of data and use of app is depicted in Fig. 1.

Outcome prediction Preliminary results on usage and acceptability of this approach are documented in this manuscript. Glycemic parameters are also explored in these cases; however, a larger study would be needed to comment on statistically significant improvement in care standards. Glycemic data shown in this report is only from CGMS readings for uniformity sake. Data was analysed descriptively using Python 3.7.0 and Jupyter 5.5.0 platforms with the help of various open source tools and libraries including Pandas, NumPy, collections, Counter, matplotlib, sklearn.cross_validation, sklearn.preprocessing, PolynomialFeatures, and sklearn.linear_mode. Data retrieval and analysis was done adhering to principles outlined in the World Medical Association's Declaration of Helsinki.

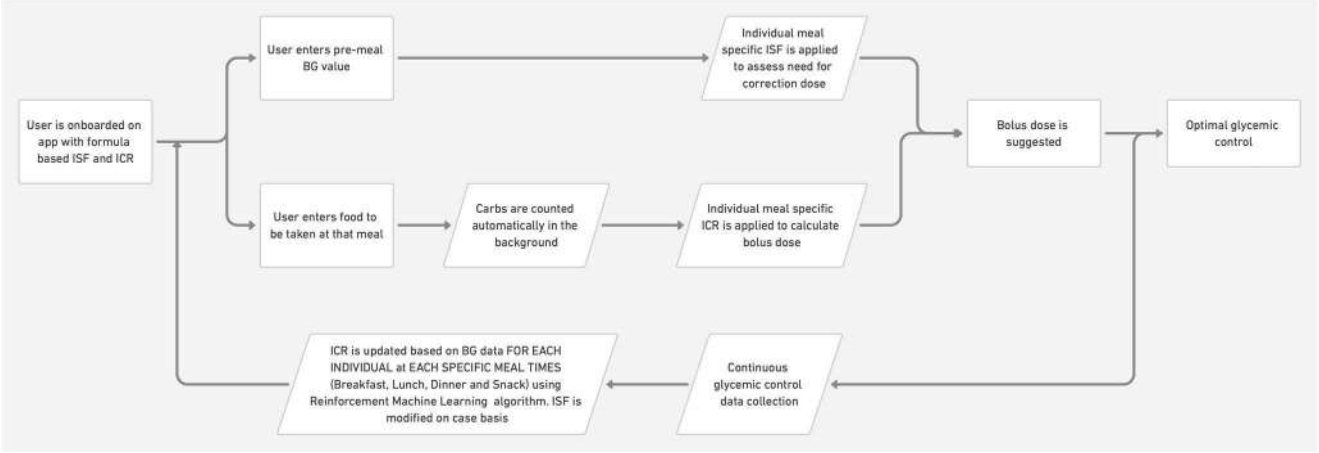

Fig. 1 Architecture of mobile application and steps involved in insulin bolus dose prediction

Results

Five patients have continuously used this app for 3 months or more and their results are being reported. Table 1 shows the baseline characteristics of app users as well as quantum of data entry done by them. It also documents acceptance rates of insulin dose predicted by app for patients. Pilot phase of current study shows excellent patient adherence and acceptance. Overall, among five people with combined usage over 115 patient-weeks, a total of 2661 insulin dose predictions were made. This translates to 3.31 bolus dose predictions per day and means that patients on an average tested their blood glucose values at least 3 times a day and entered their food and blood glucose data in app to take guidance on bolus insulin dose. There is no difference in data entry in the first and last week of their usage.

Interestingly, there is a huge difference in ICRs as per their different meal timings. At start, each meal of the day has the same ICR as per 500 rule. However, on reinforcement, ICR at each meal differed even for an individual person. Different ICRs were not explained by difference in food macronutrient composition at different meals times. From the predicted bolus insulin doses, 82% were accepted, and 18% were rejected. However, the acceptance rate is skewed by data of first patient where acceptance rate is 46.23% only. In this particular patient, on analysis of rejected doses, median difference of dose was only 0.5 units (25th percentile −0.5 unit; 75th percentile +0.5 unit).

With current approach, there is a gradual trend for improved glycemic control with lowering of mean blood glucose over time and also increase in percentage time in range. Figure 2 shows CGMS data from the patient with the longest experience on the app.

Time in range (70–180 mg/dl) increased by an average of 16.67% in three children who used CGMS in their first week as well as last week of observation. Overall, these five children were 46.86% (range 39–66%) of the time in range when analysed in their last week of app usage irrespective of their total duration of app usage. Time in hypoglycemia (<70 mg/dl) was 8.83% (range 2.3–13.6%) when analysed similarly.

Discussion

Automation of processes that are predictable and require considerable effort in daily care could ease the burden of disease and can help in improving the quality of life in people with T1D.

Care for people with type 1 diabetes poses greater challenges in India due to multiple reasons. Firstly, care in India is physician centric where insulin dose is largely determined by physicians during clinic visits based on SMBG records. Carbohydrate counting, as standard of care, is not imparted to and discussed with people with type 1 diabetes. Secondly, there is a lack of validated food databases containing

Table 1 Baseline characters, app usability, and acceptability data

Person	Age/ gender	Duration of diabetes (years)	Weeks on app	Data entry per day	Total predictions made	Acceptance rate (%)
1	7.5/M	2	36	3.27	824	46.23
2	20/F	16	15	3.31	391	95.14
3	18.5/F	5	27	3.28	648	91.82
4	8/F	2	18	2.9	372	83.33
5	6/M	1	19	2.82	426	93.66

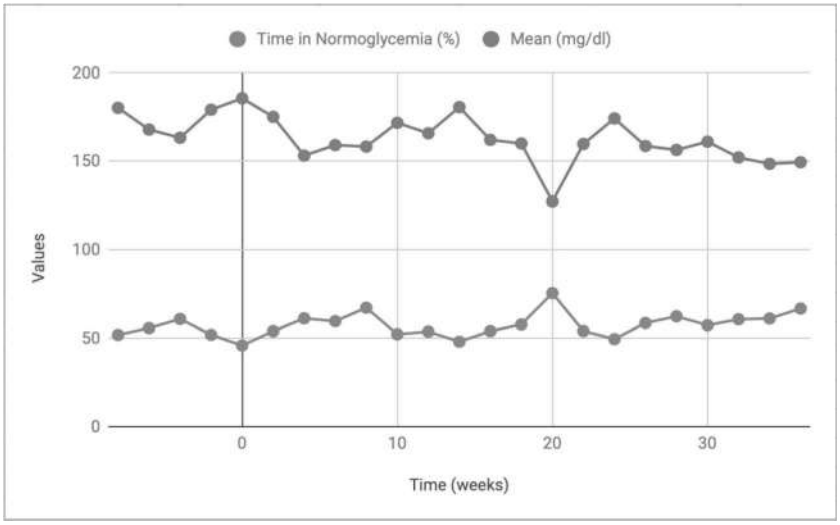

Fig. 2 Change in mean blood glucose values and time in normoglycaemia over 36 weeks of app usage in index patient

compositions of macronutrients of Indian food items. Thirdly, Indian food is largely cooked at home, and portion size of each food item varies in each household, and that makes it even harder to create a uniform food database for practical use. Fourthly, in most clinic settings in India, there is lack of availability of trained support staff who are accessible to patients from home. Fifth, standardised food labels with clear serving size, carb content, and dietary fibre are not uniformly available on packaged food. Sixth, self-monitoring of blood glucose at home also has low penetrance in India. The reasons are high cost (an average Indian pays all expenses out of his own pocket) and needle phobia.

An attempt is being made to apply machine learning for bolus dose prediction by researchers across the globe. Most notable among these would be European Union-funded patient empowerment through predictive personalised decision support (PEPPER) project, being conducted at Imperial College, London [13]. PEPPER system is based on case-based reasoning (CBR), and the first of randomised control trials is already underway. This study is recruiting people with more than 18 years of age who are proficient in carbohydrate counting and are already using insulin carbohydrates ratio (ICR) and insulin sensitivity factor (ISF) to calculate the meal-time bolus [14]. Our approach differs from case-based reasoning adopted by PEPPER for multiple reasons. Firstly, from our preliminary data, it is clear that insulin dose would have to be individualised at patient level and also at meal-time level. This variation in ICR at different meal times strengthens our belief in the correctness of our current approach over other machine learning (ML) approaches. For example, even if data is collected for 1 year from an individual person, it would only provide a maximum of 1095 (365×3) data points. Further, if data points needs to be meal specific, that would leave only 365 meal specific data points, collected over a year for an individual patient. We believe that CBR, SVM, or other ML algorithms like neural networks would require a much larger number of data points to provide practically useful clinical application. Secondly, data collected from one person cannot be used in another person. And there can be so many case scenarios in each individual that it may not be meaningfully possible to collect that amount of data. Thirdly, with change in age, height, weight, and food habits, these case scenarios are likely to keep on evolving further. Fourthly, while data is being collected for any of these ML algorithms, patients are not provided with any clinical guidance, and this results in loss of compliance and follow-up. However, as more data is compiled for each individual patient, an amalgamation of current strategy of rule-based reinforcement algorithms with other ML algorithms may definitely add more insights and increase accuracy in the future.

Table 2 Initial insulin carbohydrate ratio (ICR) and last ICR at different meals after application of reinforcement algorithm

Person	Initial ICR	Final breakfast ICR	Breakfast macronutrient distribution	Final lunch ICR	Lunch macronutrient distribution	Final dinner ICR	Dinner macronutrient distribution
1	18.19	16.371	60:20:20	16.371	62:18:20	18.19	55:25:20
2	13.157	10.65	67:16:17	13.01	62:20:18	13.01	64:18:18
3	7.54	3.84	64:18:18	4.14	59:23:18	6.6	56:25:19
4	34.48	19.41	62:20:18	13.2	67:16:17	49.65	66:17:17
5	29.411	10.33	65:19:16	10.18	61:19:20	28.58	58:24:18

Patients initiated 3.31 bolus dose prediction per day were made. That translates to at least 3 SMBG data points per day. This is contrary to expectations with previous publications reporting poor adherence to SMBG. Pesl et al reported similar adherence of 2.7 to 2.9 predictions made per week [15]. Acceptability of predicted bolus insulin dose stands at 82% in current study. Pesl el al reported an overall acceptance rate of 90% in a similar endeavour [15]. Apart from automating the bolus dose calculation, app has been able to educate people about the entire process as carb content of their food is shown each time on their screen as well as their ICRs. Absence of any difference in data entry in the first and last week of usage indicates a value perceived by patients in continued use of the app.

Alcohol, exercise, pre-meal blood glucose values, and glycemic index of food are known to impact ICR for each meal [16, 17]. Pesl et al demonstrated that exercise and alcohol consumption have inverse association with ICR (i.e. reduced insulin dose requirement), while high pre-meal blood glucose values led to reduction in ICR [16]. Taking glycemic index into consideration for calculating bolus dose may improve post prandial blood glucose control in people with type 1 diabetes [17]. In the current study, ICR varied markedly with each meal time in each individual as shown in Table 2. Macronutrient composition of diet was analysed to assess this difference, and no correlation was found. Exercise seems to be an important factor here as four out of five children reported physical activity before meal with highest ICR (i.e. dinner). While the platform has the option of entering physical activity voluntarily, entries made were too few for analysis. This is one area where further improvement is needed to make bolus dose advice more objective. Still, as reinforcement algorithms are being used for each individual meal, clinical impact would be minimal as long as timing and type of physical activity remain constant. Reinforcement principle also negates the effect of different portion sizes of same homemade food as portion size in one household largely remains the same.

Glycemic parameters are difficult to report in such a small number of patients, and their clinical veracity would have to be established with further follow-up. Figure 2 shows gradual improvement in mean BG and time in normoglycemia for the child who has used the mobile application for the longest time. Increase in time in range by 10% indicates an approximate improvement in HbA1c by 0.5–0.8% [18–20]. In current limited data, time in range improved by 16%, thus indicating a potential to improve HbA1c in long-term studies.

Conclusion and directions for the future

T1-life, integrated carbohydrate counting and reinforcement-based insulin bolus dose prediction system, has good patient usability and acceptability. Though initial results are encouraging, there is still a lot of ground to be covered. Changes in dosing based on positive or negative reinforcement need to graduate from current rule based algorithms to machine learning based algorithms in future as more data is available over time. A robust testing model needs to be developed to test out different analytical strategies in silico. Difference in composition of macronutrients other than proteins may be responsible for different ICRs at different meal times, and this need to be factored in bolus dose calculations. Similarly, objective measurement of physical activity and its impact would have to be calculated and incorporated in larger algorithms for insulin dose calculation. Finally, only a randomised controlled trial can demonstrate the clinical utility and cost-effectiveness of this approach. In a resource limited setting like India, scalable technological solutions are the way forward to ensure quality healthcare and health education to people suffering from type 1 diabetes.

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Author contribution R.S., G.G., J.B., and A.S. performed the research. R.S., A.S., and Y.G. designed the research study. R.S., J.B., S.A., and A.S. analysed the data. R.S., J.B., and Y.G. wrote the paper. All authors critically edited and endorsed the manuscript.

Declarations

Ethics approval This study was carried out as part of routine clinical care and is automation of clinical care of type 1 diabetes participants. Data was collated retrospectively.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Does famine exposure in early life modify risks of metabolic diseases in Chinese adults? Evidence based on YiduCloud clinic data

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Abstract

Background Due to inconclusive findings of Chinese famine exposure to risk of metabolic diseases, we investigate the relationship between famine exposure in early stage of life and risk of metabolic diseases in middle-aged adults in southwest China by using the novel YiduCloud big clinic data.

Methods In this retrospective study, electronic clinic records of those born between 1949 and 1978, which covers the years of the Chinese Famine in 1959–1961, were retrieved from the YiduCloud big database which has encompassed seven teaching hospitals from 1999 to the present day. Patients' gender, birth year, admitted year, height, and weight were extracted, and diagnosis of hypertension (HTN) and diabetes mellitus (DM) were identified. Relationships between birth year and risk of HTN and DM were analyzed by using linear regression. Height, weight, and BMI (body mass index) were also compared by birth year with linear regression analysis.

Results More than 360 thousand patients born in year from 1949 to 1978 were included. Those born at the first year of famine experienced longer after birth exposure did not show increased risks of HTN and DM with lower average height, weight and BMI than born year predicted values, whereas those born in the second or third year of famine followed by shorter after birth exposure actually had increased risks of HTN and DM with higher average height, weight and BMI than born year predicted values. All of the above mentioned differences were more pronounced in male than those in female.

Conclusion People who were born in years of the Chinese Famine had different risks of metabolic diseases like HTN and DM in middle ages. Selection of persistent undernutrition pressure after birth and in time replenishment of food with acquired catch-up growth after birth may help explain those different risks of metabolic diseases in adults who were born in early or later famine years. Anyway, malnutrition exposure in early stage of life alone does not likely change too much the risk of metabolic diseases. This study provides some details to fetal origins hypothesis of metabolic diseases.

Keywords Famine · Metabolic disease · Fetal origins hypothesis, Big clinic data

Abbreviations

HTN Hypertension
DM Diabetes mellitus

Introduction

Many developing countries including China are now facing severe epidemics of metabolic diseases like hypertension and diabetes with the national prevalence of 27.8% [1] and 11.9% [2], respectively. According to fetal origins hypothesis proposed by Baker in 1990, which is based on their study on birth weight and CAD (coronary artery disease) risks, malnutrition in early stage of life, especially in fetus, would increase the risk of metabolic diseases in adult life [3]. Therefore, many believed undernutrition in the past might be a major contributor fueling the epidemics of metabolic diseases nowadays in developing countries around the world [4]. But it is difficult to prove it.

Usually, lower birth weight in a baby is considered as a useful surrogate of poor nutrition status. However, it is

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generally not feasible to access birth weight records generated many decades ago or link those to adults' health information today.

Big famine, especially those covering wide populations, and big time interval could be a useful alternation to test fetal origins hypothesis, since birth year, which can be easily identified, itself may be related to the early nutritional status. At the same time, study on famine also provides evidence from an ecological standpoint.

There were several contemporary famines due to natural and social disasters worldwide, such as the Finnish Famine (1866–1868) [5], the Ukraine Famine (1932) [6], the Dutch Famine (1944–1945) [7], the Chinese Famine (1959–1961) [8], the Biafran Famine (1967–1970) [9], the Russian Leningrad Siege (1941–1944) [10], and the UK Channel Islands occupation (1940–1945) [11]. However, the Chinese Famine, which lasted nearly 3 years and leading to more than 30 million deaths across the whole country, provides a unique and invaluable opportunity to study the long-term metabolic health effects of famine exposure.

Evidence from some studies, including the latest one [12] on the Chinese famines and the Dutch famine, suggested that individuals born in famine time had improved risks for a variety of diseases, including metabolic diseases such as obesity, hypertension, and diabetes. However, based on reanalysis of 17 published studies on the Chinese Famine, Lumey recently challenged that those studies do not provide substantial evidence that prenatal famine exposure is related to type 2 diabetes mellitus (T2DM) development [6]. Xu et al., by re-examining the long-term effect of China's 1959–1961 famine, claimed that the Chinese famine exposure actually reduced risk of disease in certain cases [13]. Other researchers also denied any effect of famine based on the Dutch famine study [14]. The reasons for such a judgment include misclassification of prenatal famine exposure and unbalanced control such as pre-, post-, or pre- plus post-famine birth. Other researchers mentioned that sample bias, sample size, unreliable data source, etc. in published famine studies based on communities may also be central to the inconsistency of results [15].

Therefore, whether the Chinese Famine which occurred 5 decades ago has substantially contributed to current T2DM or HTN (hypertension) epidemic in China remains an open question. Because of weaknesses of studies based on communities, such as lacking necessary historic archives to well-defined prenatal famine exposure and obvious selective bias, a different study strategy is warranted, and well-documented clinic records should be a premium choice. However, to the best of our knowledge, no studies of the kind has been published to date.

In order to test the hypothesis of fetal original of metabolic diseases, a novel clinic big data in which medical records of a group of hospitals affiliated to Chongqing Medical University has continually been added, processed, and stored was used to measure famine exposure in early life time on hypertension and diabetes risks among hospitalized elderly adults.

Methods

YiduCloud database

YiduCloud is a program tasked with collecting, processing, and maintaining clinic records of seven teaching hospitals affiliated to Chongqing Medical University. It is a platform intended to provide data service for versatile purposes. It has more than 36,812,353 records available from July 27, 1999, to November 15, 2018, and record numbers are continuing to increase. All information of those clinic records were processed and organized in structured form for authorized users to access.

Records querying and retrieving

The YiduCloud database was queried with the limitation of "birth year" after January 01, 1949, and before December 30, 1978. Records were then retrieved one birth year after another (to avoid the maximum download limitation) as Excel tables, which include variables like birth year, age of admitted patient, discharge diagnosis, height, and weight. All sensitive information like name, ID, case number, and date of birth were masked, while a unique 32-character string number, named Patients Serial Number (PSN), was generated by machine to each patient. Because value of birth year was not available before the admitted year of 2009 in our pilot database examination, only those records of patients admitted in hospital from 2009 to 2018 were included in this study.

Data analysis

The Chinese language terms for "hypertension" and "diabetes" were used to automatically identify patients diagnosed with HTN and/or DM in Microsoft Excel table, respectively. Variables were then linked by PSN using the Query function of Microsoft Access. Pivot table of Microsoft Excel was used to check errors of age at admission year, and mismatched records were then dropped. Duplicate cases (due to multiple admissions of one person) with same PSN were also removed by using function of "remove duplicate values" of Microsoft Excel.

Proportion of case by birth year was calculated and adjusted with local census population in 2010. Prevalence of HTN and DM in hospitalized population, average height, weight, and BMI were also calculated by birth year and by

gender. The relationships between birth year and prevalence of HTN and DM were determined by linear regression analysis; UICI and LICI (upper and lower of 95% individual confidence interval) of regression were used as significant interval.

In order to explain any changes of prevalence of diseases with famine exposure in early stage of life, height, weight, and BMI by birth year were also analyzed by line regression and compared with the prevalence of HTN and DM by birth year.

Ethical issues

Because all sensitive information which can be used to trace patients in records from the YiduCloud database were properly masked, this study does not pose any ethically related concerns.

Results

Overall

From YiduCloud database, we queried and retrieved records of patients who were born in year of 1949–1978 and who were admitted in hospital in year of 2009–2018. After removal of registered errors, 514,542 out of 516,955 records remained. Among them, 361,639 patients (male/female = 175,326/186,779) had a unique PSN; 69,457 of them (male/female = 34,664/34793) were diagnosed with hypertension (HTN), and 42,684 (male/female = 22,136/20548) were diagnosed with diabetes mellitus (DM).

The admission age of hospitalization in year of 2009–2018 and case numbers by birth year were shown in Fig. 1. For patients who were born in famine year (1959, 1960, and 1961), the admission age ranges were 50–59, 49–58, and 48–57 years old, respectively. The numbers of patients born in those three years were also smaller than those born before or after the famine period.

Proportions of hospitalization by birth year

For patients born in famine year of 1959–1961, the proportions of hospitalizations by birth year from 1949 to 1978 were obviously lower than those who were born in years before or after famine, with similar pattern as those of 2010 local census of Chongqing City (Fig. 2A).

After adjusting patient proportions with population proportions to remove the influence of population size born in each year, adjusted patient proportions were negatively and linearly related with birth year in good fitness ($\beta = -0.008$, $p < 0.0001$, $R^2 = 0.9168$). For those born in the first and third famine year (i.e., 1959, 1961) and those born soon after famine (i.e., 1962), the adjusted proportions were higher than its UICI (upper of 95% individual confidence interval) of predicted value, showing significantly increased hospitalization (Fig. 2B).

Prevalence of HTN and DM in hospitalization by birth year

The overall prevalence of HTN and DM in male and female patients were 19.8%, 18.6%, 12.6%, and 11.0%, respectively. Prevalence were linearly and negatively related to birth year in both genders (all p value < 0.0001). The fitness of both

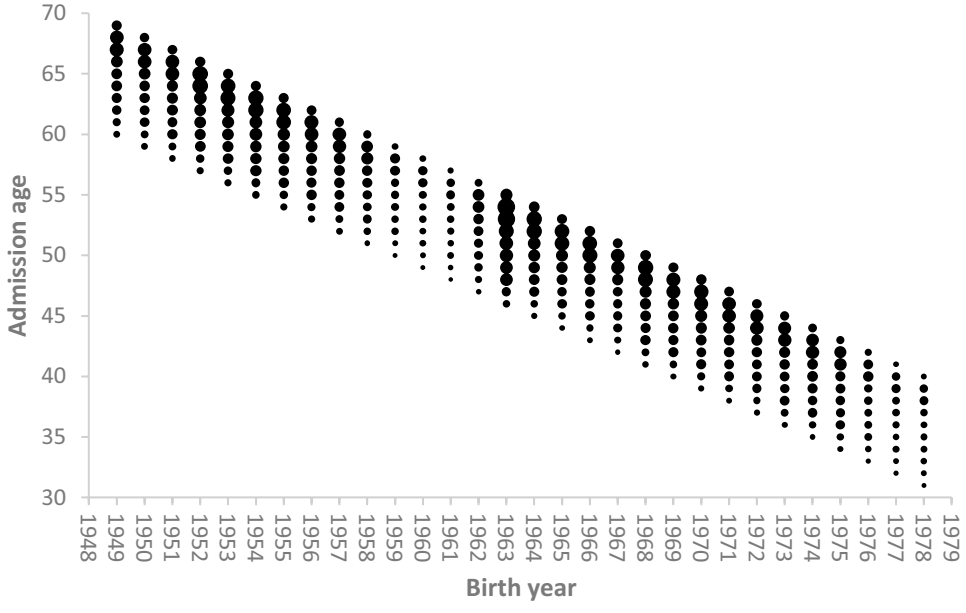


Fig. 1 The summary of age and numbers of patients admitted in 2009 ~ 2018 from YiduCloud database by birth year. Bubble's area represents the volume of case number

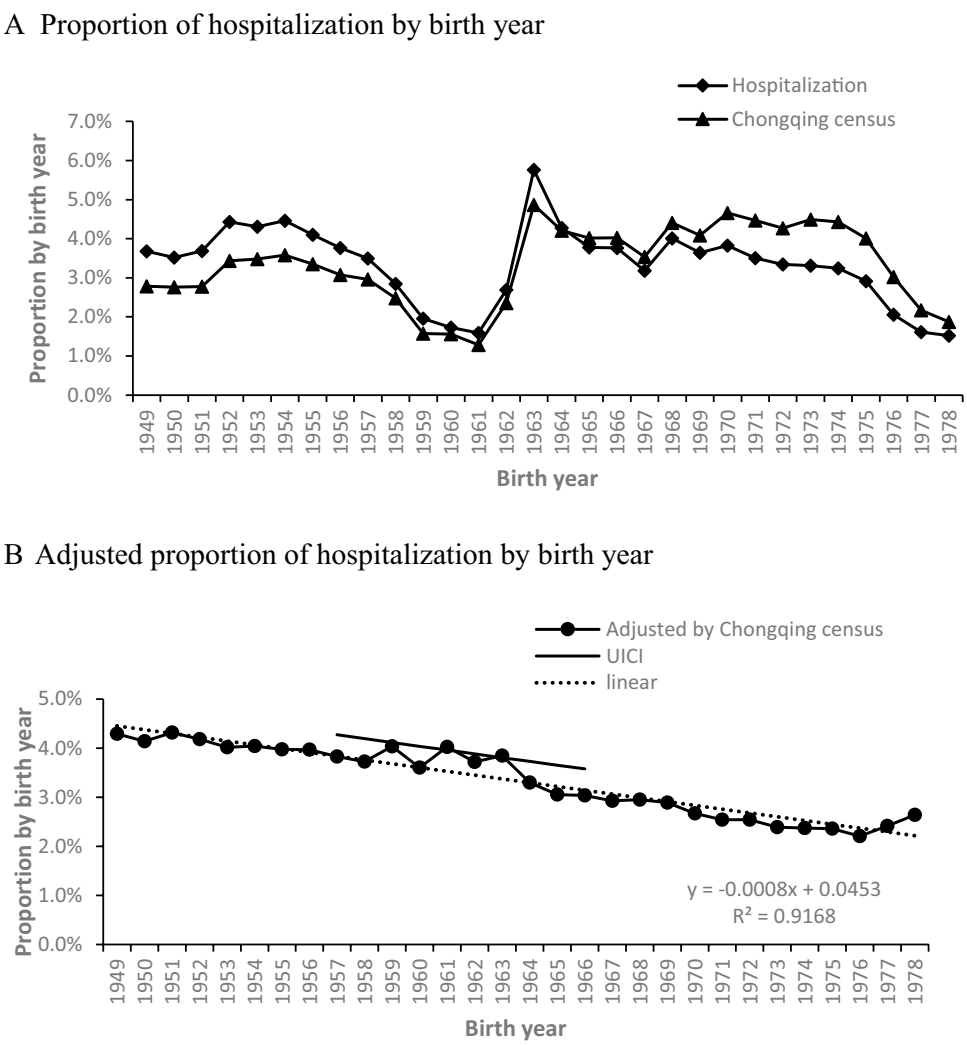


Fig. 2 **A** Proportion of hospitalization by birth year. **B** Adjusted proportion of hospitalization by birth year

HTN and DM in female were better than those in male, with higher R^2 values. The regression line predicted the average prevalence of HTN or DM by considering the influence of born year. Patients born in the first famine year (i.e., 1959) had significant lower prevalence of HTN, whereas those born in the third famine year (i.e., 1961) had significant higher prevalence of HTN when compared with the linearly predicted values in both genders (Fig. 3A–B). However, patients born in the second and third famine year (i.e., 1960–1961) had significantly higher prevalence of DM, as compared to the linearly predicted values in both genders (Fig. 3C–D).

Height, weight, and BMI of patients

There were 18.2% and 41.3% of patients with values of height and weight, respectively. The average height, weight, and BMI of all patients increased along birth year linearly ($p < 0.05$), except the BMIs for female. When compared with the linear regression predicted values, male patients born in the first famine year (i.e., 1959) usually had significant or close to significant lower height, weight, and BMI (Fig. 4A–C), whereas female patients born in the second famine year (i.e., 1960) had the significant or close to significant lower height, weight, and BMI (Fig. 4D–F).

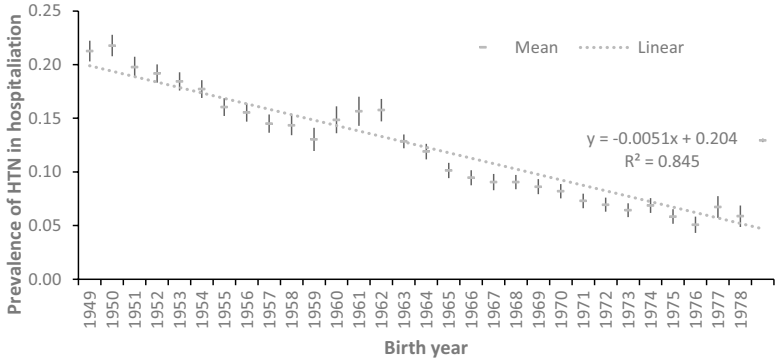
Height, weight, and BMI by HTN and DM

Patients with HTN or DM had almost the same height as those without HTN or DM, but higher in weight or BMI, wholly (Fig. 5A–F).

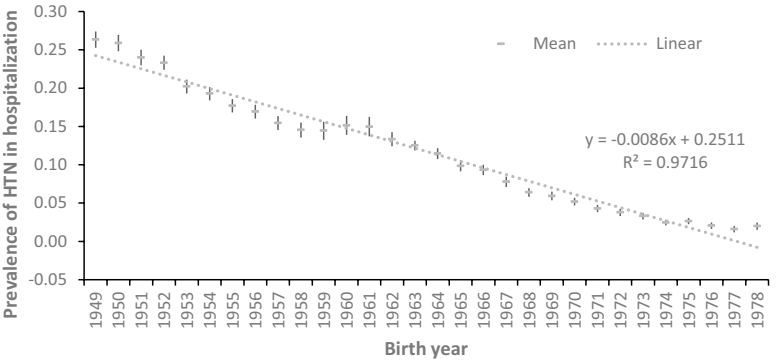
Discussion

The metabolic syndrome represents truncal obesity, in concert with combinations of HTN, hypercholesterolemia, hypertriglyceridemia, and insulin resistance [16, 17], and more than one third of US adults meet the criteria for the syndrome [18]. Several hypotheses have been generated on

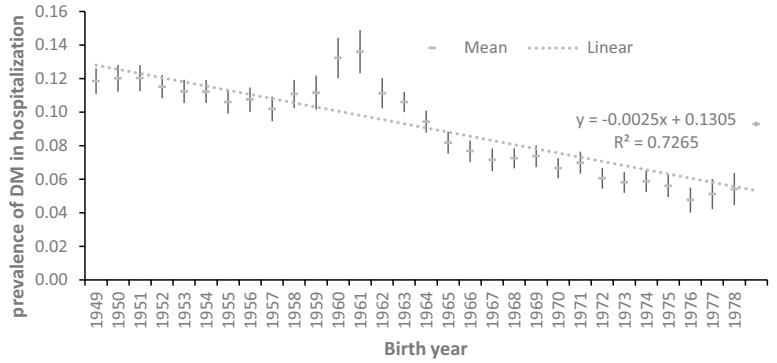
A HTN prevalence in male patients



B HTN prevalence in female patients



C DM prevalence in male patients



D DM prevalence in female patients

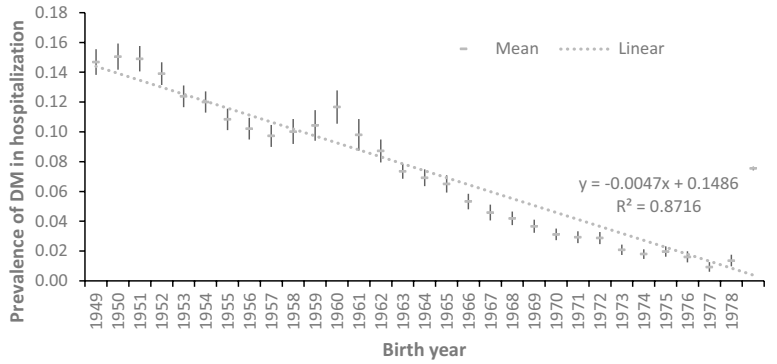


Fig. 3 **A** HTN prevalence in male patients. **B** HTN prevalence in female patients. **C** DM prevalence in male patients. **D** DM prevalence in female patients

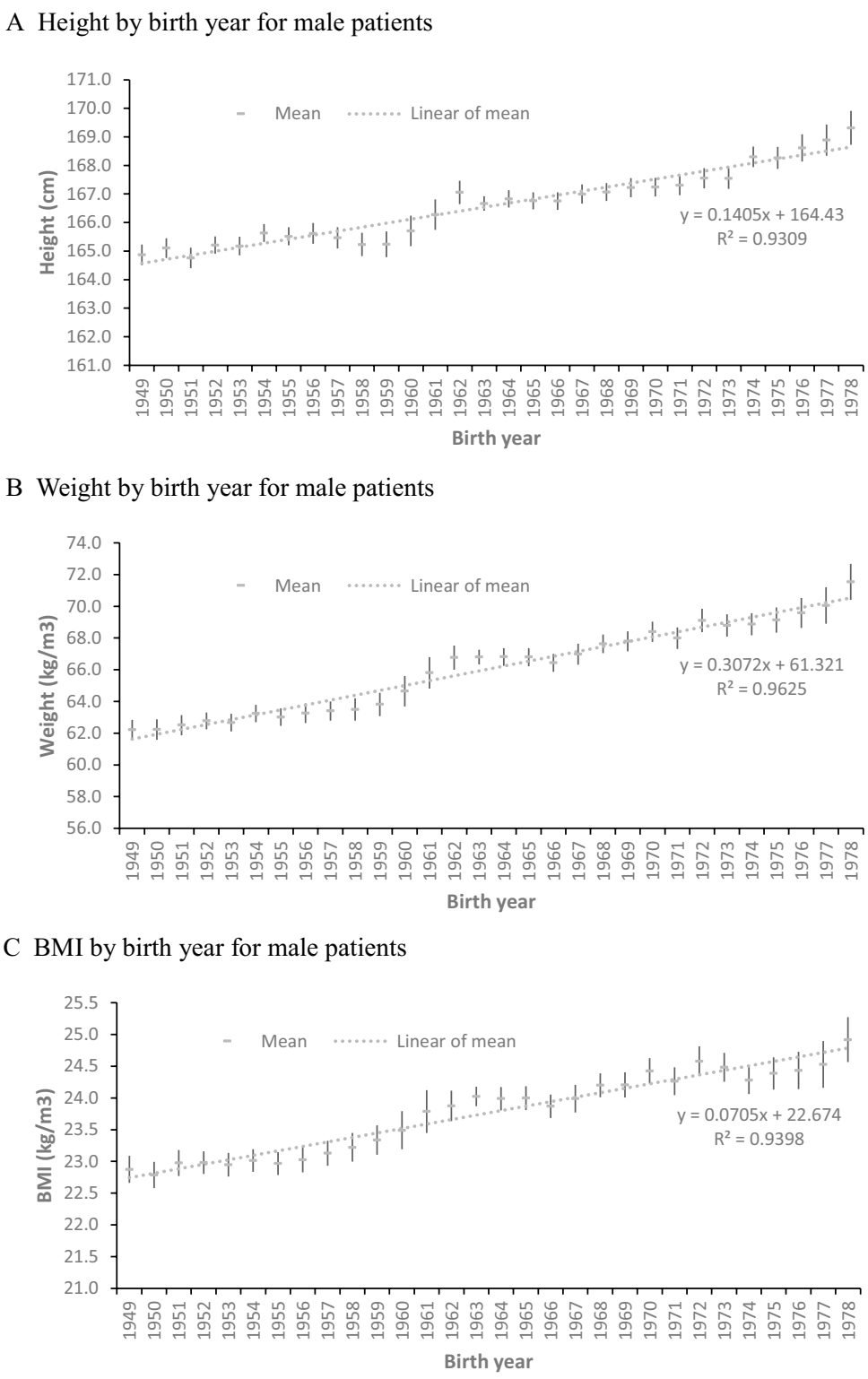


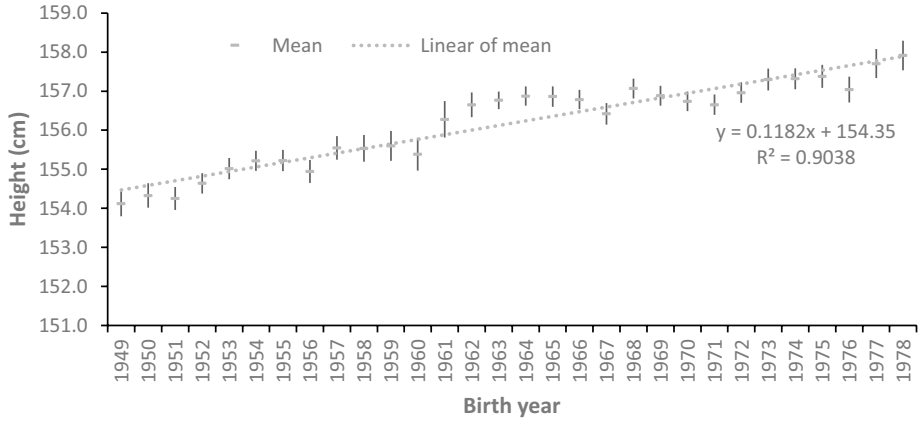
Fig. 4 **A** Height by birth year for male patients. **B** Weight by birth year for male patients. **C** BMI by birth year for male patients. **D** Height by birth year for female. **E** Weight by birth year for female patients. **F** BMI by birth year for female patients

the origins and propagation of this consortium of cardiometabolic diseases. One such theory, commonly referred to as the fetal origins of disease hypothesis, dictates that malnutrition during gestational development and early life preprograms cardiometabolic dysfunction in later life [19]. The current study aimed to assess the validity of this hypothesis by investigating the health status, in terms of HTN and DM, of a naturally occurring famine-exposed Chinese population.

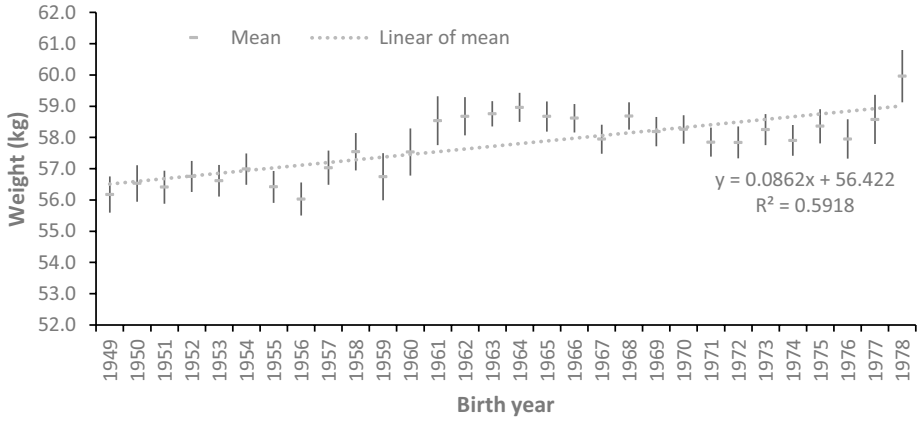
The Chongqing census data demonstrate that birth year population decreased sharply during the famine years of

Fig. 4 (continued)

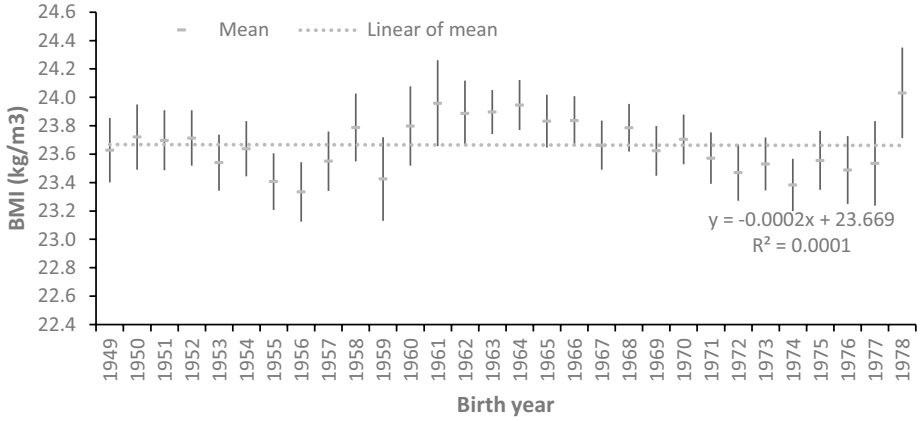
D Height by birth year for female



E Weight by birth year for female patients

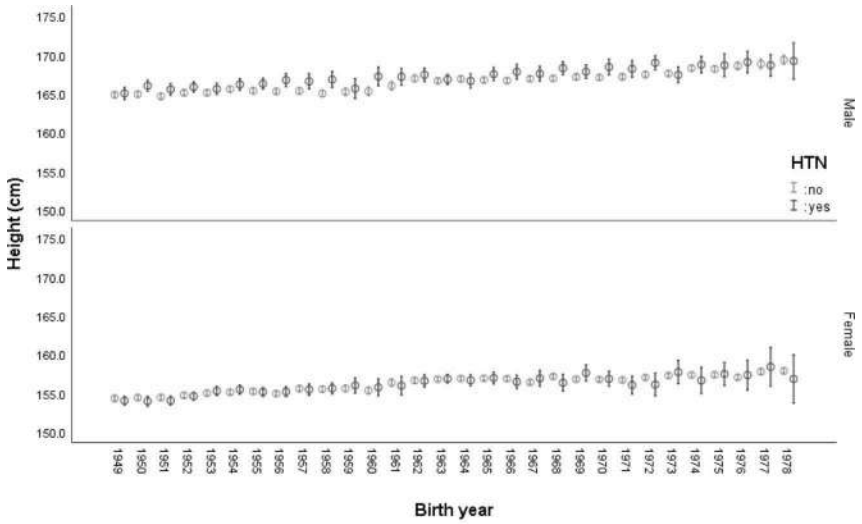


F BMI by birth year for female patients

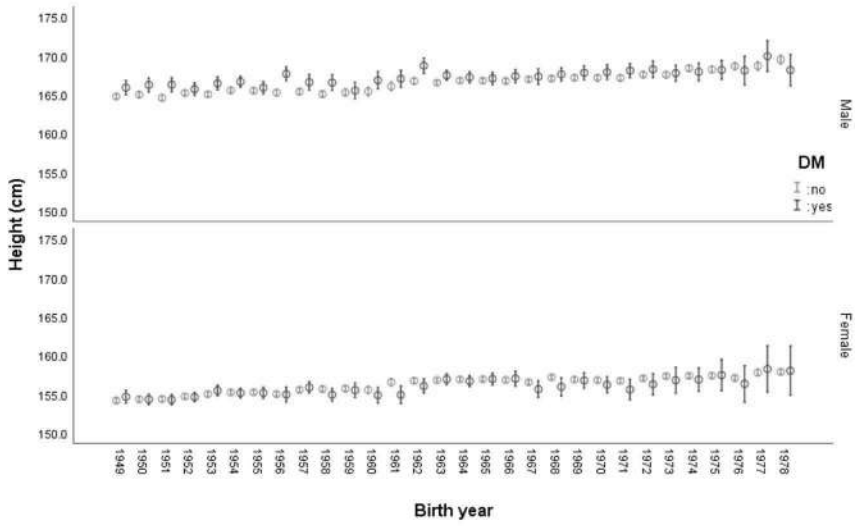


1959–1961, reflecting the serious food shortage and subsequent malnutrition of pregnant mothers and their newborn infants during this period. In conjunction with this, we observe a near perfect mirroring of hospitalization trends by birth year, indicating that the YiduCloud dataset is likely representative of the wider Chongqing population which is over 34 million. Several studies have investigated datasets pertinent to the Chinese famine period with the aim of examining the long-term health and socioeconomically implications of the event [20, 21], with some even demonstrating intergenerational effects of famine on aspects related to cognitive performance [22]. However, the sample sizes

A the comparisons of height between patients with or without HTN by birth year



B the comparisons of height between patients with or without DM by birth year



C the comparisons of weight between patients with or without HTN by birth year

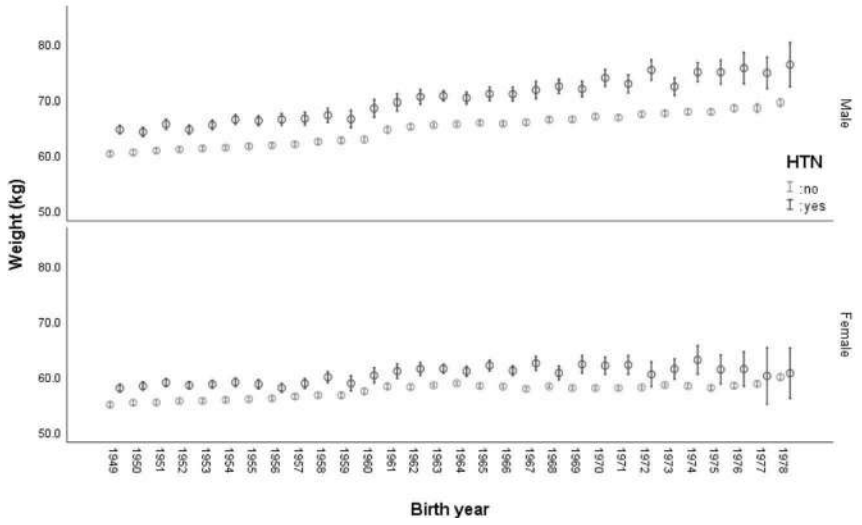
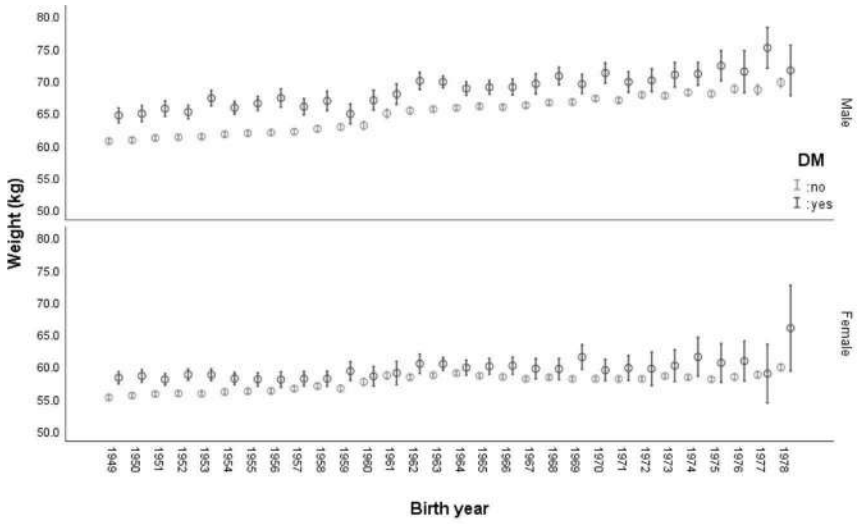


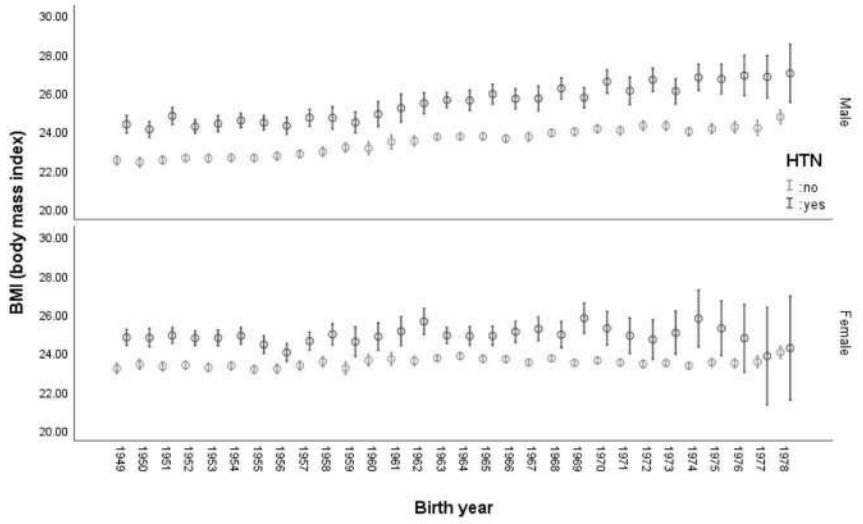
Fig. 5 **A** The comparisons of height between patients with or without HTN by birth year. **B** The comparisons of height between patients with or without DM by birth year. **C** The comparisons of weight between patients with or without HTN by birth year. **D** The comparisons of weight between patients with or without DM by birth year. **E** The comparisons of BMI between patients with or without HTN by birth year. **F** The comparisons of BMI between patients with or without DM by birth year

Fig. 5 (continued)

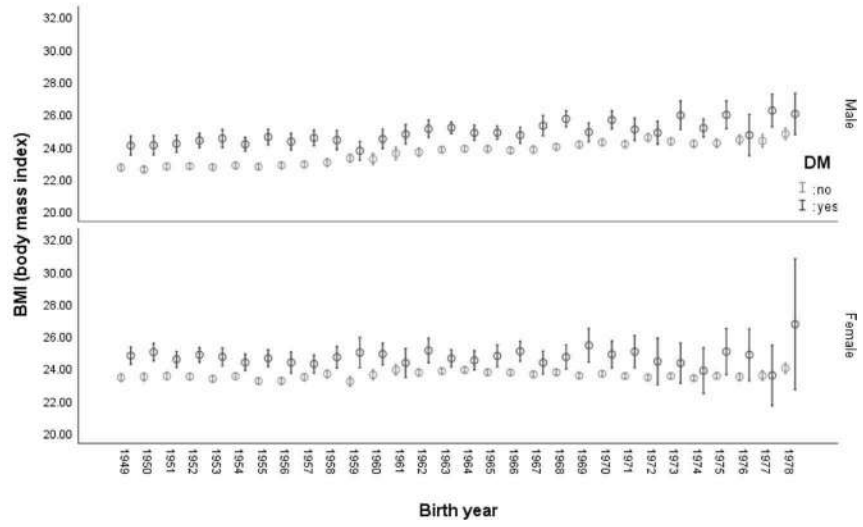
D the comparisons of weight between patients with or without DM by birth year



E the comparisons of BMI between patients with or without HTN by birth year



F the comparisons of BMI between patients with or without DM by birth year



have generally been comparatively modest, and data were collected by community sampling survey, making the results less conclusive and even inconsistent in some instances. In fact, one such study even rejected the fetal origins hypothesis, suggesting that there may in fact be a metabolic benefit to famine as a selective force at the population level [13].

In addition to census population adjusted hospitalizations, we observed that diagnosis of HTN and DM in hospitalizations was linearly and negatively associated with birth year, emphasizing the importance of age as a primary factor in the development of such chronic diseases and well predicting the prevalence of each birth year. However, people born at the beginning of the famine period tended to have a lower than predicted risk of HTN and DM, whereas those born at the end of famine displayed an increased risk of disease with heavier body weight. This important trend implies that those in gestation prior to famine, but born into famine, are less likely to develop HTN and DM, while those who are in gestation during famine and spend the early years of life in famine are at higher risk of developing diseases of cardiometabolic dysfunction. Therefore, it is plausible that both food replenishment and "catch-up" growth for those born at the end of famine may have played an important role in the increased risk of HTN and DM. This concept of catch-up growth in those who experienced unfavorable intrauterine conditions is not a new one. Indeed, this form of steep growth trajectory appears to be easily overshot and has been shown to result in higher rates of truncal obesity [23, 24], HTN [25], and insulin resistance [26, 27]. The data presented herein corroborates this theory neatly.

Interestingly, the current data suggest that males were more sensitive to the risk of HTN and DM than female following famine exposure. It is postulated that this may be due to the Chinese tradition around the perceived preference of male babies over female babies [28], which may conceivably in turn have given priority of food to the males, once available, and therefore, prompted the "catch-up" growth in male. Curiously, a previous investigation into data from the Chinese Famine, the SPECT-China study which included six and a half thousand individuals, found that the increase of metabolic syndrome associated with this early life food scarcity was more pronounced in females [29]. This may reflect the inappropriate adjustment of age, because famine exposure subgroups were no overlap in age [12].

Strengths and limitations

In the present study, the use of clinic history data allowed us to observe the long-term effects of famine exposure, over a 10-year time window nearly 50 years after the actual event. Although we investigated solely clinic data in the present analysis and have little data available for some potentially important confounding variables, the vast study population included in this analysis of more than 350,000 patients lends reliability and confidence to the conclusions. Of course, due to the retrospective observational nature of the study design, the present study is limited in that we cannot designate causation. However, in contrast to community-derived data, which usually have obvious selection biases and are often generated through self-reporting, the clinic data are of a much higher quality in terms of documentation and curation. While the current data is derived from a more urban-dwelling cohort, previous data has demonstrated that those who were situated in rural communities were the people that suffered the greatest [21]. Therefore, it is possible that the effect might have been more powerful if data was available for these rural communities. Importantly, regression analysis of 10-year hospital records covering 30 years of birth year was enough to well predict the risks of HTN and DM with good fitness and therefore avoid the bias created by differences in mortality and risk of metabolic diseases associated with age which was common in other related studies. Finally, the same observation window was utilized in order to avoid the potential bias in health care quality, hospitalization, and diagnosis of disease which might otherwise have been introduced. Therefore, the results of this study were solid enough to show that fetal famine exposure plus later "catch-up" growth chance might be the contributions of increased risk of HTN and DM in those born at the end of famine.

Conclusion

Herein, by examining the YiduCloud hospitalizations born in the years between 1949 and 1978 over the decade between 1999 and 2018, we observed those born just prior to the onset or at the beginning with longer early life famine exposure of famine presented with less than predicted rates of HTN and DM, while those with gestational and short early life famine exposure displayed a greater than predicted risk of disease development, particularly males. Indeed, these findings are ostensibly consistent with fetal origins hypothesis. Famine exposure, particularly in the prenatal period, can increase the risk of certain cardiometabolic diseases when combined with nutrition replenishment and catch-up growth after birth. However, due to the relatively small proportion of famine exposure in whole population, this is not likely to be the primary cause of the current epidemic of metabolic disease, such as HTN and DM.

Acknowledgements This study would not have been possible without the assistance of Jia Wang from Department of Medical Information and staffs from YiduCloud (Beijing) Technology Co., Ltd.

Declarations

Ethics approval Because all sensitive information which can be used to trace patients in records from the YiduCloud database were properly masked, this study does not pose any ethically related concerns.

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Effectiveness of a diabetes educational intervention at primary school

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Abstract

Background Brazil ranks fifth in the world in the number of adults with diabetes, and third for type 1 diabetes. Conducting educational actions on this topic in public schools in this country is extremely important, since it can assist in the early adoption of good life habits and in a better care for students in this condition.

Objective The aim of this study was to assess the effectiveness of an educational intervention about diabetes for students and school staff.

Methods This is an interventional non-randomized longitudinal study, in which interviews were conducted before and after a playful intervention with the use of theater play and games for students and plus a training for the school staff.

Results A total of 89 participants completed the study, being 73 students aged 7 to 12 years old, and 16 school staff. As a result, there was a positive change in knowledge and perception of diabetes by the students. The greatest changes in the answers among the participants, at the post-intervention period, were related to the possibility of consuming something with sugar by those with diabetes, and particularly how to cope in hypoglycemia situations by the school staff.

Conclusions Actions like these must be encouraged within the school environment, especially in countries with high prevalence of diabetes.

Keywords Diabetes mellitus · Health education · School health services · Primary school · Community networks

Introduction

Promoting health education in public schools in Brazil is an extremely important challenge, since it allows for the early adoption of good life habits and helps the school staff to take better care for their students. One in eleven people in the world has diabetes mellitus (DM) and, although type 1 (T1DM) is one of the most common chronic disease in childhood, type 2 (T2DM) is also increasing considerably in older children and adolescents, due to the increase in overweight and obesity in these population groups [1].

Some attitudes reflect the level of knowledge on diabetes [2, 3], so family members of affected children are constantly concerned about the management of this condition in the schools, as they recognize lack of preparedness in the school staff regarding support for self-care [4, 5], and classmates help [6]. In this way, a recent systematic review has shown association between bullying and T1DM when compared to individuals with no such condition in public environments, such as school [6].

The Children and Diabetes in Schools (KiDS) program has been used in several countries to promote diabetes education in the schools [7]. In Brazil, it was implemented in 2014 [6], being one of the basic tools used by the Diabetes Reference Centers in schools in the country [8, 9]. In addition to the KiDS educational package, it is recommended to use an interactive methodology in the schools, such as training with the school staff [8, 9], and/or playful actions with the students [10].

The use of performing arts, specifically theater play, has been shown to be very efficient for children and youth audiences [10–12]. In Brazil, few studies used theater play as a playful tool for diabetes education in schools, although without assessing the change in the participants' level of knowledge after its application [11, 12].

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When considering that children and adolescents can spend more than 30 h per week in the school environment [13], that there will be a probable 50% increase in the number of DM cases by 2040 [1], and that diabetes education is a fundamental component of the treatment [14] as well as for the prevention of T2DM, it is necessary to adopt efficient educational practices in the schools. In this way, the aim of this study was to assess the effectiveness of using a playful intervention, associated with other tools in diabetes education for students and school staff.

Materials and methods

Design

This is an interventional non-randomized longitudinal study design (pre- and post-intervention) carried out in a public Primary Education School in Teixeira de Freitas, Bahia, Brazil. The school staff and the students were invited to participate in this study at the school, after presentation by the researchers, in addition to sending an invitation letter to the guardians.

Sampling and research team

The sample of the study was a non-probabilistic one, with students from second to the fifth grade were included and school staff. In the data collection period, the school had a total of 238 students enrolled, of which 186 were between second and fifth grade, and 21 employees (10 teachers, five general service assistants, two teaching assistants, a principal and a vice director, a secretary, and an administrative assistant).

The inclusion criteria were as follows: *Students*: being enrolled from second to fifth grade of primary school, attending classes regularly; *School staff*: being part of the school permanent team. The exclusion criteria for students and employees were as follows: not accepting to participate in the study, not being present at the time of data collection, and, also for students, not having signed the Assent Form or not having the Free and Informed Consent Form signed by one of the guardians. In the pre-intervention interviews, 83 students and 19 members of the school staff participated, and in the post-intervention interviews, 73 students and 16 members of the school staff participated of the study.

The entire study was conducted by members of the Diabetes Reference Center in the Schools of Teixeira de Freitas (CRDE-TxF), by students and professors from the courses of Medicine, Psychology and Interdisciplinary Bachelor's Degree in Health of the Federal University of Southern Bahia (UFSB).

Study procedure

To check the participants' knowledge and perception of diabetes, a one-on-one face-to-face structured interview was examined twice for each participant, before and after intervention. To promote diabetes education, an intervention was conducted in the form of a theater play for the entire school community. Two days later, the school staff also had a training course. Finally, 2 months after the intervention, the same interview was re-applied in the same sample group (school staff and students), to verify changes in perception based on the educational actions. The methodological path of the study is shown in Fig. 1.

Interview and data collection

A reserved room was used for the one-on-one face-to-face structured interviews, between October and December 2019. The questions used were elaborated by the CRDE-TxF team itself, based on a review literature and on KiDS educational package [15]. For the students, 20 questions were used to assess diabetes general perceptions, signs and symptoms of hypoglycemia and hyperglycemia, and diet and diabetes management at school. For the school staff, 24 questions were used, being 20 the same as those for the students, only adapting the sentence *the classmate with diabetes* to *the student with diabetes*, in addition to other four questions about what to do during hyperglycemia and hypoglycemia crises.

The participants had the following answer options: *yes*, *no*, and *I don't know*. For the students, there were also the option to answer by pointing a corresponding picture answer, with the thumb up for *yes*, the thumb down for *no*, and both hands open and palms up for *I do not know*, in the case he/she did not want to verbally answer the questions.

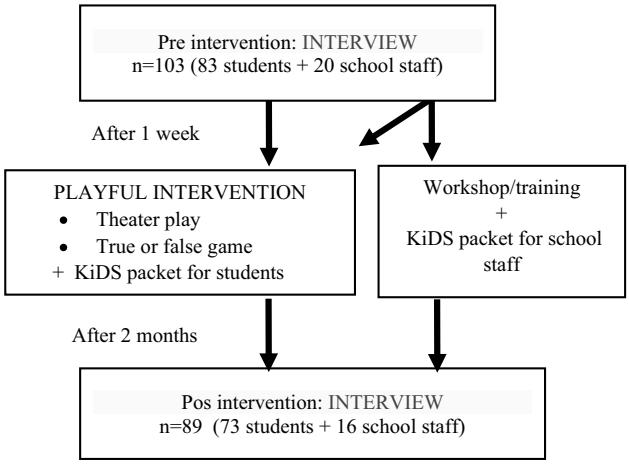

Fig. 1 Methodological path of the study

Interventions

The playful educational interventions used with the students and the school staff were a theater play [10] and a true and false dynamic game with statements questions about diabetes inside balloons, during 40 min. The script content of the theater play was about a T1DM student story starting in a new school, and his challenges with his classmates and teacher to do his diabetes self-care at school. There were specific scenes about the use of a glucometer, insulin shot, diet and consumption of sweets (candies), physical activity, unrestricted use of bathroom and access to drink water in times of hyperglycemia, signs and symptoms of a hypoglycemic crisis, among others.

The school staff training occurs in a workshop format with dynamics about diabetes care at school, highlighting the following topics: symptoms of hyperglycemia and hypoglycemia recognition and how to cope in these situations, diabetes kit bag, and the responsibilities and assignment of the stakeholders (parent/guardian, school staff, student) [16–18]. In addition, they had a practical workshop about glucometer uses and the results interpretation. All training lasted about an hour and a half.

The printed educational package with information about diabetes in schools, donated by ADJ Diabetes Brasil, was distributed at the end of the interventions to all present participants; in their *students* and *school staff* [15] versions, to help reinforcing the knowledge acquired during the interventions.

Data analysis and ethical aspects

The pre- and post-intervention answer comparison was analyzed using the PSPP software developed by the GNU (Gnuis Not Unix) project. The McNemar test was used to verify changes in the answers, with a significance level of 0.05%. The interview answers were categorized as a dichotomous variable (yes/no) option, with the *I don't know* answers being considered *not adequate*.

The study was approved by the Ethics Committee on Human Research of the UFSB-Brazil, under CAAE number 17382619.4.0000.8467. Number of Approval: 3.609.716, September, 2019.

Results

Participant profiles

A total of 89 individuals finished the study, 73 students and 16 school staff. Among students, there were 42 (57.5%) female and 31 (42.5%) male, aged 7 to 12 years ($M=9$; $SD=1$). Regarding the school grade, 22 (30.1%) were in second grade, 24 (32.9%) in third grade, 13 (17.8%) in fourth grade, and 14 (19.2%) in fifth grade. All school staff were female, aged 32 to 59 years ($M=45$; $SD=6$), and with education level as follows: completed under graduation, 7 (43.7%); completed high school, 1 (6.3%); uncompleted high school, 6 (37.5%); and completed primary school, 2 (12.5%).

Relationship with diabetes

Among students, most of them, 71 (97.2%), answered that they did not have diabetes, one had the disease but did not know the type, and other had T1DM. Additionally, 52 (71.2%) students reported having someone with diabetes close to them, family member or friend. None school staff participant stated having diabetes, but 15 of them (93.7%) answered that they had someone close with this condition.

Pre- and post-intervention knowledge assessment

The results of the interview questions with the students and school staff are shown in the following tables: Table 1 for diabetes *general knowledge*, Table 2 for *diabetes signs and symptoms recognition*, and Table 3 for *diabetes management in the school*, expressed in answers frequency and percentage, and significance level between pre- and post-intervention. The questions regarding the knowledge on behaviors and attitudes about hypoglycemia showed greater changes after the intervention.

There was a positive change in the diabetes sign and symptom recognition by the students. In the case of the school staff, adequate answers had already been given in the pre-intervention, with a significant change only for *headache* ($p=0.035$).

In relation to the *diabetes management in the schools*, there were changes for almost all the answers among students and, for the school staff, only in those related to the correction of hypoglycemia events (Table 3).

Discussion

This study evaluated the effectiveness of a diabetes intervention in school environment, and it was able to cause important changes in the students' and the school staff's knowledge and perception, with the main changes related with hypoglycemia and sugar intake by people with diabetes.

With regard to general knowledge about diabetes, the possibility of consuming something with sugar by those with diabetes is one of the issues where the majority of the lay public and even health professionals are mistaken. In our study, after the interventions, there was a greater number of correct answers in all the questions related to this topic, both for the students and for the school staff, including the

Table 1 Diabetes general knowledge of students ($n=73$) and school staff ($n=16$)

Question		Pre-intervention			Post-intervention			McNemar test
		Yes	No	I don't know	Yes	No	I don't know	*p value*
Can children and adolescents have diabetes?	Students	60 (82.2%)	7 (9.6%)	6 (8.2%)	65 (89%)	6 (8.2%)	2 (2.8%)	0.133
	School staff	16 (100%)	0	0	16 (100%)	0	0	0.001
Is diabetes contagious from one person to another?	Students	19 (26%)	40 (54.8%)	14 (19.2%)	5 (6.8%)	57 (78.1%)	11 (15.1%)	0.001
	School staff	1 (6.3%)	15(93.7%)	0	0	16 (100%)	0	0.5
Is there diabetes cure?	Students	40 (54.8%)	18 (24.7%)	15 (20.5%)	29 (39.7%)	32 (43.8%)	12 (16.5%)	0.005
	School staff	3 (18.7%)	12 (75%)	1 (6.3%)	2 (12.55)	13 (81.2%)	1 (6.3%)	0.5
Is there diabetes treatment?	Students	57 (78.1%)	6 (8.2%)	10 (13.7%)	56 (76.7%)	6 (8.2%)	11 (15.1%)	0.5
	School staff	15 (93.7%)	0	1 (6.3%)	16 (100%)	0	0	0.5
Can the person who has diabetes eat sweets?	Students	1 (1.4%)	70 (95.9%)	2 (2.7%)	33 (45.2%)	37 (50.7%)	3 (4.1%)	0.001
	School staff	4 (25%)	10 (62.5%)	2 (12.5%)	12 (75%)	4 (25%)	0	0.011
Can the "blood sugar" level get to low in a classmate/person with diabetes?	Students	42 (57.5%)	14 (19.25)	17 (23.3%)	65 (89%)	7 (9.6%)	1 (1.4%)	0.001
	School staff	9 (56.2%)	1 (6.3%)	6 (37.5%)	16 (100%)	0	0	0.008

Table 2 Diabetes sign and symptom recognition by students ($n=73$) and school staff ($n=16$)

Question		Pre-intervention			Post-intervention			McNemar test
		Yes	No	I don't know	Yes	No	I don't know	*p value*
Can a classmate/student with diabetes may need to leave the classroom several times to pee?	Students	35 (47.9%)	15 (20.6%)	23 (31.5%)	63 (86.3%)	8 (11%)	2 (2.7%)	0.001
	School staff	15 (93.7%)	0	1 (6.3%)	16 (100%)	0	0	0.5
Can a classmate/student with diabetes suddenly feel headache?	Students	45 (61.6%)	11 (15.1%)	17 (23.3%)	48 (65.8%)	10 (13.7%)	15 (20.5%)	0.356
	School staff	8 (50%)	0	8 (50%)	14 (87.5%)	1 (6.25%)	1 (6.25%)	0.035
Can a classmate/student suddenly be very irritability?	Students	21 (28.85%)	32 (43.8%)	20 (27.4%)	47 (64.4%)	16 (21.9%)	10 (13.7%)	0.001
	School staff	7 (43.7%)	3 (18.8%)	6 (37.5%)	13 (81.2%)	2 (12.5%)	1 (6.3%)	0.688
Can a classmate/student with diabetes suddenly feel dizzyness?	Students	50 (68.5%)	11 (15.1%)	12 (16.4%)	65 (89%)	5 (6.9%)	3 (4.1%)	0.001
	School staff	15 (93.7%)	0	1 (6.3%)	16 (100%)	0	0	0.5
Can a classmate/student with diabetes suddenly have blurry vision?	Students	33 (45.2%)	19 (26%)	21 (28.8%)	40 (54.8%)	16 (21.9%)	17 (23.3%)	0.072
	School staff	14 (87.5%)	0	2 (12.55)	16 (100%)	0	0	0.25
Can a classmate/student with diabetes pass out suddenly?	Students	46 (63%)	14 (19.2%)	13 (17.8%)	57 (78%)	8 (11%)	8 (11%)	0.031
	School staff	15 (93.7%)	0	1 (6.3%)	16 (100%)	0	0	0.5
Can a classmate/student with diabetes be very sleepy during class?	Students	28 (38.4%)	25 (34.2%)	20 (27.4%)	48 (65.8%)	13 (17.8%)	12 (16.4%)	0.001
	School staff	12 (75%)	0	4 (25%)	14 (87.5%)	1 (6.25%)	1 (6.25%)	0.312

questions related to behavior and attitudes in hypoglycemia, which showed the greatest changes. In this aspect, the importance of highlighting this topic in the interventions was verified, contextualizing it, as recommended, within a healthy diet and in the case of correction of hypoglycemic crises [15].

Some studies have used theater plays as a tool to stimulate behavioral change and promote diabetes education, but mostly directed to people with diabetes [19–21]. Promoting diabetes education among school community is of value to provide support and assistance to the family and T1DM student, when it is needed, as well to stimulate prevention of obesity and T2DM. In this context, it is important to consider the substantial increase in independence and classmates behavior influences in the adolescents' life [22].

The two questions that could be related to bullying, *whether diabetes is contagious from one person to another*, and *whether the classmate with diabetes can have a snack together with the others*, also presented an increase of correct answers after the intervention among the students. These

Table 3 Diabetes management in the school of students ($n = 73$) and school staff ($n = 16$)

Question		Pre-intervention			Post-intervention			McNemar test
		Yes	No	I don't know	Yes	No	I don't know	*p value*
Can a classmate/student with diabetes need to have a different snack than the other students?	Students	61 (83.6)%	6 (8.2%)	6 (8.2%)	32 (43.8%)	37 (50.7%)	4 (5.5%)	0.001
	School staff	16 (100%)	0	0	12 (75%)	4 (25%)	0	0.062
Can a classmate/student with diabetes dance, jump and run?	Students	27 (37%)	24 (32.9%)	22 (30.1%)	61 (83.5%)	8 (11%)	4 (5.5%)	0.001
	School staff	15 (93.7%)	1 (6.3%)	0	16 (100%)	0	0	0.5
Can a classmate/student with diabetes have a snack with others (in the same place and at the same time)?	Students	50 (68.5%)	15 (20.5%)	8 (11%)	63 (86.3%)	8 (11%)	2 (2.7%)	0.004
	School staff	13 (81.2%)	2 (12.5%)	1 (6.3%)	15 (93.7%)	1 (6.3%)	0	0.25
From time to time, does the classmate/student with diabetes have to prick his finger to check his diabetes?	Students	56 (76.7%)	5 (6.9%)	12 (16.4%)	71 (97.2%)	1 (1.4%)	1 (1.4%)	0.001
	School staff	15 (93.75)	0	1 (6.3%)	16 (100%)	0	0	0.5
Does the classmate/student have to have always candies with him in case he feels bad?	Students	21 (28.8%)	42 (57.5%)	10 (13.7%)	64 (87.7%)	9 (12.3%)	0	0.001
	School staff	4 (25%)	9 (56.3%)	3 (18.7%)	14 (87.5%)	1 (6.25%)	1 (6.25%)	0.001
Can a classmate/student with diabetes may need an insulin shot in the school?	Students	31 (42.5%)	20 (27.4%)	22 (30.1%)	55 (75.3%)	7 (9.6%)	11 (15.1%)	0.001
	School staff	12 (75%)	1 (6.3%)	3 (18.7%)	15 (93.7%)	1 (6.3%)	0	0.125
Can a classmate/student with diabetes may need to eat in different times than his classmates?	Students	28 (38.4%)	29 (39.7%)	16 (21.9%)	27 (37%)	36 (49.3%)	10 (13.7%)	0.5
	School staff	14 (87.5%)	1 (6.25%)	1 (6.25%)	15 (93.7%)	1 (6.3%)	0	0.5
Questions applied exclusively to the School Staff								
When a student with diabetes has very low blood sugar (hypoglycemia), should you offer him water with sugar?		5 (31.3%)	7 (43.7%)	4 (25%)	14 (87.5%)	2 (12.5%)	0	0.002
When a student with diabetes has very low blood sugar (hypoglycemia), should you offer him candies or sweets?		3 (18.75%)	10 (62.5%)	3 (18.75%)	12 (75%)	3 (18.7%)	1 (6.3%)	0.002
When a student with diabetes gets very high blood sugar (hyperglycemia), does he need an insulin shot right away?		7 (43.8%)	3 (18.7%)	6 (37.5%)	10 (62.5%)	1 (6.3%)	5 (31.2%)	0.227
When a student with diabetes gets very low blood sugar (hypoglycemia), can he die if he doesn't have help quickly?		10 (62.5%)	1 (6.3%)	5 (31.2%)	15 (93.7%)	1 (6.3%)	0	0.063

findings are important since episodes/behaviors of exclusion and/or segregation are frequently reported by children and adolescents with diabetes [6], especially at school [23, 24].

There was a significant recognition of the insulin shot necessity at school, as part of diabetes management, by the interviewed students. However, there was one staff school participant who still negatively answered about this subject after the training. This is other important subject in diabetes education, since unfortunately there are reports from people with diabetes (personal communication) that it has been mistaken for an illicit drug user while inject insulin with a syringe [25].

A number of studies have pointed out the children and adolescents with T1DM get shamed and embarrassed when measuring their blood glucose and inject insulin, both in and out of the schools [23, 26], especially with the use of a syringe or infusion pump, aggravating social stigma [23]. The use of insulin in multiple doses per day has been recognized already for some years as an efficient treatment in T1DM; therefore, there is often a need for at least one application at school, especially before eating [15, 18].

Another important result was acknowledging that the classmate with diabetes can practice physical and recreational activities. This understanding assists in the proper management of diabetes, since physical activity is an important part of the treatment [15, 27].

Not recognizing the main signs and symptoms in diabetes, both in hyperglycemia and hypoglycemia events, is admittedly another obstacle in social life [23, 26, 28]. This issue was highlighted in the theater play. Although these symptoms are very individualized, *polyuria* and *polydipsia* were highlighted for *hyperglycemia*, and *weakness fatigue*,

drowsiness, *hunger*, *blurry vision*, *sweating*, *shakiness*, *dizziness*, *fast heartbeat*, and *fainting* (when more severe) in *hypoglycemia*. Thus, only for the questions about *headache* and *blurry vision*, no differences were verified after the intervention among the interviewed students.

Learning how to acknowledge hyperglycemia symptoms is very important at the school environment. It can make a way for an early diabetes screening, often avoiding the sudden onset of T1DM in a severe and traumatic way such as ketoacidosis [29].

There was also a significant increase in the recognition of mood changes, *irritability*, by the students after the intervention. This finding is important because it facilitates support and reception of students with diabetes by their classmates during a glycemic imbalance, which can occur both in hypoglycemia [15, 30] and in hyperglycemia [15].

Understanding why students with T1DM may need to eat at different times is important to avoid discrimination and facilitate management in the school environment. This sometimes should happen to prevent hypoglycemic crisis, and the most common reasons which it can occur are as follows: prolonged fasting status, skipped missed or delayed meals/snacks, unfinished meals (for little kids), too much insulin on board, too little carbohydrate, unplanned exercise and activities [15, 22, 30–32].

Thus, generally when blood glucose is below 90 mL/dL and/or dropping, for those who use glucose sensor with trend arrows, and the next meal will be in a long time or the person will have a physical exercise, it is suggested to eat a small snack [22, 27].

The questions related to how to cope in a hypoglycemic crisis stood out among the most relevant results after the intervention for the school staff. It was acknowledged that offering something with sugar is important to revert the situation. It has been established that 15 g of quick-acting carbohydrate must be ingested at these moments [22, 31], unless loss of conscious happens [15].

Other important aspects of the diabetes care at school were highlighted for the school staff, including the encouragement of students with diabetes to have always with them the *diabetes kit bag*, which included 15 or 30 g of fast-absorbing carbohydrate and an extra snack to correct or prevent a hypoglycemia event, glucometer, and insulin, in addition to the relevant supplies, such as reagent strips for measuring glucose and syringe or pen for insulin application [13, 15, 18]. And also, it has at school the update personal diabetes management plan for each student with diabetes, where is written down all details of his/her treatment [15].

One strength of our study was to use the KiDS educational package, a validate material from International Diabetes Federation. Other strength was to work diabetes education with all school employee categories, plus the majority of students enrolled, encouraging a healthy lifestyle and reducing bullying about self-care diabetes. Thus, we stimulate the replicability of the methodology used to other schools, not only in Brazil but also in other countries, in order to better disseminate issues related to the management of T1DM within the school environment.

A limitation of this study was to conduct it only in one school, due to the restrictions resulting from the COVID-19 pandemic, with just 16 school staff finishing the post-intervention interview, thus limiting generalization of the results. Also, a longer additional interview, 6 or 9 months later, could give information about the long-term memory or impact of the intervention.

Conclusion

The educational intervention, with the use of the theater play, playful games, KiDS educational package, and workshop training, was effective in changing the students' and school staff's knowledge and perception, especially about the main changes related with hypoglycemia and sugar intake by people with diabetes, and can be used as a methodology for diabetes education. These actions contributed to stimulating the children and adolescents to become replicators of the knowledge acquired about diabetes in the school environment.

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Data availability Not applicable for that section.

Code availability Not applicable for that section.

Declarations

Ethical approval Ethical approval to report this study was obtained from the Federal University of Southern Bahia ETHICS COMMITTEE'S (APPROVAL NUMBER 3.609.716./CAAE: 17382619.4.0000.8467).

Statement of human All procedures in this study were conducted in accordance with the Federal University of Southern Bahia ETHICS COMMITTEE'S (APPROVAL NUMBER 3.609.716./CAAE: 17382619.4.0000.8467).

Statement of informed consent Written informed consent was obtained from the subjects for their anonymized information to be published in this article.

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

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Association of fasting glucagon-like peptide-1 and glucose dependent insulinotropic polypeptide with dyslipidemia in newly diagnosed diabetes

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Abstract

Aim To determine the association of fasting glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) with dyslipidemia in newly diagnosed diabetes (NDD).

Method This collaborative prospective interventional clinical study was carried out by the Biochemistry Department, at the University of Karachi-Pakistan, Baqai Institute of Diabetology and Endocrinology, Karachi-Pakistan and Kansai Electric Power Medical Research Institute, Osaka, Japan. The study duration was from March 2019 to May 2020. Participants were identified on the basis of oral glucose tolerance test (OGTT) results. Of 34 participants, 17 were NDD and 17 were healthy. Demographics and anthropometric details were noted on a predesigned questionnaire. Blood samples were collected at fasting state for plasma glucose, lipid profile, HbA1c, and hormones (GIP and GLP-1).

Results The healthy participants included nine males and eight females, and the NDD participants included ten males and seven females. Mean age was 50.78±2.44 years and BMI was 28.41±0.76 kg/m^2. Among healthy participants, both GIP and GLP-1 showed non-significant weak correlation for biochemical parameters. In NDD participants, GIP showed significantly negative moderate correlation with age and non-HDL and positive moderate correlation with LDL only. However, GLP-1 showed significantly positive moderate correlation with total cholesterol (r = 0.564, $p = 0.018$), LDL (r = 0.498, $p = 0.042$), HDL (r = 0.50, $p = 0.041$) and non-HDL (r = 0.503, $p = 0.047$) in NDD participants.

Conclusion The effect of GIP and GLP-1 measures on dyslipidemia were substantially different among NDD participants in our population. In the NDD group, GLP-1 is significantly moderately associated with dyslipidemia compared to GIP.

Keywords NDD · GLP-1 · GIP · dyslipidemia

Introduction

In lower middle-income countries, especially Pakistan, an epidemic of diabetes is one of the most alarming public health issues. The International Diabetes Federation has reported a 19.9% prevalence of diabetes in Pakistan, while a recent second National Diabetes Survey of Pakistan (NDSP) 2016–2017 has estimated it as 26% using the oral glucose tolerance test [1, 2].

It is widely accepted that the pathophysiological defects of type 2 diabetes mellitus (T2DM) results from a combination of increased resistance to insulin action in peripheral tissues, inadequate insulin secretion, increased hepatic glucose production, and pancreatic β cell dysfunctions [3]. In people with T2DM, dyslipidemia is strikingly common as a traditional risk factor for CVD and affects almost 50% of this population. Dyslipidemia, hyperglycemia, and hypertension

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are modifiable CVD risk factors specifically in people with T2DM that remain largely uncontrolled. Recent evidence indicates the pathogenetic mechanism causing T2DM includes a significant reduction of incretin hormone effect in the gastrointestinal tract [4].

Gastrointestinal incretin hormones, including glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are released from the gut to regulate the amount of insulin and glucagon to control blood glucose levels, and it is confirmed that GIP and GLP-1 act as an incretin [5, 6]. The incretin effect is impaired in patients with T2DM [7, 8]. In addition to enhancing insulin secretion, GIP and GLP-1 have been shown to have an effect on lipid metabolism. GIP regulates triglyceride turnover and may promote triglycerides clearance from the blood by increasing deposition of fat in adipocytes [9]. Importantly, the discovery of the specific incretin hormones, their signaling pathways, and their physiology has culminated in the development of new pharmacological agents to treat diabetes and related complications [10]. Recent data suggest that GIP does not have an effect on plasma triglyceride clearance, but may act synergistically with insulin to increase free fatty acid re-esterification in subcutaneous adipose tissue in lean but not in obese humans [6]. GLP-1 is also involved in energy metabolism via adipocyte development regulation, acceleration of plasma glucose and derived fatty acids of triacylglycerol, signaling of insulin, and stimulation of adipose tissue (brown) thermogenesis [11]. Enhancing GLP-1 receptor (GLP-1R) activity improves intestinal lipoprotein metabolism and decreases postprandial containing apoB48 triglyceride-rich lipoproteins. It results in low gastric emptying, improved insulin secretion or clearance of chylomicron [12]. In adipocytes, GLP-1R also activates the signaling pathway of cyclic adenosine monophosphate to regulate apoptosis and proliferation of pre-adipocyte via various cell signaling cascades. Therefore, GLP-1R agonists are widely used as regulators of adipogenesis [13]. In people with T2DM, GLP-1-mediated regulation of postprandial chylomicron may be lost because the incretin response is impaired and results in an excess of atherogenic chylomicron remnants [12]. However, GLP-1 induces an increase in the production and function of insulin and peripheral utilization of triglycerides also increases for energy production [14].

Literature suggests that people with T2DM who have a high risk of cardiovascular diseases are also treated by GLP-1R agonists to reduce the adverse cardiovascular events [13]. Incretin hormones have received consideration based on recent findings that they have an important role in the pathophysiology of high-risk patients with T2DM and have broad pharmacological potential [15]. In Pakistan, data is scarce and there is a need to explore the role of incretin hormones in diabetes. To our knowledge, the association between incretin hormones and lipid metabolism has not yet been examined in newly diagnosed diabetes (NDD) in our population; therefore, we aim to determine the association between fasting GLP-1 and GIP with dyslipidemia in NDD patients.

Methodology

This collaborative prospective interventional clinical study was carried out by the Biochemistry Department, at the University of Karachi-Pakistan, Baqai Institute of Diabetology and Endocrinology, Baqai Medical University, Karachi-Pakistan and Kansai Electric Power Medical Research Institute, Osaka, Japan. The study duration was from March 2019 to May 2020. Participants were recruited who attended the outpatient department of BIDE. A total of 34 participants were recruited and categorized into two groups: cases (17 participants with NDD) and control (17 healthy participants without diabetes). Diabetes was defined as fasting plasma glucose $\geq$126 mg/dl and 2-hour plasma glucose as $\geq$200 mg/dl [16]. Participants with impaired glucose tolerance, diagnosed diabetes, and related comorbidities were excluded from the study.

Participants were identified on the basis of oral glucose tolerance test (OGTT) results according to World Health Organization criteria. Participants who gave their informed consent and satisfied the inclusion criteria were instructed to be in a fasting state of at least 8 hours at a specified date and time. A predesigned questionnaire was used for data collection, and with the help of paramedical staff anthropometric measurements such as height, weight, and blood pressure were obtained. Blood samples were collected at fasting state for plasma glucose, lipid profile, HbA1c, and hormones (GIP and GLP-1). Analyses of preliminary tests were performed at BIDE laboratory, Pakistan, and blood samples for GLP-1 and GIP were transported to Osaka, Japan, for analysis following laboratory standard operating procedure [17].

Techniques used for biochemical parameters

Plasma levels of GLP-1 were measured using an enzyme-linked immunesorbent assay (ELISA) Meso Scale Discovery® V-plex GLP-1 total kit (catalogue number K1503PD), and plasma GIP levels were measured using an ELISA Merck® Human GIP (total) ELISA kit (catalogue number EZHGIP-54K). The glucose oxidase peroxidase technique using a SBio glucose kit (catalogue number 90504250) was used for plasma glucose, and high-performance liquid chromatography using Bio Rad D-10 program 220-0101 (Lot number L20025301) was used for HbA1c. Cholesterol (serum) was measured by the phenol oxidase peroxidase 4-amino antipyrine method using an SBio cholesterol kit (catalogue number 90282150), triglycerides (serum) were

measured by the oxidase-p-amino phenazone glycerol phosphate method using an SBio triglycerides kit (catalogue number 90810075), lipoprotein high density-cholesterol (HDL-C) and lipoprotein low density-cholesterol (LDL-C) were measured according to the enzymatic calorimetric homogeneous technique (direct method) using an SBio HDL-D cholesterol kit (catalogue number 90910040) and an SBio LDL-Cholesterol kit (catalogue number 90920040). Serum creatinine was measured by an Erba Transasia auto-analyzer (XL-600) modified Jaffe's kinetic method using an SBio creatinine kit (catalogue number 90452275).

A standardized technique was used to measure height and body weight to compute body mass index (BMI). BMI was calculated by dividing weight (kg) by height in meter square (m^2). Obesity was defined as BMI $\geq$ 25 (kg/m^2) or higher for both males and females, as explicated by the Asia Pacific guidelines. Hypertension was defined as systolic/diastolic blood pressure ≥140/90 (mmHg) on two different occasions [18].

Measurement of GLP-1

The Meso Scale Discovery (MSD) V-plex GLP-1 total assay provides a small spot plate pre-coated with the captured antibody. Calibrators and/or samples are sequentially added and a solution containing the side viewing detection antibody conjugated with electrochemiluminescent labels (MSD SULFO TAGTM) during two incubation periods is separated by wash steps. GLP-1 in the calibrator (inactive form) and in the sample (inactive and active form) binds to the GLP-1 C-terminal specific captured antibody immobilized on the working electrode surface and the detection antibody completes the sandwich. MSD was then added to the buffer that creates the appropriate chemical environment for the electrochemiluminescent reaction. An MSD instrument was used to load the plate and the voltage applied to the electrodes plate will trigger light emittance. The instrument measures the intensity of emitted light to provide the quantitative measure of analytes in the sample with a standard curve range of 0.136–1000 pg/ml.

Measurement of GIP

This ELISA kit for determination of human total GIP is based on a sandwich enzyme immunoassay. Standards or samples are added to the wells of a microtiter plate coated by a pre-titered amount of anti-GIP monoclonal antibodies for the 1st step of the immunoreaction. After incubation and plate washing in the 1st step, Streptavidin-horseradish peroxidase (HRP) labeled antibody solution was added against human GIP as the 2nd step. It forms antibody–antigen, labeled as the antibody complex, on surface of the wells. After the 2nd step incubation and rinsing to remove excess labeled antibody, the HRP enzyme activity was determined using 3,3',5,5'-tetramethylbenzidine and the concentration of human total GIP was calculated with a standard curve range of 61.7–5000 pg/ml.

Ethical Approval and Informed Consent

The study was approved by the ethical review committee of BIDE (IRB no.: BIDE/IRB/NWARIS/10/26/18/0206) and Osaka, Japan ((IRB no. 26-15). Experimental procedures and study purpose were described to each participant prior to obtaining informed written consent.

Statistical Analysis

Statistical analyses were conducted using the statistical package for social science (SPSS) version 20. Data was presented as mean ± standard error or median (interquartile range). The Shapiro–Wilk test was used to check the normality of the data. The Mann–Whitney U test was applied to compare various parameters between groups. The Spearman correlation (r) was used to check the relationship between variables. A correlation coefficient <0.3 was considered a weak correlation, a correlation coefficient between 0.3 and 0.7 was considered a moderate correlation, and a correlation coefficient of >0.7 was considered a strong correlation. Statistical significance was set at p value < 0.05.

Results

Of the 34 participants, there were nine (26.5%) male and eight (23.5%) female healthy participants and ten (29.4%) male and seven (20.6%) female NDD participants. Mean age was 50.78±2.44 years and BMI was 28.41±0.76 kg/m^2. Table 1 presents the baseline and biochemical parameters for males and females among NDD and healthy participants. Systolic and diastolic blood pressure was within the normal range in both sexes of healthy and NDD participants. Levels of triglycerides (mg/dl) in male (217.8±40.75) and female (192.71±40.79) NDD participants were found to be higher compared to healthy individuals. Similarly, very low-density lipoprotein was also elevated in male and female NDD participants compared to healthy individuals. Both GIP and GLP-1 levels were higher in male healthy individuals and female NDD participants.

Table 2 shows the correlation of fasting GIP with basic characteristics and clinical parameters. Among healthy participants, GIP showed a non-significant weak correlation for age, BMI, blood pressure, HbA1c, lipid parameters, and serum creatinine. In NDD participants, GIP showed a significantly negative moderate correlation with age ($r = -0.483$, $p = 0.049$) and non-HDL ($r = -0.524$, $p = 0.037$) and a significantly positive moderate correlation with LDL only.

Table 1 Baseline characteristics of healthy and newly diagnosed with diabetes participants

Parameters	Healthy participants		Newly diagnosed diabetes		Overall
	Male	Female	Male	Female	
n	**9 (26.5%)**	**8 (23.5%)**	**10 (29.4%)**	**7 (20.6%)**	**34**
Age (years)	55.89±4.4	46.38±3.89	49±3.56	46.29±2.93	50.43±1.59
Body mass index (kg/m^2)	25±0.9	25.47±0.99	30.02±1.69	32.43±2.63	28.41±0.76
Systolic blood pressure (mmHg)	113.33±2.89	110±2.67	125±4.77	121±1.56	116.67±1.89
Diastolic blood pressure (mmHg)	75.56±1.76	76.25±1.83	83±3.67	82±3.71	77.48±1.36
Total cholesterol (mg/dl)	199.78±12.01	206.5±20.63	181±13.25	175.86±23.66	184.09±7.59
Triglyceride (mg/dl)	164.56±23.33	120.25±15.57	217.8±40.75	192.71±40.79	167.83±13.7
Low density lipoprotein (mg/dl)	126.22±12.96	135.13±13.33	113.9±13.05	105.57±21.89	115.43±6.64
High density lipoprotein (mg/dl)	30.33±2.48	36±2.41	30±4.21	36.86±12.18	32±2.2
Very low-density lipoprotein (mg/dl)	32.82±4.63	24.1±3.11	43.59±8.1	38.49±8.16	33.89±2.77
Non- high-density lipoprotein (mg/dl)	169.44±11.11	170.5±19.3	154.44±14.06	150.43±22.32	153.64±7.34
HbA1c (%)	5.33±0.15	5.51±0.13	7.51±0.46	7.76±0.4	6.63±0.28
Serum creatinine (mg/dl)	1.1±0.1	0.99±0.06	1.12±0.08	1.01±0.07	1.1±0.03
Glucose dependent insulinotropic polypeptide (pg/ml)	146.84±40.45	109.83±32.03	136.71±50.59	111.39±28.27	126.11±16.07
Glucagon like peptide-1 (pg/ml)	37.67±9.78	20.36±3.54	35.25±4.35	42.27±7.74	33.15±2.83

Data presented as mean ± SE; Mann Whitney U test was applied; p value < 0.05 considered to be statistically significant

Table 2 Correlation of fasting glucose-dependent insulinotropic polypeptide with basic characteristics and clinical parameters

Variables	Healthy participants		NDD participants	
	r	p value	r	p value
Age (years)	0.007	0.978	**−0.483**	**0.049**
Body mass index (kg/m^2)	−0.119	0.649	−0.096	0.715
Systolic blood pressure (mmHg)	0.283	0.271	−0.049	0.852
Diastolic blood pressure (mmHg)	0.268	0.298	0.247	0.339
HbA1c (%)	−0.345	0.175	−0.113	0.666
Cholesterol (mg/dl)	−0.273	0.288	0.475	0.056
Triglyceride (mg/dl)	0.238	0.358	0.407	0.105
Low density lipoprotein (mg/dl)	−0.282	0.273	**0.506**	**0.038**
High density lipoprotein (mg/dl)	−0.244	0.346	0.181	0.486
Very low-density lipoprotein (mg/dl)	0.237	0.36	0.4	0.112
Non-high-density lipoprotein (mg/dl)	−0.26	0.314	**−0.524**	**0.037**
Serum creatinine (mg/dl)	−0.111	0.672	−0.093	0.722

r = spearman's correlation coefficient; p value < 0.05 considered to be statistically significant

Table 3 Correlation of fasting glucagon-like peptide-1 with basic characteristics and clinical parameters

Variables	Healthy participants		NDD participants	
	r	p value	r	p value
Age (years)	0.022	0.933	−0.048	0.855
Body mass index (kg/m^2)	−0.155	0.554	−0.189	0.468
Systolic blood pressure (mmHg)	0.236	0.363	0.178	0.494
Diastolic blood pressure (mmHg)	0.098	0.709	0.134	0.609
HbA1c (%)	−0.423	0.091	0.091	0.729
Cholesterol (mg/dl)	−0.272	0.29	**0.564**	**0.018**
Triglyceride (mg/dl)	0.238	0.358	0.392	0.119
Low density lipoprotein (mg/dl)	−0.4	0.112	**0.498**	**0.042**
High density lipoprotein (mg/dl)	−0.2	0.443	**0.50**	**0.041**
Very low-density lipoprotein (mg/dl)	0.243	0.348	0.396	0.116
Non-high-density lipoprotein (mg/dl)	−0.277	0.282	**0.503**	**0.047**
Serum creatinine (mg/dl)	−0.122	0.642	0.177	0.498

r = spearman's correlation coefficient; p value < 0.05 considered to be statistically significant

Table 3 shows the correlation of fasting GLP-1 with basic characteristics and clinical parameters. Similar to GIP, GLP-1 also showed a non-significant weak correlation in healthy participants for age, BMI, blood pressure, lipid parameters, and serum creatinine, while a non-significant moderate negative correlation was only observed for HbA1c levels. Among NDD participants, a positive moderate correlation was observed with total cholesterol (r = 0.564, p = 0.018), LDL (r = 0.498, p = 0.042), HDL (r = 0.50, p = 0.041), and non-HDL (r = 0.503, p = 0.047) levels.

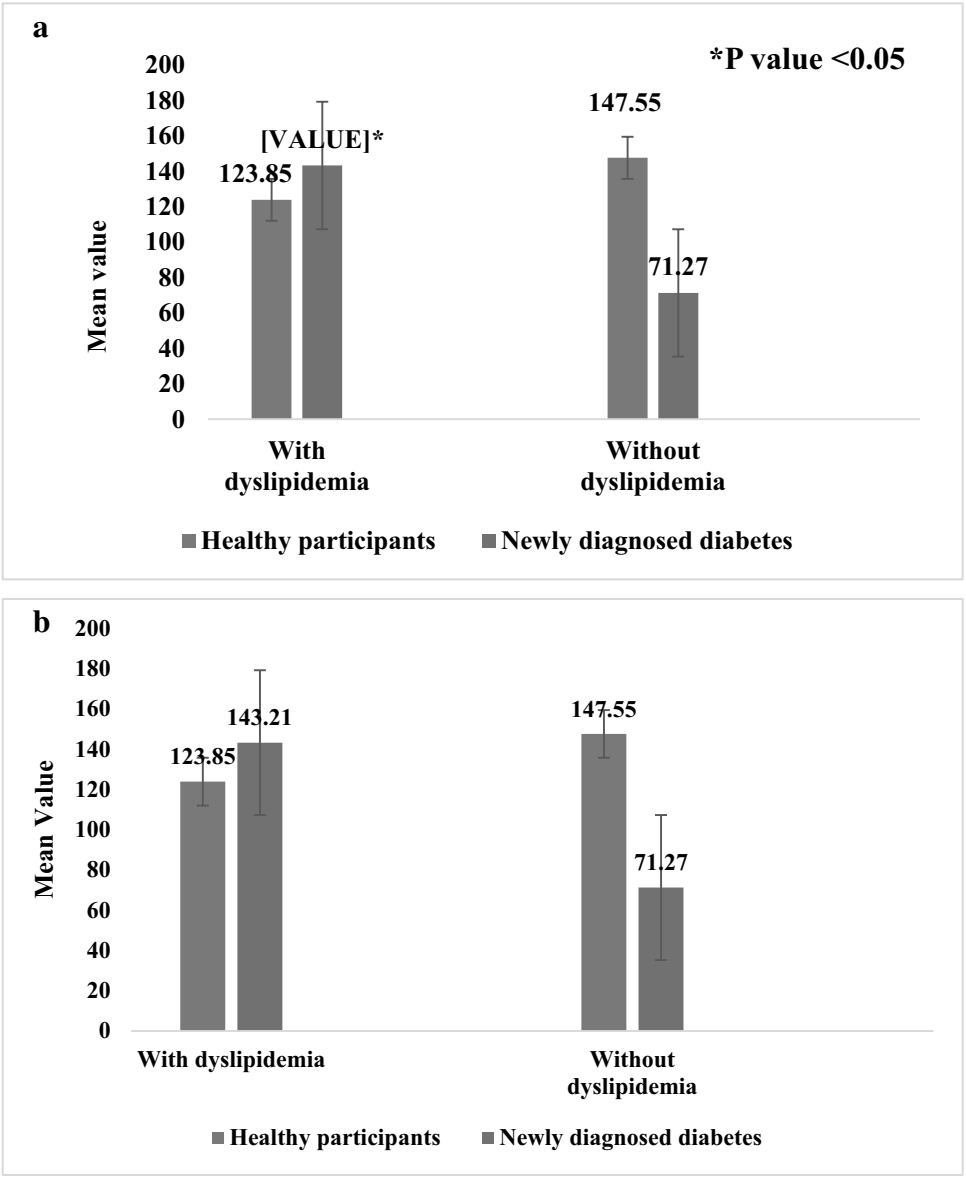

Fig. 1 **a** Association of GLP-1 with dyslipidemia in newly diagnosed with diabetes and healthy participants. Mann–Whitney U test was applied. *Significantly different from healthy participants. **b** Association of GIP with dyslipidemia in newly diagnosed with diabetes and healthy participants. Mann–Whitney U test was applied. *Significantly different from healthy participants

Figure 1 shows the association of GLP-1 and GIP with dyslipidemia in NDD and healthy individuals. GLP-1 was more significantly associated with dyslipidemia in NDD compared to healthy participants (Fig. 1a). Moreover, GIP showed non-significant results with dyslipidemia (Fig. 1b).

Discussion

Abnormal lipid metabolism or lipotoxicity usually contributes to the development of T2DM. Both GLP-1 and GIP anti-diabetic drugs are used to treat people with diabetes in different perspectives, such as in improving lipid accumulation and inflammation in hepatocytes. Because data on the Pakistani population is scarce, this study mainly explores the novelty of incretin hormones with lipid parameters. Overall, the study results demonstrate that GLP-1 had a significantly positive moderate correlation with total cholesterol, LDL, HDL and non-HDL compared to the GIP hormone in NDD participants.

It was also reported previously that GLP-1 levels in people with T2DM retain their stimulatory activity, while GIP levels are almost lost [19]. A greater association of GLP-1 with lipid parameters in our study is also consistent with previously reported data that GLP-1 modulates lipid metabolism. It affects assimilation and transport of lipids, formation and decomposition of fat, metabolism of lipids in hepatic tissue, and reverse cholesterol transport [20]. However, the exact molecular mechanism of GLP-1 on lipid metabolism was not well discussed in literature. It was previously

presented that GLP-1 decreases hypercholesterolemia by regulating microRNA (miR)-19b levels, which improves ATP-binding cassette transporter A1 (ABCA1) expression to reverse disorders by the lipid mechanism [20]. Our results are consistent with a recent study showing that the insulin secretagogue GLP-1 acts as an anti-diabetic therapy for regulating glycaemia and helps as a regulator of lipid and lipoprotein metabolism [21]. Also, dipeptidyl peptidase 4 inhibitors that raise the endogenous bioactive levels of GLP-1 and GLP-1 receptor agonists that directly stimulate GLP-1 receptors have been assessed in both preclinical and clinical trials for their ability to modulate plasma lipid parameters [13]. Apart from the direct effects on lipid metabolism, clinically, GLP-1 receptor agonists not only control triglycerides lipoprotein production but are also used to treat obesity, to reduce atherosclerotic events by inhibiting expression of atherogenic inflammatory mediators, and to prevent major adverse cardiovascular events in participants with T2DM [22, 23]. Akhter et al. reported that a single injection of a GLP-1 receptor agonist reduces the transfer of intestinally packaged fatty acids more rapidly than GLP-2 receptor activation in plasma [24].

In adipose tissues, regulation of the uptake and release of lipids plays an essential role in energy homeostasis. Previously, GIP importance in relation to nutrient intake has been discussed more than its role in the fasting state with lipid metabolism [24]. Conflicting evidence has been found regarding the role of GIP in the dyslipidemia of people with T2DM [25]. We observed positive significant association between GIP and LDL only in NDD participants consistent with Møller et al. and Thondam et al., who also reported an association between GIP and lower LDL in people with T2DM due in part to reduced islet cell expression of GIP receptors (GIPR) secondary to chronic hyperglycemia [9, 26]. In previous studies, GIP is known to regulate triglycerides turnover and lipid clearance from the blood independent of fasting insulin levels by enhancing the activity of lipoprotein lipase [27, 28]. However, we did not find any correlation between GIP with triglycerides and VLDL in NDD as well as in healthy participants contrary to a previous study presenting that GIP in healthy participants acts on its receptors on β-cells and adipocytes to produce adipogenic action (lipid deposition) [26]. We found increased levels of GIP and GLP-1 in healthy male participants and NDD female participants. However, overall incretin levels were higher in NDD participants compared to healthy participants, while previously, low levels of GIP in people with T2DM were observed, reducing visceral adipose tissue storage and improving insulin sensitivity compared to healthy individuals [29]. In our study, GIP levels showed significant negative moderate correlation with age among NDD participants. Moreover, we did not find a significant association of GLP-1 and GIP with metabolic parameters such as weight, blood pressure, and HbA1c in NDD as well as in healthy participants, but literature exemplify that using GLP-1 agonist in people with T2DM improves these metabolic parameters [30]. We also found the NDD group had high BMI compared to healthy participants. It may be because in Pakistan the prevalence of obesity and dyslipidemia are rising trends along with diabetes reported recently [31]. Calanna et al. reported that, in general, people with T2DM exhibit normal GIP secretion in response to OGTT. However, high BMI, young age, and low HbA1c levels positively affect plasma GIP responses in people with T2DM. We also found overall increased GIP levels and BMI in NDD participants compared to healthy individuals [32].

A small sample size is a limitation of our study. Most studies show the relation of incretin with T2DM. However, this study contains simultaneous measures of GLP-1 and GIP with parameters of lipid metabolism in NDD participants of our population, which is our study strength. Other gut hormones relevant for the regulation of energy balance not measured in this study need future evaluation. Future studies with large sample size on genetic levels are recommended to know the exact mechanism of action of incretin on lipid metabolism in our population.

Conclusion

GLP-1 was significantly highly associated with dyslipidemia compared to GIP among NDD participants in our population. GLP-1-based therapies appear promising in the management of diabetic dyslipidemia, and further studies are warranted to elucidate their mechanisms of action in both the intestine and liver.

Abbreviations NDSP: National Diabetes Survey of Pakistan; T2DM: Type 2 diabetes mellitus; GIP: Glucose dependent insulinotropic polypeptide; GLP-1: Glucagon-like peptide-1; NDD: Newly diagnosed diabetes; OGTT: Oral glucose tolerance test; ELISA: Enzyme-linked immunesorbent assay; HRP: Horseradish peroxidase; MSD: Meso scale discovery; HDL-C: Lipoprotein high density-cholesterol; LDL-C: Lipoprotein low density-cholesterol; BMI: Body mass index; GLP-1R: GLP-1 receptor

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Availability of data material All data relevant to the study are presented in the tables.

Author contributions Waris N: Concept and design, literature search, interpretation of data, and wrote the manuscript.

Bano S: Concept and design, interpretation of data, edited, and approved the final manuscript.

Fawwad A: Concept and design, edited, reviewed and approved the final manuscript.

Basit A: Reviewed and approved the final manuscript

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Declarations

Conflict of interest All authors declare that they have no conflict of interest

Ethics approval The study was approved by the ethical review committee of BIDE (IRB no.: BIDE/IRB/NWARIS/10/26/18/0206) and Osaka, Japan (IRB no. 26-15).

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ORIGINAL ARTICLE

The relationship between treatment response and precursors of advanced glycation end-products in type 2 diabetes: a prospective case-control study

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Abstract

Background In glycolysis, hydroxyl radicals emerge via the auto-oxidation pathway in a status of hyperglycemia, and by binding to proteins or lipids, form advanced glycation end-products (AGE). Glyoxal (GO) and methylglyoxal (MGO) are precursors of AGEs. The aim of this prospective, case-control study was to investigate the difference in levels of AGE precursors in patients with and without diabetes and to investigate the relationship between the change in rates of the AGE precursors and treatment in diabetic patients.

Methods The study included 21 treatment-naive patients diagnosed with type 2 diabetes and 21 age and gender-matched healthy control subjects. Throughout an observation period of 3 months, the diabetic patients were started on anti-diabetic treatment and used no additional treatment or supplementary products. The GO and MGO levels were examined with the HPLC method.

Results The GO and MGO levels were determined to be 1.74 and 0.024μg/mL respectively in the diabetic patients and 1.14 and 0.002 μg/mL in the control group. After 3 months of treatment, a statistically significant decrease compared to pre-treatment values was determined of 0.684 μg/mL in GO and 0.01989 μg/mL in MGO (p=0.001, p<0.01). No statistically significant relationship was determined between the change in HbA1c at 3 months compared to pre-treatment and the changes in GO and MGO (p>0.05).

Conclusion The level of AGE precursors was found to be significantly higher in diabetic patients than in the healthy control group. Although there was a significant decrease in AGE precursors when the HbA1c level fell in patients who followed anti-diabetic treatment and diabetes-appropriate diet recommendations, this decrease was at different rates in each patient, which demonstrated that the HbA1c level is not the only determinant of AGE level in plasma. It can be concluded that with appropriate diet and antidiabetic treatment, significant reductions can be obtained in several risk factors which increase the level of AGE precursors.

Keywords Advanced glycation end-products · Glyoxal · Methylglyoxal · Diabetes

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Introduction

Diabetes mellitus, which is basically seen as a result of insulin deficiency or insufficient insulin function, is a chronic metabolic disease characterized by impairments in the metabolism of carbohydrates, fats, and proteins, with the main finding of chronic hyperglycemia [1].

In glycolysis, hydroxyl radicals emerge via the auto-oxidation pathway in a status of hyperglycemia, and by binding to proteins or lipids, form advanced glycation end-products (AGEs). This is a non-enzymatic reaction and causes oxidative stress in the environment where there are AGEs [2, 3]. Glyoxal (GO) and methylglyoxal (MGO) are precursors of AGEs and most often occur as a result of lipid peroxidation, glucose autoxidation, and microbial fermentation [4, 5]. AGEs, which are glycotoxins, are thought to play a role in the pathogenesis of many chronic diseases, especially diabetes [6, 7]. By binding with cross-links to various body proteins or cell surface receptors, these molecules change their structures and functions. All of these events cause oxidative stress and inflammation [8]. It is accepted that oxidative stress has a significant role in the pathogenesis of complications developing associated with diabetes [9].

Glycated hemoglobin A1c (HbA1c), which is used for the diagnosis of diabetes, is an example of a glycated protein that shows average blood sugar concentrations in the previous 2- to 3-month period. HbA1c has also been shown to be correlated to complications that develop associated with diabetes [10–12]. The aim of this prospective, case-controlled, observational study was to investigate the relationship between the levels of AGE precursors and diabetes with real-life data. The difference between levels of AGE precursors was examined in patients with and without diabetes and the relationship between the change in rates of the AGE precursors and treatment in diabetic patients. It was believed that significant results would be obtained from this study that would be able to shed light on the estimated prognosis course and follow-up of diabetes.

Materials and methods

This prospective study was approved by the Ethics Committee of Bakirköy Sadi Konuk Training and Research Hospital, and in accordance with the Helsinki Declaration and its later amendments or comparable ethical standards (No:2019/312). Written informed consent was obtained from all the participants after a detailed explanation of the study protocol and it was explained that they could leave the study at any time they wished.

Study design and participants

The study included patients aged >18 years, who were being followed up in the General Internal Polyclinic between January 2019 and December 2019, had a new diagnosis of type 2 diabetes, were not yet receiving any antidiabetic treatment and had HbA1c value >8. Study exclusion criteria were defined as the presence of additional disease other than regulated hypertension, the emergence of any new disease throughout the 3-month follow-up period, or starting any additional treatment, including antibiotics, other than the antidiabetic treatment prescribed.

A control group was composed of 21 healthy volunteers, matched for age and gender to the patient group, selected from those presenting at the General Internal Polyclinic for a general check-up, with no insulin resistance, diabetes, or any other disease, including hypertension. From both groups, subjects were excluded if they had any malignancy, were using drugs that would interact with the study tests, if they had chronic diarrhea or malabsorption syndrome for any reason, inflammatory disease, or active infection, if they were smokers, were obese (BMI>30), or were taking supplementary products.

The patients diagnosed with type 2 diabetes in Group 1 were given information about the disease, were informed about the standard nutrition appropriate to diabetes and were instructed that they would maintain a diabetes-appropriate diet throughout the 3 months, not follow a different diet, not take any supplementary products, and not take any drugs other than the prescribed medication. The patients were instructed that in any unusual circumstances or when there was a need for additional drugs, they should contact the researchers and in such an event they would be withdrawn from the study.

On the day of the first presentation at the hospital and at the 3-month follow-up examination, a 2-ml venous blood sample was taken by an experienced nurse in the morning after at least 8 hours fasting. The HbA1c measurement was performed with the HPLC method on an Adams Premier Hb 9210 device (Trinity Biotech, USA).

Blood samples were taken from 40 patients initially included in the patient group. Then, a total of 19 were excluded; 5 patients had an infection that required antibiotics, 6 wished to take supplementary products, 3 started to follow a special diet program for various reasons, and 5 could not be contacted after 3 months. The blood samples taken before treatment were disposed of appropriately, and the study analysis continued with a patient group of 21 subjects.

Glyoxal, methylglyoxal, methanol, sodium acetate, 4-nitro-1,2-phenylenediamine, acetonitrile, fructose, glucose, and sucrose were obtained from Sigma-Aldrich (St. Louis, MO, USA).

HPLC determination of GO and MGO

The HPLC conditions in our study were referenced to the HPLC conditions detailed in the study of Cengiz et al. [13]. Determination of the most potent precursors of AGEs in chips,

crackers, and breakfast cereals by HPLC using precolumn derivatization with 4-nitro-1, 2-phenylenediamine.

A Shimadzu SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan) and a Shimadzu LC 20AT pump were used when setting up the HPLC system. The mobile phase consisted of methanol:water:acetonitrile (42:56:2, v/v/v) and had a wavelength of 255 nm. GO and MGO were separated with an Inersil ODS-3, 250 × 4.6 mm, 5 μm column with a flow rate of 1 mL/min.

Quality assurance/quality control (QA/QC)

AOAC guidelines [14] were used for method validation of GO and MGO analysis. Linearity was determined between 0.2 and 2.0 μg/mL for GO and MGO using five levels of calibration in triplicate. The precision of GO and MGO was evaluated for repeatability and reproducibility by analyzing one of the test samples ten times on the same day and three times on different 3 days, respectively. In addition, 2 μg/mL of GO and MGO were spiked to the test sample to check the recovery of the method. To monitor the quality control of the analyzes, the quality control material prepared in the laboratory condition was analyzed with each batch of samples. The quality control samples were prepared as follows: 1 μg/mL of GO and MGO were spiked to the test sample used for the recovery and analyzed ten times. Then, the average value was calculated with standard deviation (SD) using one-way analysis of variance (ANOVA; $p < 0.05$, Tukey's test). The upper and lower acceptable warning limits are determined by calculating the average ±2SD values, while the lower and upper precaution limits are determined by calculating the average±3SD values and recorded in the Quality Control Graph Chart [15]. All analyses were performed in triplicate ($n = 3$).

Statistical analysis

Data obtained in the study were analyzed statistically using the NCSS 2007 software (Number Cruncher Statistical System, Kaysville, UT, USA). The conformity of quantitative data to normal distribution was assessed with the Shapiro-Wilk test and graphic examinations. In the comparisons of two groups of quantitative data, the Student's *t* test was applied to data showing normal distribution and the Mann-Whitney *U* test when the data did not show normal distribution. The Wilcoxon signed-rank test was applied in the intra-group comparisons of quantitative data not conforming to normal distribution. In the comparison of qualitative data, the Pearson Chi-square test was used. Relationships between quantitative data were evaluated using Spearman correlation analysis. A value of $p<0.05$ was accepted as statistically significant.

Power analysis was applied with the G*Power (version 3.1.7) software to determine the sample number. In the pilot study applied to 10 subjects in each group, power was calculated as 0.928 so to obtain 80% power at $\alpha=0.05$, it was calculated to be necessary to have a total of 40 subjects as 20 in each group.

Results

The evaluation was made of a total of 42 subjects, comprising 26 (61.9%) females and 16 (38.1%) males with a mean age of 54.45 years (range, 39–78 years) (Table 1).

In Group 1, 10 patients with a tendency for hypertension were taking angiotensin-converting enzyme inhibitor (ACEi) treatment. All the patients diagnosed with diabetes were started on the treatment of metformin and DPP4 inhibitor. Insulin treatment was added to this treatment for 8 patients.

A statistically significant difference was determined between the groups in respect of the GO and MGO measurements ($p<0.01$). The GO and MGO levels were determined to be statistically significantly higher at 1.74 and 0.024μg/mL respectively in the diabetic patients compared to 1.14 and 0.002 μg/mL in the control group (Table 2).

After 3 months of treatment, a statistically significant decrease compared to pre-treatment values was determined of 0.684 μg/mL in GO and 0.01989 μg/mL in MGO ($p=0.001$, $p<0.01$).

A statistically significant decrease of mean 2.90 units was determined in the HbA1c measurements at 3 months compared to the pre-treatment values ($p=0.001$, $p<0.01$) (Table 3).

Table 1 Age and gender distribution by groups

		Group 1 (*n*=21)	Group 2 (*n*=21)	Total
Age (year)	*Min–max (median)*	39–71 (52)	41–78 (52)	39–78 (52)
	Mean±SD	54.10±9.43	54.81±9.93	54.45±9.57
Gender	**Women (%)**	13	13	26 (61.9)
	Men (%)	8	8	16 (38.1)

SD standard deviation

Table 2 Comparison of glyoxal and methylglyoxal levels by groups

		Group 1 (*n*=21)	Group 2 (*n*=21)	*p*
Glyoxal	*Min–max (median)*	0.87–4.2 (1.5)	0.3–1.8 (1.12)	[c]***0.004****
μg/mL	*Mean±SD*	1.74±0.79	1.14±0.35	
Methylglyoxal	*Min–max (median)*	0.006–0.203 (0.011)	0.0001–0.008 (0.0008)	[c]***0.001****
μg/mL	*Mean±SD*	0.024±0.045	0.002±0.003	

[c] Mann-Whitney *U* test, *$p<0.01$, *SD* standard deviation

The mean HbA1c of the control group without any additional disease, diabetes, or prediabetes was 4.8±0.18 (range 4–5.4%).

No statistically significant relationship was determined between the change in HbA1c at 3 months compared to pre-treatment and the changes in GO and MGO ($p>0.05$) (Table 4).

No statistically significant relationship was determined in the pre-treatment and post-treatment GO and MGO values according to the presence or absence of hypertension in the patient group ($p>0.05$) (Table 5).

Discussion

In addition to the formation of AGE by hyperglycemia, the intake of food with a high amount of AGE can cause insulin resistance and ultimately diabetes. In the most striking study on this subject, insulin resistance was seen to develop much earlier in mice fed with feed containing high amounts of AGE compared to mice fed a normal diet [16]. Several studies have shown AGE of exogenous origin to be associated with a decreased peripheral insulin response [17, 18].

The current study results demonstrated significantly higher levels of GO and MGO, which are AGE precursors, in the diabetic patients compared to the healthy control subjects with no diabetes and no insulin resistance. In the light of these findings and those of previous studies, it can be said that both GO and MGO were taken exogenously and the hyperglycemia in these patients were effective in the high GO and MGO levels determined in the current study. The effect of patient age on AGE level was taken into consideration [19] and care was taken to match the ages of the two groups. As the GO and MGO levels could be higher in smokers [20], any subjects who smoked were not included in the study.

There is known to be a relationship between tissue damage of high blood levels of AGEs and diabetic complications [21, 22]. Just as for a reduction in HbA1c, a reduction in serum AGE amounts with treatment or lifestyle changes could be a predictor of a positive response to treatment and a disease course with fewer complications. In the patient group of this study, significant reductions were observed in the GO, MGO, and HbA1c levels with antidiabetic treatment and the recommended diabetes-appropriate diet. In a previous study, type 1 and type 2 diabetes patients were given a low AGE level diet for 6 weeks, after which there was seen to be a reduction in inflammation markers together with reduced serum AGE levels [23]. In the current study, a significant decrease was determined in GO and MGO in Group 1 with diet and treatment, but it is not known to what extent this reduction can be associated with the antidiabetic drugs or the reduction in hyperglycemia with the dietary changes.

An interesting point of the current study findings was that there was no correlation between the pre-treatment HbA1c level and the reduction obtained in HbA1c with treatment and the reduction in GO and MGO levels. This demonstrates

Table 3 Comparison of glyoxal, methylglyoxal, and HbA1c levels in Group 1 before and at the 3rd month of treatment

Group 1 (*n*=21)		Before treatment	3rd month	Difference	*p*
Glyoxal μg/mL	*Min–max (median)*	0.87–4.2 (1.5)	0.4–1.61 (1.0)	0.10–3.23 (0.44)	[d]***0.001****
	Mean±SD	1.74±0.79	1.05±0.32	0.684±0.71	
Methylglyoxal μg/mL	*Min–max (median)*	0.006–0.203 (0.011)	0.0001–0.0096 (0.00001)	0.0004–0.20299 (0.00679)	[d]***0.001****
	Mean±SD	0.024±0.045	0.0025±0.0036	0.01989±0.046	
HbA1c %	*Min–max (median)*	8–15.8 (10)	6.1–9.2 (7.2)	0.3–8.6 (2.2)	[d]***0.001****
	Mean±SD	10.25±1.97	7.36±1.04	2.90±2.11	

[d] Wilcoxon signed-rank test, **$p<0.01$, *SD* standard deviation

Table 4 The relationship between HbA1c changes and glyoxal and methylglyoxal changes

	Difference HbA1c	
	r	*p*
Difference glyoxal	0.014	*0.951*
Difference methylglyoxal	0.143	*0.736*

r Spearman's correlation coefficient

that the blood glucose level reflected by the HbA1c level is not the only factor affecting the GO and MGO levels.

Studies conducted with the aim of decreasing the AGE level have shown interest in the blockage associated with the renin-angiotensin-aldosterone system in addition to nutrition with antioxidant molecules or a diet poor in AGE. The use of olmesartan (angiotensin II receptor blocker: ARB) and temocaprilate (an ACEi), which are antihypertensive agents, has been shown to have AGE-lowering effects. The effect mechanism is thought to be that these agents can have an effect by retaining carbonyl components [24]. In another study, it was demonstrated that the ARB valsartan could improve in vitro protein glycation and oxidation in various conditions and this was thought to be associated with the positive effect on the redox balance and the reduction in protein glycation [25].

In contrast to these data in the literature, no significant difference was seen between those with and without hypertension in the current study population in respect of the level of AGE precursors before or after treatment. The patients in this study were taking ACEi as an antihypertensive drug and the basic design of the study was not for the evaluation of the effect of antihypertensive drugs. Throughout the follow-up period of the study, no patient started any new antihypertensive drug or changed the dose of their existing medication. Only patients with regulated hypertension were accepted in the study. To be able to draw clearer conclusions about the effects of antihypertensive treatment on AGE precursor levels, there is a need for further studies designed accordingly with a greater number of patients.

The main limitation of this study was that although it was planned to include 40 patients, 21 could not continue for various reasons. Although the selection criteria were applied as far as possible in respect of comorbidities, smoking, and BMI, evaluations were made according to the patients' own statements of dietary habits and whether or not they were taking additional drugs.

In conclusion, with real-life data in this prospective, case-controlled study, it was shown that AGE precursor levels are significantly higher in diabetic patients than in healthy control subjects. When the HbA1c level fell in patients with antidiabetic treatment and diabetes-appropriate diet recommendations, although there was a significant decrease in the level of AGE precursors, this decrease was at different rates in each patient. This indicated that the HbA1c level is not the only determinant of plasma AGE level. Patient age, genetic factors, dietary habits, microbiota, and many other reasons such as these may have an effect on the plasma AGE level. In this study, no special diet other than a standard diabetic patient diet was applied. If factors that can be changed are focussed on, it can be said that in parallel with the reduction in HbA1c obtained with appropriate antidiabetic treatment and diet, significant reductions could be obtained in several risk factors that increase the level of AGE precursors.

Table 5 Glyoxal and methylglyoxal levels according to hypertension

		Hypertension		*p*
		No (*n*=11)	Yes (*n*=10)	
Glyoxal-1 μg/mL	*Min–max (median)*	0.203–4.23 (1.4595)	0.872–3.076 (1.423)	*0.36*
	Mean±SD	1.9178±0.94	1.5849±0.63	
Glyoxal-2 μg/mL	*Min–max (median)*	0.616–1.49 (0.9925)	0.374–1.612 (0.946)	*0.31*
	Mean±SD	1.068±0.3	1.014±0.37	
Methylglyoxal-1 μg/mL	*Min–max (median)*	0.0064–0.094 (0.508)	0.203–0.059 (0.0068)	*0.7*
	Mean±SD	0.018±0.026	0.264±0.0586	
Methylglyoxal-2 μg/mL	*Min–max (median)*	0.00001–0.0087 (0.00001)	0.00001–0.0096 (0.00001)	*0.83*
	Mean±SD	0.0021±0.003	0.0029±0.0041	

[c] Mann-Whitney *U* test, *SD* standard deviation

Author contribution All authors contributed to the study conception and design, material preparation, data collection and analysis. All authors read and approved the final manuscript.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Bakirköy Sadi Konuk Training and Research Hospital (No:2019/312).

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

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Lipocalin-2 levels increase in plasma of non-alcoholic fatty liver disease patients with metabolic syndrome

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Abstract

Introduction Non-alcoholic fatty liver disease (NAFLD) has emerged as major health risk in metabolic syndrome. The liver biopsy is the golden standard for the diagnosis and differentiation of all NAFLD stages, but its invasiveness poses a risk for patients, which is why new, non-invasive ways of diagnostics ought to be discovered. Lipocalin-2 (LCN2), which is a part of the lipocalin transport protein family, is a protein formally known for its role in iron transport and in inflammatory response. In the present study, we evaluated the levels of LCN2 a marker of liver inflammation and injury in the blood of patients with ultrasonography proven NAFLD.

Materials and methods A total of 94 (24 control and 70 cases with different grade of NAFLD) aged and sex matched patients participated in the single-centre, open, hospital-based case control cross-sectional study. All anthropometric, biochemical and hematological assessment was done after proper consent. The metabolic syndrome was determined by new IDF criteria. Plasma LCN2 levels were measured by ELISA method.

Results The LCN2 levels were increased in patients with NAFLD (170.38±114.43 ng/ml) as compared to healthy volunteers (40.46±6.44 ng/ml) (p<0.001). The LCN2 levels increase as the level of steatosis increases from grade I (143.99±90.04), grade II (205.78±143.07) to grade III (219.49±146.30) of fatty liver even though there was no difference in BMI showing it is specific to liver injury than obesity. The ROC curve analysis predicted sensitivity of 96.9% and predicted specificity of 91.7%. These changes were detected before derangement in liver enzymes.

Conclusion The plasma lipocalin levels increase in NAFLD with metabolic syndrome and it can be considered a good blood biomarker. The early detection in NAFLD cases may improve prognosis.

Keywords NGAL · Lipocalin-2 · Non-alcoholic steatohepatitis · Insulin resistance · Obesity · Diabetes

Introduction

NAFLD, defined as an extensive accumulation of fat in the liver, is a fast-growing form of new world's disease caused by excess calorie intake and a sedentary lifestyle. Since NAFLD seems to be associated with classical indicators of metabolic syndrome (e.g. insulin resistance, type 2 diabetes and dyslipidemia), it is no wonder that LCN2 overexpression was lately defined as a hepatic manifestation of a metabolic syndrome [1]. Epidemiological studies suggest the prevalence of NAFLD in around 9–32% of the general population with higher prevalence in those with overweight or obesity and those with diabetes or prediabetes [2].

The biggest dilemma in the diagnosis of these patients is that they may be completely normal and thus it may be suspected by the accidental discovery of hepatomegaly or a

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radiological test carried out for showing suggestive changes of fatty liver. The final diagnosis is confirmed by liver biopsy but it has certain limitations of morbidity mortality due to invasive nature. It may also give false-negative results if needle punctured in non-fibrotic areas. This limits liver biopsy as routine investigation for diagnostic purposes. So it is important to determine some non-invasive biomarkers which can help in early detection of the disease and thus assist in the timely management of patients. NAFLD cannot be diagnosed at early stage because very few people do ultrasound for routine or preventive check-up. Most of the preventive check-up packages include blood test only to keep it as low cost [3, 4].

LCN2 has been proven to be a good target to study as a possible biomarker for NAFLD and subsequent liver pathology. It has been proven by independent research teams that its high levels can indicate liver damage [3, 4]. Since it can be detected in bodily fluids such as blood and urine, it can be routinely used as a part of laboratory tests. To prove it, one study examined the levels of circulating LCN2 as well as its gene expression in obese women with NAFLD (with either NASH or simple steatosis) and normal liver [5]. The research concluded that both gene expression and protein levels were upregulated in obese women with NAFLD. However, gene expression correlated with simple steatosis while protein levels correlated with NASH. The same study showed that treatment with proinflammatory TNF, IL6 and resistin causes the upregulation of LCN2 in HepG2 cells [5]. Therefore, LCN2 is considered to be a liver's protective response to inflammation. Another study, done on Chinese subjects, proved that LCN2 serum levels are elevated in patients with NAFLD as opposed to the control and that they highly correlate with both inflammation (C-reactive protein) and insulin resistance [6]. Therefore, LCN2 may serve as a valuable biomarker for monitoring the initiation, clinical diagnosis and progression of NASH [7].

In support of the above study, more research is still needed in different populations to know the effects of lipocalin-2 levels in MS patients with different grades of NAFLD. The aim of this study is to establish a correlation between the levels of lipocalin as a useful biomarker with increasing severity of steatosis in NAFLD patients so it can be used with adjunct methods as alternative method to liver biopsy.

Methodology

Study design

This is a single-centre, open, hospital-based case control cross-sectional study. This study was conducted at the Department of Medicine, King George's Medical University (KGMU), Lucknow, in collaboration with the Department of Radiodiagnosis.

The present study was conducted on the indoor and outdoor patients of medicine and gastroenterology. The patients who visited the KGMU, Lucknow, were divided by age and sex matched into two groups: healthy controls without NAFLD and MS cases with different grades I, II and III of NAFLD. A written informed consent was obtained from all patients. The clinical examination, medical history and anthropometric and demographic profile of patients with past and current medications were recorded. Following inclusion/exclusion criteria, the subjects were enrolled in respective groups according to the decision of clinician. The blood samples were collected at the KGMU from 24 healthy patients termed as control group and 70 with MS group (case group, who were found to have ultrasound proven fatty liver) according to new IDF-International Diabetes Federation definition Criteria [8]. The study was duly approved by the institutional ethics committee. The study was conducted according to the ethical guidelines of the Declaration of Helsinki 1964 and its amendments. The decision of clinician was final in the allocation of patients to its groups.

Sample collection and processing

Serum and plasma were collected from clotted blood using serum separator tubes, EDTA tubes centrifuged at 1000–2000 rpm for 10 min. The serum was snap-frozen and stored at −80 °C until required and plasma was transferred into a clean polypropylene tube. All serum samples were thawed only once.

Determination of biochemical measurements

The clinical serum samples were also used to measure fasting glucose, total bilirubin (Bil. Tot), alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT), alkaline phosphatase (ALP), HbA1c and lipid profile. All of the parameters were measured using fully automated clinical chemistry analyzer (TRANSASIA Bio-medical Ltd, India, Erba Manheim). Insulin resistance is being calculated by homeostasis model assessment-insulin resistance (HOMA-IR) level index: as HOMA IR= (fasting insulin (IU/mL) × fasting glucose (mg/dL))/405 [9, 10].

Determination of hematological measurements

The clinical blood samples were used to measure WBC, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and PLT using hematolyzer (Sysmex, Japan).

Determination of biomarker (lipocalin-2 or NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) was measured by Human Lipocalin-2 ELISA kit (RAB0332, Sigma-Aldrich) which is an in vitro enzyme-linked immunosorbent assay for quantitative measurement of human lipocalin [11]. This kit is showing the inter-assay cv <12% and intra-assay cv <10%.

Grading of liver

Ultrasonography of liver was done by radiologist with high-end ultrasound equipment. The radiologist was same to avoid interpersonal bias. The echo pattern of the liver was compared with renal parenchyma along with visualization of periportal echogenicity and obscuration of the diaphragm and was classified into normal, grade I, grade II and grade III steatosis [12]: *normal liver (grade 0)*, echotexture was recorded in the absence of steatosis; *mild steatosis (grade I)*, increased hepatic echogenicity with visible periportal and diaphragmatic echogenicity; *moderate steatosis (grade II)*, increased hepatic echogenicity with imperceptible periportal echogenicity, without obscuration of the diaphragm; and *severe steatosis (grade III)*, increased hepatic echogenicity with imperceptible periportal echogenicity and obscuration of the diaphragm. Diffuse involvement of the liver was given consideration for assessment and any focal changes were not considered valid. The liver was scored by the most affected area.

Statistical analysis

Continuous data were summarized as mean ± SD (standard deviation) whereas discrete (categorical) in number (n) and percentage (%). Continuous two independent groups were compared by independent Student's t test whereas. Continuous more than two independent groups were compared by one factor analysis of variance (ANOVA) and the significance of mean difference between (inter) the groups was done by Tukey's HSD (honestly significant difference) post hoc test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variances between the groups by Levene's test. Categorical groups were compared by chi-square (χ^2) test. A two-tailed (α=2) $p < 0.05$ was considered statistically significant. Analyses were performed on SPSS software (Windows version 22.0).

Observations and results

Demographic

A total of 94 individuals were included in this study. Out of these, 24 were healthy controls and 70 were patients diagnosed with different grades of NAFLD which are as follows: grade I NAFLD (31, 44.28%), grade II NAFLD (26, 37.1%) and grade III NAFLD (13, 18.5%).

Anthropometric characteristics

The disease remains uninfluenced with the height of patients. However, a significant increase in the body weight of group II patients confirms the role of BMI among NAFLD patients. The BMI of patients was significantly higher than healthy shown in Table 1. The blood pressure and blood sugar in cases as well as in controls are given in Table 2. There was no significant difference between the number of males and females in cases and control group. The mean age in cases was 50.96±11.53 years (36 were males and 34 were females) whereas in control group was 43.04±8.28 years of which 11 were males and 13 were females. Overall, control group included a relatively younger age group which was significant (p=0.003). BMI was significantly higher in patients with steatosis compared with patients without any steatosis (p<0.001). Waist circumference was significantly on the higher side in NAFLD patients as compared to controls (p<0.001). Patients of NAFLD were mostly hypertensive with overall increased mean blood pressure levels both systolic and diastolic as compared to controls (Table 1). Around 77% of patients were hypertensive as defined by IDF criteria of metabolic syndrome. Among those who were hypertensive, 74% were controlled with antihypertensive treatment (Table 1). Also, cases were reportedly having increased fasting blood glucose levels when compared to controls and this difference was also significant (p<0.001) as seen in Table 1 and Figure 1. Of the NAFLD patients with metabolic syndrome, 71.4% had clinical diabetes, out of which 60% of them were already controlled on antidiabetic treatment. Among the 70 NAFLD patients, a total of 55 patients (78.6%) were dyslipidemic and among them, 28 (40% of total) patients were on regular treatment.

In the hospitalized complicated patients, BMI was significantly lower in both male and female patients than MS. This could be the effect of severe trauma of stroke or CVD or DN. The complicated patients were admitted cases of hospital. They were under clinical supervision before and after the episode. It might be this reason that their BMI was affected with suitable diet chart as preclinical advice.

Hematological characteristics

The WBC (9298.29±3359.25) and MCV (84.55±6.85) of NAFLD cases increased significantly as compared to control (Table 3). However, hemoglobin (11.56 ± 2.27), MCH (27.89 ±3.02) and platelet (1.99±0.89) decreased significantly in the NAFLD cases from control (Table 3).

Table 1 Demographic and biochemical characteristics comparison of cases and controls

Demographic and biochemical characteristic	Cases (n=70)	Controls (n=24)	Statistical significance	
	Mean ± SD	Mean ± SD	't/χ^2'	'p'
Mean age±SD (range) in years	50.96 ± 11.53 (30–80)	43.04 ± 8.28 (27–66)	3.095	0.003
Gender				
Male Female	36 (51.43%) 34 (48.57%)	11(45.83%) 13(54.17%)	0.220	0.636
Bil. Tot	1.20 ± 3.32	0.76 ± 0.29	0.645	0.521
AST	59.62 ± 58.10	36.88 ± 16.97	1.884	0.063
ALT	65.60 ± 62.50	56.25 ± 21.21	0.716	0.476
ALP	222.22 ± 169.66	123.17 ± 43.00	2.820	0.006
TC	168.28 ± 81.86	91.54 ± 26.20	4.500	<0.001
TG	186.86 ± 157.93	81.46 ± 29.85	3.239	0.002
HDL	33.15 ± 20.98	52.75 ± 21.31	−3.934	<0.001
LDL	87.54 ± 49.66	88.21 ± 36.53	−0.060	0.952
Fasting glucose	153.93 ± 80.16	92.96 ± 14.48	3.693	<0.001
HbA_{1c}	8.34 ± 2.60	5.68 ± 0.69	4.949	<0.001
Waist circumference	94.39 ± 8.79	83.13 ± 6.40	5.769	<0.001
Hip circumference	96.63 ± 6.91	86.13 ± 6.31	6.569	<0.001
SBP	141.39 ± 5.18	134.25 ± 6.44	5.459	<0.001
DBP	90.69 ± 3.54	83.42 ± 7.56	6.315	<0.001
BMI	34.21 ± 4.63	23.59 ± 1.81	10.926	<0.001
HOMA-IR	7.66 ± 5.88	1.46 ± 1.04	5.119	<0.001
Lipocalin	170.38 ± 114.43	40.46 ± 6.44	5.543	<0.001

Correlation of lipid profile, liver function tests in controls and in NAFLD patients

Cholesterol, triglycerides and HDL levels were significantly increased in cases as compared to controls (p<0.001) as in Figure 2. AST and ALT levels were not significantly different between the groups (Table 2). Also in cases, AST and ALT levels do not correlate with different grades of steatosis (p value for AST is 0.812, p value for ALT is 0.684—Table 2). Thus ALT and AST levels do not have any correlation with increasing grade of steatosis. Lipocalin levels were significantly raised in NAFLD patients as shown in Table 2; mean lipocalin levels were significantly on the higher side (p<0.001) in cases (170.38±114.43) as compared to controls (40.46±6.44) [Figure 2]. The correlation of metabolic syndrome parameters with increasing grade of steatosis is given in Table 2. In our study, we found out that with increasing grade of steatosis, there is a statistically significant increase in fasting glucose levels and HbA1c levels (Table 2). Serum HbA1c levels were significantly on the higher side in patients (8.34±2.60) than in controls (5.68±0.69) a with p value of <0.05. With increasing grade of steatosis, there is no significant increase in levels of lipid profile parameters, viz. HDL, LDL, cholesterol and triglycerides level. As seen in Table 2, systolic BP, as well as diastolic BP, did not correlate with increasing grade of hepatic steatosis and there was no significant difference.

The sensitivity and specificity of lipocalin levels for diagnosing steatosis were evaluated on the basis of ROC curve analysis. As in Figure 1, on ROC curve, the area under the curve was 0.992, suggesting a high predictive value in differentiating between cases and controls. The cut-off value of lipocalin was ≥46.5 ng/ml which was found to have predicted sensitivity of 96.9% and predicted specificity of 91.7% (Figure 1). This suggests that lipocalin can be used with great accuracy in determining the presence or absence of hepatic steatosis.

Discussion

Excess calorie intake and a sedentary lifestyle have made NAFLD one of the fastest growing forms of liver disease of the modern world. It is characterized by abnormal accumulation of fat in the liver and can range from simple steatosis and non-alcoholic steatohepatitis (NASH) to cirrhosis as well as development of hepatocellular carcinoma (HCC). Lipocalin-2, which is a part of the lipocalin transport protein family, is a

Table 2 Demographic and biochemical characteristics comparison of NAFLD cases with different grades I, II and III

Demographic and biochemical characteristic	Grade I (n=31)	Grade II (n=26)	Grade III (n=13)	Statistical significance	
	Mean	Mean	Mean	'F/χ^2'	'p'
Gender					
Male	16 (51.61%)	11 (42.31%)	9 (69.23%)	2.52	0.284
Female	15 (48.39%)	15 (57.69%)	4 (30.77%)		
Bil. Tot	0.90 ± 0.89	0.81 ± 0.40	0.61 ± 0.39	0.901	0.411
AST	55.04 ± 62.33	61.40 ±55.64	67.01 ± 55.94	0.209	0.812
ALT	71.22 ± 79.46	65.18 ± 43.45	53.04 ± 49.91	0.381	0.684
ALP	241.33 ± 188.26	203.73 ± 130.80	213.62 ± 198.76	0.361	0.698
TC	173.16 ± 60.22	154.69 ± 53.33	183.83 ± 151.05	0.641	0.530
TG	174.32 ± 91.00	162.31 ± 67.59	265.85 ± 323.62	2.103	0.130
HDL	33.19 ± 16.64	31.56 ± 18.32	36.25 ± 33.50	0.211	0.810
LDL	91.35 ± 47.80	79.16 ± 42.17	95.23 ± 67.14	0.611	0.546
Fasting glucose	145.03 ± 63.45	139.51 ± 49.56	203.97 ± 135.65	3.359	0.041
HbA_{1c}	7.81 ± 2.32	8.05 ± 2.27	10.19 ± 3.18	4.483	0.015
Waist circumference	94.69 ± 6.71	94.97 ± 12.32	92.54 ± 3.30	0.356	0.701
SBP	142.77 ± 6.46	139.92 ± 3.64	141.00 ± 3.56	2.262	0.112
DBP	90.39 ± 3.98	90.69 ± 3.34	91.38 ± 2.87	0.358	0.701
BMI	33.70 ± 4.81	35.21 ± 5.04	33.43 ±3.02	0.980	0.381
HOMA-IR	7.03 ± 6.14	6.94 ± 4.39	10.58± 7.28	2.306	0.138
Lipocalin	143.99 ± 90.04	205.78 ± 143.07	219.49 ± 146.30	1.283	0.292

protein formally known for its role in iron transport and in inflammatory response. However, in recent years, its implication in the pathogenesis of NAFLD has become apparent. LCN2 shows significant upregulation in several benign and malignant liver diseases, making it a good candidate for the NAFLD biomarker or even a therapeutic target [13].

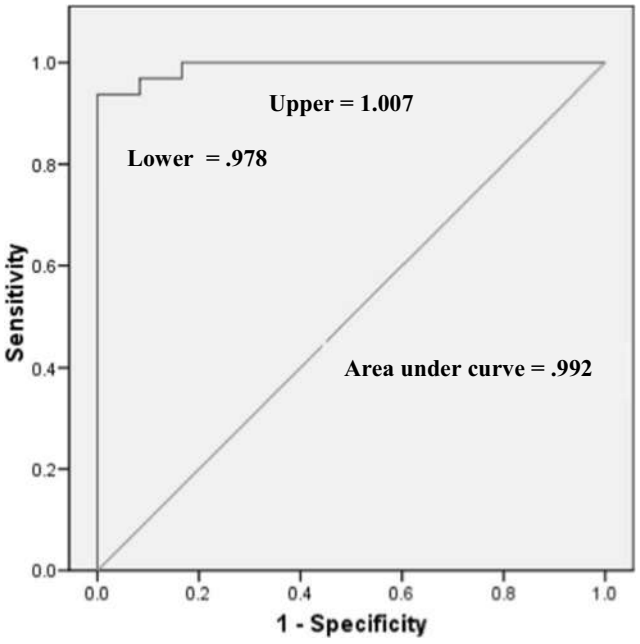

Fig. 1 Receiver-operator curve analysis for deriving a cut-off value to differentiate between cases and controls

The NAFLD is a major health burden and emerged as an important cause of morbidity and mortality throughout the world. It has an estimated worldwide prevalence ranging from 20 to 46%, varying with study population and diagnostic criteria used. NAFLD is associated with cardiovascular disease, obstructive sleep apnoea, type 2 diabetes mellitus and other manifestations of the metabolic syndrome. A very important subset of patients which are clinically relevant include the patients with NAFLD specifically those with non-alcoholic steatohepatitis. In these patients, in whom NAFLD-associated cirrhosis develops, the outcome may be similar to that for other causes of cirrhosis. It is of major importance to diagnose steatosis in patients with metabolic syndrome and early intervention may prevent further progression and reversal of steatosis. Though radiological investigations can confirm fatty changes in the liver, liver biopsy can confirm the diagnosis which is an invasive procedure. Hence, our study was conducted to find the correlation of a non-invasive biomarker, lipocalin, with hepatic steatosis and to establish its role as a diagnostic and prognostic marker in NAFLD patients [14].

Patients with steatosis had higher BMI, systolic and diastolic blood pressure and waist circumference as compared to controls. The BMI did not differ in NAFLD patients significantly

Table 3 Haematological characteristics comparison of cases and controls

Parameters (unit)	Cases	Control	Statistical significance	
			'*t*'	'*p*'
WBC (10^3/μl)	9298.29±3359.25	8354.17±2209.77	1.282	0.202
HGB (g/dl)	11.56 ± 2.27	12.16±1.55	1.200	0.233
MCV (Fl)	84.55±6.85	81.81±7.56	1..646	0.103
MCH (Pg)	27.89±3.02	28.28±2.40	0.573	0.568
PLT (10^3/μl)	1.98±0.88	1.86±0.59	0.620	0.536

irrespective of grade of fatty liver. Patients with steatosis had higher NGAL levels and it increases as the level of steatosis increases from grade I to grade III of fatty liver even though there was no difference in BMI showing it is related to liver injury more than obesity. As studied by Tekkesin et al. [15], BMI, HOMA-IR and waist circumference are significantly higher in patients with NAFLD as compared to controls. It is also found that patients with steatosis had higher urinary NGAL levels in their patients. A study done by Auguet et al. found that liver LCN2 gene expression correlated with simple steatosis [5]. This genetic association explains the high predicted sensitivity of 96.9% and predicted specificity of 91.7% of lipocalin-2 in our study. It can be also added to composite scores of diagnosing NAFLD with other tests. Recently showed that hepatocyte-derived LCN2 plays a key role in protection against diet-induced NAFLD by regulating lipid metabolism, lipid peroxidation and apoptosis [16]. The rise in level of lipocalin may be due to body response against hepatic injury. Anastasia Asimakopoulou et al. suggested that LCN2 played a major role in the pathogenesis of fatty liver in NAFLD patients and levels of LCN2 were statistically on the higher side in NAFLD patients [17]. As studied by Milner et al., LCN2 levels were significantly on the higher side in NAFLD patients as compared to controls [18]. Another study done by Z et al. showed that circulating LCN2, produced by adipocytes, are elevated and may contribute to the development of NAFLD [19].

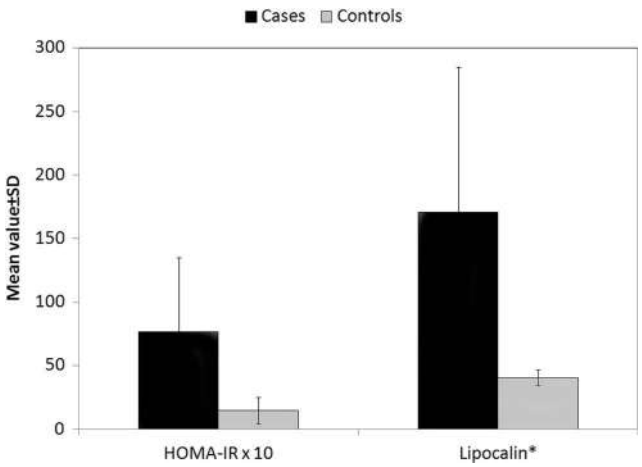

Fig. 2 Comparison of values of HOMA-IR and lipocalin levels in cases and controls

In our study, we have found a positive correlation between the lipocalin levels and severity of liver dysfunction as assessed by ultrasonographically defined grade of hepatic steatosis. This is in accordance with the study done by Tekkesin et al., which showed that urinary NGAL levels were higher in patients with steatosis compared to those with no steatosis (49.8 ng/mL and 22.7 ng/mL, respectively) with a statistically significant difference ($p<0.001$) [15].

Injured hepatocytes have been identified as the main producers of LCN2 in the liver. Inflammatory cytokines but not profibrotic factors induce hepatocyte LCN2. LCN2 is, therefore, an indicator of liver inflammation and its function is to limit tissue damage. As liver biopsy remains the standard for diagnosing NAFLD, the use of adipokines as the non-invasive diagnostic test is of interest. From this and other recent animal and human studies on adipose tissue contributions to the pathogenesis of hepatic steatosis and steatohepatitis, it is suggested that elevated LCN2 levels suggest hepatic injury in NAFLD patients. With our study, we have opened new realms in a study of NAFLD patients with non-invasive biomarkers by which we can correlate the severity of disease in the patient with LCN2 and avoid invasive procedures like a liver biopsy. The strength of our study is that we have compared lipocalin levels between cases as well as healthy controls and also compared these parameters with different grades of hepatic steatosis and parameters of metabolic syndrome. The limitation of our study is we used only ultrasonography and not biopsy which is invasive technique associated with some morbidity. We preferred ultrasonography because as per meta-analysis of 49 studies by Ruben et al., ultrasonography allows for reliable and accurate detection of moderate-severe fatty liver, compared to histology [20]. The sensitivity and specificity of ultrasound were similar to that of other imaging techniques, i.e. computed tomography or magnetic resonance imaging [20]. Due to changing physiology, individuals are moving towards metabolic syndrome easily and need frequent monitoring, preventive check-ups and lifestyle changes to prevent complications [21]. Lipocalin-2 measurement may be added to screening as well as preventive check-up and other biochemical tests. In the future, larger studies are needed to further establish the role of lipocalin-2 in NAFLD.

NAFLD, defined as an extensive accumulation of fat in the liver, is a fast-growing form of new world's disease caused by

excess calorie intake and a sedentary lifestyle. Since NAFLD seems to be associated with classical indicators of metabolic syndrome (e.g. insulin resistance, type 2 diabetes and dyslipidemia), it is no wonder that LCN2 overexpression was lately defined as a hepatic manifestation of a metabolic syndrome.

As mentioned above, major sources of NAFLD pathogenesis seem to be glucose and fructose lipogenesis-derived FFA. However, a series of recent studies have indicated that sugars can induce NAFLD by means independent of de novo lipogenesis. A study conducted in our group showed how excess fructose leads to hepatic steatosis. However, in this context, fructose appears to directly affect liver homeostasis, thereby manipulating fat metabolism [22]. Fructose might disturb liver homeostasis by promoting lipid uptake into the liver, while LCN2 counteracts lipid uptake. The same study showed there are potential differences between the sexes in LCN2-mediated lipid metabolism. This finding is of the utmost importance because it also shows a potential influence of estrogens on LCN2-mediated lipid homeostasis. This goes in agreement with another study made by Alwash and colleagues who fed rats a fructose high diet that provoked a gradual increase of the LCN2 level over the course of 8 weeks [23].

Conclusion

From this study, it is concluded that lipocalin-2 is a good blood biomarker for detection of hepatic steatosis. The lipocalin levels increase with increasing severity of steatosis. In addition to this, lipocalin will be useful for early diagnosis of NAFLD and convenient to do with other blood tests. The early intervention may prevent high morbidity and mortality and to manage the disease properly.

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Author contribution Vivek Bhosale, Hirdesh Chawla—concept, design, literature search, data collection and analysis, clinical studies, experimental studies, manuscript writing

Ravi Misra, Satyendra Kumar Sonkar, Ashim Ghatak—concept, design, manuscript review

Mahendra Pal Singh Negi, Kavita Durgapal, Shail Singh, Shobhit Vimal, Banwari Dangi—bioanalysis, data analysis

Neera Kohli, Naseem Jamal—radiodiagnosis, ultrasonography data acquisition

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Data availability Data will be available upon request from the corresponding author.

Declarations

Ethics approval and consent to participate Ethical clearance was obtained from the institutional review board (IRB) of the King George Medical University, Lucknow, India. Purpose and significance of the study were explained, and informed consent was taken from each study participant. Respondent's confidentiality was ensured during the study period.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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SHORT ARTICLE

Correlation between insulin resistance score and daily total insulin dosage in patient with type 1 diabetes mellitus: a pilot study

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Abstract

Purpose of the study Although insulin resistance is the pathogenic basis of type 2 diabetes mellitus (T2DM), it can also affect patients with type 1 diabetes mellitus (T1DM). In this clinical study, we investigated the relationship between insulin resistance grade and daily insulin dosage in order to clarify whether the approach to improving insulin resistance along with insulin therapy should be considered to treat T1DM and T2DM.

Methods As the means of insulin resistance estimation, we selected the insulin resistance score because patients use insulin therapy and homeostatic model assessment insulin resistance (HOMA-IR) is not appropriate for those patients. The insulin resistance score was calculated as 24.31 – (12.22 × WHR) – (3.29 × HT) – (0.57 × HbA1c), where WHR is the waist-to-hip ratio, HT is hypertension, and HbA1c is glycated hemoglobin (%).

Results The insulin resistance score was negatively correlated with the body mass index (BMI; $r = –0.511$) and the WHR ($r = –0.773$). The total insulin dosage was positively correlated with the BMI ($r = 0.734$) but negatively correlated with the insulin resistance score ($r = –0.540$).

Conclusion Insulin resistance estimation is necessary for T1DM treatment, and the insulin resistance score is a useful tool for estimating insulin resistance in patients with T1DM accompanied with insulin resistance.

Keywords Insulin resistance · Insulin resistance score · Type 1 diabetes mellitus · Insulin therapy

Introduction

Type 1 diabetes mellitus (T1DM) results from primary loss of β-cell mass due to complex autoimmune processes with consecutive insulin deficiency. For long the presence of insulin resistance in T1DM has been unclear. However, recent clinical and experimental evidence suggests that insulin resistance can indeed be present in T1DM [1]. In this clinical study, we studied the relationship between insulin resistance (IR) grade and daily insulin dosage in order to clarify whether the approach to improving IR along with insulin therapy should be considered to treat T1DM.

Materials and methods

Subjects

The study protocol used was reviewed and approved by the review boards of Hidaka Hospital (Takasaki City, Gunma

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Prefecture, Japan; #335) and the Saku Central Hospital Advanced Care Center (Saku City, Nagano Prefecture, Japan; R201811-03) in accordance with the Declaration of Helsinki.

In this two-center retrospective study, we used the electronic records of 32 patients who visited Hidaka Hospital and the Saku Central Hospital Advanced Care Center every month between 2020 and 2021 for T1DM treatment.

The exclusion criteria were as follows: patients treated with glucocorticoids, those who were anemic (hematocrit < 39% in men and < 36% in women), and those with compromised glucose levels (such as pregnant women). In addition, patients with cancer were also excluded.

T1DM was diagnosed if there was history of ketoacidosis or fasting C-peptide <0.3 PMol/mL and stimulated C-peptide <0.6 PMol/mL or if insulin treatment was required from the time of diagnosis [2].

Blood examination

Venous blood samples were collected into tubes containing EDTA and fluoride. Plasma was separated from whole blood within 1 h after collection. The casual plasma glucose (PG) and glycated hemoglobin (HbA1c; %) were measured according to the hexokinase method using a Synchro CX4/CX5 glucose analyzer (Beckman Coulter Inc., Fullerton, CA, USA) and Glycohemoglobin Analyzer RC20 (Sekisui Medical Co., Ltd, Tokyo, Japan), respectively. Both intra- and inter-assay coefficients of variation were ≤ 2% at PG values of < 126 mg/dL.

Estimation of the IR score

The IR score (estimated glucose disposal rate (eGDR), a validated, inverse measure of insulin resistance derived from hyperinsulinemic-euglycemic clamp studies) was calculated as 24.395 – (12.971 × WHR) – (3.388 × HT) – (0.601 × HbA1c), where WHR is the waist-to-hip ratio and HT is hypertension [3]. Hypertension is blood pressure ≥140/90 mmHg or use of blood pressure lowering medication (0= no, 1 = yes) [3]. The IR score was presented in milligrams per kilogram per minute [3].

Statistical analysis

All statistical data were analyzed using SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA). To estimate the linear correlation between variables, we calculated Pearson's correlation coefficient.

Results

Characteristics of subjects at baseline measurements

Table 1 presents the patients' characteristics at baseline measurements. The median duration with T1DM was 20 years (range ~ 5–42). The patients' median age was 59 (range ~ 29–85) years. The median body height (BH) was 161.2 (range ~ 151.6–175.0) cm, the median body weight (BW) was 57.1 (range ~ 45.9–123.0) kg, and the median body mass index (BMI) was 23.4 (range ~ 17.9–40.2) kg/m^2. The median estimated glomerular filtration rate (eGFR) of the patients was 68.0 (range ~ 32.0–128.0) mL/min/1.73 m^2. The median systolic blood pressure (SBP) was 124.0 (range ~ 92–198) mmHg, the median diastolic blood pressure (DBP) was 69.0 (range ~ 47–95) mmHg, and the median HbA1c was 7.65 (range ~ 5.5–12.7) %. The median total insulin dosage was

Table 1 Patient characteristics

	Median	Min.	Max.
Duration with T1DM (year)	20	5	42
Age (years old)	59	29	85
BH (cm)	161.2	151.6	175
BW (kg)	57.1	45.9	123
BMI (kg/cm^2)	23.4	17.9	40.2
WHR	0.91	0.68	1.04
SBP (mmHg)	124	92	198
DBP (mmHg)	69	47	95
GOT (IU/L)	20	9	33
GPT (IU/L)	18	7	23
SCr (mg/dL)	0.74	0.53	1.46
eGFR (mL/Min/1.73m^2)	68	32	128
HDL (mg/dL)	59.5	30	103
LDL (mg/dL)	103	60	146
TG (mg/dL)	110	51	326
PG (mg/dL)	167	77	375
HbA1c (%)	7.65	5.5	12.7
Total insulin dosage (unit/day)	28	8.4	99
IR score (mg/kg/min)	6.09	1.85	11.78
Sex (F/M)	16/16		
Antihyperlipidemic drugs (Y/N)	8/32		
Antihypertensive drugs (Y/N)	17/32		

The characteristics of the participants at baseline measurements were summarized

BH, body height; *BW*, body weight; *BMI*, body mass index; *WHR*, waist-to-hip ratio; *SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *GOT*, glutamic oxaloacetic transaminase; *GPT*, glutamic pyruvic transaminase; *SCr*, serum creatine; *eGFR*, estimated glomerular filtration rate; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *TG*, triglyceride; *PG*, plasma glucose; *HbA1c*, glycated hemoglobin

28.0 (range ~ 8.4–99) units/day, and the median IR score was 6.09 (range ~ 1.85–11.78) mg/kg/min.

Relationship between IR score and BMI, and WHR in patients with T1DM

Figure 1 illustrates the regression coefficients of the univariate linear regression between the IR score and the BMI (Fig. 1A) and the WHR (Fig. 1B). The IR score was negatively correlated with the BMI (r = –0.511) and the WHR (r = –0.773).

Relationship between total insulin dosage and BMI, and IR score in patients with T1DM

Figure 2 illustrates the regression coefficients of the univariate linear regression between the total insulin dosage and the BMI (Fig. 2A) and the IR score (Fig. 2B). The total insulin dosage was positively correlated with the BMI (r = 0.734) but negatively correlated with the IR score (r = –0.540).

Discussion

The euglycemic-hyperinsulinemic clamp is a gold standard method to estimate insulin resistance [4]. However, the euglycemic-hyperinsulinemic clamp is a costly and invasive procedure, and sample size is limited [4]. Therefore, based on the clinical characteristics of hypertension, WHR, triglyceride and HDL cholesterol levels, family history of type 2 diabetes, and glycemic control, an IR score was developed and validated using the euglycemic-hyperinsulinemic clamp in a subset (n = 24) of the Pittsburgh Epidemiology of Diabetes Complications (EDC) population [4]. The results from the IR score and estimated glucose disposal rate (GDR) are remarkably consistent [4]. Alternatively, the frequently sampled intravenous glucose tolerance test could have been used to assess insulin resistance [5]. In type 1 diabetes, this testing would have also required an overnight hospital admission for discontinuation of long-acting insulin and stabilization of glucose levels, a practice that could also limit sample size. Thus, the IR score is a validated clinical tool for estimating insulin sensitivity in T1DM [3, 4].

Several studies have assessed IR score cutoffs. Tam et al. found that patients with an IR score of < 5.6 mg/kg/min are insulin resistant [6]. Epstein et al. found that most patients with IR have an IR score of < 5.39 mg/kg/min [7]. Šimonienė et al. estimated that the IR score cutoff that reflects IR is < 6.4 mg/kg/min [8]. In this study, the median IR score was 6.09 (range ~1.85–11.78), suggesting that some of the patients had IR.

Obesity is related to IR. Low-grade inflammatory cells (e.g., adiponectins, tumor necrosis factor) in adipose tissue cause IR [9]. Although the frequency of T1DM with obesity is rare compared to that of T2DM with obesity, obesity in T1DM has become a clinical problem [10, 11]. Obesity in T1DM might be explained, in part, by intensive insulin therapy, which causes insulin-induced IR. The distribution of the BMI in our study was ~17.9–40.2 kg/m^2, suggesting that some of the patients were obese. Importantly, we found a strong negative correlation between the IR score and the BMI (Fig. 1A) and the WHR (Fig. 1B). Thus, consistent with previous studies [6–8], the IR score showed a good negative correlation with representative indicators of obesity, suggesting that the IR score is a good indicator of IR and is affected by obesity.

We discovered that the daily total insulin dosage is significantly positively correlated with the BMI (Fig. 2A) and significantly negatively correlated with the IR score (Fig. 2B). Thus, overweight patients tend to be prescribed more insulin, and patients with severe IR also tend to use more insulin. Over-insulinization induces overweight [9, 10], and there seems to be a vicious circle between overweight and over-insulinization, which can be avoided with appropriate insulin dosage. As our current data indicated that the IR score is significantly negatively correlated with the daily total insulin dosage, a reduction in IR is required for treating patients with T1DM. This approach will increase the treatment efficacy of insulin therapy and reduce the risk of hypoglycemia and inappropriate weight gain. In addition, conventional risk factors

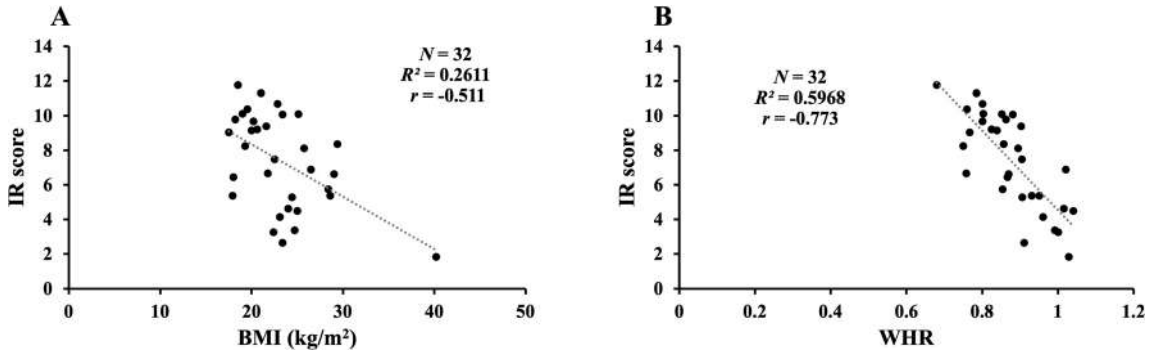

Fig. 1 Correlation between the IR score and the BMI and the WHR in patients with T1DM. The regression coefficients of the univariate linear regression between the IR score demonstrated a negative correlation with **A** the BMI (r = –0.511) and **B** the WHR (r = – 0.773). T1DM, type 1 diabetes mellitus; IR score, insulin resistance score; BMI, body mass index; WHR, waist-to-hip ratio

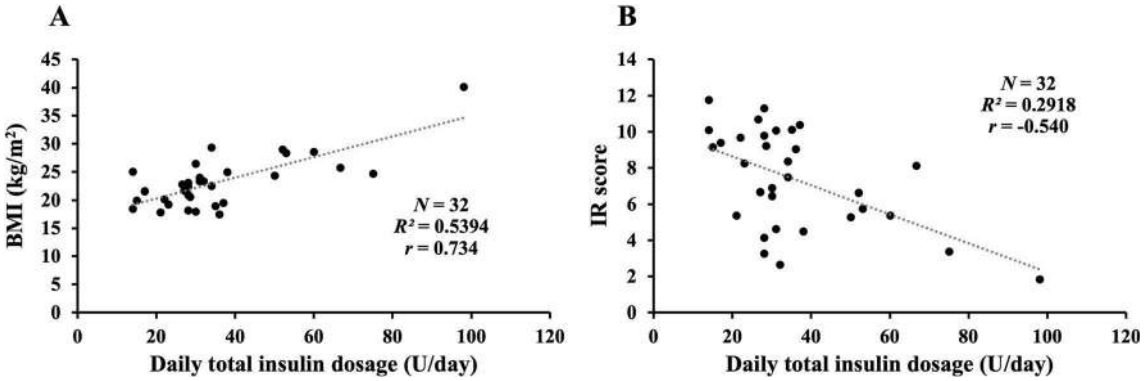

Fig. 2 Correlation between the total insulin dosage and the BMI and the IR score in patients with T1DM. The regression coefficients of the univariate linear regression between the total insulin dosage demonstrated **A** a positive correlation with the BMI (r = 0.734) and **B** a negative correlation with the IR score (r = –0.540). T1DM, type 1 diabetes mellitus; BMI, body mass index; IR score, insulin resistance score

(e.g., HT, overweight, dyslipidemia) generally predict adverse cardiovascular disease (CVD) outcomes, and the IR score or IR is the strongest independent factor for CVD [12]. Therefore, the estimation of IR is important and the IR score is a validated clinical tool for patients with T1DM.

How do we improve IR in patients with T1DM? Numerous small trials on people with T1DM have evaluated metformin, with the hope that its insulin-sensitizing properties would improve glycemic management or reduce CVD risk [13, 14]. The largest study to date assessed the use of 1 g of metformin, twice daily, in 428 patients with T1DM who were treated for 3 years, with the primary end point being changes in the mean carotid intima–media thickness, a marker of CVD risk. The study ultimately found no difference in the primary end point, minimal and nonsustained effects on HbA1c, minimal effects on weight (~1-kg reduction), and no change in the total daily insulin dose [15]. Thus, we need to focus on appropriate diet therapy and physical exercise to avoid obesity and improve IR. In addition, new medicine to improve IR in patients with TIDM needs to be developed.

The limitation of this study was the relatively small sample size, which could decrease the probability to detect real differences between groups.

Conclusion

IR estimation is necessary for T1DM treatment, and the IR score is a useful tool for estimating IR in patients with T1DM accompanied with IR.

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Author contribution TW and SO collected the data. EY, KO (Okada), KK, KO (Ohshima), and SO analyzed the data. JO and SO prepared the manuscript.

Data availability The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval The ethics committees at Hidaka Hospital and Saku Central Hospital Advanced Care Center approved our study, which conformed to the Declaration of Helsinki (as #355 and R201811-03, respectively).

Informed consent All patients provided written informed consent to analyze and present their clinical laboratory data.

Consent for publication All of the authors have also agreed to submit our manuscript to your journal.

Conflict of interest The authors declare no competing interests.

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LETTER TO THE EDITOR

Persistence of new-onset diabetes in the post-acute phase of COVID-19

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Keywords COVID-19 · new-onset diabetes · diabetes · long-covid

To the Editor,

The article by Madhu [1] discusses the challenges in providing post-COVID-19 diabetes care. In addition to these challenges, we like to point out that the persistence of new-onset diabetes (NOD) after recovery from COVID-19 is a crucial challenge. A meta-analysis of 8 studies with 3711 COVID-19 patients (492 NOD cases) showed that 14% of hospitalized COVID-19 patients had NOD during the acute phase of the illness [2]. Since studies included in this meta-analysis were conducted in mid-2020, it was not known whether NOD is transient or if it would persist after recovery. Given the pandemic is ongoing for more than 2 years, reports on the long-term effects of COVID-19 are emerging.

In a study by Farag et al. [3], of 570 COVID-19 patients (mean age: 47.9 years) admitted to two hospitals in Egypt, 65 (11.4%) were diagnosed with NOD (fasting plasma glucose [FPG] ≥126 mg/dl or RBG ≥200 mg/dl and HbA1c <6.5%). Among the 54 NOD survivors, diabetes persisted in 37 (68.5%) patients after 3 months. In a study by Montefusco et al. [4], of 551 patients (mean age: 61 years) hospitalized for COVID-19 in a single center in Italy, 65 (11.8%) had NOD (definition not given) during their in-hospital stay. Diabetes persisted in approximately 2% of NOD patients at 6 months. In a more recent study by Cromer et al. [5], of 1902 COVID-19 patients (median age: 59.1 years) admitted to a single tertiary care hospital in Boston, 77 (13.0%) were diagnosed with NOD. NOD was defined if the patient had no prior history of diabetes (based on self-reports or clinical notes), no HbA1c values ≥6.5%, no random blood glucose [RBG] values >200 mg/dl, and had never taken non-metformin diabetes medications. More than half (56.3%) of the 64 survivors with NOD continued to have diabetes (HbA1c ≥6.5%, use of diabetes medications, or diabetes diagnosis in clinical notes) at a median follow-up of 323 days.

Potential mechanisms contributing to NOD in the post-acute phase may include virus-induced ß-cell cytotoxicity, insulin resistance, and dysregulation of the immune and renin-angiotensin systems [2]. The persistence of NOD in the post-acute phase of COVID-19 is alarming, as this might put enormous burden on individuals and health systems in terms of increased morbidity and cost of care, particularly in developing countries. Therefore, clinicians should be aware of this emerging challenge and must be prepared to tackle this as part of the post-COVID-19 management strategies.

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Declarations

Competing interests The authors declare no competing interests.

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CASE REPORT

Brentuximab vedotin-associated diabetic ketoacidosis: a case report

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Abstract

Background Diabetic ketoacidosis (DKA) is a life-threatening complication of diabetes mellitus (DM). It is characterized by hyperglycemia, metabolic acidosis, and ketonemia. Fortunately, drug-induced hyperglycemias are usually mild and not life-threatening. However, rarely some cases may present with ketoacidosis. In this case report, we aimed to present a brentuximab vedotin (BV) associated with DKA.

Case presentation A 23-year-old Caucasian man presented with abdominal pain, nausea, and vomiting for 1–2 weeks. The patient had a previous diagnosis of Hodgkin's lymphoma and primer hypothyroidism. He is using levothyroxine 150 μg per day and received BV treatment for Hodgkin lymphoma (HL) 10 days ago. No steroid treatment was administered for premedication before BV. Except for obesity, all system examinations are normal. There were no signs of any infection. Laboratory data revealed hyperglycemia, metabolic acidosis, and ketonemia. The patient was admitted to the service with a diagnosis of DKA. After the patient was admitted to our clinic, insulin treatment and hydration started immediately. Despite the insulin infusion reaching 1700 units per day, the patient's diabetic ketoacidosis extended to 1 week. Anti-insulin, anti-glutamic acid decarboxylase, and islet cell autoantibodies were negative, which were checked to exclude type 1 DM. Fasting C-peptide was 28 ng/mL (normal range, 0.9–7.1 ng/mL). With all these, the diabetic ketoacidosis status of the patient was evaluated as a BV side effect.

Conclusion This patient is a rare case of BV-associated DKA. It is very important to know this relationship since BV treatment has turned into a standard treatment for relapsed Hodgkin lymphoma. Our case highlights that this diagnosis should be kept in mind as a complication of each dose of BV administration.

Keywords Diabetic ketoacidosis · Brentuximab vedotin · Drug-induced hyperglycemia

Background

Diabetic ketoacidosis (DKA) is one of the most important emergencies of diabetes mellitus (DM). It is characterized by hyperglycemia, metabolic acidosis, and ketonemia. Although it is more common in type 1 DM, it can also be seen in all types of DM. DKA occurs due to absolute/relative insulin deficiency with or without increased counter-regulatory hormones and is often precipitated by external factors like acute major illnesses, trauma, dehydration or drugs [1].

Brentuximab vedotin (BV) is a drug used in Hodgkin lymphoma (HL) containing an anti-CD30 antibody linked to the anti-tubulin agent monomethyl auristatin [2]. Although generally well-tolerated, there are also dose-limiting side effects. Hyperglycemia is one of them, especially in high-dose therapy [3]. In this article, we presented a case of diabetic ketoacidosis after BV treatment.

Case report

A 23-year-old Caucasian man presented emergency room with abdominal pain, nausea, and vomiting. The pain was dull, continuous, and localized to the periumbilical region, without any bowel or bladder complaints. He had a previous diagnosis of HL for 4 years, primary hypothyroidism for ten years and obesity for 5 years. He was using levothyroxine 150 μg per day and received a fourth BV infusion for HL 10

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days ago. He had no history of alcohol and smoking. The patient was not working.

On a physical examination, his weight was 135 kg, height 1.78 m, and body mass index 42.7 kg/m^2. His vital signs were normal besides sinus tachycardia (194/min). There was no high fever and no focus of infection was detected. Throat, respiratory tract, and abdominal examinations were normal. The perianal region was normal. No acanthosis nigricans, lipodystrophy, or signs of cortisol excess existed. His skin and mouth were dry due to dehydration.

Laboratory tests showed hyperglycemia, acidosis, and ketonemia at emergency admission [glucose: 306 mg/dL (normal range, 74–106 mg/dL), ph: 7.25 (normal range, HCO_3: 14.9 mmol/L (normal range, 22–26 mmol/L), serum ketone level: 6.2 mmoL/L (normal range, 0.02–0.27 mmol/L)]. He internalized to the endocrine clinic with a DKA diagnosis. Laboratory tests performed in our clinic are in Table 1. There was no pathology on the chest X-ray and the COVID-19 polymerase chain reaction was negative.

In the detailed history, the patient did not have a covid infection and had the COVID-19 mRNA vaccine 6 months ago. He has no history of DKA and HHS but he had hyperglycemia 2 years ago after his first dose of BV therapy. The patient's hyperglycemia at that time had started at a similar timing to now, 1 week after BV treatment. In the patient with polyuria polydipsia, HbA1c: 6.8% and fasting blood glucose: 206 mg/dl were found, respectively. The ketone was not detected in the complete urinalysis. Linagliptin was prescribed to the patient with a DM diagnosis whose general condition was good. Before 1 month of the first BV treatment, the patient's glycemic course was normal and Hba1c was 5.3%. The patient was also obese and had a body mass index of 42.7 at that time. The patient who used linagliptin for 1 month was normoglycemic during home follow-ups, so the drug was discontinued and HbA1c was detected at 5.3%, 3 months later (Fig. 1).

Table 1 Laboratory results

Laboratory tests	Results	Reference range
Hgb (g/dL)	15.3	12.5–16.3
WBC (×10^3/μL)	6.22	3.6–10.2
Plt (x10^3 /μL)	243	152–348
Glucose (mg/dL)	450	74–106
HbA1c (%)	8.0	< 6.5
Creatinine (mg/dL)	0.8	0.67–1.17
ALT (U/L)	16	< 33
Albumin (g/dL)	3,7	3.5–5.2
Potassium (mmol/L)	4.2	3.5–5.1
Sodium (mmol/L)	133	136–145
TSH (mIU/L)	2,2	0.27–4.2
CRP (mg/L)	84	< 0.5
Insulin (μIU/mL)	668	2.6–24.9
C-peptide (ng/mL)	28	0.9-7.1
pH	7.25	7.35–7.45
HCO_3 (mmol/L)	16	22–26
pCO_2 (mmHg)	20	35–45
Anion gap (mmol/L)	17	8–12
Serum ketone (mmol/L)	6.9	0.02–0.27
Anti-Insulin (U/mL)	1.12	< 10
Anti-GAD (IU/mL)	Negative	
Islet cell antibodies (IU/mL)	Negative	

The treatment of DKA was initiated as, insulin infusion (0.15 IU/kg/hour), 0.9% NaCl (1 L/h) therapy, and potassium replacement (20 mEq each liter of iv fluid) immediately. The daily insulin need of the patient, whose DKA did not improve despite 1 week of appropriate treatment, reached 1700 units/per 24 h. DKA resolved on the 8th day of hospitalization and insulin infusion continued for three more days due to the intense insulin need. When DKA resolved, metformin was added to the treatment. After the addition of metformin, the patient's insulin requirement gradually decreased (Fig. 2). After insulin infusion stopped, basal-bolus insulin therapy started at 0.5 IU/kg/day. Euglycemia was achieved with dose titration. The patient was discharged on the 12th hospital day. The patient was discharged with basal-bolus insulin therapy. The dose requirement gradually decreased, and after 2 months, insulin and metformin treatment were completely discontinued. At the end of 3 months without treatment, the patient's current Hba1c is 5.3%. He continues to be followed up.

Discussion

Despite the widespread use of BV and its use as a first-line treatment for relapsed HL, the reported case of BV-associated DKA is extremely rare. Awareness of the hyperglycemia side effect of BV would be effective in early diagnosis and reduction of mortality, especially for DKA.

BV is an antibody-drug conjugate directed against the CD-30 antigen expressed on classical HL and anaplastic large cell lymphoma. It is leading to subsequent internalization of the anti-tubulin agent and cell death [4]. In the BV phase I clinical trial, treatment doses ranged from 0.1 to 3.6 mg/kg. With these doses, the most common adverse effects were fatigue (36%), fever (33%), nausea (22%), neutropenia (22%), and peripheral neuropathy (22%), followed by headache, vomiting, back pain, anemia, and alopecia. One patient had grade 3 hyperglycemia at a dose of 2.7 milligrams per kilogram, while patients treated with 1.8 mg/kg did not [5]. In our case, the patient's treatment dose was 1.8 mg/kg. Perhaps this dose was as much

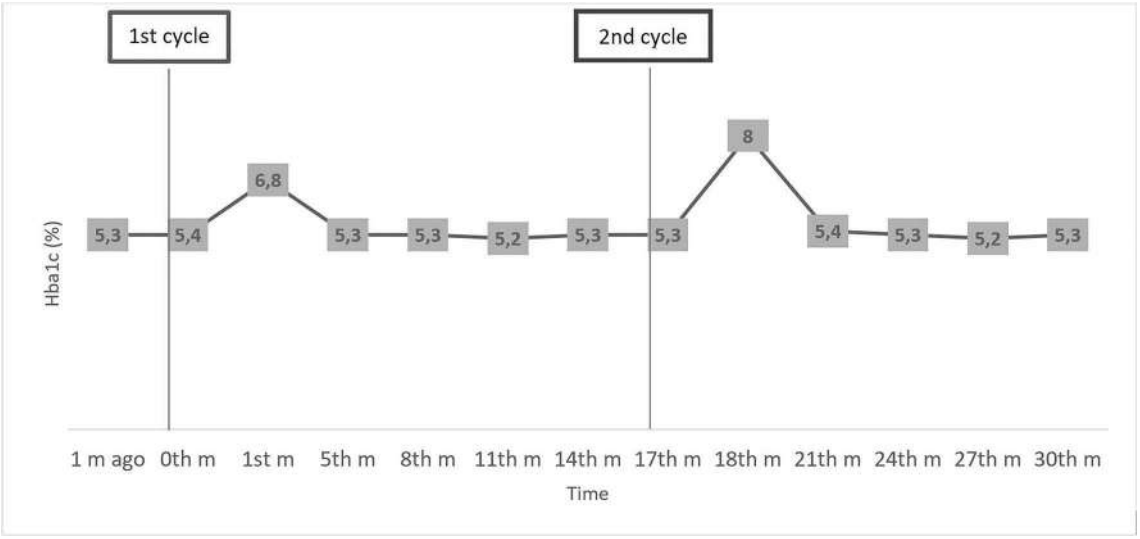

Fig. 1 Normoglycemic cycle

as the maximum total dose given in the 2.7 mg/kg treatment arm in the phase 1 study due to patient obesity. A search of the U.S. Food and Drug Administration Adverse Events Reporting System showed 58 reported reactions of hyperglycemia with resulted in 10 patient death and 20 DKA with 6 death [6].

Drug-induced hyperglycemia is usually mild and reversible. Glucocorticoids, somatostatin analogs, diuretics, statins, and antipsychotics are well-known diabetogenic drugs [7, 8]. However, with the development of new immunotherapy agents and chemotherapy regimens recently, many new diabetogenic drugs have also been defined. Drugs may cause hyperglycemia with a variety of mechanisms, including alterations in insulin secretion and sensitivity, direct cytotoxic effects on pancreatic beta cells, and increases in glucose production [7]. We suspected DKA with severe insulin resistance due to diminution of peripheral insulin sensitivity in our case. He had hyperinsulinemia in his biochemical test done before insulin infusion treatment and also insulin requirement decreased rapidly after initiation of metformin therapy,

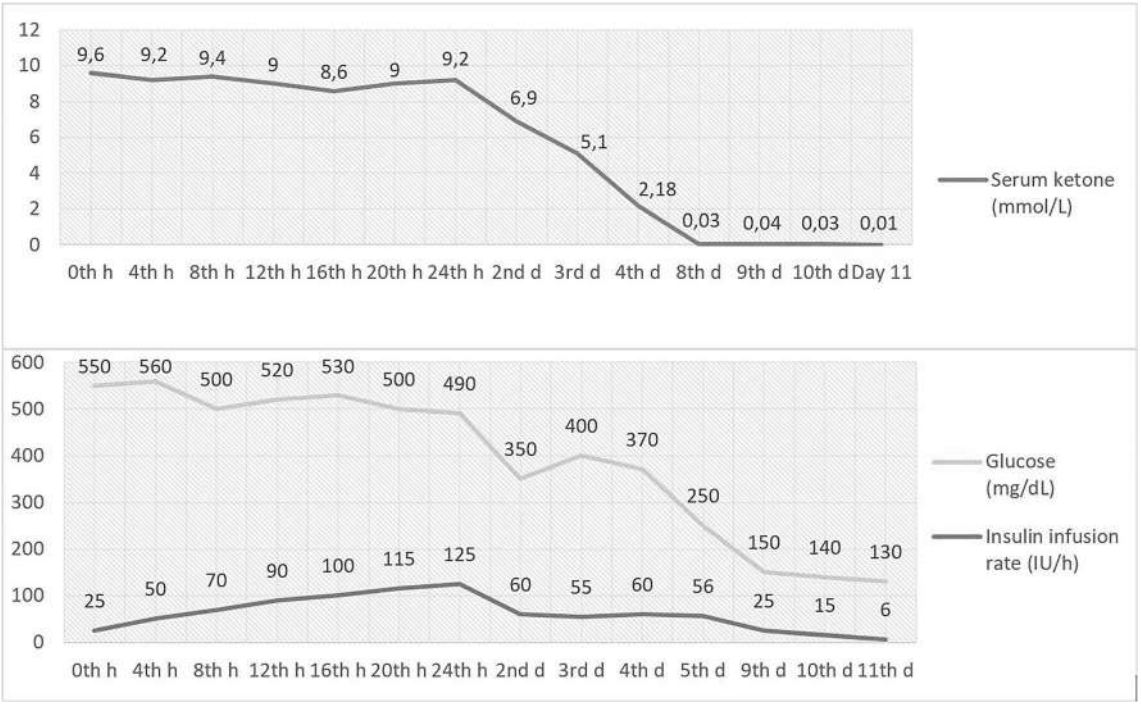

Fig. 2 Insulin requirement

suggesting insulin resistance. There was no finding suggestive of pancreatitis. Like many diabetogenic drugs, BV also causes temporary DM. Our patient had hyperglycemia after his first BV therapy 2 years ago and used linagliptin for 1 month. His weight has been stable for the last 6 years and he did not have DM before that despite obesity and metabolic syndrome.

Apart from our case, there is only one case report related to DKA after BV treatment. That patient also had human immunodeficiency virus infection and he died due to multiple organ failures. In that case, after the first dose of BV administration, the patient had a systemic cytokine release with extreme insulin resistance. Although IV insulin was titrated to > 600 units/h, blood glucose regulation could not be achieved [9]. The patient's diagnosis of AIDS and cytokine storm at presentation may have played a role in the etiology of diabetic ketoacidosis in that case. Because the patient died, it could not be predicted whether the diabetes mellitus would be temporary or permanent. Since we had the chance of long-term follow-up with our patient, we were able to document the hyperglycemic periods that developed after both cycles and that these conditions were temporary, in addition to those presented in this case. No hyperglycemia was detected in another case report in which cytokines storm was described with BV [10]. The first case has class 2 obesity as in our case [9]. However, we do not know about the body mass index of another case[10]. The resulting IL-6 was 17.7 pg/mL (normal < 5.9 pg/mL) and there was no cytokine storm clinic in our patient. Although there are cases of type 1 DM reported in drug-induced hyperglycemias, we excluded this with negative antibodies and sufficient C-peptide levels in our patient. There is no reported type 1 DM associated with BV.

We think that the condition is related to brentuximab, as our patient developed hyperglycemia after both treatment cycles and regressed within a few months after the treatment. However, while the hyperglycemic state that occurred the first time was milder, we could not explain his application with diabetic ketoacidosis the second time. In both cases, the patient did not have an infection or systemic comorbidity. His weight and total treatment doses were the same in both treatments. Maybe this diabetic process was also related to an antibody that we do not know about and caused a more severe response in the body of the sensitized individual in the 2nd cycle.

Finally, BV is a drug that is increasingly used in hematologic malignancies today. Although the side effects are mostly tolerable, we should also be aware of life-threatening situations like DKA.

Conclusion

DKA due to BV therapy is a very rare condition. However, these cases may increase relatively due to the frequent use of BV. Since this situation may be temporary, it should be considered in the etiology of DM. These patients should be followed closely and should be reevaluated after BV therapy. Currently, treatment is the same as standard DM and DKA besides severe insulin resistance and high insulin need. However, as molecular studies are carried out to define the hyperglycemia mechanism, specific treatments for the mechanism can be developed.

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Author contribution DK, MS, and AS performed concept, design, and data acquisition. DK, MS, and EG performed clinical studies, literature search, and manuscript review. DK, MS, BÇ, and ZC performed manuscript preparation, and manuscript editing. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication A written consent was signed by the patient for publication.

Competing interests The authors declare no competing interests.

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ORIGINAL ARTICLE

An increased disulfide/native thiol ratio and oxidative stress index in metabolic syndrome patients with postprandial lipemia

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Abstract

Background Metabolic syndrome (MetS) is closely related to lipid disorders and increased oxidant stress, and is associated with cardiovascular diseases.

Objective The purpose of this research was to examine thiol/disulfide homeostasis and oxidative stress in MetS patients with postprandial lipemia (PPL) during fasting by considering time-dependent changes in the postprandial period.

Methods Twenty-five patients with MetS and 25 healthy controls underwent a 6-h oral fat tolerance test. Dynamic thiol/disulfide homeostasis (native thiol, disulfide, and total thiol) values and total oxidant status (TOS), total antioxidant status (TAS), and Oxidative Stress Index (OSI): (TOS/TAS) were evaluated.

Results Increased levels of disulfide, and higher disulfide/native thiol ratio, TOS, and OSI values were observed at fasting and in the postprandial period in MetS compared to the control group, peaking at the 4th hour in both groups ($p < 0.05$). ROC analysis showed that both fasting and 4th hour disulfide/native thiol ratios exhibited the highest values. Higher disulfide/native thiol ratio values were observed at the 4th hour and higher OSI in the 2nd hour in the upper tertiles for MetS ($p < 0.05$).

Conclusions An increased disulfide/native thiol ratio and OSI level elevation in MetS may be closely associated with PPL. The disulfide/native thiol ratio in MetS subjects with PPL may play a role for evaluating oxidative stress, especially in postprandial 4th hour.

Keywords Metabolic syndrome · Postprandial lipemia · Thiol/disulfide homeostasis · Oxidative stress

Introduction

Metabolic syndrome (MetS) is a degenerative disease accompanied by abdominal obesity, blood lipid disorders, inflammation, insulin resistance or full-blown diabetes, and cardiovascular diseases [1].

Postprandial triglyceride (TG) concentrations have been identified as a clinically significant and independent predictor of the risk of cardiovascular disease. Postprandial lipemia (PPL) refers to elevated and prolonged changes in plasma TG levels, > 2.5 mmol/L (220 mg/dL) [2] at any time in the postprandial period, after a fatty meal [3]. Depending on the frequency of eating, spending a large part of the day in a state of satiety causes an increase in atherogenic lipoprotein particles such as remnants [4]. PPL is therefore regarded as an independent risk factor for the development of cardiovascular diseases and atherosclerosis [5, 6]. In addition, the consumption of large amounts of food, oxidative stress induced by

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hyperlipidemia, and/or hyperglycemia are directly related to PPL [7]. High TG levels, which are common in PPL, are thought to increase oxidative stress and inflammation in the postprandial period and are also associated with the development of atherosclerosis [8].

Oxidative stress refers to the state that occurs as a result of disruption of the balance between reactive oxygen and nitrogen species levels and the antioxidant defense system [9]. Oxidative stress is reported to play an important role in the pathophysiology of diabetes, cardiovascular diseases, MetS, age-related decreased immunity, as well as degenerative diseases of the nervous system [10].

Antioxidant defense systems reduce oxidative stress through both enzymatic and non-enzymatic mechanisms. One of these antioxidant molecules is thiols, which play a critical importance in preventing cellular oxidative stress [11, 12]. Thiols containing sulfhydryl (SH) groups are most commonly observed in plasma and other proteins [11]. Fewer thiol groups are seen in cysteine-containing molecules such as glutathione, cysteine, homocysteine, N-acetylcysteine, and gamma-glutamylcysteine. Reactive oxygen species (ROS), which form as a result of oxidative stress, cause damage to cells and tissues. Thiols react with free radicals to prevent ROS-mediated cell and tissue damage [13, 14]. Free radicals lead to oxidation of thiol groups of sulfur-containing amino acids. Disulfide bonds thus form as a result of this oxidation. Disulfide bonds can be converted to thiols by means of reduction. Dynamic thiol/disulfide homeostasis has critical functions as in the regulation of detoxification, antioxidant protection, enzyme activity, apoptosis, and intracellular signal transduction mechanisms. Thiol/disulfide balance is thus maintained in cells and tissues. It has therefore been suggested that thiol/disulfide homeostasis can be used to assess the organism's oxidant-antioxidant status [14].

Measurement of total antioxidant status (TAS) represents a valuable tool in the diagnosis and treatment of conditions such as cardiovascular diseases and diabetes mellitus [15]. In addition, total oxidant status (TOS) levels are used as one of the biomarkers for monitoring oxidative stress in humans. Similarly, the Oxidative Stress Index (OSI) (TOS/TAS ratio) described by Erel is a widely recognized parameter for evaluating the body's general oxidation state [16].

Although several studies have evaluated oxidative stress in individuals with MetS [17–19], no previous research has investigated thiol/disulfide homeostasis as an important component of oxidant-antioxidant status in MetS patients with PPL by considering time-dependent changes in the postprandial period. The purpose of this study was thus to investigate thiol/disulfide homeostasis together with classic oxidant-antioxidant parameters including TAS, TOS, and OSI in MetS patients with PPL and in a control group in the fasting and postprandial periods after the oral fat tolerance test (OFTT).

Materials and methods

Subject recruitment

Fifty individuals, 25 patients (13 female, 12 male) fulfilling the American Heart Association criteria for MetS and 25 clinically healthy controls (13 female, 12 male) paired by age and gender, were randomly enrolled. All participants were recruited from Karadeniz Technical University Faculty of Medicine Cardiology outpatient clinic. MetS inclusion criteria were waist circumference exceeding 102 cm/40 in. (men) or 88 cm/35 in. (women), blood pressure exceeding 130/85 mmHg, fasting serum TG level exceeding 1.69 mmol/L (150 mg/dL), fasting HDL-cholesterol less than 1.03 mmol/L (40 mg/dL) (men) or 1.29 mmol/L (50 mg/dL) (women), and fasting glucose exceeding 5.55 mmol/L (100 mg/dL) [20, 21].

Participants' health status was assessed through detailed medical history, lifestyle factors, eating habits, medication, treatment, physical examination, complete blood cell count (CBC), and lipids, lipoproteins, and diabetes, thyroid function (TSH, free-T4), liver (ALT, AST), and kidney function (BUN, creatinine) tests.

The participants completed a questionnaire concerning medical history, lifestyle factors, eating habits, medication, and treatment.

Exclusion criteria were use of cholesterol, lipid, or lipoprotein concentration–lowering drugs, alcohol or drug use, smoking, pregnancy, recent history of acute illness, chronic liver disease, thyroid disease, or acute-chronic kidney failure, history of pancreatic disease, presence of digestive-absorption disorder or any inflammatory condition, recent history of acute illness, heavy exercise, or use of any dietary supplement.

Anthropometric measurements

Anthropometric measurements were recorded before the test for each participant attending the clinical center. Anthropometric data (weight, height, waist circumference) and systemic and diastolic BP were measured. Prior to the OFTT, each participant filled out a form involving lifestyle factors (including nutrition), medications used, and medical history. In addition, body weights were measured using a digital scale in the morning following overnight fasting (Tanita WB-110MA, Japan). Waist circumferences were measured in the horizontal plane midway between the lowest rib and iliac crest. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Resting systolic and diastolic BP was measured using a standard mercury sphygmomanometer device (ERKA, Berlin, Germany).

Dietary intervention (oral fat tolerance test)

The study groups fasted for 12 h before OFTT administration. The participants were asked to refrain from long-term and strenuous physical activity the day before the test. Fasting blood samples were also collected and biochemical measurements were performed. Participants consumed the standardized OFTT meal described in our previous study [4, 6]. The meal contained a total of 75 g fat (62.5% fat, 24.1% carbohydrate, and 13.4% protein) and was consumed in 20 min, as recommended by the expert panel [2]. Participants consumed no food or drink, except for water, for 6 h. For biochemical testing, blood samples were collected before the meal (fasting) and 2, 4, and 6 h after the OFTT. These blood samples were collected using the OFTT recommended by the expert panel [2]. In the light of the information provided by the expert panel, patients with PPL testing positive for MetS (TG concentration > 2.5 mmol/L (220 mg/dL) at any time after the OFTT meal), and individuals testing negative in the case of the control group (TG concentration ≤ 2.5 mmol/L (220 mg/dL) at any time after the OFTT meal) were included.

As in our previous studies, the total (AUC) and increasing (iAUC) area under the curves of TG concentrations were calculated following the trapezoidal rule to assess the magnitude of change during fasting and at 2, 4, and 6 h after OFTT (postprandial state) [4, 6]. AUC and iAUC were expressed as mg * min * dL^{-1}.

Biochemical measurements

Venous blood samples were collected in the morning after at 12 h of fasting and all postprandial time points. All blood samples were centrifuged at 1800 g for 10 min, and the separated serum and plasma were kept frozen at −80 °C until assay. Glucose, TC, TG, LDL-C, and HDL-C were measured on an auto analyzer (AU 5800, Beckman Coulter, Shizuoka, Japan). Insulin levels were determined with an IMMULITE 2000 XPi analyzer (Siemens, Munich, Germany). The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using the following formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5 [22].

Measurement of thiol/disulfide homeostasis parameters

Serum thiol/disulfide homeostasis was measured using the newly developed automated spectrophotometric method previously described by Erel et al. [14]. Essentially, disulfide bonds were reduced to give rise to free functional thiol groups through the presence of sodium borohydride. The remaining reductant sodium borohydride was then consumed and eliminated using formaldehyde to prevent reduction of DTNB (5,5′-dithiobis-(2-nitrobenzoic) acid). Reduced or non-reduced thiol groups (native thiol groups) were determined in the light of the DTNB reaction. Half of the difference between the total thiols and native thiols yields the amount of dynamic disulfide present. Disulfide/native thiol percentage ratios ([SS] / [SH] × 100) were then calculated following the determination of native and total thiols [14].

Measurement of TAS, TOS, and OSI parameters

Serum TAS and TOS values were calculated using commercial colorimetric kits (Rel Assay Diagnostics, Gaziantep, Turkey) in line with the manufacturer's instructions. Results were expressed in μmol H_2O_2 equivalent/L and mmol trolox equivalent/L, respectively. The TOS/TAS ratio represented OSI and was calculated using the following formula OSI = [(TOS, μmol H_2O_2 equivalent/L)/(TAS, μmol Trolox equivalent/L)×100].

Statistical analysis

All statistical calculations were performed using an appropriate software package (SPSS 23.0 for Windows; SPSS Inc., Chicago, IL, USA). Normally distributed variables were expressed as mean ± standard deviation and non-normally distributed variables as median (interquartile range (IQR)). The distribution of variables was evaluated using the Kolmogorov-Smirnov test. Student's t test was applied in the comparison of two groups exhibiting normal distribution, or the Mann-Whitney U test in case of two groups with non-normal distribution. Each MetS group was categorized into 3 equal sub-groups based on tertiles of AUC values and was assessed using the Kruskal-Wallis test. Chi-square analysis was used to examine gender data between the groups. Analyses were performed on MedCalc Statistical Software version 19.1 (Medcalc software BVBA, Belgium), and results were evaluated using the receiver operating characteristic (ROC) curve analysis. Diagnostic accuracy for disulfide/natural thiol starvation, OSI starvation, and disulfide/natural thiol values at the 4^{th} hour and OSI levels at the 4^{th} hour were analyzed using AUC values yielded by the ROC analysis. Spearman's correlation coefficient was applied between numerical variables. The sample size was calculated using G*PowerSoftware 3.1 (Heinrich-Heine Universitat, Dusseldorf, Germany). Previously reported serum concentrations of disulfide/native thiol ratio in T1DM patients and healthy subjects were used to calculate the sample size requirements for this research [23]. To detect significant difference between the groups according to disulfide/native thiol ratio with a moderate effect size (Cohen's d = 0.869), the minimum required sample size was estimated as 22 for each group (α = 0.05, 1-β = 0.80, a sample size ratio = 1).

Results

The study population's anthropometric measurements, and biochemical and fasting oxidative stress data are given in Table 1. No significant difference was observed in terms of gender or age between MetS and the control groups ($p = 0.697$ and $p = 0.638$, respectively) (Table 1). However, the AUC and TG levels in the MetS group were almost twice as high as those in the control group. TG levels were significantly higher in the MetS group than in the control group at all postprandial time points, peaking at the 4th hour in both groups ($p = 0.0001$, respectively) (Table 1). Statistically significant differences were found between the control and MetS groups in terms of the other parameters. HOMA-IR was significantly higher in the MetS group than in the control group ($p = 0.0001$).

The oxidative stress variables were measured with patients fasting and also at 2, 4, and 6 h postprandially in both groups in order to identify baseline levels and time-dependent changes. The mean total thiol levels in the MetS group were significantly higher at 2, 4, and 6 h in the postprandial period than those in the control group ($p < 0.05$). Levels of native thiol were significantly lower during fasting and at 2 and 4 h in the postprandial period in the MetS group, while disulfide levels were significantly higher at each time point in the postprandial period ($p < 0.05$) (Table 2).

Table 1 Anthropometric measurements and biochemical variables in the study group

Parameter	Control	MetS	*p*
n (F/M)	25 (13/12)	25 (13/12)	0.697*
Age (years)	45.3±6.77	48.3±6.25	0.638
BMI (kg/m^2)	26.6±1.58	30.2±4.23	0.0001
WHR	0.778±0.065	0.992±0.051	0.0001
WHtR	51.7±6.22	70.2±7.08	0.0001
Glucose (mmol/L)	3.88 (2.97–4.53)	6.16 (4.83–6.87)	0.0001**
Insulin (mIU/L)	6.46 (4.53–14.5)	19.8 (9.54 –26.3)	0.0001**
HOMA-IR	1.85 (0.836–3.05)	5.81 (2.81–6.40)	0.0001**
TG fasting (mmol/L)	0.876±0.191	2.35±0.512	0.0001
TG 2nd hour (mmol/L)	1.29±0.293	3.07±0.809	0.0001
TG 4th hour (mmol/L)	1.94±0.406	4.66±1.02	0.0001
TG 6th hour (mmol/L)	1.54±0.423	3.63±0.575	0.0001
TC (mmol/L)	4.24±0.860	6.21±0.916	0.0001
HDL-C (mmol/L)	1.34±0.200	0.951±0.105	0.012
LDL-C (mmol/L)	2.54±0.621	5.11±0.915	0.001
AUC	788±202	1678±359	0.0001
iAUC	703±158	1442±298	0.0001

p shows differences between control and MetS according to Student's *t* test. Data were expressed as mean ± SD

**p* shows gender differences between control and MetS according to chi-square test

***p* shows differences between control and MetS according to the Mann-Whitney *U* test. Data were expressed as median (interquartile range for 25–75%)

BMI body mass index, *WHR* waist to hip ratio, *WHtR* waist to height ratio, *HOMA-IR* homeostatic model assessment for insulin resistance, *TG* triglyceride, *TC* total cholesterol, *HDL-C* high-density lipoprotein-cholesterol, *LDL-C* low-density lipoprotein-cholesterol, *AUC* area under the curve, *iAUC* incremental area under $p < 0.05$

The disulfide/native thiol ratio in the MetS group was significantly higher than in the control group at all postprandial time points, peaking at 4 h in both groups ($p = 0.004$, $p = 0.001$, $p = 0.0001$, and $p = 0.010$, respectively) (Figure 1A) (Table 2). Both the MetS and control groups also exhibited a gradual increase at each time point in the postprandial period compared to fasting state ($p = 0.0001$). The most significant change in the disulfide/native thiol ratio in both groups was observed at the 4th hour ($p = 0.0001$) (Figure 1A).

TAS levels were statistically significantly lower during fasting and at 2 and 4 h in the postprandial period in the MetS group than in the control group ($p < 0.05$). TOS levels were significantly higher in the MetS group in the fasting and at 2, 4, and 6 h in the postprandial period than in the control group, especially at the postprandial 4th hour ($p < 0.05$) (Table 2).

OSI levels in the MetS group increased significantly compared to the control group during fasting and at all postprandial time points ($p < 0.001$). OSI levels peaked at 4 h postprandially, and then decreased at 6 h postprandially in both the MetS and control groups ($p = 0.0001$, respectively) (Figure 1B). However, these did not return to the fasting levels (Figure 1B).

ROC curve analysis was performed for the disulfide/native thiol ratio and OSI at fasting and at 4 h postprandially in the MetS group. The values for AUC, sensitivity, and specificity are shown in Figure 2. The highest differences between the disulfide/native thiol ratio and the OSI parameter were observed during fasting and at the 4th hour.

The correlations between the disulfide/native thiol ratio during fasting and at the 4th postprandial hour and age, BMI, OSI fasting, AUC, 4th hour TG, and 4th hour OSI in the MetS group are shown in Table 3.

Fourth-hour disulfide/native thiol ratio and 2nd hour OSI values in the MetS group increased approximately 2-fold in the third tertile compared to the first tertile ($p = 0.002$ and $p = 0.027$, respectively) (Table 4).

Discussion

The main aim of the present study was to evaluate thiol/disulfide homeostasis in MetS patients with PPL during fasting and especially at postprandial hours 2, 4, and 6 in order to observe time-dependent changes. In association with increased thiol oxidation, the native thiols were lower, while

Table 2 The level of all oxidative stress variables in control and MetS groups in the postprandial period

Parameters	Time (h)	Control	MetS	*p*
Total thiol (μmol/L)	Fasting	252±20.6	250±24.1	0.826
Total thiol (μmol/L)	2nd	253±23.6	284±26.5	0.002
Total thiol (μmol/L)	4th	249±26.1	286±32.6	0.002
Total thiol (μmol/L)	6th	247±30.2	277±30.8	0.013
Native thiol (μmol/L)	Fasting	208±19.2	184±9.90	0.0001
Native thiol (μmol/L)	2nd	192±14.7	178±11.0	0.006
Native thiol (μmol/L)	4th	181±17.0	156±16.5	0.0001
Native thiol (μmol/L)	6th	179±23.1	170±20.8	0.267
Disulfide (μmol/L)	Fasting	21.7±11.9	31.9±11.1	0.013
Disulfide (μmol/L)	2nd	31.2±12.8	51.7±15.6	0.0001
Disulfide (μmol/L)	4th	33.7±13.8	65.2±16.9	0.0001
Disulfide (μmol/L)	6th	34.4±12.2	53.6±20.6	0.005
Disulfide/native thiol ratio (%)	Fasting	10.2 (4.5–15.4)	17.1 (11.8–21.7)	0.004*
Disulfide/native thiol ratio (%)	2nd	15.4 (11.0–20.7)	28.9 (24.0–38.4)	0.001*
Disulfide/native thiol ratio (%)	4th	16.1 (14.3–19.3)	41.5 (31.4–54.8)	0.0001*
Disulfide/native thiol ratio (%)	6th	16.0 (13.2–25.9)	29.1 (22.0–45.8)	0.010*
TOS (μmol H_2O_2 equivalent/L)	Fasting	3.43±0.542	4.36±1.42	0.004
TOS (μmol H_2O_2 equivalent/L)	2nd	4.35±0.674	5.22±1.94	0.040
TOS (μmol H_2O_2 equivalent/L)	4th	5.26±0.674	6.88±1.35	0.0001
TOS (μmol H_2O_2 equivalent/L)	6th	5.16±0.589	6.08±1.38	0.003
TAS (mmol trolox equivalent/L)	Fasting	0.490±0.234	0.257±0.082	0.0001
TAS (mmol trolox equivalent/L)	2nd	0.369±0.115	0.204±0.070	0.0001
TAS (mmol trolox equivalent/L)	4th	0.183±0.065	0.141±0.025	0.003
TAS (mmol trolox equivalent/L)	6th	0.194±0.060	0.166±0.066	0.114
OSI	Fasting	0.783 (0.475–0.952)	1.67 (1.31–2.08)	0.0001*
OSI	2nd	1.19 (0.953–1.64)	2.34 (1.67–3.67)	0.0001*
OSI	4th	2.93 (2.43–4.05)	4.56 (3.98–6.35)	0.0001*
OSI	6th	2.50 (2.08–3.56)	3.82 (3.11–4.89)	0.007*

p shows differences between control and MetS according to Student's *t* test. Data were expressed as mean ± SD

**p* shows differences between control and MetS according to the Mann-Whitney *U* test. Data were expressed as median (interquartile range for 25–75%). *TAS* total antioxidant status, *TOS* total oxidant status, *OSI* Oxidative Stress Index. $p < 0.05$

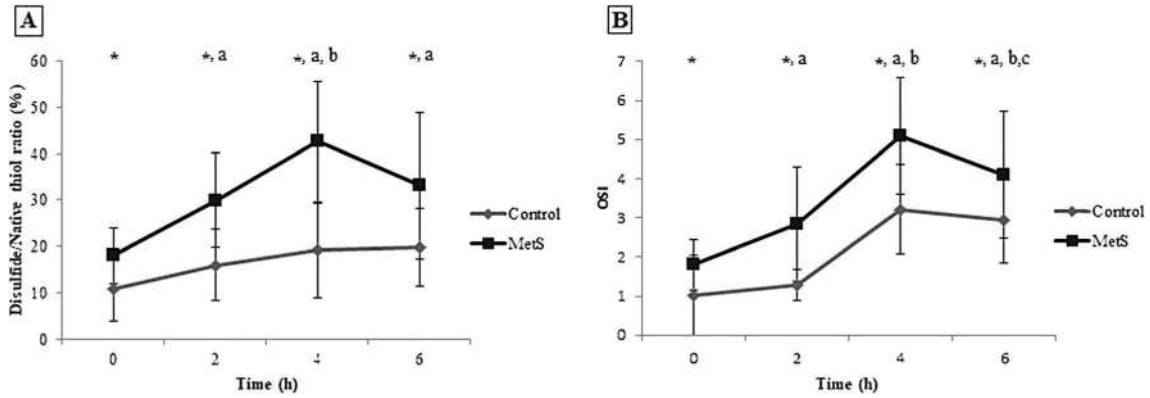
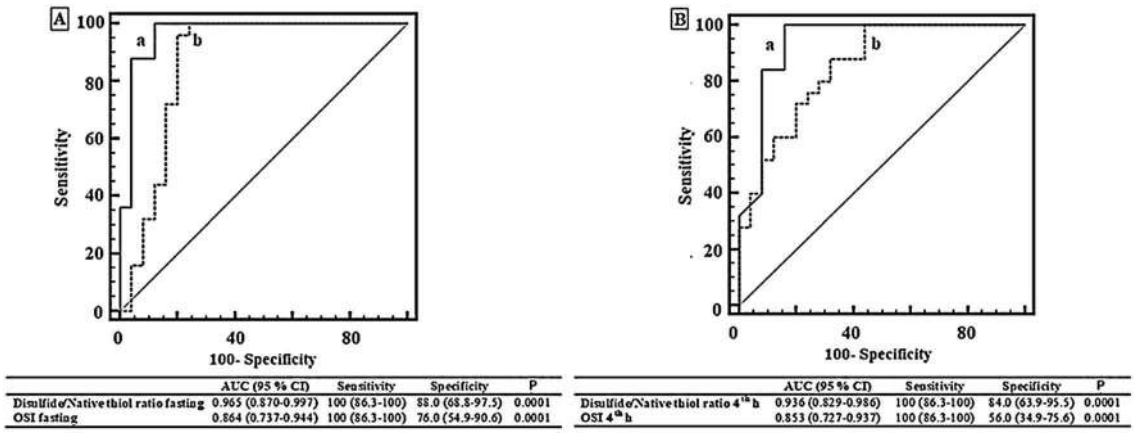


Fig. 1. Disulfide/native thiol ratio (**A**) and levels of OSI (**B**) in control and MetS groups in the postprandial period. Asterisks show that differences between control and MetS were statistically significant (*$p < 0.05$). [a]p:0.0001, significantly different from fasting, [b]p:0.0001, significantly different from 2nd hour, [c]p:0.042, significantly different from 4th hour within the group



	AUC (95 % CI)	Sensitivity	Specificity	P
Disulfide/Native thiol ratio fasting	0.965 (0.870-0.997)	100 (86.3-100)	88.0 (68.8-97.5)	0.0001
OSI fasting	0.864 (0.737-0.944)	100 (86.3-100)	76.0 (54.9-90.6)	0.0001

	AUC (95 % CI)	Sensitivity	Specificity	P
Disulfide/Native thiol ratio 4th h	0.936 (0.829-0.986)	100 (86.3-100)	84.0 (63.9-95.5)	0.0001
OSI 4th h	0.853 (0.727-0.937)	100 (86.3-100)	56.0 (34.9-75.6)	0.0001

Fig. 2. ROC curve analysis of **A**: disulfide/native thiol fasting (a), OSI fasting (b) and **B**: disulfide/native thiol 4th hour (a) OSI 4th hour (b). AUC, area under the curve

disulfide levels were higher, in the MetS group than in the control group in the fasting state and at all postprandial time points (Tables 1 and 2). The disulfide/native thiol ratio in the MetS group thus increased not only in the fasting period but also at postprandial hours 2, 4, and 6 compared to the control group.

Some recent studies exploring thiol/disulfide hemostasis have reported lower native and total thiol levels in patients with prediabetes, and type 1 and type 2 diabetes mellitus, while disulfide levels and disulfide/native thiol ratio values were higher, compared to healthy groups [23–26]. Ateş et al. demonstrated that disulfide and disulfide/native thiol increased in patients with primary and masked hypertension [27, 28]. The fasting disulfide/native thiol ratios in the above studies were similar to those in our MetS group, in which glucose, TG, BMI, and systolic and diastolic BP values were higher than in the control group (Table 1). MetS is a degenerative disease accompanied by abdominal obesity, insulin resistance, blood lipid disorders, inflammation, or diabetes. It is therefore significantly associated with oxidative stress [1]. Studies have determined that oxidative stress levels increase in MetS. This has been linked to an increase in oxidant radicals or a decrease in antioxidant capacity [1, 29]. The number of studies investigating thiol/disulfide homeostatic status in obese and diabetic patients is also limited [23, 24]. However, there are no studies of dynamic thiol/disulfide homeostatic status, which expresses the body's total thiol capacity, and thiol oxidation in MetS patients.

The present study focused on the time-dependent response of the disulfide/native thiol ratio in the postprandial period. An increasing trend was detected in the first 4 h of the postprandial period, peaking at 4 h postprandially and then gradually decreasing at 6 h postprandially in all groups. The time-dependent trend of the ratio in the postprandial period was more evident in the patients with MetS compared to the control group (Figure 1A). Oxidative stress caused by the postprandial state contributes to atherosclerotic cardiovascular disease, in which it initiates the cycle of endothelial dysfunction [30]. Studies have shown that excessive consumption of fatty meals increases serum TG levels and triggers the production of free radicals. Free radicals cause the oxidation of proteins and lipids. It has been suggested that increased TG levels in the postprandial period exacerbate a proinflammatory state that triggers oxidative stress [31]. Consuming a high-fat meal leads to an imbalance in postprandial metabolism, thus increasing susceptibility to oxidative damage. Bloomer et al. [32] and Kanner et al. [33] demonstrated that consuming high-fat meals leads to a significant increase in blood oxidative stress. Deveraj et al. showed that consumption of an energy-dense fatty meal by patients with MetS results in increased postprandial oxidative stress compared to a heart-

Table 3 Spearman's rank correlation coefficients of disulfide/native thiol fasting and 4th hour ratio with BMI, OSI fasting, AUC, TG 4th hour, and OSI 4th hour in the MetS group

	MetS (*n*:25)				
	Disulfide/native thiol fasting ratio			Disulfide/native thiol 4th hour ratio	
	r	*p*		*r*	*p*
BMI	0.636	0.001	**AUC**	0.740	0.0001
OSI fasting	0.544	0.005	**TG 4th hour**	0.758	0.0001
			OSI 4th hour	0.687	0.0001

Table 4 Disulfide/native thiol ratio and OSI levels in the MetS group according to AUC tertiles, mean ± SD [min–max]

		Tertiles			
		1 1209 ± 175 [977–1426] (*n*:8)	2 1614 ± 123 [1438–1785] (*n*:9)	3 2218 ± 296 [1889–2830] (*n*:8)	*p*
Disulfide/native thiol ratio (%)	Fasting	16.3 (11.8–21.7)	19.4 (15.4–26.1)	17.9 (12.6–20.3)	0.411
Disulfide/native thiol ratio (%)	2nd	27.4 (24.1–40.3)	29.9 (23.4–39.7)	28.9 (26.2–38.2)	0.880
Disulfide/native thiol ratio (%)	4th	30.8 (27.8–45.3)	41.5 (31.4–50.5)	57.0 (53.2–60.5)[a, b]	0.003
Disulfide/native thiol ratio (%)	6th	34.8 (27.5–45.9)	28.6 (21.4–46.3)	30.8 (26.4–45.9)	0.680
OSI	Fasting	1.95 (1.46–2.70)	1.58 (1.36–1.97)	1.36 (1.07–1.95)	0.250
OSI	2nd	1.66 (1.24–2.30)	2.97 (2.32–5.38)[a]	3.2 (1.84–3.86)[a]	0.021
OSI	4th	3.98 (3.43–5.57)	4.49 (4.20–5.59)	6.38 (4.51–6.86)	0.136
OSI	6th	3.84 (2.59–5.20)	3.49 (2.39–5.03)	4.28 (3.87–4.79)	0.243

p values according to the Kruskal-Wallis test and post hoc Mann-Whitney *U* test. Data were expressed as median (interquartile range for 25–75%)
[a] Significantly different from 1.tertile; [b] significantly different from 2.tertile

healthy meal [34]. Since postprandial oxidative stress has been proposed as the unifying mechanism in the link between MetS, CVD, insulin resistance, and type 2 diabetes, any worsening of the postprandial pro-oxidative state may lead to adverse outcomes [35–37]. Increased remnant particles in PPL are recognized as one of the important risk factors for development of atherosclerosis [38]. However, oxidative stress is crucial in all stages of atherosclerosis, although a high prevalence of TG is very common in patients with MetS [34].

In addition, OSI increased significantly in the MetS group compared to the control group during fasting and at all postprandial time points. Both groups also exhibited a gradual increase at postprandial hours 2 and 4 compared to the fasting state, followed by a marked decrease in the postprandial 6th hour. However, OSI levels in the postprandial 6th hour did not return to the fasting basal level (Table 2, Figure 2B). There are no previous studies with which to examine these findings observed for OSI in the postprandial period. The mechanisms involved in the increase in OSI may be similar to those applying to the ratio.

ROC analysis was performed to evaluate the suitability of the disulfide/native thiol ratio in terms of markers of fasting and postprandial oxidative stress. The highest ratio values were observed at fasting and at the 4th hour (AUC = 0.936, $p = 0.0001$) (Figure 2). It may therefore be concluded that this value may be used for the assessment of oxidative stress in the fasting and postprandial periods. An imbalance of thiol disulfide homeostasis may play a critical role in increased oxidative stress in the fasting and postprandial periods in patients with MetS.

In addition, the disulfide/native thiol ratio exhibited positive correlation with BMI and OSI in the fasting period and with AUC, TG, and OSI at the postprandial 4th hour (Table 3). These results may indicate that impaired thiol/disulfide homeostasis was associated with increased oxidative stress in the MetS group, not only during the fasting period but also at the postprandial 4th hour.

AUC values were subdivided into tertiles in order to better evaluate the relationship between PPL and oxidative stress in MetS. An approximately two-fold difference was observed between the lower and upper tertiles for the disulfide/native thiol ratio at the 4th hour in the patients with MetS (Table 4). This suggested that the ratio may have a close association with PPL. This in turn may indicate that accompanying increased oxidant stress (increased OSI at the 2nd hour and a higher ratio in the 4th hour) in MetS patients with PPL may be considered one of the important risk factors for the development of atherosclerosis.

The main limitation of this study is the low number of participants in the tertiles. The relationship between PPL and oxidative stress in MetS subjects could be better understood by increasing the number of participants in the tertiles. The results of the present study now require further investigation in more extensive studies. In particular, future studies should focus on the mechanisms involved in the increased postprandial oxidative stress in subjects with PPL.

Conclusion

To the best of our knowledge, this is the first study to investigate thiol/disulfide homeostasis in MetS with PPL and to compare the results with those of healthy controls in the postprandial period. An increased disulfide/native thiol ratio and OSI levels in MetS may have a close association with PPL and play critical roles in the development of atherosclerotic

lesions. The findings revealed that thiol-disulfide homeostasis deteriorated in MetS subjects with PPL in favor of disulfide amounts. The disulfide/native thiol ratio in MetS subjects with PPL may play a role for evaluating oxidative stress, especially in postprandial 4th hour. In conclusion, dynamic thiol/disulfide homeostasis in MetS patients changes in favor of oxidative stress in the postprandial period.

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Author Contribution S.O.Y., A.O., and F.B.Y. planned and designed this research. S.O.Y., A.O., and C.O. conducted the research. S.O.Y. and F.B.Y. performed and conceived the experiments. S.O.Y., F.B.Y., and H.Y. analyzed the data. S.O.Y., A.O., F.B.Y., and B.V.K. wrote the manuscript. All authors read and approved the final version.

Declarations

Ethics approval Approvals from the local Ethics Committee were obtained (Submission Number 2021/142, dated May 5, 2021).

Consent to participation Before enrolling in this study, written informed consent was obtained from all participants (both control and MetS groups).

Competing interests The authors declare no competing interests.

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ORIGINAL PAPER

Astaxanthin reduces oxidative stress and alleviates diabetic neuropathy in STZ-induced diabetic mice

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Abstract

Background Diabetic neuropathy (DN) is a condition resulting due to high glucose level. DN is closely linked to oxidative stress and mitochondrial dysfunctions by many pathophysiological pathways that lead to loss of sensation.

Aim The current study targeted to elucidate the effect of astaxanthin (AX), a potent antioxidant, on blood glucose level, behavioral impairments specifically evaluating its effects on diabetic neuropathy. We further studied its effect on oxidative stress and mitochondrial dysfunction in diabetic neuropathic mice.

Methods The study was conducted by inducing diabetes in Swiss albino mice using STZ (40 mg/kg i.p.). Neuropathy was confirmed in diabetic mice by hot hyperalgesia and cold allodynia tests. To study the protective effects of AX, we administered AX intraperitonially for 4 weeks in consecutive increasing dosage of 2 and 4 mg/kg. Blood glucose levels and body weight were monitored after AX administration. Thereafter, mice were sacrificed and used to estimate levels of different oxidative stress markers along with mitochondrial function in different sections of the brain.

Results Astaxanthin (2 and 4 mg/kg, i.p.) administration attenuated STZ-induced alternation in behavioral impairments in DN mice. AX also significantly reduced the levels of blood glucose($p < 0.01$), reactive oxygen species (ROS), malondialdehyde (MDA), nitric oxide (NO), and boosted the antioxidant defenses viz. superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH), glutathione peroxidase (GPx), thioredoxin reductase (TRR), and acetylcholinesterase (AChE) significantly ($p < 0.001$) after 4 weeks. In mitochondria, AX increased the activity of enzymes like (complexes I–III), citrate synthase (CS), succinate dehydrogenase (SDH) significantly ($p < 0.001$) in the different brain sections of DN mice.

Conclusion This study reveals that AX normalized blood glucose, oxidative stress, mitochondrial dysfunction and leads to amelioration of behavioral impairments in DN mice.

Keywords Diabetic neuropathy · Astaxanthin · Oxidative stress · Mitochondrial dysfunction

Introduction

Diabetic neuropathy (DN) is one of the common insidious complications of diabetes. According to IDF Diabetes Atlas 9th edition estimates, about 463 million people are afflicted with diabetes, and this number will double by 2025 [1]. It is one of the common chronic complications in which pain is characterized by mechanical and thermal hyperalgesia [2, 3]. Increased glucose levels mediated glycation product formation, autoxidative glycosylation, and elevated polyol pathway activity, which are some of these underlying mechanisms. Oxidative stress has devastating consequences on vascular dysfunction, resulting in lack of neurotrophic reinforcement and progressively contributing to decreased nerve conduction velocity and diminished neuronal activity [2, 4–6]. Overproduction of reactive oxygen species (ROS) may cause damage to mitochondrial DNA, and the unrepaired DNA leads to defect in complexes I–III functions, mutations, altered respiratory chain enzyme levels (succinate dehydrogenase (SDH), and citrate synthase (CS)) and weakens the mitochondrial defense system. All these changes are implicated in the pathogenesis of neurodegenerative diseases or amplifying neuronal dysfunction

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[7, 8]. Still the pathophysiology of DN has not been clearly understood; also, the current therapeutic strategies are not capable of entire relief from pain in DN.

Astaxanthin (AX) is a reddish-orange xanthophyll carotenoid potent drug for the control of progression of different diseases including cardiovascular diseases, inflammatory diseases, nephropathy, and neurodegenerative diseases. AX is also a chlorophyta member of *Haematococcus pluvialisa* alga [9–11]. However, the effects of AX on oxidative stress parameters in experimental DN have not been studied. We hypothesize that AX is expected to protect against diabetes linked neuropathy in vivo by reducing the oxidative stress and mitochondrial dysfunction. Therefore, the current study for the very first time investigates the effect of AX on biochemical markers and mitochondrial function in selected brain regions like cortex, cerebellum, striatum, and hippocampus within STZ-induced diabetic neuropathic mice.

Material and methods

Animals

Adult male Swiss albino mice (around 20–25 g) were obtained from the Lala Lajpat Rai University of Veterinary and Animal Science, Hisar (Haryana), India. The animals had free access to water and normal diet and kept at 24 ± 1 °C temperatures and of 55 ± 5% humidity with 12-h light–dark cycle. Prior to the experimentation, animals were acclimatized for at least 1 week. Streptozotocin (STZ), astaxanthin (97% HPLC grade) etc. were purchased from Sigma (St. Louis, MO).

Induction of diabetes

Diabetes was induced in mice by administration of five consecutive doses of STZ (40 mg/kg, i.p.) every day at fixed time. Age-matched control mice received the equal volume of citrate buffer (vehicle). Diabetes was confirmed 48 h after the last STZ dose.

Experimental design and drug treatment

Mice with plasma glucose level > 200 mg/dl were confirmed diabetic and subjected to behavioral test every week as described below. Animals were divided into two experimental groups of diabetic mice and non-diabetic mice. Each group was composed of 6 mice. Diabetic mice were categorized into 3 groups: (1) diabetic control, (2) diabetic mice treated with AX, 2 mg/kg, and (3) diabetic mice treated with AX, 4 mg/kg. Non-diabetic mice were also divided into 3 groups: (1) non-diabetic control, (2) non-diabetic mice treated with AX, 2 mg/kg, and (3) non-diabetic mice treated with AX, 4 mg/kg. The AX was administered through intraperitoneal injections (2 and 4 mg/kg) for 4 weeks after confirmation of DN. The controls were administered with 200 µl of DMSO. Body weight of mice was monitored weekly till 4 weeks. For experimentation, mice were sacrificed under mild anesthesia. Different sections of brain viz. cortex, cerebellum, striatum, and hippocampus were excised and processed on ice for biochemical and mitochondrial assays.

Assessment of plasma glucose level

One hundred microliters of blood was withdrawn from mice tail in centrifuge tubes containing 10 µl of heparin (1000 units/ml). The mixture was centrifuged at 5000 rpm for 5 min to isolate the plasma. Then, the GOD-POD kit was used for the assessment of glucose level using the plasma.

Behavioral tests for confirmation of diabetic neuropathy

Hot hyperalgesia

Thermal sensitivity was tested weekly among all groups of mice by a tail immersion test. Each mice tail was submerged in water kept at $52\ °C \pm 0.5\ °C$ temperature. The time taken for flicking response (tail withdrawal) was recorded. A cutoff time of 15 s was maintained in all cases [3].

Cold allodynia

Sensitivity to cold temperature was tested weekly among all groups of mice by a tail immersion test. Each mice tail was submerged in water kept at $10\ °C \pm 0.5\ °C$ temperature. The time taken for tail withdrawal (flicking response) was recorded. A cutoff time of 25 s was maintained in all cases [3].

Estimation of oxidative stress by biochemical assays

The various brain tissues extracted were subjected to homogenization and centrifugation at 5000 rpm for 10 min, and the supernatant was utilized for the estimation of various biochemical assays.

Determination of superoxide dismutase (SOD) activity

Twenty-five-microliter tissue supernatant was added to the reaction mixture containing 5 mM phosphate buffer, 1 mM EDTA, 100 mM methionine, 450 mM nitro blue tetrazolium (NBT), and 10 mM riboflavin. The samples were incubated in light for 30 min. The blue color was observed, and absorbance was taken at 560 nm [6].

Determination of catalase (CAT)

The tissue supernatant (equivalent to 50 ng protein) was added to a 1-ml reaction mixture containing 8.8 mM H_2O_2 (3%), 0.1 mM sodium phosphate buffer, and pH 7.0. The absorbance was recorded at 240 nm and expressed as U/mg protein [6].

Determination of glutathione reductase (GSH)

Formic acid (0.1 M) was added to supernatant of samples and centrifuged for 10 min at 10,000 rpm. The tissue supernatant (deproteinized) was added to the tubes containing buffered formaldehyde (1:4 (v/v) 37% formalin:0.1 M Na_2HPO_4). Each tube was filled with sodium phosphate buffer (0.1 M, pH 8.0); then, o-phthalaldehyde (OPA) (100 µg/ml) was added. The fluorescence was measured after 45 min of incubation at room temperature at excitation and emission wavelengths of 345 and 425 nm, respectively, and expressed as µg/mg protein [12].

Determination of glutathione peroxidase (GPx) activity

The supernatants (0.2 mg protein) were added to a reaction mixture of phosphate buffer (0.2 M, pH 7.0) containing 20 mM oxidized glutathione, 2 mM EDTA, and 2 mM NADPH. The absorbance was measured taken at 340 nm for 3 min and expressed in nmol NADPH oxidized/min/mg protein [12].

Determination of reactive oxygen species (ROS)

The supernatant (100 ng protein equivalent) from different brain regions was incubated in Locke's buffer (pH7.4) containing dihydrodichlorofluorescein diacetate (5 mM) at room temperature for 30 min. The dichlorofluorescein (DCF) product was formed and measured with excitation wavelength of 480 nm and emission of 530 nm. It is calculated from the standard curve and expressed as picomoles DCF/min/mg protein [3].

Determination of nitric oxide (NO)

The supernatants from different brain regions (100 ng protein) were mixed with Griess reagent, and absorbance of colored product was taken at 540 nm. NO was quantified as nitrites using the standard curve [13].

Determination of lipid peroxidation (LPO)

The supernatants (0.2 ml) were mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution changed to pH 3.4 with the help of NaOH, and 1.5 ml of 0.8% thiobarbituric acid. Thereafter, the 4 ml distilled water was added to the volume of the reaction mixture. The reaction mixture was heated for 60 min. at 95 °C. Further, the reaction mixture was cooled at room temperature and centrifuged at 10,000 rpm. The absorbance was recorded at 532 nm and expressed as µM/ml [6].

Assays to estimate the mitochondrial dysfunctions

The brain tissues extracted were subjected to homogenization by differential centrifugation at 29,000 rpm for 10 min, and the mitochondrial fraction was utilized for the estimation of mitochondrial dysfunctions.

In brief, 1000 µl buffer (1 mM NADH, 20 mM sodium succinate, mannitol-sucrose-HEPES, pH 7.4) was added to supernatants (equivalent to 10 ng protein). Fifteen microliters of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (5 mg/ml) was added to it and then incubated for 1 h at 37 °C. Formazan crystals formed were further dissolved in N,N-dimethylformamide-sodium dodecyl sulfate buffer. Thereafter, absorbance was recorded at 570 nm [14].

Phosphate buffer (0.1 M, pH 7.4) containing potassium cyanide (KCN) 1 mM and NADH 0.2 mM was added to supernatants (50 ng). The reaction mixture of 0.1 mM was initiated by addition of cytochrome C, and the reduction in absorbance was measured at 550 nm for 3 min. The activity of NADH-Cyt C reductase (complexes I–III) was expressed in nanomoles cytochrome C reduced/min/mg protein [15].

In brief, 50 mM potassium phosphate (pH 7.4) containing p-iodonitrotetrazolium violet (2.5 ng/ml) and sodium succinate (0.01 mol/l) was incubated with supernatants (50 ng) for 10 min. Ten percent trichloroacetic acid (TCA) was used to stop the reaction. The color product was observed with addition of ethyl acetate:ethanol:trichloroacetic acid (5:5:1) and was monitored at 490 nm. The activity of SDH was expressed in OD/mg protein [16].

The supernatant was added to tris HCl buffer (0.1 M, pH 8.1) which contained acetyl CoA (0.1 mM) and DTNB (0.2 mM). The reaction was started by addition of oxaloacetate (10 mM), and absorbance was measured at 412 nm for 3 min. The CS activity was expressed in nmol substrate conjugated/min/mg protein [17].

Determination of thioredoxin reductase (TRR) activity

There was a recorded decline of 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in a potassium phosphate buffer (0.1 M, pH 7.0, containing 10 mM EDTA, 0.2 mM NADPH) at 412 nm of the test samples. The values were expressed as nmol substrate reduced/min/mg protein [18].

Determination of acetylcholinesterase (AChE) activity

The reaction mixture contains phosphate buffer (0.1 M, pH 8.0), 10 mM DTNB, tissue supernatant, and acetylthiocholine iodide (150 mM). The absorbance was measured at 412 nm for 3 min and expressed as nanomoles of substrate hydrolyzed/min/mg protein [19].

Determination of protein

Protein concentration in the tissue supernatants was determined by incubating brain tissue sample with Folin–Ciocalteau phenol in an alkaline medium for 30 min and measuring the OD at 750 nm. The amount of protein was quantified with bovine serum albumin (BSA) as the standard [20].

Statistical analysis

Results of each experimental group were represented as mean ± SD ($n = 6$). The results were analyzed by using two-way analysis of variance (ANOVA) followed by a SNK (Student–Newman–Keuls) test by Sigma stat 3.5 software. The data with $p \leq 0.001$ and $p \leq 0.01$ were considered statistically significant.

Results

Effect of AX on blood glucose levels and body weight

As shown in Fig. 1a, the body weight of the non-diabetic control groups was found to be higher than the diabetic group. The AX treatment increased the body weight of diabetic mice significantly ($p < 0.001$) as compared to the respective controls.

The blood glucose levels increased four to five-fold among the diabetic mice during the experimental period after STZ induction (40 mg/kg). Treatment with AX decreased the blood glucose of diabetic mice significantly ($p < 0.01$) as compared to the controls. There was no effect of AX on non-diabetic groups as shown in Fig. 1b.

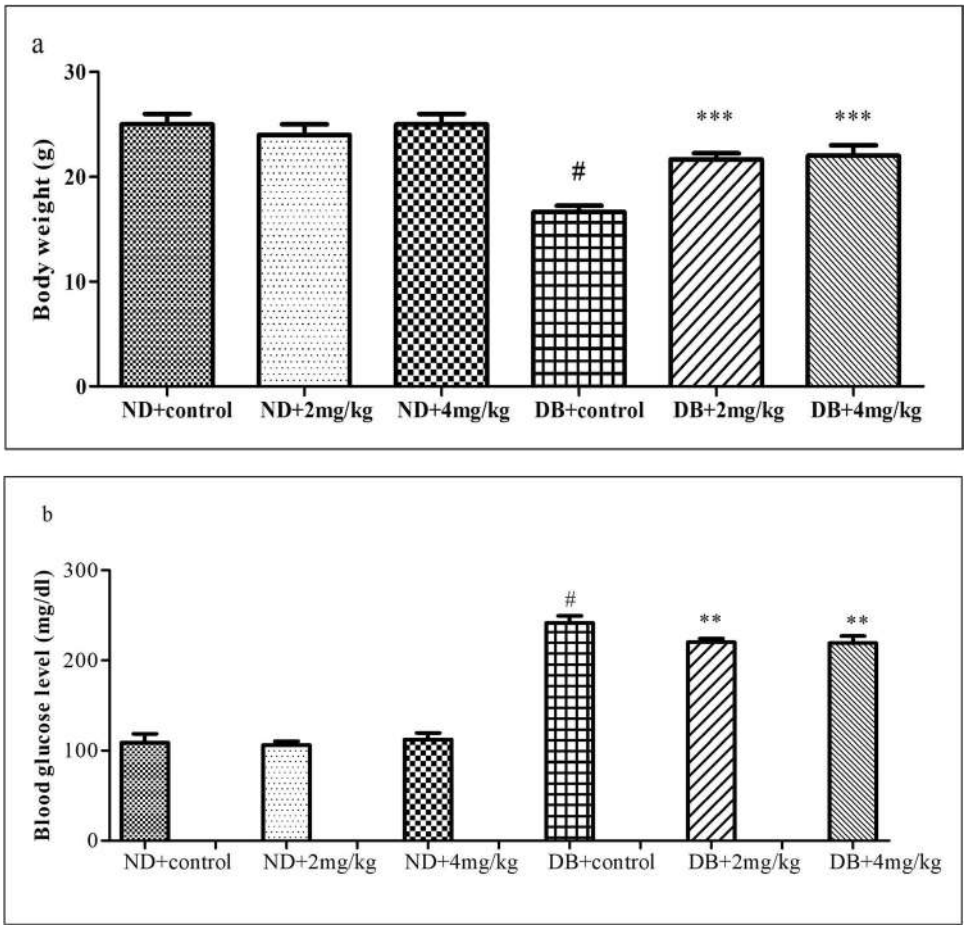

Fig. 1 Effect of 4-week treatment with AX on (**a**) body weight; (**b**) blood glucose level. Values are means ± SD ($n = 6$). Results analyzed by two-way ANOVA followed by SNK test. $^{\#}p < 0.001$ vs control mice. $^{***}p < 0.001$ vs diabetic mice $^{**}p < 0.01$ vs diabetic mice

Effect of AX on motor and sensory functions

Hyperalgesia (52 °C) and cold allodynia (10 °C) tail immersion test have shown a decreased latency period in diabetic mice as compared to non-diabetic mice, before AX treatment. Treatment with AX led to reversal of decrease in tail flick latency significantly ($p < 0.001$) on administration of both dose levels (2 and 4 mg/kg) for 4 weeks (Fig. 2a,b).

Effect of AX on oxidative stress markers

Effect of AX on SOD, GPx, GSH, and CAT activity

SOD, GPx, GSH, and catalase level in different brain regions of diabetic mice was found to be significantly decreased ($p < 0.001$) as compared to the non-diabetic mice. After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg ($p < 0.001$) exhibited a significant improvement in different brain regions as compared to controls. The changes in endogenous levels of SOD, GPx, GSH, and CAT in various brain regions of AX-treated non-diabetic mice were found to be non-significant ($p > 0.05$) (Fig. 3a, b, c, and d).

Effect of AX on ROS, LPO, and NO level

Levels of ROS, MDA, and NO in diabetic mice were found to be significantly elevated ($p < 0.001$) as compared to non-diabetic mice. After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg exhibited a significant decrease ($p < 0.001$) in different brain regions as compared to controls. The changes in endogenous levels of LPO in various brain regions of AX-treated non-diabetic mice were found to be non-significant ($p > 0.05$) (Fig. 4a, b, and c).

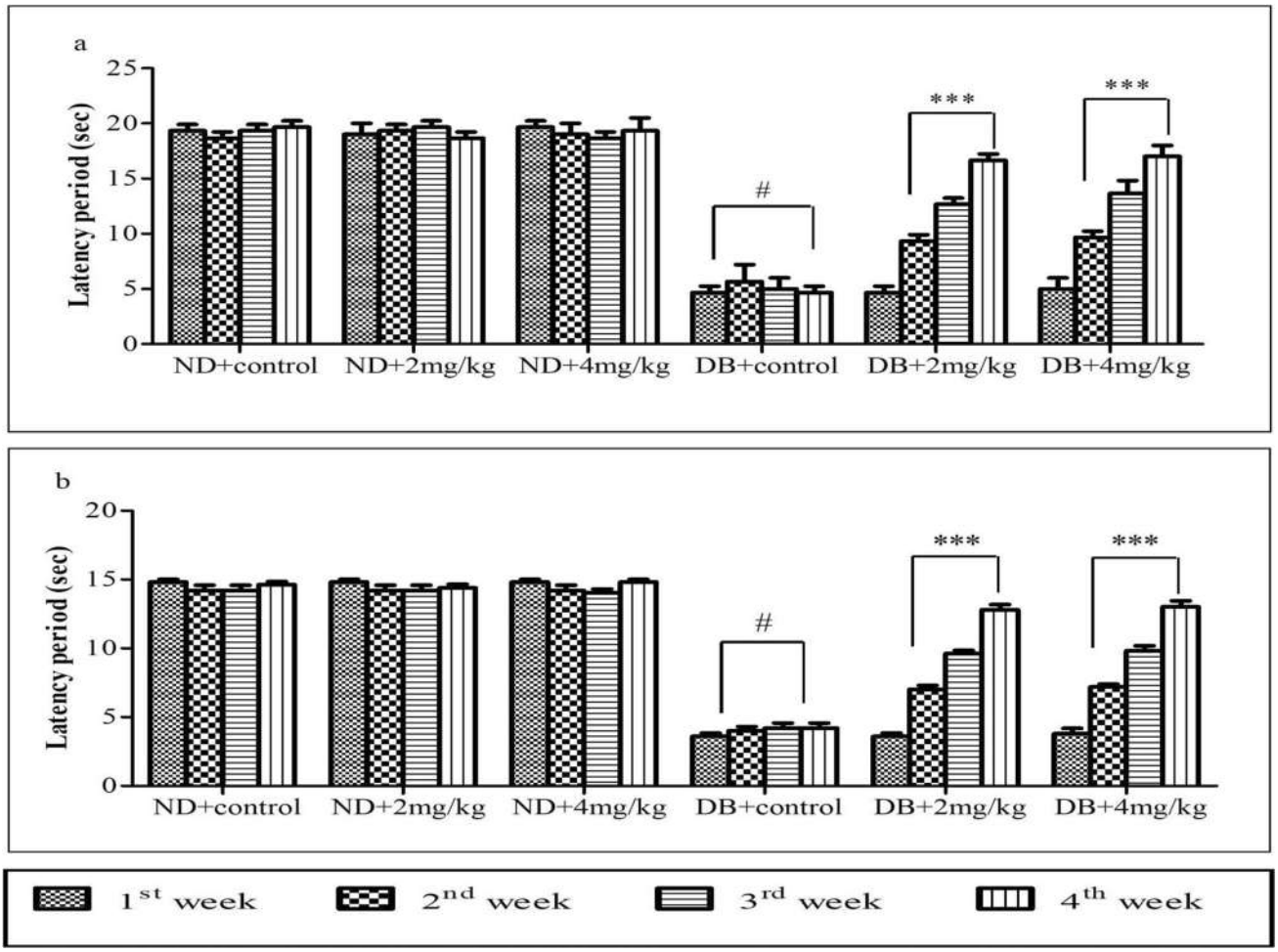

Fig. 2 Effect of AX on tail immersion test. (**a**) Allodynia and (**b**) hyperalgesia among different groups of diabetic and non-diabetic mice. Values were expressed as mean ± SD ($n = 6$). From the second to fourth week, we have seen a significant improvement in graph shown above. ***$p < 0.001$ vs diabetic mice, $^{\#}p < 0.001$ vs control mice

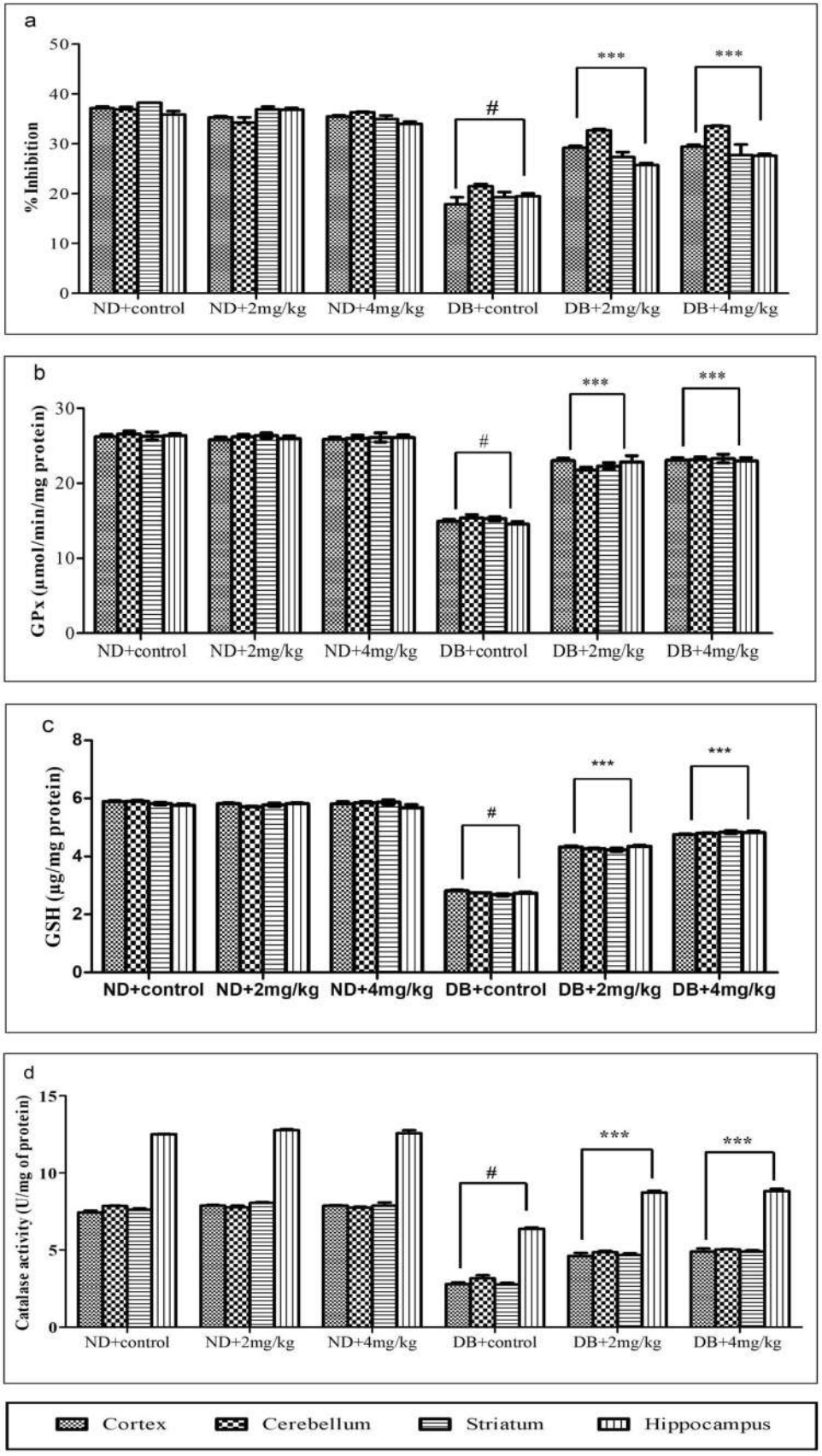

Fig. 3 Effects of 4-week treatment with AX on biochemical stress markers (**a**) SOD; (**b**) GPx; (**c**) GSH; (**d**) CAT measured in different brain regions (cortex, cerebellum, striatum, and hippocampus) of mice. Values were expressed mean ± SD ($n = 6$). ***$p < 0.001$ vs diabetic mice, $^{\#}p < 0.001$ vs control mice

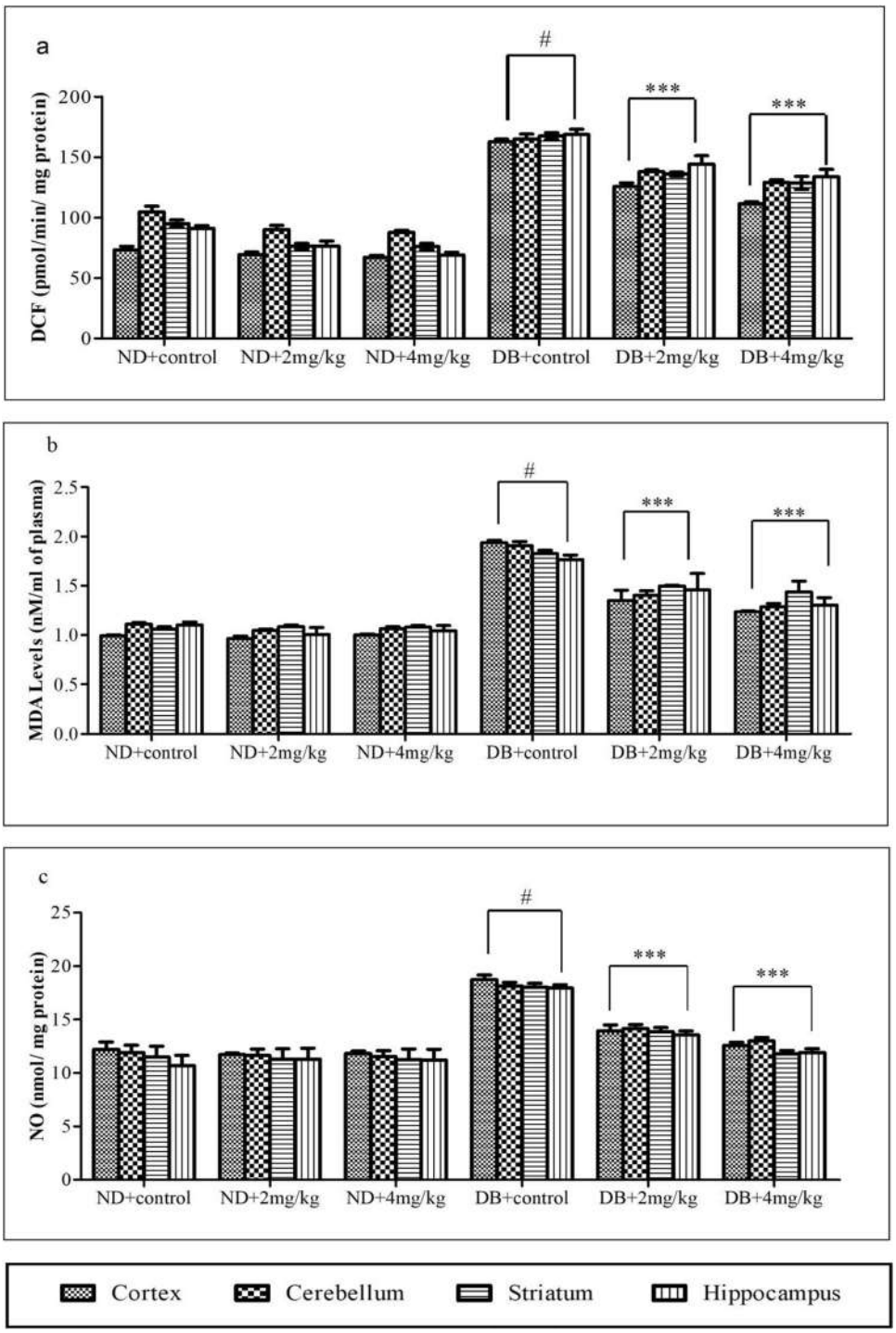

Fig. 4 Effects of 4-week treatment with AX on (**a**) ROS; (**b**) LPO; (**c**) NO; measured in different brain regions (cortex, cerebellum, striatum, and hippocampus) of mice. Values were expressed mean ± SD ($n = 6$). ***$p < 0.001$ vs diabetic mice, $^{\#}p < 0.001$ vs control mice

Effect of AX on AChE and TRR activity

AChE activity in diabetic mice was found to be significantly increased ($p < 0.001$) as compared to non-diabetic mice (except in hippocampus and cerebellum). After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg ($p < 0.001$) exhibited a significant decrease in AChE activity in brain regions like cortex and striatum as compared to controls (except in cerebellum and Hippocampus). The changes in the activity of AChE in various brain regions of AX-treated non-diabetic mice were found to be non-significant ($p > 0.05$) (Fig. 5a).

TRR level in diabetic mice was found to be significantly decreased ($p < 0.001$) as compared to non-diabetic mice. After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg ($p < 0.001$) exhibited a significant improvement in levels of TRR in different brain regions as compared to controls.

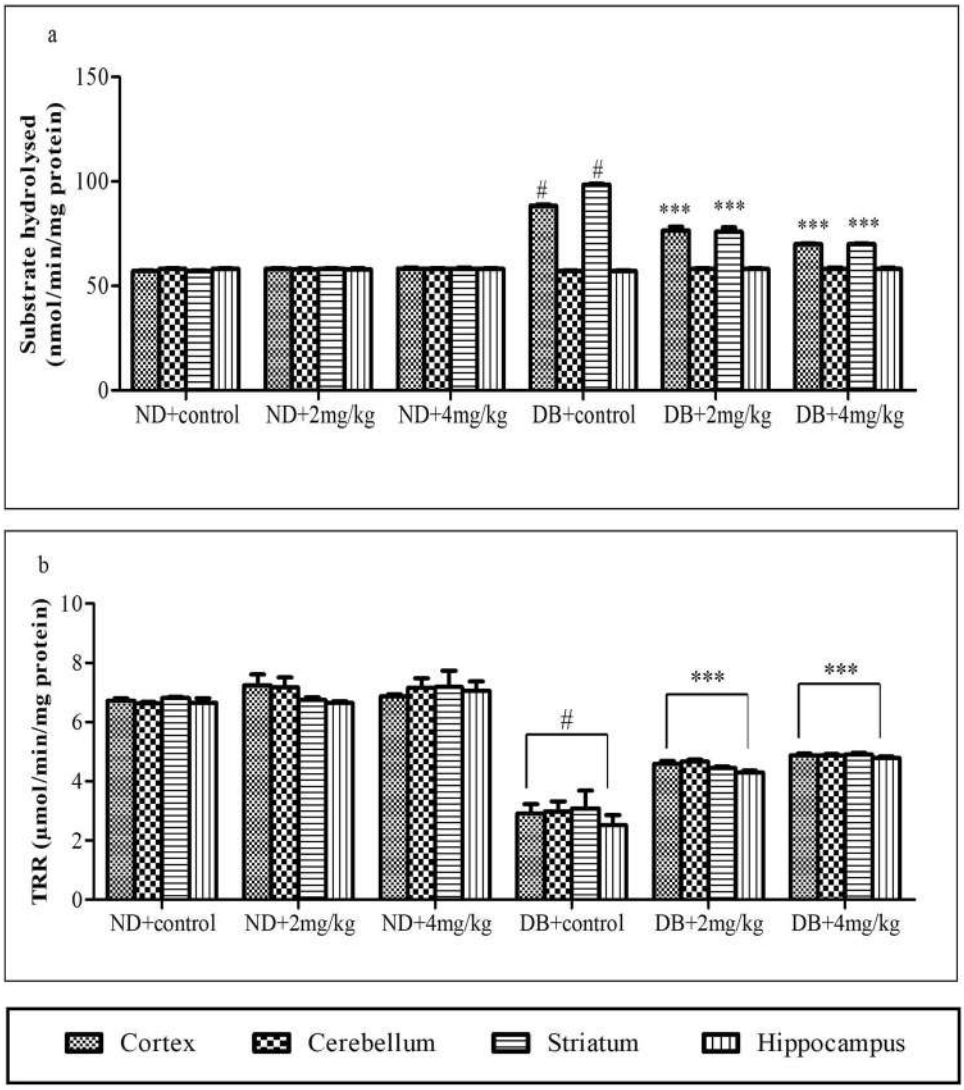

Fig. 5 Effects of 4-week treatment with AX on (**a**) AChE and (**b**) TRR activity measured in different brain regions (cortex, cerebellum, striatum, and hippocampus) of mice. Values were expressed mean ± SD ($n = 6$). ***$p < 0.001$ vs diabetic mice, $^{\#}p < 0.001$ vs control mice

The changes in endogenous levels of TRR in various brain regions of AX-treated non-diabetic mice were found to be non-significant ($p > 0.05$) (Fig. 5b).

Effect of AX on mitochondrial fractional

MTT reduction in diabetic mice was found to be significantly decreased ($p < 0.001$) as compared to the non-diabetic mice (except in hippocampus) (Fig. 6a). After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg ($p < 0.001$) exhibited a significant improvement in levels of MTT in different brain regions as compared to controls. The activities of complexes I–III, CS, and SDH in diabetic mice were found to be significantly diminished ($p < 0.001$) as compared to non-diabetic mice. After 4 weeks, diabetic mice treated with AX 2, 4 mg/kg ($p < 0.001$) exhibited a significant improvement in the level of these enzymes in different brain regions as compared to controls (Fig. 6b, c, and d).

Discussion

Earlier studies were mainly focused on curing the pains like symptoms by various medications like antidepressants, opioids, and anticonvulsants to evaluate the neuropathy in mice model [2]. In this study, we examined the effects of natural xanthophyll carotenoid potent drug, AX, on biochemical markers and mitochondrial function in selected brain regions like cortex, cerebellum, striatum, and hippocampus in STZ-induced mice model. To study above parameters, STZ-induced mice were used, which mimicked the DN features. Development of DN in mice was confirmed by behavioral tests. Sensory nerves are responsible for the pain sensation. Under diabetic conditions, sensory nerves are usually affected earlier than the motor nerves because damaged terminals end, i.e., nociceptors of nerves are the main reason of loss of sensation and delay in reflexes [3, 21]. Similar sensory dysfunctions have been observed in our

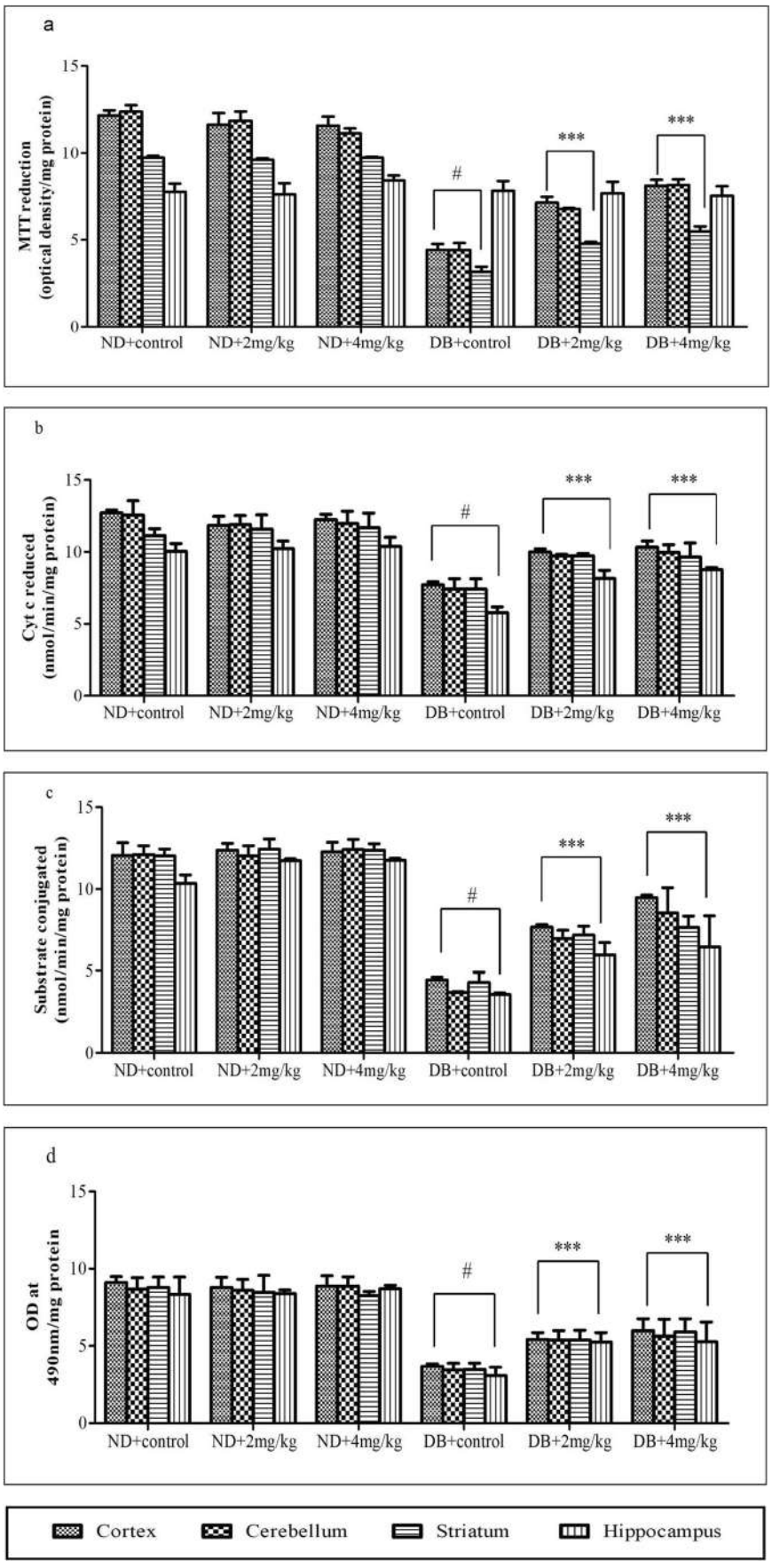

Fig. 6 Effects of 4-week treatment with AX on mitochondrial assays measured in different brain regions (cortex, cerebellum, striatum, and hippocampus) of mice. (**a**) MTT reduction; (**b**) complex I-III; (**c**) CS; (**d**) SDH. Values were expressed mean ± SD (n = 6). ***$p < 0.001$ vs diabetic mice, $^{\#}p < 0.001$ vs control mice

current diabetic neuropathic mice model as proven by results of hot hyperalgesia and cold allodynia. Diabetic mice treated with increasing dosage, i.e., 2, 4 mg/kg of AX, exhibited a significant improvement in both noxious (hyperalgesia) and non-noxious (allodynia) sensation. AX administration also resulted in decreased plasma glucose levels in our study. This may be because AX has been reported to preserve the beta-cell function of insulin secretion in the diabetic animals [13].

In our study, AX significantly stabilized or reduced the levels of ROS/RNS, due to its unique molecular structure. The presence of the hydroxyl (OH) and keto (CdO) moieties on each ionone ring of AX rationalizes some of its distinctive features, like the ability to be esterified and a higher antioxidant activity and a more polar nature than other carotenoids. In its free form, AX is highly unstable and particularly susceptible to oxidation. It can scavenge and quench ROS and free radicals in both the inner and outer layers of the membrane [9, 10, 22–24].

In addition, our findings are consistent with previous research on the efficacy of AX in balancing GPx, GSH, and TRR activities in brain parts [25]. Based on this data, we have shown that AX restored the optimum levels of these antioxidant enzymes SOD, CAT, GPx, GSH, LPO, and TRR levels which are helpful in the balancing of redox reaction in central and peripheral nervous tissue in diabetic mice.

Free radicals like superoxide anions react with nitric oxide (NO) to form peroxynitrite. It is a key mediator which causes a nitration leading to functional damage and imposes toxic effects on nerve tissues. Our study indicates AX's anti-inflammatory role in diabetic mice by inhibiting nitric oxide synthase (NOS) expression decreasing the levels of nitric oxide [13, 25, 26]. Concomitantly, our study reveals that AX administration in DN mice exhibited a significant decrease in levels of NO in different brain regions.

In the current study, we found that AX significantly improved the activity of AChE among diabetic mice in cortex and striatum. AChE is an important component of membrane that acts as neurotransmitter and neuromodulator. AChE increased level causes a reduction of cholinergic neurotransmission which affects routine functions of nervous system such as neuronal excitability and behavior [3, 19]. A similar finding was found in previous studies of AX on AChE activity [27].

In diabetic models, dysfunctional mitochondria caused a pathophysiology change in sensory neurons, such as alternation in number or size and decrease in activity. Damaged mitochondrial function/physiology impairs other surrounding cells due to excessive donation of free electron to electron chain reactions thereby causing continuous instability in the cell's redox status [7, 8, 12]. Our findings have shown a decrease in the activities of mitochondrial enzymes in DN mice. AX administration reduced the oxidative stress linked mitochondrial dysfunction by increasing activities of enzymes like complexes I–III, CS, and SDH. This decrease in mitochondrial activity may lead to imbalance in fluctuating ATP which is a remarkable feature of diabetic neuropathic conditions [17, 28].

Conclusion

In summary, the present study concluded that AX possesses modulatory potentials of normalizing glucose level, ameliorating DN-induced behavioral changes, oxidative stress, and mitochondrial dysfunction in various brain regions of STZ-induced diabetic neuropathic mice model. Therefore, AX has significant neuroprotective properties that are largely attributable to its antioxidant properties.

Author contribution SG has performed the experiments, and SG, RM, RKS, and SB have analyzed and interpreted the data. SG and SB have written the manuscript. All the authors have read and approved the final manuscript.

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Data availability All the experiments have been executed in our laboratory, and the data generated is transparent and original.

Declarations

Ethics approval All the experiments were conducted strictly in accordance with the guidelines of the Institute Ethical Committee regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Handling and experimentation of animals were strictly according to the standard guidelines of the Institutional Ethics Committee. The experimental protocol was approved by the Institutional Animal Ethical Committee (BV/IAEC/4063/2020).

Conflict of interest The authors declare no competing interests.

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ORIGINAL ARTICLE

Shotgun proteomic analysis using human serum from type 2 diabetes mellitus patients

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Abstract

Background Type 2 diabetes mellitus (T2DM), also known as adult-onset diabetes or noninsulin-dependent diabetes mellitus, is characterized by hyperglycemia and insulin resistance. Protein biomarker screening plays an essential role in different diseases. Proteomic methods such as MALDI-TOF based peptide mass fingerprinting, LC-MS/MS based peptide sequencing, and multidimensional liquid phase chromatography (MDLC) coupled with tandem mass spectrometry (MS) shotgun proteomics are used to identify biomarkers.

Methods In this study, we used a MDLC coupled with tandem MS shotgun proteomic method to demonstrate protein quantitation results by comparing human serum samples from T2DM patients with those of healthy subjects. We utilized quantitative techniques, dimethyl labeling, MDLC by hydrophilic interaction liquid chromatography separated column, and reverse-phase high-performance liquid chromatography coupled with tandem MS to identify proteins with high potential to be T2DM biomarker candidates.

Results Identified candidates included vitamin D–binding protein, apolipoprotein B-100, apolipoprotein A2, apolipoprotein A1, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, fibrinogen gamma chains, fibrinogen alpha chains, and alpha-1-antitrypsin. In addition, we also generated relative protein networks using STRING bioinformatic software.

Conclusion These potential biomarker candidates might be verified by further experiments such as an ELISA assay or multiple reaction monitoring MS screening.

Keywords Type 2 diabetes mellitus (T2DM) · Shotgun proteomics · Tandem mass spectrometer · Vitamin D–binding protein · Apolipoprotein

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Introduction

Diabetes mellitus (DM), which is commonly known as diabetes, is a group of metabolic diseases characterized by high blood sugar levels due to the cells not responding to the insulin produce or the body not producing enough insulin [1]. Diabetes is frequently observed in the people of Taiwan; the incidence rate of diabetes and associated medical expenses have rapidly grown in recent years [2, 3]. From 2000 to 2014, the incidence rate of diabetes in Taiwan was ranging from 0.6% to 0.65%, and moreover, the new cases were increasing from 130 thousand to 160 thousand. Among three main types of diabetes, type 2 diabetes mellitus (T2DM), about 90–95% in cases of diabetes [4], is a complex chronic disease associated with hyperglycemia and is a form of diabetes which is characterized by high blood glucose, insulin resistance, and insufficient insulin secretion [5]. T2DM is a multiple factor disease involved with genetic risk factors [6]. Recent genetic studies have shown that a number of genes play a variety of roles in the onset of T2DM [7, 8]. Also, T2DM is the leading cause of death in the USA since it is an important risk factor for cardiovascular disease (CVD). To date, no satisfactory medication is available to treat T2DM. Pre-diabetes is an almost asymptomatic condition, but it has been recognized with a fasting glucose level of higher than 6.10 mmol/L. Ten to 40% of pre-diabetic cases are more prone to cardiovascular diseases than normal people, and 25% of subjects with pre-diabetes will develop to T2DM within 3–5 years. Subjects were chosen from over 30-year-old volunteers, those who had the fasting plasma glucose level $\geq$ 5.55 mmol/L, the golden standard by Department of Health Taiwan, and not taking medicines that influence the blood sugar. Risk factors may vary with ethnicity and among various populations. The risk assessment is frequently reported but little evidence is reported in Asian countries. The key is to discover useful markers for early intervention and prevention of disease progression. Plasma or serum protein biomarkers are commonly used as early detection and diagnosis indicators [9, 10]. The serum concentration of protein markers reflects the progression of T2DM [11, 12]. Thus, it is important and valuable to develop T2DM biomarker screening method and characterization of these markers may provide the basis for early detection and diagnosis of diabetes.

Mass spectrometry with high sensitivity and specificity based quantitative proteomic methods, including absolute and relative quantitative proteomics, has been applied in plasma or serum protein expression profiles. These methods are also used to screen urinary or serum protein biomarkers [13, 14]. However, using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as a separation technique to reduce sample complexity coupled with LC–MS/MS on large sample sizes is time-consuming, labor-intensive, and inconvenient. Surface-enhanced laser desorption ionization mass spectrometry (SELDI MS) analysis relies on complex bioinformatics and statistical tools [15]. Quantitative proteomic reagents such as isotope-coded affinity tags are insufficient for global peptide labeling and sensitivity due to ionization impairment [16]. These limitations cause difficulties for protein quantitation and high-throughput analysis. Fortunately, bioinformatic software and MS instruments have improved significantly. Human serum biomarkers in diabetes mellitus type 2, one-dimensional PAGE, and chromatofocusing coupled with peptide mass fingerprinting (PMF) and MS have been used to reveal C-reactive proteins, leptin, and apolipoproteins as biomarker candidates [12]. Hemoglobin A1C has been reported to be a potential diabetes biomarker [17]. A pilot proteomic study showed that down-regulated apolipoprotein A1 was a possible candidate. Alpha-1 antitrypsin, vitamin D–binding protein, fibrinogen gamma chain, haptoglobin, and transthyretin have also been considered potential biomarkers with high ratios in poorly controlled diabetes patients [10]. Moreover, top-down proteomics was used to analyze a drop of blood, and finally, the authors illustrated a new assay to evaluate blood glycemia [9]. Multiple reaction monitoring (MRM), a tandem MS scan mode, was utilized to validate diabetes biomarkers using a tip-based immunoassay focusing on high-density lipoproteins and apolipoprotein A1 with post-translational modification –[18].

In this study, we selected shotgun proteomic analysis that included a dimethyl labeling technique, protein identification by tandem MS, and a quantitative software to globally quantitate protein and accurately screen and detect potential protein biomarker candidates [19]. This method uses inexpensive quantitative reagents. We used multidimensional separation systems to fractionate each serum sample into ten fractions in order to increase protein identifications [20, 21]. We concluded that potential T2DM biomarkers included vitamin D–binding protein, apolipoproteins, fibrinogen gamma and alpha chains, hemoglobin subunits, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, and alpha-1-antitrypsin.

Materials and methods

Chemicals and materials

Sodium acetate, trifluoroacetic acid (TFA), and sodium cyanoborohydride ($NaBH_3CN$) were bought from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (MeCN, for LC/MS grade) and acetone were obtained from Merck (Seelze, Germany). Formaldehyde-D_2 solution (20% solution in D_2O) was obtained from Isotec Corp. (Miamisburg, OH, USA), while formaldehyde-H_2 solution (36.5–38% in H_2O),

sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium dihydrogen phosphate (NaH_2PO_4), formic acid (FA, 98–100% for LC/MS grade), and iodoacetamide (IAM) were purchased from Sigma (St. Louis, MO, USA). Ammonium hydrogen carbonate (NH_4HCO_3), acetonitrile (gradient grade for HPLC), hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate (SDS), and ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Modified trypsin was bought from Promega (Madison, WI, USA). Deionized H_2O was obtained using a Millipore water system with characterization of 18.2 MΩ. Each protein concentration of serum samples belonging to patients and healthy subjects was determined by Bradford assay based on the measurement of bovine serum albumin (BSA) calibration curve (Thermo, Rockford, IL, USA).

Serum sample collection and pretreatment

The three samples from the diabetes mellitus type 2 patients and the three control samples from the healthy subjects were collected in Eppendorf tubes. Individual serum proteins were determined using the Bradford assay. Samples containing 100 μg of total proteins were adjusted in 60 μL solution and then reacted with 9.3 μL of 7.5% SDS and 0.7 μL of 1 M dithiothreitol (DTT) at 95 °C for 5 min. After reduction, the samples were treated with 8 μL of 50 mM IAM for alkylation at room temperature for 30 min in the dark with violent agitation. To each tube, 52 μL of 50% trichloroacetic acid (TCA) was added to regulate the TCA concentration at 20%. The serum samples were then precipitated with incubation on ice for 15 min. The supernatant was removed by 13,000 × *g* centrifugation for 10 min. The protein pellets were washed using 150 μL 10% TCA (5 min), 250 μL deionized H_2O (5 min), and 250 μL acetone (5 min). In addition, the pellets were washed twice with 250 μL deionized H_2O. The protein pellets were redissolved in 180 μL 50 mM NH_4HCO_3 (pH 8.5).

Tryptic digestion, dimethyl labeling, and HILIC column fractionation

The protein solutions were digested initially with 2 μg of trypsin at 37 °C for 4 h. Then, 2 μg of trypsin was added at 37 °C and reacted overnight. Digested solutions were dried by vacuum centrifugation in order to remove the buffer. The procedures for peptide labeling followed a previous study that demonstrated quantitative proteomics by stable-isotope dimethyl labeling [19]. The T2DM tryptic-digested samples were labeled with d_2-formedehyde, and healthy control samples were labeled with h_2-formedehyde. The T2DM samples were individually random matched and mixed with one healthy control sample. The three labeled mixtures were adjusted to pH 2.5 with utilizing 10% TFA/H_2O and combined for reverse-phase chromatography using a homemade C18 cartridge to desalt. After eluting with 50% and 100% MeCN, triplicate combined samples were dried by vacuum centrifugation. The lyophilized samples were redissolved by 20 μL 85% MeCN before hydrophilic interaction liquid chromatography (HILIC) fractionation. The HILIC separation system followed a previous study that used a Tosoh TSK HILIC column (2.0 mm × 150 mm, 3 μm particle size; Tosoh Biosciences, Tokyo, Japan) [20]. A mobile phase system was used for gradient elution (solvent bottle A containing 0.1% TFA in H_2O and solvent bottle B containing 0.1% TFA in 100% MeCN). The redissolved sample was injected into a 20 μL sample loop. The gradient was performed as follows: 95% (B) (2 min), 95–60% (B) (52 min), 60–5% (B) (20 min), 5% (B) (5 min), 5–95% (B) (1 min), and 95% (B) (10 min). The mobile phase solution containing 1.2 mL buffer was collected in each fraction; 15 fractions were dried by vacuum centrifuge.

Protein characterization by nano-LC–MS/MS

The vacuum-dried lyophilized samples were redissolved in 10 μL of 0.1% FA in H_2O. MS was performed using Thermo LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA) with positive polarity, and detection voltage 1.8 kV. MS scan mode was m/z 400–1600 Da in "Profile" for the MS spectrum and "Centroid" for the MS/MS spectrum. The MS resolution was set at 30,000 with a rate of 30 ms/scan. The data-dependent mode selected five high-intensity peptide signals in MS mode according to priority. The value of automatic gain control (AGC) was set at 10^4 and the ion injection time was set at 50–150 ms. Peptides were transferred to a collision chamber for collision-induced dissociation. Collision energy was set to 35 eV. In the MS/MS detection mode (mass range setting m/z 100–2000 Da), fragmented ions were analyzed. The repeat duration of the data-dependent mode was set to 30 s in order to exclude repeated ions with equal and similar m/z and avoid interferences. A total of 10 μL of the sample was injected into a C18 trapped column (0.3 mm × 5 mm, 5 μm, Agilent Zorbax XDB) using a HPLC binary pump (5 μL/min flow rate, 0.2% FA in water mobile phase). The nano-LC separation system utilized an Agilent 1200 series nanoflow pump (300 nL/min, Agilent Technologies, Santa Clara, CA) and a C18 column (i.d. 75 μm × 150 mm, 3 μm particle size, Micro Tech, Fontana, CA). The nano-LC mobile phases consisted of (A) 0.1% FA in water and (B) 0.1% FA in 100% MeCN. The linear gradient was 98% (A) (2 min), 98–60% (A) (40 min), 60–5% (A) (8 min), 5% (A) (2 min), 5–98% (A) (1 min), and 98% (A) (7 min).

Protein database identification and quantitation

Xcalibur software (version 2.0.7, Thermo Scientific Inc., San Jose, CA) was used to control the MS instrument and acquire MS and MS/MS data. The raw data were converted to a merged file type by Raw2msm software for protein identification [22]. Mascot Distiller software (version 2.5.1.0 [64 bits], Matrix Science Ltd., London, UK) was used to perform protein quantitation. Mascot Distiller data processing parameters were as follows: peak list transformation, "Orbitrap_res_MS2" (default parameter setting); taxonomy, "*Homo sapiens*"; tryptic digestion allowable missed cleavages, zero; quantitation, dimethylation [MD]; fixed modification (cysteine), carbamidomethyl; and peptide precursor ion tolerance, 10 ppm. MS/MS tolerance (Mascot Search Engine, Swiss-Prot database) was set to 0.8 Da. Peptide ion charge was set to 1^+, 2^+, and 3^+. The MS instrument was set to ESI-trap. Individual ion score was set to 20 (positively identified, $p < 0.05$). The quantitative ratios of proteins were listed with the values of H/L (T2DM/healthy control) as shown in Table 1.

STRING protein networks

We utilized the STRING (version 9.1, http://string-db.org) database to predict the interactions between proteins [23]. The protein quantitative result was obtained by Mascot Distiller according to deuterium-labeled/hydrogen-labeled (D/H) ratios.

Results

Sample pretreatment and shotgun proteomics

Multidimensional liquid phase chromatography (MDLC) based separation with tandem MS shotgun proteomics has advantages over gel-based separation. These advantages include a global view of protein regulation, numerous biomarker candidates, convenient procedures compared with 1D or 2D PAGE, high speed, and a high dynamic range [24, 25]. A schematic representation of the experimental procedures and apparatus is presented in Fig. 1. The conditions and concentration of reagents for protein quantitation were consistent with previous reports [19, 26]. Manual off-line HILIC fractionation was utilized to decrease the complexity of the sample mixtures and enhance protein characterization [20, 21, 27]. Protein identification and quantitation were determined and calculated using the Mascot and Distiller bioinformatic software in order to generate a protein list.

Quantitative results for serum proteins associated with T2DM

From the quantitative results based on statistical data from serum samples obtained from three T2DM patents, potential T2DM biomarker candidates were selected (Table 1).

Table 1 Identification of serum proteins in T2DM patients by MDLC coupled with tandem MS shotgun proteomic analysis

Accession[a]	Accession no.[a]	Protein name	Theoretical MW (kD)	Identification method	No. of identified peptides	Ratio	Standard deviation (RSD)
APOA2_HUMAN	P02652	Apolipoprotein A-II	11.1	MS/MS	2	0.34	–-
APOA1_HUMAN	P02647	Apolipoprotein A-I	30.8	MS/MS	33	0.53	0.11 (20.5%)
APOB_HUMAN	P04114	Apolipoprotein B-100	515.6	MS/MS	40	3.41	1.56 (61.3%)
VTDB_HUMAN	P02774	Vitamin D–binding protein	53.0	MS/MS	4	0.76	–-
IGHG4_HUMAN	P01861	Ig gamma-4 chain C region	35.9	MS/MS	16	0.47	0.25 (53.9%)
VTDB_HUMAN	P02774	Vitamin D–binding protein	53.0	MS/MS	4	0.76	–-
IGHG4_HUMAN	P01861	Ig gamma-4 chain C region	35.9	MS/MS	16	0.47	0.25 (53.9%)
HV305_HUMAN	P01766	Ig heavy-chain V–III region BRO	13.2	MS/MS	8	0.33	0.07 (22.2%)
A1AT_HUMAN	P01009	Alpha-1-antitrypsin	46.7	MS/MS	65	1.51	0.32 (21.0%)
TTHY_HUMAN	P02766	Transthyretin	15.9	MS/MS	7	0.44	–-
ANT3_HUMAN	P01008	Antithrombin-III	52.6	MS/MS	2	17.91	–-
FIBG_HUMAN	P02679	Fibrinogen gamma chain	51.5	MS/MS	3	2.01	1.80 (89.5%)
FIBA_HUMAN	P02671	Fibrinogen alpha chain	95.0	MS/MS	12	1.21	0.42 (35.0%)
K1549_HUMAN	Q9HCM3	UPF0606 protein KIAA1549	210.8	MS/MS	18	41.86	8.46 (20.2%)

[a]Accession and accession no. were obtained in UniProt database (http://www.uniprot.org/)

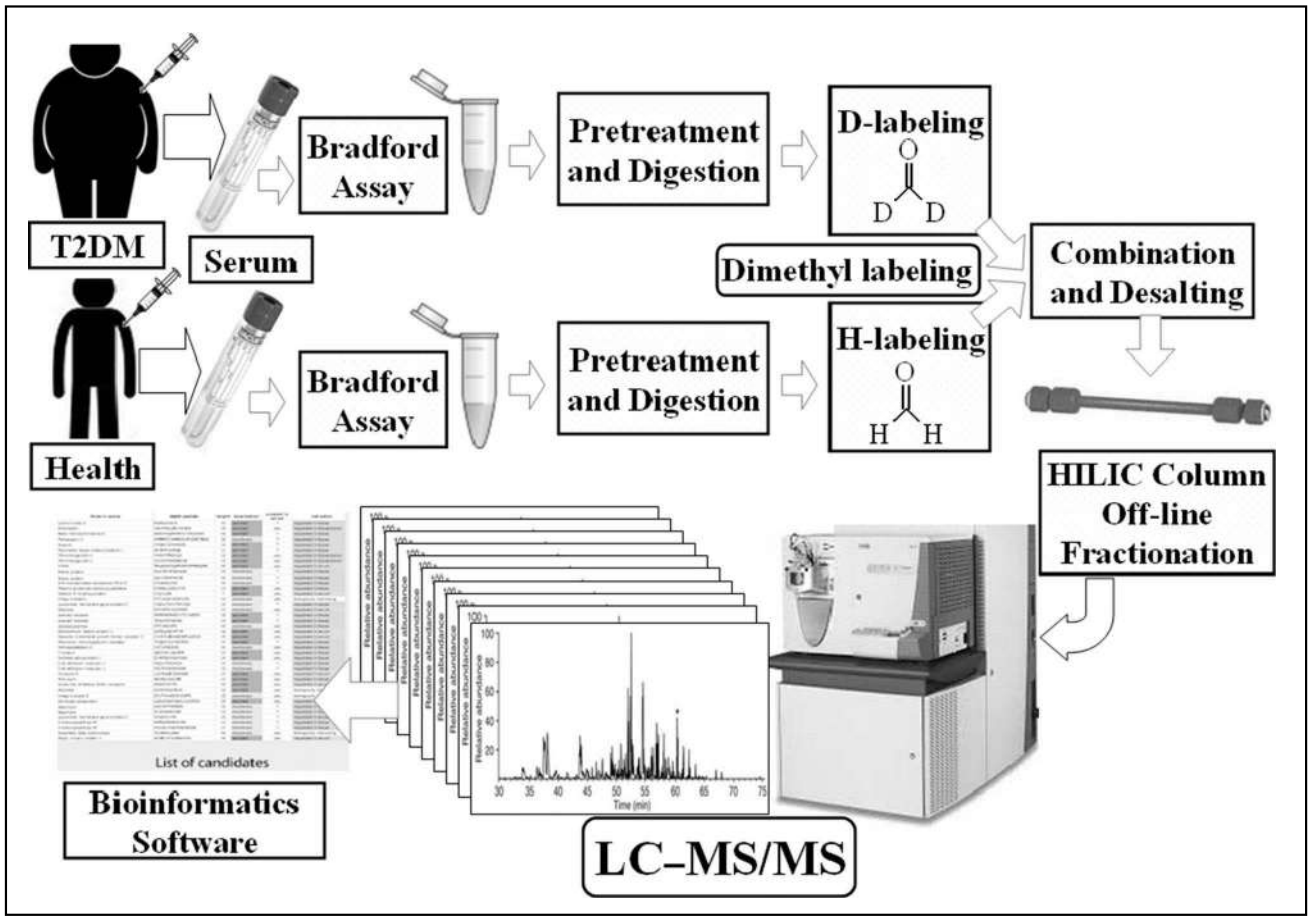

Fig. 1 Systematic flowchart of serum protein digestion including blood collection, protein pretreatment, dimethyl labeling, HILIC fractionation, tandem MS analysis, and bioinformatics software processing

MS/MS spectra of biomarker candidates

The following results were determined from qualitative and quantitative data of tandem MS. The peptide "TLADLTLLDSPIK" is an apolipoprotein B-100. As shown in Fig. 2, the MS/MS spectrum showed "TLADLTLLDSPIK" b- and y-ion patterns, which confirmed the peptide sequence (shown in Fig. 2A). The quantitative results, shown in Fig. 2B, could be calculated by LCMS peak height (AH) and peak area (AA) for which the peptides of apolipoprotein B-100 were labeled individually as formaldehyde-D_2 and formaldehyde-H_2 (dimethyl labeling method). The ratio of co-eluted deuterium-labeled peptide (m/z 732.46) and hydrogen-labeled peptide (m/z 728.44) was 2.6–2.8 ratio (Fig. 2C). Statistical results are listed in Table 2.

A vitamin D–binding protein was also characterized by tandem MS as a biomarker. MS/MS fragmentation showed the "VPTADLEDVLPLAEDITNILSK" peptide. The fragmented ions are presented in Fig. 3A, and quantitative data with AH and AA consistent with vitamin D–binding protein are shown in Fig. 3B. Deuterium- and hydrogen-labeled peptides (m/z 810.81 and 808.12, respectively) were compared and showed a ~ 0.55–0.64 fold ratio.

Apolipoprotein A-I has been identified as a T2DM biomarker candidate [10, 12]. We also identified apolipoprotein A-I protein; the MS/MS fragmentation, "VSFLSALEEYTK" peptide, is shown in Fig. 4A. With formaldehyde-D_2 and formaldehyde-H_2 labeled peptides (m/z 725.92 and 721.89, respectively) (Fig. 4C) and quantitative data AH and AA (Fig. 4B), a ratio of ~ 0.60–0.64 was determined. The AH and AA signal and the statistical ratio are listed in Table 2.

STRING network establishment by T2DM biomarker candidates

We used the STRING (version 9.1, http://string-db.org) protein interaction database to establish the relationship between twelve candidates (vitamin D–binding protein,

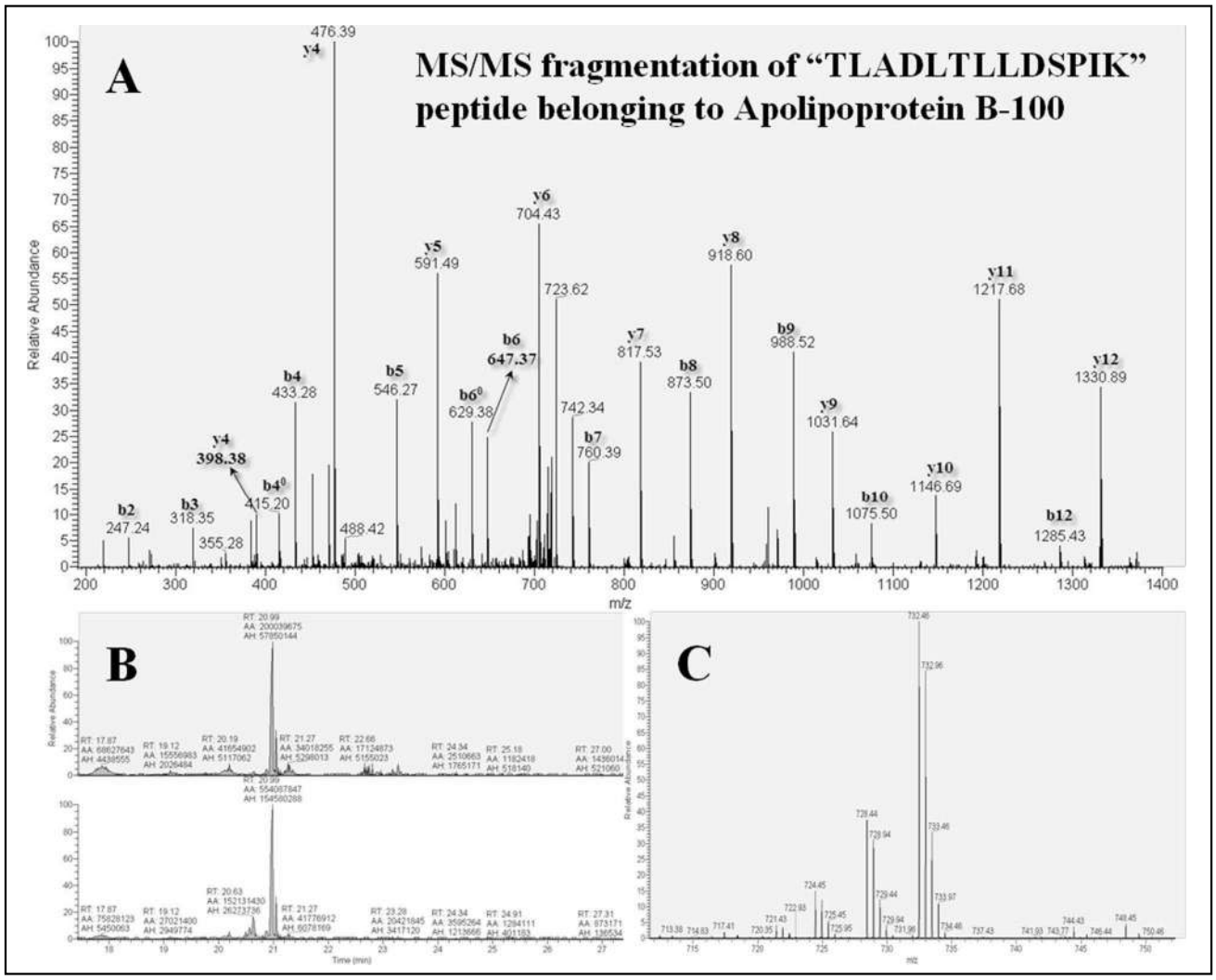

Fig. 2 MS and MS/MS spectrum of the peptide sequence TLADLTLLDSPIK belonging to apolipoprotein B-100. **A** MS/MS fragmentation of TLADLTLLDSPIK peptide showing b- and y-ion patterns. **B** Formaldehyde-H_2 labeled peptide (above) and formaldehyde-D_2 labeled peptide (below) peaks were extracted from LCMS spectrum; peak height and area show individual quantitative quality in serum from T2DM and healthy subjects. **C** Formaldehyde-D_2 labeled peptide (m/z 732.46) and formaldehyde-H_2 labeled peptide (m/z 728.44) were co-eluted under the same conditions and showed different peak intensities

Table 2 Statistical quantification results for three characterized proteins

Protein name	Peptide sequence	m/z	Charge	Peak area	Peak height	Ratio of area	Ratio of height
Apolipoprotein B-100	TLADLTLLDSPIK	728.44	2+	200,039,675	57,850,144	2.77	2.67
		732.46	2+	554,087,847	154,580,288		
Vitamin D–binding protein	VPTADLEDVL-PLAEDITNILSK	808.12	3+	776,734,809	171,876,621	0.55	0.64
		810.81	3+	425,323,077	109,906,448		
Apolipoprotein A-I	VSFLSALEEYTK	721.89	2+	6,822,440,600	375,370,811	0.60	0.64
		725.92	2+	4,117,595,256	240,180,913		

apolipoprotein B-100, apolipoprotein A2, apolipoprotein A1, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, fibrinogen gamma chains, fibrinogen alpha chains, alpha-1-antitrypsin, UPF0606 protein KIAA1549, and Ig gamma-4 chain C region proteins) shown in Fig. 5.

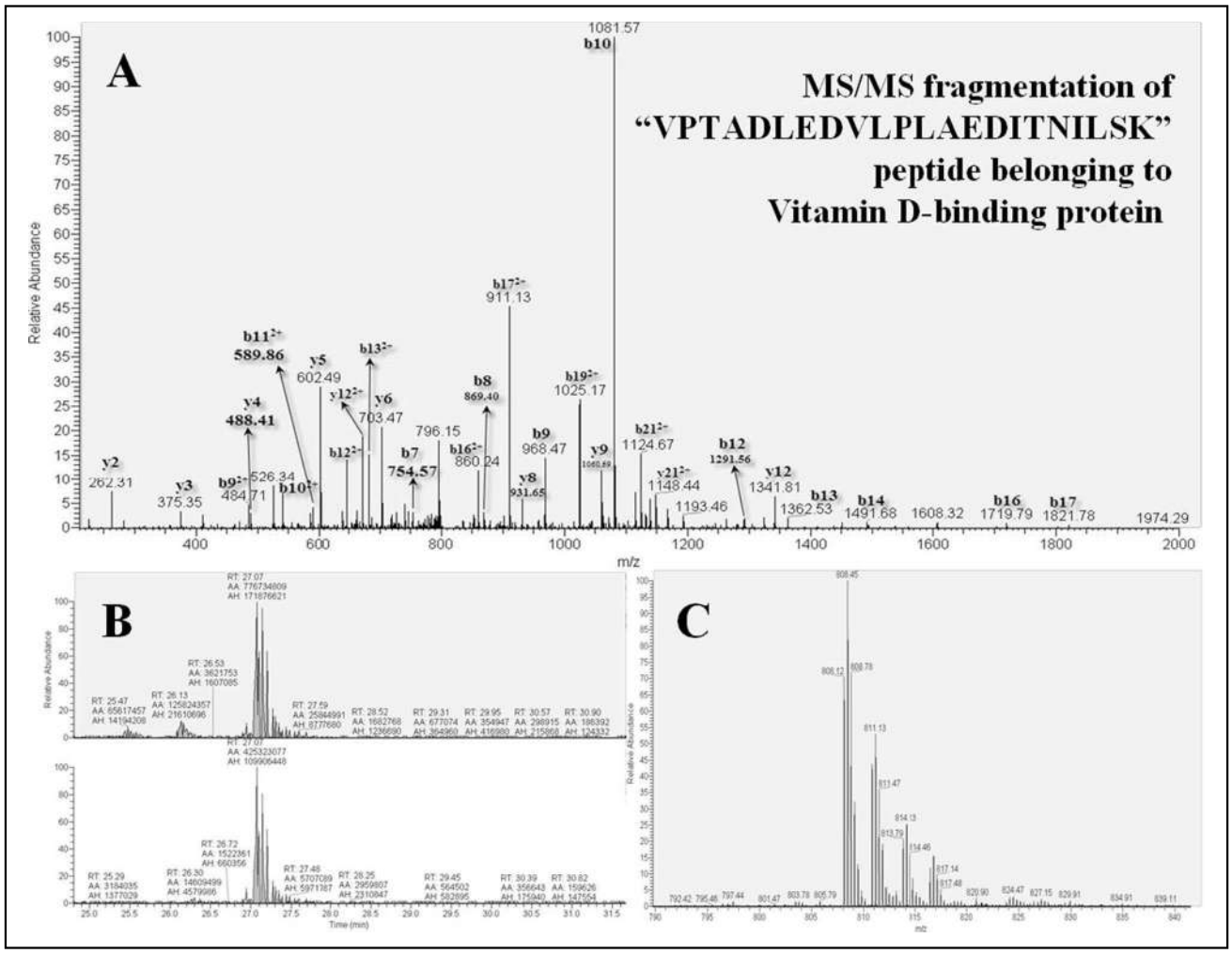

Fig. 3 MS and fragmented spectrum of vitamin D–binding protein with the peptide sequence VPTADLEDVLPLAEDITNIKSK. **A** VPTADLEDVLPLAEDITNIKSK peptide fragmentation for protein identification. **B** LC–MS extracted labeled peptides that belong to vitamin D–binding protein showed formaldehyde-H_2 labeled peptide (above) and formaldehyde-D_2 labeled peptide (below). **C** Formaldehyde-D_2 labeled peptide (m/z 810.81) and formaldehyde-H_2 labeled peptide (m/z 808.12) were co-eluted by HPLC, and the ratio was calculated as a quantitative reference

Discussion

According to previous studies, alpha-1-antitrypsin, vitamin D–binding protein, fibrinogen gamma chains, apolipoprotein A1, haptoglobin, and transthyretin have been characterized by 2D PAGE as biomarkers that can be used to evaluate diabetic patients [10]. Protein candidates, including apolipoprotein A1, apolipoprotein E, C-reactive protein, and leptin, have been identified as biomarkers by first-phase chromatofocusing, second reverse-phase HPLC, and protein identification by MALDI-TOF analysis [12]. There were several observational studies which suggested the associations between vitamin D level and components of metabolic syndrome, for example, dyslipidemia, glucose metabolism, obesity, and diabetes [28–31]. The relationship between vitamin D and T2DM is well documented. Wang et al. analyzed that the genetic polymorphism of vitamin D–binding protein was associated with increased susceptibility to T2DM in Asians, but not in Caucasians. The result of this shotgun analysis provided further evidence that vitamin D–binding protein is potentially an ethnic factor in T2DM patients. Transthyretin (TTR), a homotetrameric thyroxine transport protein, is conjugated with retinol-binding protein 4 (RBP4). TTR is involved in the regulation of glomerular filtration of RBP4 protein and in maintenance of the protein level in plasma. Only a few evidences reported that the plasma level of TTR is high in T2D, but the relation with the diabetic condition is not clear [32]. Alpha (1)-antitrypsin (AAT) is a member of the circulating serine protease inhibitor (SERPIN) superfamily. The activity of AAT is low in T2DM, and AAT therapy can hinder the progression of the disease for the type 1 in

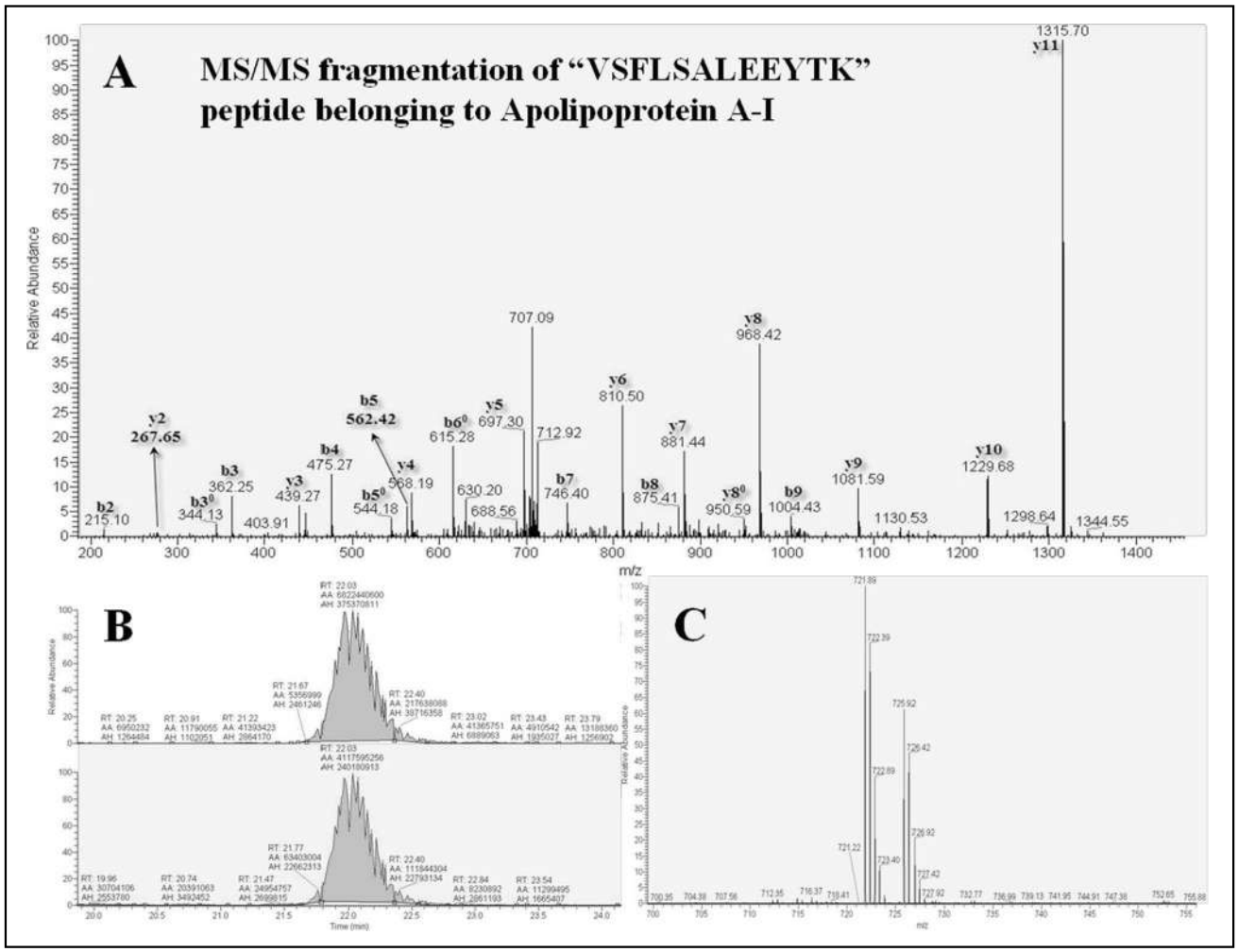

Fig. 4 MS and MS/MS spectrum of apolipoprotein A-1 with the peptide sequence VSFLSALEEYTK. **A** MS/MS fragmentation of VSFLSALEEYTK showing product ions patterns. **B** Extracted labeled peptides including formaldehyde-H_2 labeled peptide (above) and formaldehyde-D_2 labeled peptide (below); the peak area is obtained by Mascot Distiller for quantitative statistics. **C** Formaldehyde-D_2 labeled peptide (m/z 725.92) and formaldehyde-H_2 labeled peptide (m/z 721.89) were co-eluted under the same conditions and showed individual peaks for protein quantitation

mice model. The biological information and functional role of AAT in T2DM are still to be elucidated [33].

We also characterized apolipoprotein B-100, apolipoprotein A2, apolipoprotein A1, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, fibrinogen gamma chain, fibrinogen alpha chain, and alpha-1-antitrypsin proteins as biomarker candidates that could be correlated. Among the proteomic biomarkers uncovered in this study, these proteins are well characterized in numerous studies in past decades. These proteins are closely associated with the risk of type 2 diabetes mellitus. These results indicated that the shotgun approach analysis is quite reliable and reproducible in this study. However, both UPF0606 protein KIAA1549 (KIAA1549) and Ig gamma-4 chain C region proteins (IGHG4), with obvious changes in quantitative ratio, showed no relationship with the above ten proteins. Nevertheless, we selected both proteins as possible biomarker candidates.

The STRING (version 9.1, http://string-db.org) correlation illustrates that the apolipoprotein family has an evident connection, and is related to transthyretin and fibrinogen alpha chains. Furthermore, both alpha-1-antitrypsin (SERPINA1) and antithrombin-3 (SERPINC1) had a strong relationship, and they expanded to other proteins with less related strength. C-reactive protein (CRP) is correlated to fibrinogen, plasminogen activator inhibitor-1, antithrombin 3, and coagulation factors in patients with T2DM who had microvascular complications. It is suggested that CRP is linked with increased risk of thrombus formation [34]. The STRING suggests several proteins are inter-related and multi-linked with each other to contribute to the pathogenesis of T2DM. This study gives valuable

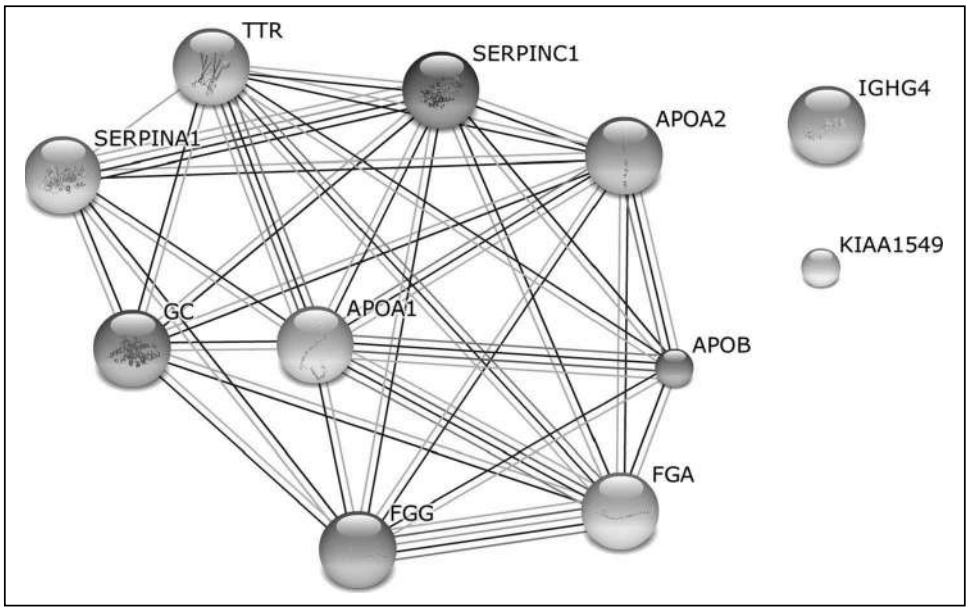

Fig. 5 STRING protein interaction database software for determining relationships between apolipoprotein family, vitamin D–binding proteins, fibrinogen gamma chains, fibrinogen alpha chains, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, alpha-1-antitrypsin, UPF0606 protein KIAA1549, and Ig gamma-4 chain C region

insights of additional biomarker information of the disease, and further investigations are required to demonstrate their clinical significance.

Diabetes frequently occurs in Taiwan; the incidence rate of diabetes and associated medical expenses are greatly growing in recent years. Type 2 diabetes is the most common chronic metabolic disease and its syndrome is associated with hyperglycemia, insufficient insulin secretion, and increased blood glucose. To date, there is still no satisfactory medicine available to treat the disease. Risk factors may vary with ethnicity and among various populations. Therefore, it is important and valuable to identify useful biomarkers for subjects with diabetic disease.

We have identified that T2DM biomarker candidates include vitamin D–binding proteins, proteins belonging to the apolipoprotein family, fibrinogen gamma chains, fibrinogen alpha chains, transthyretin, Ig heavy-chain V–III region BRO, antithrombin-3, and alpha-1-antitrypsin. UPF0606 protein KIAA1549 with a high quantitative ratio could not be linked to any other proteins. Moreover, Ig gamma-4 chain C region with a low ratio was not related to any proteins. In the future, we consider that the confirmation of potential biomarker candidates may require additional validation, such as an ELISA assay or MRM MS scanning. Nevertheless, the results will be provided in the early intervention of diabetes prevention and diagnosis.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval All serum samples and procedures were approved by the clinical research ethics committee at Kaohsiung Medical University Hospital.

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ORIGINAL ARTICLE

SNPs in the *catalase* promoter: a study based on Indian diabetic individuals

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Abstract

Background It is known that anti-oxidant defense is compromised in diabetic individuals. In our study, we indeed found decreased catalase activity in diabetic individuals compared to non-diabetic controls. Therefore, we analyzed the single nucleotide polymorphisms (SNPs) in the promoter region of catalase in diabetic individuals. To investigate catalase promoter SNP's contribution (if any) to the low catalase activity and assess their functional significance by reporter assay.

Methods The activity of catalase was quantitated from 109 non-diabetic and 138 diabetic individuals. Genomic DNA isolated from these individuals was screened for SNPs in the *catalase* promoter by direct sequence analysis of PCR products. The functional effect of these polymorphisms was checked by reporter assay.

Results We found six reported SNPs of which, three were polymorphic in our study groups at −330, −89, and −20 position viz. rs1001179: C>T, rs7943316: A>T, and rs1049982: T>C. Out of the three SNPs, only rs1001179: C>T showed a positive association with the occurrence of low activity of catalase in diabetic individuals ($p<0.05$). However, reporter assay confirmed that the presence of these SNPs does not significantly affect the transcriptional activity of the gene ($p>0.05$). Furthermore, our in silico analysis revealed that the presence of these SNPs did not significantly affect the binding of transcription factors except at −330 position.

Conclusion Our reporter assay suggesting no functional relevance of promoter SNPs in reduced catalase activity observed in Indian diabetic individuals is a novel finding.

Keywords Diabetes · SNP · Single nucleotide polymorphisms · Catalase

Introduction

Reactive oxygen species (ROS) are formed continuously during metabolic reactions and are removed by anti-oxidant defense mechanism, including enzymatic and non-enzymatic systems. Although low ROS levels are necessary for cell growth, differentiation, apoptosis, and immune response, excess ROS cause oxidative stress (OS) [1, 2]. OS is known to contribute to the pathophysiology of several neurodegenerative and cardiovascular diseases, cancer, and metabolic disorders such as type 2 diabetes (T2D) [3].

T2D, characterized by hyperglycemia, occurs due to insulin insufficiency or insulin resistance. It is well established that OS is involved in establishing T2D and, more significantly, in the development of diabetic complications [4]. In particular, islets are known to succumb more easily to oxidative damage due to their poor anti-oxidant defense [5]. This results in further deterioration and loss of function of the insulin-producing β cells, leading to hyperglycemia. Persistent hyperglycemia leads to microvascular diabetic complications such as retinopathy, neuropathy, and nephropathy in diabetic individuals [6, 7]

Among the anti-oxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) form the first line of the anti-oxidant defense and are primarily crucial in removing ROS. SOD dismutates O_2^- to H_2O_2, which in turn is degraded by catalase. The efficient activity of these enzymes is necessary for the removal of excess ROS. Individuals with reduced catalase and SOD activity have an increased incidence of OS-related diseases, such as atherosclerosis, diabetes, dyslipidemia, and neurodegenerative diseases. Our previous study found that catalase activity in newly diagnosed T2D individuals was significantly low compared to non-diabetic healthy individuals [8]. While this could be due to an increased level of ROS under hyperglycemic conditions, genetic variations both in the coding and promoter regions of the *catalase* (*CAT*) gene can also lead to decreased or impaired enzymatic

activity. Several polymorphisms in the gene encoding for catalase and its promoter region have been identified. Some of these have been shown to be associated with reduced enzyme activity in many diseased conditions such as vitiligo, depressive disorder, bronchial asthma, and diabetes, [9–12]. However, the mechanism by which each of these variants might influence the expression and hence the enzyme activity has not been elucidated.

The present study is based on our finding of significantly low catalase activity in Indian diabetic individuals compared to healthy non-diabetic controls. The study was undertaken to check whether SNPs in the *CAT* promoter region could be contributing to low catalase activity observed in diabetic individuals. For this, we scanned the *CAT* promoter for both known and unknown SNPs and investigated whether these SNPs have an association (if any) with diabetes occurrence. Furthermore, a functional reporter assay was also performed to check the influence of SNPs, if any, in the *CAT* promoter region on catalase expression.

Materials and methods

Sample collection and estimation of catalase activity

Samples used in this study are from those collected in our earlier work and approved by the Institutional Ethics committee. The sample size was calculated using G* power software 3.1.9.4, setting the alpha value at 0.05. A total of 247 samples were used for the present work. Fifty-three samples were from study Ref no. KEMHRC/VSP/Dir.Off/EC/661 and 194 samples from another study Ref. no. ECN- ECR/352/Inst/NIH/2013. Non-diabetic controls (n=109) with fasting blood sugar level < 125 mg/dL and HbA1c < 6.5%, and T2D individuals were (n=138) with fasting blood sugar level > 126 mg/dL and HbA1c ≥ 6.5%. From all the subjects, 10-ml fasting blood sample were collected by Golwilkar Metropolis, Pune – 411004, and fasting glucose and HbA1c were measured at Golwilkar Metropolis on an automated analyzer. Whole blood samples were centrifuged at 4000 rpm for 10 min to separate plasma and erythrocyte fraction. For further analysis, plasma, serum, and erythrocyte lysate were stored separately at −80°C. We used erythrocyte lysate to measure the catalase activity, which was determined following the standard protocol [13]. The decomposition rate of H_2O_2 by catalase was monitored at 240 nm. One unit of catalase is equal to 1 mmol of H_2O_2 decomposed per minute. The enzyme activity was expressed as units per mg protein.

DNA extraction and PCR-sequencing

Genomic DNA was extracted from venous blood by phenol-chloroform extraction and ethanol precipitation [14]. Using the gene sequence of *CAT* promoter from the NCBI website, primers were designed for a region of 921 bps (−834 bp to +87 bp) relative to the transcription start site (TSS) (forward primer 5′-GGGGAAGCAGATTTCTCCAGTGTTT-3′ and reverse primer 3′- CTCGGGGAGCACAGAGTGTAC-5′). To design forward and reverse primers, we used nucleotide BLAST (BLASTn) from NCBI (http://www.ncbi.nlm.nih.gov/), and Primer3 (Version 0.4.0) [15, 16] (Fig. 1a,b). This region of 921 base-pairs includes six known SNPs (rs148068536:del>T, rs17883920:G>A, rs1001179:C>T, rs57470823:C>T, rs7943316:A>T, and rs1049982:T>C) as listed in Table 1. The total reaction volume of 25 μl for PCR contained 10–15 ng of genomic DNA template, 20 pM of forward and reverse primers each, and 200 μM dNTP master mix (Bangalore Genei Pvt. Ltd., India). The thermal cycle conditions were set as initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 59.6°C for 40 s, extension at 72°C for 35 s, and a final extension at 72°C for 5 min. PCR products were purified to remove dimers or non-specific bands using polyethylene glycol (PEG 8000; Sigma-Aldrich, USA). The purified PCR products were resolved in 2% agarose gel with a 100 bp ladder (Fig. 1c). The purified amplicons were sequenced using the BigDye Terminator v3.1 chemistry in ABI3700 capillary sequencer (Applied Biosystems, USA) according to manufacturer's protocols at National Centre for Microbial Resource (NCMR), National Center for Cell Sciences (NCCS), Pune, India.

Reporter gene constructs, cell culture, and transfection

The human *CAT* core promoter region (−848 to +49) was amplified using a DNA template from control non-diabetic and diabetic individuals with forward primer 5′-CCCT CGAGATTTGGGGAAGCAGATTT-3′ and reverse primer 5′-GATCAAGCTTCTAAACGGACCTTCGGGC-3′ containing restriction sites Xho I and Hind III complementary with pGL3 enhancer vector. The PCR reaction was carried out using high fidelity polymerase (New England Biolabs, UK) with initial denaturation at 94°C for 5 mins, 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s with 45-s extension at 72°C. The final extension was carried out at 72°C for 5 min. PCR product of 1072 bp was *A*-tailed with Taq polymerase (New England Biolabs, UK) and cloned into a pGEM-T easy vector (Promega, USA). Both *CAT* core promoter region containing plasmids were digested with Xho I and Hind III and gel purified by QIAGEN endotoxin-free kit (Qiagen, Germany). Furthermore, product was subcloned into Xho I and Hind III sites of pGL3 enhancer vector (Promega, USA), resulting in two constructs pGL3-enhancer-control and pGL3-enhancer-SNP. These two constructs contain a transcription initiation site that was mutated to AAG using site-directed mutagenesis (Stragene quick change kit, USA) to

Table 1 SNPs in the promoter region of the *CAT* gene

Sr. no.	NCBI ref. SNP ID	Bases upstream to TSS	Clinical association	Nucleotide	Ancestral allele	Significance (p value)	Reference
1	rs148068536	−542	Unknown	-/T	T	–	1000Genomes Consortium (Mar 11, 2011)
2	rs17883920	−533	Unknown	G/A	G	–	Deborah Nickerson (Feb 03, 2005)
3	**rs1001179**	−330	Known	C/T	C	0.03	Chambliss et. al. 2020
4	rs57470823	−254	Unknown	C/T	C	–	Ning Z et. al. (Nov 26, 2007)
5	**rs7943316**	−89	Unknown	A/T	A	0.51	Michael Z. et. al. (Jul 04, 2003)
6	**rs1049982**	−20	Unknown	C/T	T	0.43	Christopher Lee (Sep 13, 2000)

avoid early transcription of luciferase. The sequences of the constructs were verified by sequencing, and plasmids were purified using the QIAGEN endotoxin-free kit (Qiagen, Germany).

Human hepatoma S10-3 cells obtained from National Institute of Virology, Pune were cultured in DMEM, containing 1× penicillin-streptomycin-glutamine solution (Gibco, USA) and 10% FBS at 37°C and 5% CO_2. S10-3 cells were seeded in 6-well plate at a density of 5×10^5 cells per well and incubated overnight. These cells were transfected with 500 ng of either pGL3-enhancer-control or pGL3-enhancer-SNP using lipofectamine3000 reagent (Invitrogen, USA). The transfection solution was replaced after 4 h with a fresh medium containing 10% fetal bovine serum. Forty-eight hours later, cells were harvested, and according to the manufacturer's protocol, luciferase activities were determined (PerkinElmer Victor X3, USA). The pGL3-enhancer derived plasmids were always co-transfected with 100 ng of pGL4.74 vector for variable transfection efficiency correction. The data of at least three independent experiments were expressed as mean ± standard deviation.

Statistical analyses

ClustalW was used for multiple sequence alignment from BioEdit Sequence Alignment Editor (version 7.1.3.0). A Chi-square test was performed to determine if SNPs' allele frequencies conformed to Hardy-Weinberg equilibrium (HWE). Since the population belongs to a common geographical area, the effects of admixture and mixed ancestry on the allele frequencies cannot be ruled out. Hence, we employed STRUCTURE program version 2.3.4 for inferring population ancestry (Q) and divergence (Fst), keeping 10,000 burn-in periods and 100,000 repetitions [17]. The software has two programs: (1) admixture model/ancestry model, which considers Dirichlet's parameter for the degree of admixture (α) as 1 as an initial value. Degree of admixture ranges from >1 to <1, indicating either the individuals are admixed or essentially from one population respectively; (2) allele frequencies model assumes that allele frequencies are correlated among populations if they have recent shared ancestry or have different Fst value for different subpopulations. Fst value ranges from 0 to

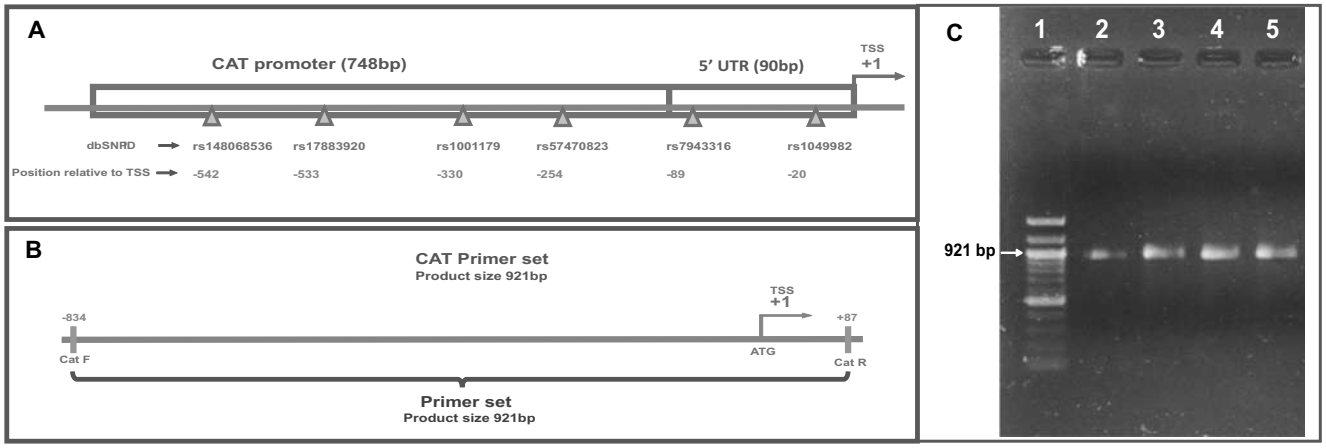

Fig. 1 **a** Structure of *CAT* promoter showing the relative position of all six known SNPs reported in dbSNP to transcription start site. **b** *CAT* promoter region amplified using specific primer pair covering the entire core promoter. **c** Amplified PCR product electrophoresed on 2% agarose gel, lane 1—100 bp ladder, lanes 2–5 PCR product of 921 bp

1, indicating free interbreeding in the population or the two groups do not share any genetic diversity, respectively [17]. Linkage disequilibrium (LD) analysis was carried out between the polymorphic SNPs to determine the disequilibrium coefficient (D') and correlation coefficient (r). The pair of SNPs were considered to be in high LD if $D' > 0.8$ and $r > 0.5$ [18]. McNemar's 2×2 contingency tests using a multiplicative genetic model based on allele frequencies were used for odds ratio estimation to check the association between *CAT* promoter SNP and disease occurrence [19]. An independent samples *t*-test for finding an association between catalase activity and allele frequency of SNPs with the diabetic condition was carried out using SPSS software (SPSS Statistics 27). A Student's *t*-test (two-tailed) was used for reporter assay to determine significant differences at $p < 0.05$. In silico analysis of differential binding of transcription factors (TFs) to the *CAT* promoter's polymorphic sites was carried out by transcription factor affinity prediction (TRAP) Web Tool [20].

Results

Clinical characteristics

Diabetic individuals (n= 138; 74 males and 64 females) had a significantly high concentration of fasting glucose and HbA1c, 161.44 ± 61.79 mg/dL and 8.41 ± 1.91 %, respectively, compared to control non-diabetic individuals with fasting glucose 89.78 ± 8.64 mg/dL and HbA1c 5.59 ± 0.38 % ($p < 0.01$).

Catalase activity was significantly low (2840.23 IU/ml ± 1480.23) in diabetic individuals than (41,31.01 IU/ml ± 4897.51) in control non-diabetic individuals ($p < 0.01$) (Supplementary table-1).

Polymorphisms in the CAT promoter and allele frequencies

PCR products were obtained after amplifying 921 bps promoter sequence of *CAT* (Gene ID: 847) of all the control non-diabetic and diabetic samples. PCR products were subjected to sequencing, and sequences obtained were scanned for SNPs' presence. We detected only six SNPs reported in dbSNP, of which only three were found to be polymorphic among our studied groups *viz*., rs1001179:C>T, rs7943316: A>T, and rs1049982: T>C. Allele frequency distribution for polymorphic SNPs across two study groups is given in Table 2. LD was calculated between the polymorphic SNPs rs1001179:C>T and rs1049982:T>C that are farthest from each other to check the influence of allele distribution pattern of one over other using 2×2 contingency model [18]. LD coefficient (D'=0.096) and correlation coefficient (r^2=1.58) between the two SNPs indicated that the pair of SNPs are in LD. The study groups were also tested for Hardy-Weinberg equilibrium (HWE) and found obeying HWE across the three SNP loci.

Among the three SNPs, allele T of rs1001179:C>T was found to be predominant in the diabetic group compared to the control non-diabetic group. Chi-square (χ^2) value of this rs1001179:C>T (χ^2 table value = 5.18 df = 1, $p < 0.05$) also suggested significant difference between control non-diabetic and diabetic individuals. Chi-square (χ^2) values of other two SNPs rs7943316:A>T (χ^2 table value = 0.480 df = 1, p= 0.488) and rs1049982:T>C (χ^2 table value = 0.798 df = 1, p= 0.371) do not suggest significant difference between control non-diabetic and diabetic individuals. To further verify, the allele frequency data of rs1001179:C>T was used to calculate risk association using McNemar's 2×2 contingency table. A positive coefficient of association (ϕ=+0.14) and risk ratio (1.9443) was found with rs1001179:C>T with a 95% confidence interval (1.0738–3.5204). It suggested a slight positive association of allele T of rs1001179:C>T with diabetes occurrence. We also tried to determine the correlation between the occurrence of alleles of rs1001179:C>T, rs7943316: A>T, and rs1049982: T>C and catalase enzyme activity among control non-diabetic and diabetic individuals. The presence of allele T of rs1001179:C>T showed a significant association with decreased catalase activity in control non-diabetic (p=0.04) and diabetic (p=0.00) individuals. However, the presence of alleles T and C of rs7943316: A>T and rs1049982: T>C, respectively, did not show any association in both the study groups (p>0.05).

Since the sampled individuals were from the same geographical area (Pune), it is essential to rule out sampling bias, which may influence the distribution of alleles of the SNP and the allele frequency. Therefore, ancestry/admixture model was used in the STRUCTURE software, which takes into account, the sampling location, i.e., individuals from the same sampling location often come from the same population. Here, for K = 2 (for two study groups), the mean alpha—value was estimated to be +0.2765, implying that participants of the study belonged to an admixed population, i.e., they are of mixed ancestry (indicated by two different colors in Fig. 2) and do not share a common ancestor. The STRUCTURE

Table 2 Allele frequency distribution for three polymorphic SNPs in *CAT* promoter

Locus	Control (n=109)	Disease (n=138)
−330 (rs1001179)	C=0.881	C=0.768
	T=0.119	T=0.232
−89 (rs7943316)	A=0.587	A=0.630
	T=0.413	T=0.370
−20 (rs1049982)	C=0.56	C=0.616
	T=0.44	T=0.384

software's allele frequencies model was employed to estimate Fst, which was found to be 0.5358 ± 0.12, suggesting gene flow and heterozygosity in the sampled population.

Effect of polymorphic SNPs (rs1001179:C>T, rs7943316:A>T, and rs1049982:T>C) on transcriptional activity

To understand the SNPs functional relevance in the promoter region, reporter assay was carried out using pGL3-enhancer-control and pGL3-enhancer-SNP. A significant difference was not observed between normalized luciferase activities of pGL3-enhancer-control and pGL3-enhancer-SNP in S103 cells (Fig. 3a, b). This indicates that these SNPs do not significantly affect the *CAT* expression in diabetic individuals.

Furthermore, in silico analysis was performed, using TRAP Web Tool [20] to detect potential TFs binding in the *CAT* promoter region, specifically at −330, −89, −20. In both control non-diabetic and diabetic groups, similar transcription factors were found to bind at −89 and −20 positions. However, at position −330 rs1001179 $C^{-330}T$, c-REL (NF-κB member) TF was found to be binding in the diabetic group instead of GATA1 found in non-diabetic controls. However, both GATA1 and c-REL have not been reported to activate *CAT* transcription (Fig. 4).

Discussion

Several studies have reported the involvement of OS in the development of T2D and, more importantly, in its complications[21–23]. Therefore, the anti-oxidant defense of an individual, including anti-oxidant enzymes and anti-oxidant molecules, becomes very important. We observed a significant decrease in the activity of one of the important anti-oxidant enzymes CAT in the diabetic population. One of the reasons for this decrease in the enzyme activity could be the presence of SNPs in the gene and its promoter region. In this study, we have examined SNPs in the promoter region of *CAT* in both control non-diabetic and diabetic individuals. The *CAT* promoter (from −834 bp to +87 bp to TSS) was PCR amplified and sequenced. Out of six reported SNPs in this region, only three, viz. rs1001179:C>T, rs7943316:A>T, and rs1049982:T>C, situated upstream of the transcription start site (TSS), at −330, −89, and −20 bp, respectively, were found to be polymorphic within the two study groups.

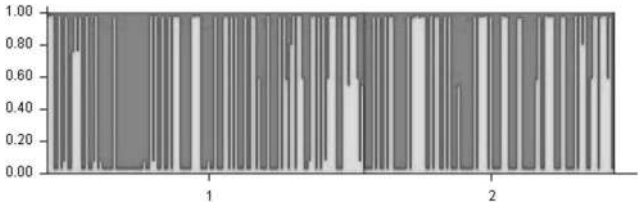

Fig. 2 Summary plot of admixture estimation (*Q*), each individual is represented by a single vertical line broken into two colored segments, with length proportional to each of the two inferred clusters, diabetic (red) and control non-diabetic (green). The numbers 1 and 2 correspond to the pre-defined clusters, diabetic, and control non-diabetic, respectively

Among the observed polymorphic SNPs (rs1001179:C>T, rs7943316:A>T, and rs1049982:T>C), the predominance of allele T of rs1001179:C>T was found to be positively associated with the incidence of diabetes. This SNP observed in our diabetic population has been previously reported to be associated with several disease conditions. These include RSV Bronchiolitis and pancreatic cancer in the USA population [24, 25], cervical cancer in Portugal population [26], prostate cancer in Turkey population [27], and breast cancer in the Iran population [28]. Association between type 1 diabetic neuropathy and this SNP has also been reported previously in the Russian population [29]. However, our risk association analysis suggests that allele C/T of rs1001179:C>T has a marginal positive association with the occurrence of type 2 diabetes. Further study with large sample size is required to ascertain these findings, including diabetic individuals with microvascular complications.

Earlier studies have reported the presence of SNP rs769217 in the coding region of *CAT* (exon 9) and its association with various diseases. SNP rs769217 showed association with decreased catalase and increased glucose and HbA1c in the Hungarian population [30], with primary open-angle glaucoma in a Chinese population [31], increased risk of type 1 diabetes in the Hungarian and Russian population [32, 33], increased risk of vitiligo in UK population [34], increased risk of osteonecrosis in Korean population [35], and higher bone mineral density in Korean women population [36]. In this study, we have looked at SNPs in the promoter region of the *CAT*. Since SNPs in the promoter region could also influence the expression of the gene, and hence the enzyme's activity, we were curious to know whether these three polymorphic SNPs in the promoter of the *CAT* influence catalase activity. Therefore, further analysis of the correlation between the occurrence of allele C or T of rs1001179:C>T among control non-diabetic and diabetic individuals with catalase activity ($p<0.05$) was carried out. It revealed that allele T of rs1001179:C>T is associated with a significant decrease in catalase activity in the diabetic group ($p=0.00$). However, the presence of alleles T and C of the other two SNPs rs7943316:A>T and rs1049982:T>C, respectively, were not found to be associated with a decrease in catalase activity in the diabetic groups ($p>0.05$).

We therefore, further investigated whether SNP rs1001179:C>T in the promoter region has any influence on the transcriptional activity of *CAT* using a reporter assay. Results obtained indicated that three SNPs together did not significantly affect the transcriptional activity of a gene. Forsberg et al. in 2001 showed a non-significant association

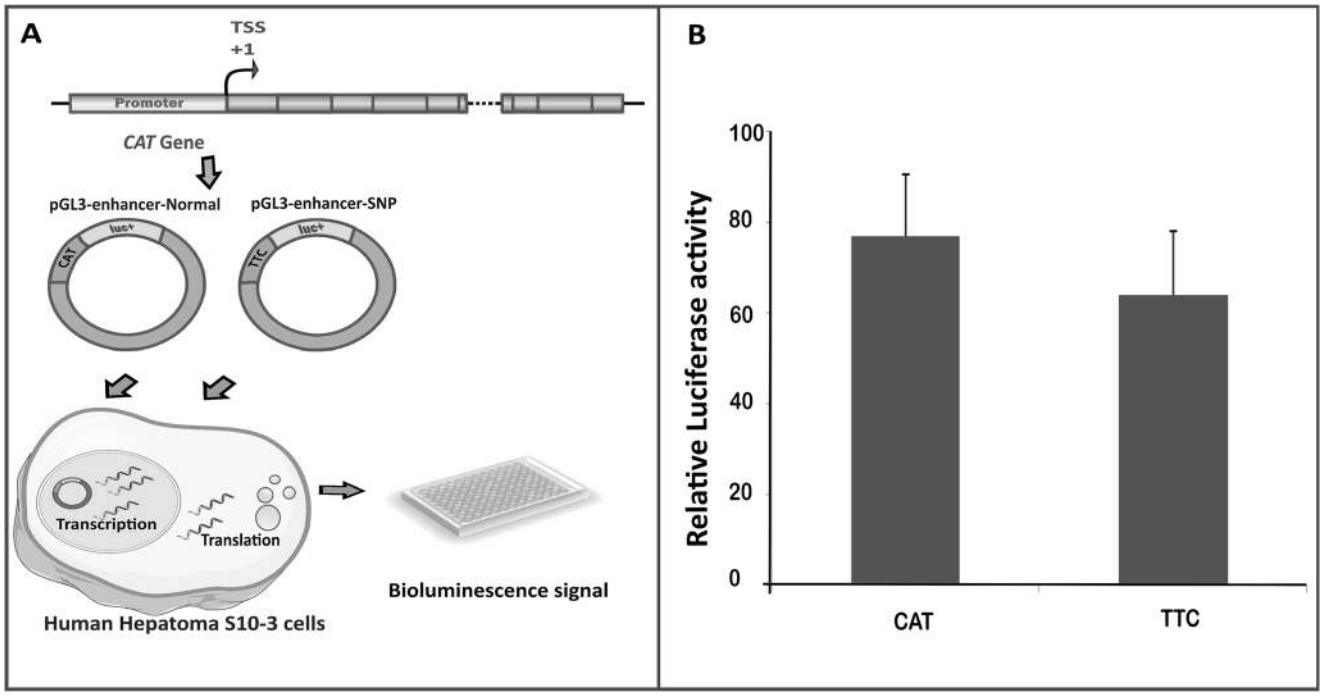

Fig. 3 **a** Schematic diagram for reporter gene assay **b** Significant difference in the transcriptional activity of human *CAT* promoter variants was not observed

between increased blood catalase protein level and the presence of allele T of rs1001179:C>T SNP in the promoter region of the *CAT* in a healthy Swedish population (n=29) [37]. In our reporter assay, we did not find a significant increase or decrease in transcriptional activity in the presence of all three SNPs in the *CAT* promoter. This could be because of population-based differences and the use of all three SNPs together for the reporter assay.

Further in silico analysis of promoter region for binding of TFs using TRAP Web Tool revealed that majority of TFs (SP1, NF-Y, and C/EBP-β) binding in promoter region remained same except at −330 position where c-REL was found to be binding in place of GATA1 in the diabetic group. GATA-1 is a cis-regulatory transcription factor of RNA polymerase-II and its role in activating catalase expression is unknown. c-REL transcription factor belongs to the NF-kB family of dimeric transcription factors and requires dimerization with its protein family member p50, p52, p65, c-REL, and RelB to become functionally active. Since at the −330 position only one subunit of NF-κB family transcription factor, c-REL was observed to bind, significant effect on the transcriptional activity of CAT was not seen.

In summary, six reported SNPs were observed in the 921bps sequenced *CAT* promoter region, out of which three were polymorphic, namely rs1001179:C>T, rs7943316:A>T, and rs1049982:T>C, in control non-diabetic and diabetic groups. Furthermore, allele T, of rs1001179:C>T, was found to be significantly associated with the decreased catalase

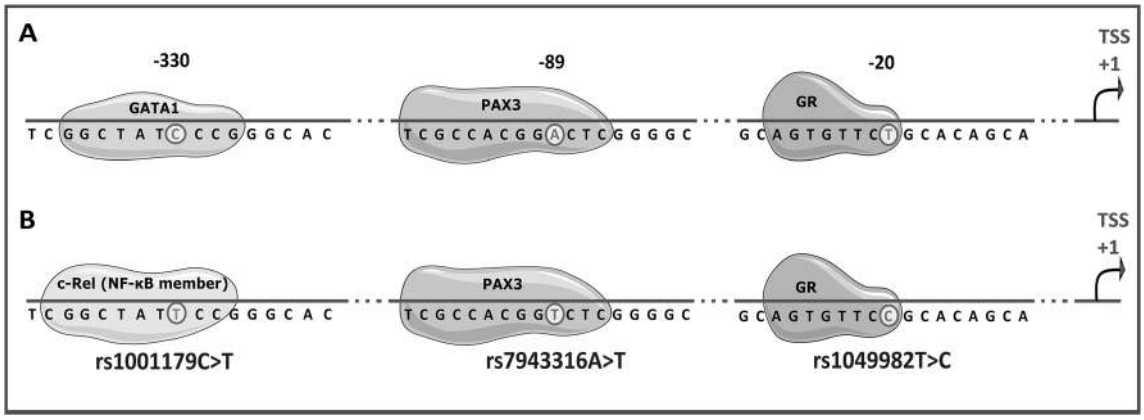

Fig. 4 Transcription factors binding at three polymorphic SNPs found in our population using TRAP web tool. **a** Control non-diabetic. **b** Diabetic. The difference in the binding of transcription factors was observed only at the −330 position

activity in diabetic individuals. However, in functional reporter assay, with haplotype CAT (control) or TTC (diabetic), a significant difference in the transcriptional activity was not observed. Ours is the first study reporting three polymorphic SNPs in the promoter region of CAT in the Indian diabetic population with no functional relevance in reducing catalase activity observed in these individuals.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13410-022-01051-w.

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Author contributions Dipak Kadam: formal analysis; methodology; resources; writing—review and editing. Saurabh D. Kalamkar: Resources. Amit Saraf: methodology. Imran Pathan: investigation, methodology, writing—original draft preparation. Jhankar Acharya: formal analysis; writing—review and editing. Komal Pekhale: formal analysis; methodology. Yogesh Shouche: resources. Kavita Lole: resources. Richa Ashma: conceptualization; formal analysis; supervision; validation; writing—original draft preparation; writing—review and editing. Saroj Ghaskadbi: conceptualization; funding acquisition; supervision; validation; writing—review and editing.

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Data availability The data that supports the findings of this study are available in Appendix, table-1 of this article.

Declarations

Conflict of interest The authors declare no competing interests.

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

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ORIGINAL ARTICLE

Basic fibroblast growth factor alleviates metabolic abnormalities in the heart of streptozotocin-induced diabetic rats

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Abstract

Background Basic fibroblast growth factor (bFGF) has the ability to treat diabetic cardiomyopathy, but its therapeutic mechanism is still far from being fully understood. The aim of this study was to investigate the impact of bFGF on heart metabolism in streptozotocin-induced diabetic rats and explore possible metabolic mechanisms.

Methods We analyzed metabolic profile in the heart of the normal control, diabetic, and bFGF-treated diabetic rats by using a nuclear magnetic resonance (NMR)–based metabolomics approach. Principal component analysis was used to examine metabolic pattern changes and identify important metabolites. Metabolic pathway analysis was carried out using significantly altered metabolites.

Results We found that bFGF treatment can effectively attenuate myocardial fibrosis in the diabetic rats. Metabolomics results show that metabolic phenotypes were significantly changed in the heart among normal control, diabetic, and bFGF-treated diabetic rats. Diabetic rats had significantly higher levels of taurine, leucine, isoleucine, valine, and glutamate, but lower levels of acetate, aspartate, creatine, succinate, glutamine, and high-energy phosphate pool (AXP) in the heart than normal control rats. Of note, these metabolic disorders can be partly reversed in the heart of diabetic rats after bFGF treatment.

Conclusions Our results reveal that bFGF attenuated diabetic cardiac fibrosis and metabolic disorders mainly involving amino acid metabolism and energy metabolism. However, their causal associations still need to be further investigated.

Keywords Diabetes · bFGF · Heart · Metabolomics · Metabolic disorder

Introduction

Diabetic cardiomyopathy (DCM), one of the serious diabetic complications, is a leading cause of death in diabetic patients [1]. An epidemiological report has shown that about 16.9% of diabetic patients suffered from DCM [2]. So far, several potential mechanisms underlying DCM were elucidated, such as myocardial structural injury [3, 4], lipid accumulation [5], energy metabolism disturbance [6], oxidative stress [7, 8], cardiocyte apoptosis [9], and myocardial fibrosis [10]. Since the prevalence of DCM is increasing worldwide, its prevention and treatment have attracted considerable attention. So far, several chemicals or drugs have been used to ameliorate DCM including breviscapine [11], matrine [12], and pioglitazone [13]. However, it is still urgent to develop the new potential therapy for DCM.

Basic fibroblast growth factor (bFGF) is one of the members in the FGF family and exerts a promising ability to treat diabetes and its complications. For example, Li et al. reported that implantation of bFGF-treated islet progenitor cells into the diabetic rats was capable of ameliorating diabetes [14]. A reduction in serum bFGF level was found to increase the risk of cardiovascular disease in diabetic patients [15]. Facchiano et al. revealed that glycation reduced bFGF level and thereby impaired angiogenesis in diabetes mellitus [16]. Moreover, the regulation of cardiac angiogenesis and repair by bFGF was also reported by Zhao et al. [17] and Landau et al. [18]. Treatment with bFGF also exerted a protective effect on ischemia/reperfusion-induced cardiac damage in diabetic rats [19]. Xu et al. revealed that bFGF treatment can achieve an early repair of ischemic heart by preventing

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cardiomyocyte apoptosis [20]. In addition, Cui et al. found that treatment with bFGF restored myocardial infarction and improved cardiac function via mediating angiogenic effect [21]. These findings imply that bFGF may have a great potential in the treatment of DCM, but its metabolic mechanisms are still far from being fully understood.

Metabolomics attempts to analyze all low molecular weight metabolites in biological samples under a particular condition, such as disease or drug treatment [22], and has been used to analyze drug-induced metabolic changes and elucidate pharmacological mechanisms [23]. In the present study, therefore, we analyzed the metabolic changes in the heart of streptozotocin-induced diabetic rats after bFGF treatment using a nuclear magnetic resonance (NMR)–based metabolomics approach and aimed to explore the protective mechanism of bFGF on DCM.

Methods

Animals

Male Sprague-Dawley (SD) rats (body weight = 180–200 g) were purchased from the SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in a specific pathogen-free colony under a fully controlled condition (temperature, 22 ± 2 °C; humidity, 47 ± 2%) and 12-h/12-h light/dark cycle (lights on at 8:00 a.m.) at the Laboratory Animal Center of Wenzhou Medical University (WMU, Wenzhou, China). All rats had free access to standard rat chow and tap water. This study was performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of WMU (No.: wydw-2020-0332). Experiments were reported according to the ARRIVE guidelines.

Streptozotocin-induced diabetic rat model

After 1 week of acclimation, all rats were weighed and randomly divided into the control (Con) and diabetic (DM) groups. After a 12-h fasting, rats in the DM group received intraperitoneal (i.p.) injection of streptozotocin (STZ, Sigma-Aldrich) solution prepared in citrate buffer (0.1 M, pH 4.5) at a single dosage of 65 mg/kg of body weight. Rats in the Con group were injected with the same volume of sodium citrate. After 3 days of STZ injection, blood glucose level was measured using a handheld glucometer (One Touch Ultra, LifeScan) via tail nick and the DM rat was defined when its blood glucose level was > 16.70 mmol/l. Moreover, body weight was determined by a digital balance (JY, Shanghai Minqiao Precise Science Co. Ltd., China).

Treatment with bFGF

According to the previous study by Zhao et al. [24], rats were suffered from cardiac dysfunction after 8 weeks of STZ injection. Therefore, in the present study, the DM rats after 9 weeks of STZ injection were randomly assigned into the DM and bFGF groups. Rats in the bFGF group were treated with bFGF solution by tail intravenous (i.v.) administration at 5 μg/kg of body weight for 7 consecutive days. In addition, rats in the Con and DM groups were injected the same volume of normal saline as the control.

Masson staining analysis

Masson staining was used to examine collagen deposition in the heart of rats [25]. Paraffin sections (5 μm) were prepared and stained with the Masson stain kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturers' instruction. Finally, an Eclipse 80i fluorescence microscope (Nikon, Japan) was applied to capture the stained cardiac sections at 400 × magnification.

Sample collection and preparation

After a 7-day treatment of bFGF, rats were overnight fasted and sacrificed by rapid decapitation under isoflurane anesthesia (5% induction; 2% maintenance) to avoid stress responses. Heart tissues were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until use. The frozen heart tissue was weighed into an Eppendorf tube and ground using an electric homogenizer (FLUKO, Shanghai, China). Then, ice-cold methanol (4 ml/g) and distilled water (0.85 ml/g) were added and homogenized by vortex. Next, ice-cold chloroform (2 ml/g) and distilled water (2 ml/g) were added consecutively and mixed by vortex. After standing on ice for 15 min, the mixture was centrifuged at 1000 *g* at 4 °C for 15 min. The supernatant was transferred into a fresh Eppendorf tube, lyophilized for 36 h, and stored at −80 °C until analysis. The lyophilized extract was redissolved in 500 μl D_2O containing sodium trimethylsilyl propionate-d_4 (TSP, 0.42 mM) and transferred to a 5-mm NMR tube for NMR analysis.

NMR spectral acquisition and preprocessing

^{1}H NMR spectra were acquired on a Bruker AVANCE III 600-MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) with a 5-mm TXI probe and a *z*-axis pulsed field gradient at 298 K. A standard single-pulse sequence, "ZGPR," with water signal pre-saturation was used to acquire ^{1}H NMR spectra of heart tissue extracts in rats. Moreover, typical acquisition parameters were set as follows: spectral width = 12,000 Hz; data points = 64 K; relaxation delay = 6 s; acquisition time = 2.65 s per scan.

^{1}H NMR spectra were manually phase- and baseline-corrected and referenced to the methyl peak of lactate at 1.33 ppm (CH_3) [26] in the TopSpin software (v.2.1, Bruker BioSpin, Germany). The "icoshift" procedure was used to align NMR spectra using MATLAB software (R2012a, The MathWorks Inc., Natick, MA, USA) [27]. The spectral region from 0.0 to 4.5 ppm was subdivided with a size of 0.01 ppm and integrated to binning data for multivariate analysis. Metabolite signals were assigned according to Chenomx NMR suite 7.5 software (Chenomx Inc., Alberta, Canada) and the Human Metabolome Database [28].

Multivariate analysis

Principal component analysis (PCA) was used to examine the metabolic changes between different groups based on the Pareto-scaled metabolite data using the SIMCA 12.0 software (Umetrics, Umeå, Sweden). The leave-one-out cross-validation (LOOCV) method was employed for PCA development. The PCA score plot shows the differences in metabolic patterns between different groups, while the corresponding loading plot identifies the specific metabolites that mainly contributed to the separation of metabolic patterns.

Statistical analysis

In this study, all rats were randomly assigned to the experimental procedures including housing and feeding, STZ injection, and bFGF treatment. NMR data acquisition was performed by masking the group labels of the animals. The levels of metabolites identified from PCA model were quantified according to the known concentration of internal standard (TSP) and presented as mean ± SD. The difference between two groups was analyzed by using Student's *t* test with SPSS software (v.13.0, SPSS Inc, USA), and a p value < 0.05 was considered as a statistically significant difference.

Results

bFGF attenuates myocardial fibrosis in diabetic rats

In this study, rats after STZ treatment exhibited characteristic diabetic symptoms such as significantly higher blood glucose level (Figure 1A) and lower body weight ((Figure 1B) than the Con rats. However, of note, treatment with bFGF for 7 days significantly reduced blood glucose level in the DM rats (Figure 1A), while no significant alteration in body weight of the DM rats after bFGF treatment (Figure 1B). Figure 1C illustrates the change of myocardial collagen deposition (blue region) in the Con, DM, and bFGF-treated rats using Masson staining. The quantitative analysis shows that the DM rats had a significantly higher myocardial collagen deposition than the Con rats, indicating that myocardial fibrosis presented in the DM rats (Figure 1D). However, interestingly, the degree of myocardial fibrosis can be significantly attenuated in the DM rats after bFGF treatment (Figure 1D).

bFGF alters metabolic phenotype in the heart of diabetic rats

Figure 2A–C illustrates typical ^{1}H NMR spectra obtained from heart tissues in the Con, DM, and bFGF-treated rats, respectively. We identified a series of metabolites mainly involving energy metabolism (acetate, lactate, succinate, creatine, and high-energy phosphate pool (AXP)), amino acid metabolism (valine, isoleucine, leucine, taurine, alanine, glutamate, glutamine, aspartate, and glycine), and membrane metabolism (choline). The detailed assignments of these metabolites in ^{1}H NMR spectrum are listed in Table 1.

To examine the effect of bFGF treatment on metabolic phenotype in diabetic rats, PCA was performed on the basis of heart metabolome in the Con, DM, and bFGF-treated rats. We found that the Con rats were clearly separated from the DM and bFGF-treated rats along PC1 (Figure 3A), indicating diabetes-induced shift in metabolic phenotype, which might be ascribed to higher levels of lactate, acetate, glutamine, aspartate, and creatine as well as lower levels of leucine, isoleucine, valine, glutamate, and taurine in the Con rats as shown in the PCA loading plot in Figure 3B. Moreover, an apparent separation in metabolic phenotypes between the DM and bFGF-treated rats along PC2 could be due to reduced levels of leucine, isoleucine, valine, lactate, glutamate, and taurine and increased levels of acetate, glutamine, aspartate, and creatine in the bFGF-treated rats, as shown in Figure 3C.

bFGF recovers metabolic disorders in the heart of diabetic rats

Furthermore, we quantified the relative concentrations of metabolites identified from PCA and performed metabolic pathway analysis using metabolites with significant changes between different groups, as illustrated in Figure 4. The results show that the levels of taurine, leucine, isoleucine, valine, and glutamate were significantly increased in the heart of the DM rats when compared with the Con rats, while their levels were significantly reduced in the DM mice after bFGF treatment (Figure 4). However, the DM rats had significantly lower levels of another two amino acids (aspartate and glutamine) in the heart than the Con rats, but treatment with bFGF significantly increased their levels in the DM rats. Relative to the Con rats, we observed that energy metabolism was downregulated in the heart of the DM rats as indicated by significantly decreased levels of acetate, creatine, succinate, and AXP (Figure 4). However, these energy metabolism–related metabolites were significantly increased in the heart of the DM rats

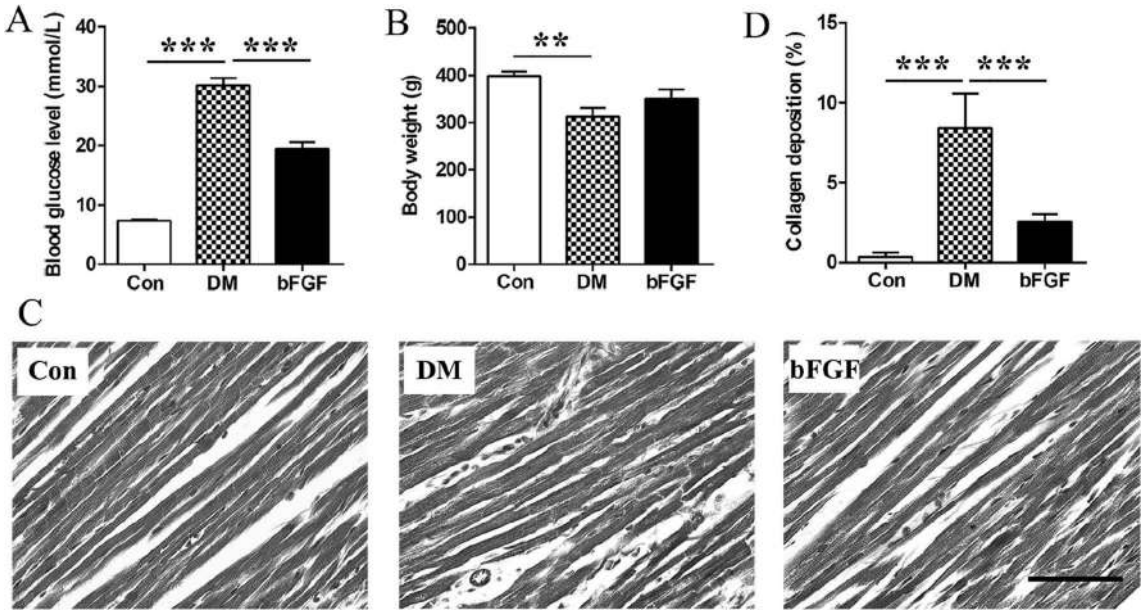

Fig. 1 Treatment with bFGF attenuates myocardial fibrosis in diabetic rats. In this study, diabetic rats were induced by intraperitoneal injection of streptozotocin and the DM rats after 9 weeks were treated with bFGF solution by tail intravenous for 7 consecutive days. (**A**) Changes in blood glucose level in the normal control (Con), diabetic (DM), and bFGF-treated DM (bFGF) rats. (**B**) Changes in body weight in the Con, DM, and bFGF-treated rats. (**C**) The myocardial collagen deposition in the heart of the Con, DM, and bFGF-treated rats examined by Masson staining. Scale bar = 600 μm. (**D**) The percentages of collagen deposition in the heart of the Con, DM, and bFGF-treated rats. The difference between the two groups was analyzed by Student's *t* test. Significant level: $^{**}p < 0.01$; $^{***}p < 0.001$

after bFGF treatment, excepting succinate. Therefore, our results suggest that bFGF treatment can recover disorders in amino acid metabolism and energy metabolism in the heart of the DM rats.

Discussion

Myocardial fibrosis is an important marker for diabetic cardiomyopathy (DCM) [29, 30]. Of note, metabolic disorders have been associated with tissue fibrosis, so targeting metabolism might be a promising strategy for fibrosis therapy [31, 32]. Herein, we revealed that bFGF can significantly reduce myocardial fibrosis in diabetic rats and also alleviate metabolic disturbance in the heart especially amino acid metabolism and energy metabolism. Our results suggest that bFGF might be a potential drug to target metabolism for treatment of diabetic myocardial fibrosis.

Amino acids are main components of collagen [33] and an excess of collagen synthesis and deposition contributes to

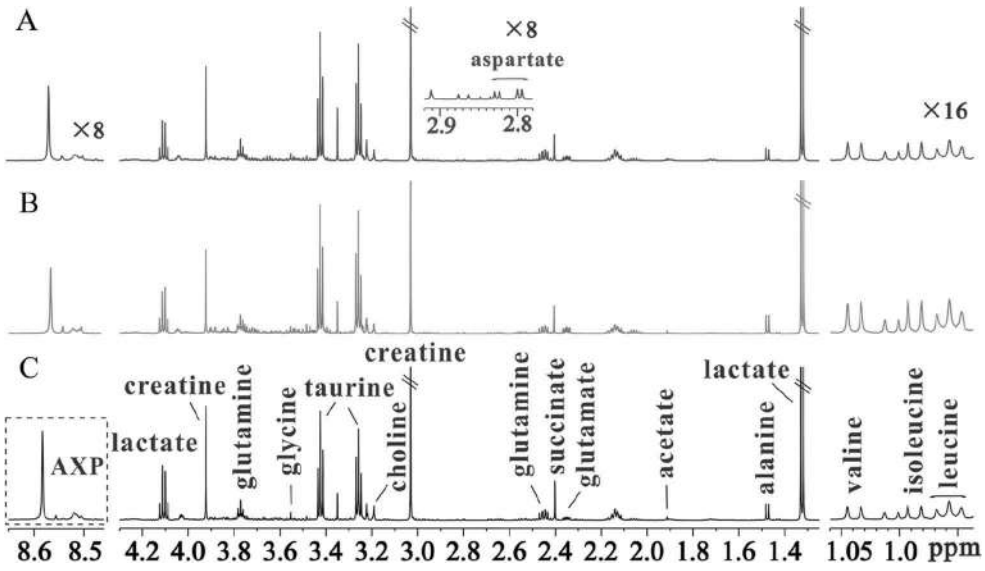

Fig. 2 Nuclear magnetic resonance (NMR)–based metabolomics analysis. Typical 600-MHz ^{1}H NMR spectra of the heart tissues obtained from (**A**) normal control, (**B**) diabetic, and (**C**) bFGF-treated diabetic rats. AXP, high-energy phosphate pool. ×8, magnification 8 times relative to the original spectrum; ×16, magnification 16 times relative to the original spectrum

Table 1 Assignment of metabolites in ^{1}H NMR spectra from rat heart samples

No.	Compound	δ^1H (ppm)	Assignment	Multiplicity
1	Leucine	0.96	δ-CH_3	d, d
2	Isoleucine	0.94, 1.01	δ-CH_3, β-CH_3	t, d
3	Valine	0.99, 1.04	γ-CH_3	d, d
4	Lactate	1.33, 4.12	CH_3, CH	d, q
5	Alanine	1.48	CH_3	d
6	Acetate	1.91	CH_3	s
7	Glutamate	2.12, 2.36	β-CH_2	m
8	Succinate	2.43	CH	s
9	Glutamine	2.13, 2.46	γ-CH_2	m
10	Aspartate	2.82	β-CH_2	dd
11	TMA	2.91	CH_3	s
12	Creatine	3.03, 3.92	CH_3	s
13	Choline	3.21	$N(CH_3)_3$	s
14	Taurine	3.25, 3.41	CH_2	t
15	Glycine	3.56	CH_2	s
16	AXP	8.45–8.57	-	m

Abbreviations: *s*, singlet; *d*, doublet; *dd*, doublet of doublets; *t*, triplet; *q*, quartet; *m*, multiplet; *TMA*, trimethylamine

myocardial fibrosis [34]. In this study, disrupted amino acid metabolism was observed in the diabetic heart with fibrosis, as characterized by higher levels of taurine, leucine, isoleucine, valine, and glutamate as well as lower levels of glutamine and aspartate in the heart of the DM rats. Taurine, as a sulfur-containing amino acid, is highly rich in the heart muscle, and has been associated with the development of DCM [35, 36]. Militante et al. reported that an increase in cardiac taurine level was implicated in the DCM development of insulin-dependent diabetes mellitus by inhibiting cardiac PDH activity [37]. Branched-chain amino acids (BCAAs) including leucine, isoleucine, and valine have been revealed as the key metabolic link between diabetes and cardiovascular disease [38]. Wang et al. found that BCAAs had the ability to affect cardiac dysfunction and fibrosis via the mTOR signaling pathway [39]. Besides, glutamine is one of the most abundant amino acids in the heart tissues and plays an important role in cardiovascular diseases [40]. Moreover, glutamine has been reported to possess a cardioprotective activity in diabetic rats by modulation of endogenous antioxidant defense system [41]. Glutamate as an excitotoxic amino acid can be metabolized from glutamine by glutaminase [42]. Herein, we observed a higher glutamate level but lower glutamine level in the heart of the DM rats relative to the Con rats, suggesting that diabetic heart may have an enhanced metabolic activity from glutamine to glutamate. Of note, high plasma glutamate and low ratio of glutamine-to-glutamate have been associated with increased risk of heart failure in humans [43]. Thus, our results may indicate diabetes as a risk factor for heart failure

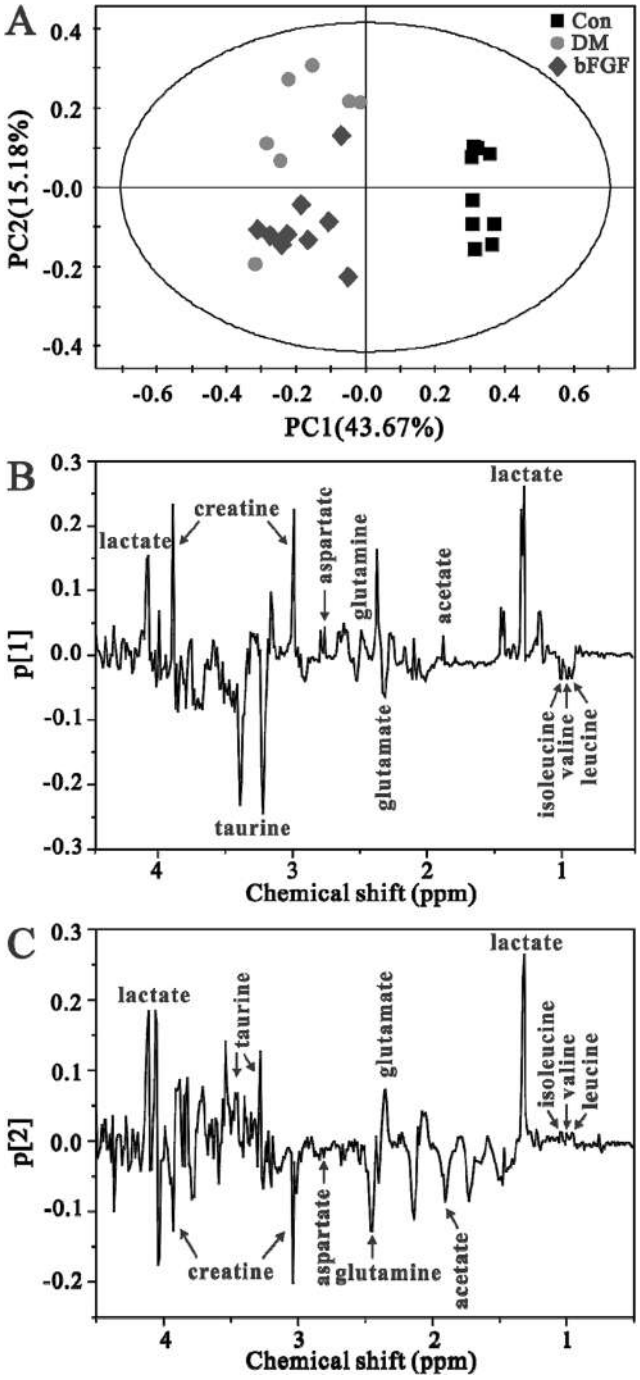

Fig. 3 Multivariate data analysis. (**A**) Principal component analysis showing the differences of metabolic patterns among the normal control (Con), diabetic (DM), and bFGF-treated DM (bFGF) rats; each point represents one of the rats. (**B**) Principal component 1 (p[1]) and 2 (p[2]) loading plots showing the specific metabolites that mainly contributed to the separation of metabolic patterns along PC1 and PC2, respectively

[44]. Additionally, aspartate as a non-essential amino acid is derived from oxaloacetate, one of the intermediates in TCA cycle, by transamination. Although little information is available about the role of aspartate in DCM, a reduced aspartate level in the heart of the DM rats may indicate a defective

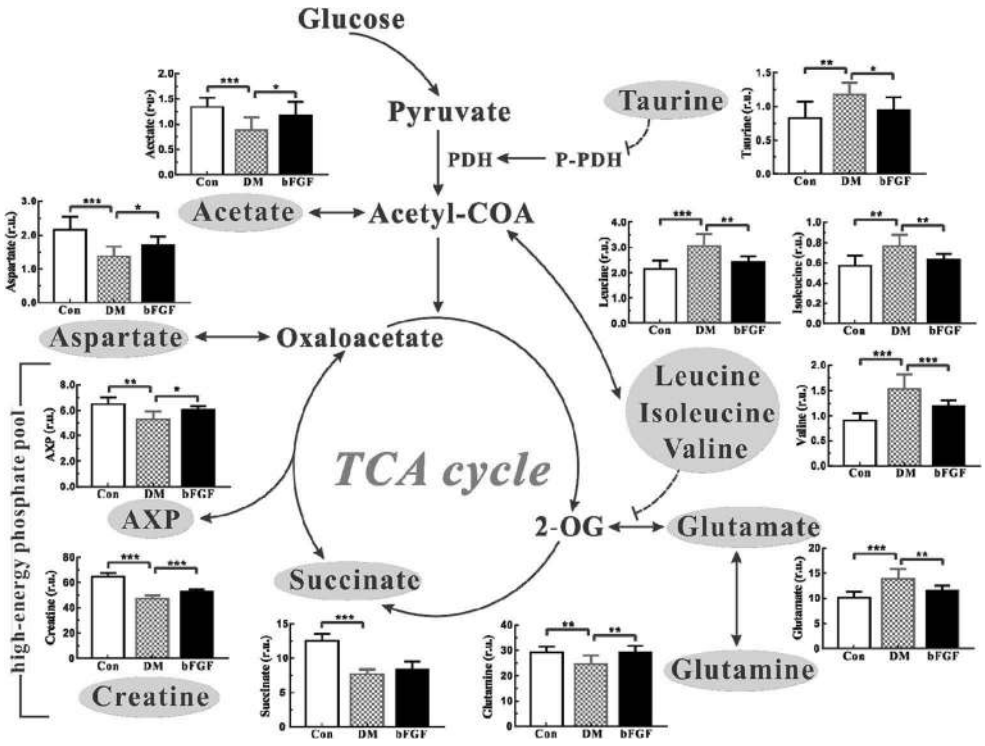

Fig. 4 Metabolic pathway analysis. Metabolic changes in the heart of the normal control (Con), diabetic (DM), and bFGF-treated DM (bFGF) rats. AXP, high-energy phosphate pool. r.u., relative unit. The difference between two groups was analyzed by Student's *t* test. Significant level: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

energy metabolism in the diabetic heart. Most importantly, these adverse metabolic features in the heart of the DM rats can be alleviated after bFGF treatment. In our previous study, we reported that bFGF can protect against diabetic nephropathy by reshaping metabolic phenotype in mice [45]. Herein, our results suggest that bFGF may have the ability to alleviate myocardial fibrosis via reversing disorders in amino acid metabolism in the heart of diabetic rats.

In this study, we also found that energy metabolism was downregulated in the heart of the DM rats, as indicated by significantly lower levels of TCA cycle metabolites, such as succinate and high-energy phosphate pool (AXP). This finding indicates that mitochondrial dysfunction occurs in the diabetic heart and results in cardiac dysfunction [46, 47]. Creatine also plays a key role in maintaining energy homeostasis via the creatine-phosphocreatine cycle [48]. Yet, a lower creatine level was observed in the heart of the DM rats. Lorentzon et al. reported that creatine depletion may be associated with left ventricular dysfunction and thereby cause heart failure in rats [49]. Besides, acetate can be converted to acetyl-CoA and flowed into the TCA cycle [50], and herein we also found a significantly reduced level of acetate in the heart of the DM rats. It is worth noting that these metabolic disorders can be reversed in the heart of the DM rats after bFGF treatment. In addition, of note, disrupted energy metabolism may contribute to cardiac fibrosis [51, 52], and improvement of energy metabolism has been shown to suppress cardiac fibrosis [53]. Our results suggest that bFGF may have the ability to alleviate diabetic myocardial fibrosis by regulating energy metabolism.

In conclusion, bFGF treatment can effectively attenuate myocardial fibrosis in the DM rats and the potential metabolic mechanisms may be implicated in reversing abnormal amino acid metabolism and energy metabolism in the diabetic heart. Several limitations or further works need to be considered: (1) Diabetes is often accompanied by hypoxia, so the impact of hypoxia on metabolic changes in the diabetic heart and whether bFGF has a positive effect on hypoxia-induced shifts in heart metabolism still need to be investigated; (2) It could be interesting to investigate metabolic changes in other tissues in order to examine whether bFGF can improve systemic metabolic disorders in diabetes; (3) A multi-analytical platform is recommended to achieve more detailed metabolic analysis; (4) Key enzymes or proteins involved in these metabolic pathways should be further explored in order to facilitate better understanding of the protective effect of bFGF on diabetic cardiomyopathy.

Code availability Not applicable.

Author contribution HZ and HCG contributed to the experimental design. YLH, WD, MJL, and HZ contributed to animal experiment and NMR analysis. HZ contributed to data analysis, result interpretation, and writing. All authors have read, revised, and approved the final manuscript.

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Data availability All data in this study are present in the main text. Additional data and materials can also be requested from the first author or corresponding author.

Declarations

Ethics approval All animals were strictly treated in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of WMU (No.: wydw-2020-0332).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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To be recognized as a global leader for clinical care, education, training, research, advocacy and capacity building in the field of diabetes.

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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

NEW EXECUTIVE COMMITTEE AND OFFICE BEARERS 2022-2023

Patrons of RSSDI

Dr. H.B. Chandalia, Mumbai
Dr. C. Munichhoodappa, Bengaluru
Dr. Ashok K. Das, Puducherry
Dr. Siddarth Das, Cuttack
Dr. Binode K. Sahay, Hyderabad
Dr. V. Seshiah, Chennai
Dr. P.V Rao, Hyderabad
Dr. Jitendra Singh, New Delhi
Dr. V Mohan, Chennai
Dr. Vinod Kumar, New Delhi

President

Dr. Brij Makkar, Delhi

President Elect

Dr. Rakesh Sahay, Hyderabad

Immediate Past President

Dr. Vasanth Kumar, Hyderabad

Secretary-General

Dr. Sanjay Agarwal, Pune

Vice-President

Dr. Sujoy Ghosh, Kolkata

Vice-President

Dr. L. Sreenivasamurthy, Bengaluru

Joint Secretary

Dr. Pratap Jethwani, Rajkot

Treasurer

Dr. J.K. Sharma, Delhi

Executive Committee

Dr. J Aravinda, Bengaluru
Dr. Manoj Chawla, Mumbai
Dr. N.K. Singh, Dhanbad
Dr. M. Shunmugavelu, Trichy
Dr. Amit Gupta, Greater Noida
Dr. Jothydev Kesavadev, Kerala
Dr. Rakesh Parikh, Jaipur
Dr. Anil Virmani, Jamshedpur

Co-opted

Dr.Vijay Viswanathan, Chennai
Dr. Anuj Maheshwari, Lucknow
Dr. Sunil Gupta, Nagpur

TRAINEE GRANTS (Up to 10 grants)

Research Grants upto INR 200000 to support outstanding thesis/ research work by first year MD/DNB/ PHD students/Research fellows from India.

Eligibility Criteria
All Postgraduates in First year MD, DM /DNB from any of the institutions in the country are eligible to apply

How to apply?
Upload your Research proposals on the RSSDI Online Research Grant Platform.

Research proposal should have following proofs-

1. A supporting letter from your guide/ head of department stating that this is a bonafide project for your thesis and also mentioning the dates of you joining the program and expected date of graduation. The guide must also state that he/she will stand guarantee for the work done
2. A detailed budget
3. Thesis proposal approved by the department/appropriate institutional authority
4. Approval by the ethics committee

Selection Process
Proposals will be reviewed by the research committee of the RSSDI.

Disbursement of Grant
20% of the grant amount will be disbursed initially. 30% of payment after receiving your project status report and utilisation of sanctioned amount, 25% on further completion and pending 25% on final submission of your project. All reports must be uploaded on the RSSDI Online Research Grant Platform.

Responsibility:
All grant awardees are expected to present their work at RSSDI Annual Conference during research presentation's session. Failure to file progress reports annually and when requested by the RSSDI and failure to present progress at RSSDI Annual conference may result in the forfeiture of the grant.

All awardees are expected to follow the tenets of responsible and ethical conduct of research. Unethical or fraudulent use of RSSDI research funds will warrant adverse action from the society including forfeiture of grant, black listing in the society's databases and other legal recourses that are available to the society.

Publication
The RSSDI expects that the grant source be acknowledged in all publications and submissions made with regards to the research done with the grant.

All awardees are encouraged to submit their work to the RSDDI Journal IJDDC

CALL for RESEARCH PROPOSALS for GRANTS (up to 5 lacs)

Research proposals are invited from Indian scientists, who are members of RSSDI interested in conducting research in the field of Diabetes, Endocrinology& Metabolism, for funding by RSSDI

The proposals may of clinical or translational research importance. A maximum grant amount of INR 5 Lakhs will be sanctioned. All grants will be reviewed by the research committee.

The detailed proposals should include the following:
Title, names of principal and co investigators, summary, introduction/ background, review of literature, aims, methodology, study design and detailed plan of work & bibliography.

Brief biodata of principal investigator and other co-investigators.

Importance of work
Detailed Budget sought along with full justification/ proposed utilization, of funding sought from RSSDI

Whether the project is being partly funded from any other source? If yes, please mention the source and the amount received.

Ethics Committee clearance of the Institution or other bonafide body.

How to apply
Upload your Research proposals on the RSSDI Online Research Grant Platform.

When to apply
Proposals will be accepted every quarter of a year. The first month will be for the proposal submission, the second month for the scrutiny of the submitted proposals and the third month for the grant disbursement. This cycle will repeat for each quarter.

MAJOR RESEARCH GRANT PROPOSALS- usually not more than one at a given time.

Above 10 Lacs upto a total amount of 50 Lacs will be Granted to RSSDI initiated, owned, multi-centric, clinical or translational research, having long term application of scientific and clinical findings, which can translate into strategies for improving health-care delivery, patient outcomes, and community health in India.

Such research proposals will be carried out in only centres with research capabilities across India.

TRAVEL GRANTS FOR YOUNG DIABETES RESEARCHERS TO ATTEND INTERNATIONAL CONFERENCES

Criteria for the travel grant are as follows:

- Applicant should apply 2 months in advance.
- Travel Grant is open only to the RSSDI members.
- Applicant should submit Oral paper / Poster acceptance document to RSSDI Secretariat.
- Applicant should submit Declaration that he/she has not receiving grant from any other agency / Organization – In case of receiving grant from any other Organization, RSSDI shall pay only the exceeding amount not covered by that agency.

ADVANCED CERTIFICATE COURSE IN DIABETOLOGY

(IN ASSOCIATION WITH JAIPUR NATIONAL UNIVERSITY)

Research Society for the Study of Diabetes in India (RSSDI) was founded by Prof. M.M.S. Ahuja in 1972. RSSDI is the largest body of professional doctors and researchers in Asia, working in the area of Diabetes & is the National Body recognized by IDF (International Diabetes Federation). One of the key areas of focus is to train doctors at all levels to better manage Diabetes and its complications. RSSDI recognizes this problem and runs a well-structured, full time, residential "Advanced Certificate Course in Diabetology". This two-year course is like any other post graduate course and has immensely helped doctors to practice better diabetes care. RSSDI has

List of RSSDI Accredited Centres

Sl. No	Institute Name	Institute Location
1.	Diacon Hospital	Bangalore, Karnataka
2.	North Delhi Diabetes Centre	New Delhi, Delhi
3.	Prithvi Hospital	Tumkur, Karnataka
4.	Total Diabetes Hormone Institute	Indore, Madhya Pradesh
5.	Dia Care - A Complete Diabetes Care Centre	Ahemdabad, Gujarat
6.	Sonal Diabetes Hospital	Surat, Gujarat
7.	Jothydev's Diabetes and Research Center	Trivandrum, Kerala
8.	Advanced Endocrine & Diabetes Hospital	Hyderabad, Telangana
9.	Sunil's Diabetes Care N' Research Centre	Nagpur, Maharashtra
10.	Marwari Hospital and Research Centre	Guwahati, Assam
11.	Down Town Hospital	Guwahati, Assam
12.	St.Theresa's Hospital	Hyderabad, Telangana
13.	Aegle Clinic	Pune, Maharashtra
14.	Lilavati Hospital & Research Centre	Bandra West, Mumbai
15.	Srajan Hospital	Udaipur, Rajasthan
16.	Endeavour Clinics & Dr. Sambit's Centre of Diabetes and Endocrinology	Bhubaneswar, Odisha
17.	ILS Hospital, Salt Lake	Salt Lake City, Kolkata
18.	Belle Vue Clinic	Dr. U N Brahmachari Sreet, Kolkata
19.	Arthur Asirvatham Hospital	Mdurai, Tamil Nadu
20.	M V Hospital for Diabetes	Chennai, Tamilnadu
21.	Sarvodaya Hospital and Research Centre	Faridabad, Uttar Pradesh

22.	Galaxy Speciality Centre	Sodala, Jaipur
23.	SL Raheja Hospital	Mumbai, Maharashtra

carefully looked into all aspects of this course & has accredited & recognized 23 centres across India at present and more centers are being inspected for accreditation. National Faculties and experts of RSSDI chosen from Academia visit these centers from time to time to ensure high standards. Now this Advanced Certificate Course has Dual Accreditation from RSSDI and Jaipur National University.

COURSE DETAILS

Name of the Course: Advanced Certificate Course in Diabetology

Duration: 2 Years – Post MBBS & 1 Year - Post MD / DNB (Gen - Medicine)* (Full Time) Educational.

Qualification: A candidate must possess MBBS degree from ANY of the recognized university approved by Medical Council of India (*The duration of the course is 1 Year for those with MD/ DNB in Internal Medicine. Candidates having MD degree in other specialties will have to do the course over 2 Years).

Number of seats: 2 seats per year for every eligible teacher as per rules of Medical Council of India (MCI).

Selection of Candidates: Selection for the Certificate course is through a performance evaluation by screening interview which will be conducted by the centre coordinator of all respective accredited centers. The results will be declared in a week's time. A maximum of 50 marks will be scored for this assessment. Those who have scored at least 50%, will be initially considered based on their merit.

NOTE : Post MD (Internal Medicine) will be given preference.

FEES FOR APPLICATION FORM: RS 1500/-

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)

Applications are taken twice a year - June and December

Check the RSSDI website for the dates

Click on the link to apply: https://rssdi.in/rssdi-accd/

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.

RSSDI 50th Golden Jubilee Year Celebrations
(look out for more details on our website)

INCENTIVES FOR REVIEWERS AND SUBEDITORS OF THE IJDDC (the official journal of RSSDI)

Based on the annual reviewer performance data, top 10 reviewers of IJDDC, selected by the journal's editorial team, shall be honored at the RSSDI annual conference with certificate/awards. Reviewers submitting thorough critical evaluation comments of assigned articles would also have the leverage of coming onboard as part of the core editorial team of IJDDC.

The best three subeditors of IJDDC, displaying outstanding contribution and consistent support to the associate editors, shall be conferred with certificate/awards at the RSSDI annual conference and will also have the leverage of joining the main editorial team of IJDDC.

TRANSITION OF IJDDC FROM PRINT TO ONLINE

IJDDC, the journal of the society, will now be available in full-text pdf format on the RSSDI website for all RSSDI Members for easy access.

Print copies will also be available. However, members keen to move from print to online only may submit their request by visiting the RSSDI website https://www.rssdi.in and following instructions on the RSSDI homepage

Those who wish to continue receiving the print copy may also submit their request choosing the appropriate option through the same link on the RSSDI website https://www.rssdi.in

The deadline for submitting request for print copy will be 7th April, 2023.

RSSDI Research Retreat 2023

The 2nd RSSDI Research Retreat scheduled for 8th and 9th April, 2023, in Hyderabad provides a unique opportunity for young investigators/ researches to present their published original research work as well as network and collaborate with fellow researchers.

Registered with Registrar of News Papers, India vide no. RNI-43019/85

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