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EDITORIAL

Monogenic diabetes—diagnostic conundrums

Aaron Chapla[1] · **Felix K. Jebasingh**[1] · **Nihal Thomas**[1]

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Diabetes mellitus (DM) is a global pandemic [1] that affects nearly 382 million people worldwide [2]. The vast majority of patients (approximately 85 %) are classified into polygenic type 1 diabetes (T1D) or type 2 diabetes (T2D). However, with growing evidence from genomic research, several monogenic causes of diabetes have emerged. Monogenic forms of diabetes include maturity onset of diabetes of young (MODY), neonatal diabetes and rare syndromic forms of diabetes [3].

Maturity onset of diabetes of young

Mutations involving 13 different genes have been reported to cause MODY and more than 20 genes have been reported to be implicated in neonatal diabetes and rare syndromic forms of diabetes [3]. However, till recently, the molecular diagnosis of these monogenic disorders included sequential screening of only a few related genes based on the phenotype [4]. Moreover, due to the prohibitive cost and limitations associated with the scalability of Sanger sequencing, most diagnostic laboratories screen for hepatocyte nuclear factor 1alpha (*HNF1A*), glucokinase (*GCK*) and hepatocyte nuclear factor 4 alpha (*HNF4A*) mutations in MODY [5] or the ATP-sensitive potassium channels, KCNJ11 and ABCC8, and the insulin gene (INS) mutations in neonatal diabetes [3]. Only a few of those patients who test negative for mutations in these genes are subjected to further genetic testing of the less common monogenic forms of diabetes [4]. In developing countries, due to a paucity of clinician-related awareness, limited genetic diagnostic facilities and affordability, patients with diabetes are often misdiagnosed as T1D or T2D and may potentially receive inappropriate therapy [6]. Furthermore, with the overlapping clinical features with common forms of polygenic diabetes, the diagnosis of monogenic diabetes becomes challenging [7].

Neonatal diabetes mellitus

Neonatal diabetes mellitus (NDM) is a rare monogenic form of diabetes that occurs within 6 months of infancy. The incidence of NDM is 1 in 500,000 live births. Most of these infants are misdiagnosed as having type 1 diabetes mellitus and have been advised long-term insulin. The major difference between NDM and type 1 diabetes is the age of onset of the disease. Usually T1DM occurs after the first 6 months of life owing to increased activation of the immune system which occurs after 6 months. There are two types of NMD, permanent neonatal diabetes mellitus (PNDM) wherein the disease is lifelong and transient neonatal diabetes mellitus (TNDM) in which the diabetes disappears during infancy but can reappear later in life [8, 9].

The clinical symptoms of NDM include frequent micturition, dehydration and failure to thrive. These symptoms mimic those of distal or proximal renal tubular acidosis of congenital origin. However, the major differentiating feature between NDM and renal tubular dysfunction is the elevated level of plasma glucose in NDM. Some infants may present with frank diabetic ketoacidosis. A small for gestational-age baby may be associated with NDM due to the presence of insulinopenia. Following delivery, these babies fail to gain weight when

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compared to the infants of the same age and sex. Appropriate therapy (insulin initially, then followed by sulphonylurea agents) improves and may normalize the growth and development of the infant. Therefore, genetic screening may help in confirming the diagnosis of these disorders and potentially evading long-term insulin therapy [8, 9].

NDM is a true monogenic condition where hyperglycaemia is related to a single-gene mutation [8]. Recently, Al-Agha et al. from Saudi Arabia has performed NDM gene screening in eight children with hyperglycaemia diagnosed during 1 to 17 weeks of birth. Initial screening revealed one patient with a KCNJ11 mutation and one with an insulin gene mutation. Furthermore, screening of ABCC8 and FOXP3 did not reveal any mutations, therefore yielding a mutation-positive rate of 25 % when screened for specific genes in patients with permanent NDM [9]. Another study from China performed by Huang et.al. screened four cases of PNDM for mutations in ABCC8, KCNJ11 and INS. However, they could not find any causative mutations and therefore could not provide a definitive diagnosis [10]. Therefore, in the developing countries, various mutation screening studies yielded 0–33 % mutation-positive rates when screened for 3 to 4 genes (ABCC8, KCNJ11, INS and FOXP3) [11]. However, the rarer forms of known ND genes further need to be tested to increase the likelihood of making a confirmed genetic diagnosis.

Recent studies utilizing next-generation sequencing (NGS)-based parallel multi-gene testing have shown promising results [3, 5], and the testing has been proven to find the genetic cause even with limited phenotypic information and also in the absence of characteristic features in monogenic diabetes-related subtypes [12]. Further, with the identification of digenic mutations in MODY [5] and also often with overlapping clinical features [7], project the need for parallel multigene testing in monogenic diabetes which could provide a comprehensive genetic portrait.

Wolfram syndrome

Wolfram or DIDMOAD is an uncommon disorder which has been considered as a differential diagnosis in young patient with diabetes mellitus. The clinical diagnosis of Wolfram syndrome can be clenched if routine funduscopy is practiced in all patients with young onset diabetes mellitus. DIDMOAD stands for diabetes insipidus, diabetes mellitus, optic atrophy and deafness. The temporal profile of this disorder is that diabetes mellitus presents around the age of 6 years followed by optic atrophy at around 11 years. Most patients will have loss of vision after 8 to 10 years after signs of optic atrophy first begin. Around three fourths of patients effected by Wolfram syndrome will have diabetes insipidus and sensory neural deafness. Moreover, up to 90 percent of these patients can have various urinary tract problems ranging from bladder outlet obstruction to an atonic bladder [13].

In case of syndromic forms of diabetes, phenotype guided specific gene sequencing could be adopted. A study published by Abbasi et al. have studied two Iranian patients with Wolfram syndrome which is a rare neurodegenerative disorder [14]. Wolfram syndrome (WFS1) gene has eight exons and screening of only the eighth exon in two patients revealed a pathogenic variant providing a confirmed diagnosis [14]. However, in case of variable expression of this gene, it is important to note that there could be heterozygote carriers [15] or the mutation could be present in other exons of WFS1 or in WFS2 gene in which few mutations have been reported. Till date, there are around 230 WFS1 gene mutations that have been reported; however, there is a poor understanding of the relationship between the non-synonymous coding variants and the phenotype. Therefore, studies looking at short fragments or partial gene require extensive bioinformatics predication and careful interpretation of the novel variants identified [16].

Even with a considerable phenotypic heterogeneity in monogenic diabetes [3], a confirmed genetic diagnosis may help in understanding molecular cause for diabetes and also help in cases where there is significant change in medical management. Patients' families could benefit through genetic counselling and also to predict their clinical course and familial risk. Therefore, a heterogeneous disorder like MODY or neonatal diabetes would require NGS-based multi-gene testing to uncover the molecular basis and pave a way to personalized genetic medicine through improved glycaemic control and quality of life.

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ORIGINAL ARTICLE

Comparison of sulfur transferases in various tissue and mitochondria of rats with type 1 diabetes mellitus induced by streptozotocin

Hüseyin Aydın[1] · Veysel Kenan Çelik[1] · İsmail Sarı[1] · Yusuf Kenan Tekin[1] · Özlem Demirpençe[1] · Sevtap Bakır[1]

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Abstract This study aims to investigate the relationship between sulfurtransferase (STS) activities [rhodanese (TST), mercaptopyruvate sulfurtransferase (MST)] involved in the catalysis of several biochemical reactions including detoxification of cyanide (CN^-), restructuring of Fe-S cluster in proteins, and detoxification of oxygen radicals. Rats with type 1 diabetes mellitus induced by streptozotocin (STZ) were anesthetized at 14th day, and liver, lung, kidney, and heart tissues were extracted. All samples were homogenized, and mitochondrial parts were separated. Same processes were performed also in the control group, and TST and MST activities were measured in each part. The homogenate MST (MST_{Homo}.) activities of the type 1 diabetes mellitus group were compared with the control group, and a decrease was observed in the lung, liver, and kidney, respectively; at the same time, an increase was seen in the heart tissue. The mitochondrial MST (MST_{Mito}.) activities of rats with type 1 diabetes mellitus group were compared with the control group, and a decrease was found in all tissues. The highest decrease in the TST_{Mito}. level of rats with type 1 diabetes mellitus was observed in kidney tissue. The TST activities of the type 1 diabetes mellitus group were compared with the control group, and a decrease was observed in the liver, lung, and kidney, respectively; at the same time, an increase was seen in the heart tissue. It is demonstrated in the present study that decreases occur both in enzyme levels of tissue homogenates and in mitochondria, of rats with induced type 1 diabetes mellitus. However, these results were not statistically significant. In the presence of these findings, we think that kidney, liver, lung, and heart tissue can be affected by type 1 diabetes in the long term.

Keywords Type 1 diabetes mellitus · Sulfurtransferase · Rhodanese · Mercaptopyruvate sulfurtransferase

Introduction

STSs including several different enzymes are found extensively in various living organisms including archaebacteria, bacteria, plants, and animals. Sulfurtransferases catalyze the transfer of sulfane sulfur (SSO_3^{2-}) atom to thiophilic substrates including thiols, cyanide, sulfide, and sulfinates. Enzymes involved in this process are TST and MST. TST (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) is the first sulfurtransferase [1] found in rat liver prior to plant and bacteria and catalyzes the following reaction.

$$SSO3^{2-} + CN^- \rightarrow SO3^{2-} + SCN^-$$

This enzyme has been purified and examined in several animal tissues including the rat liver, cattle liver, and kidney [2]. TST is localized both in mitochondrial membrane of liver and kidney tissues as well as mitochondrial matrix [3, 4]. Additionally, recombinant TST has been overexpressed in several animal tissues, and its crystal structure has been examined [5–7]. The *rhdA* gene has been identified and cloned [8] which encodes TST in gram-negative (*A. Vinelandii* [9], *Acinetobacter calcoaceticus lwoffi* [10], *Pseudomonas* spp. [11], *E. coli* [12]) and gram-positive bacteria (*Bacillus subtilis* [13]). Although the in vivo biochemical role of TST has not

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been elucidated completely, it is has been demonstrated to be involved in the formation of iron-sulfur complexes [14] and cyanide detoxification [15, 16]. Studies on the molecular features of TST have demonstrated that the enzyme also has important metabolic function in the regulation of mitochondrial respiration rate [17].

3-Mercaptopyruvate: cyanide sulfurtransferase (MST, EC 2.8.1.2) is another STS that catalyzes the separation of carbon-sulfur bond and transfers of one sulfur atom from 3-merkaptopyruvate to various thiosulfates (including thiols, cyanide, sulfide, and sulfanide).

$$HSCH2COCOO^{-} + CN^{-} \rightarrow SCN^{--} + CH3COCOO^{-}$$

or

$$HSCH2COCOO^{-} + RSH \rightarrow RSSH + CH3COCOO^{-}$$

MST was first discovered in the rat liver about 60 years ago but has not attracted the interest it deserves compared with TST [6]. MST is specific to 3-mercaptopyruvate [18], 3,3′-dimercaptopyruvate (*bis*(2-carboxy-2-oxoethyl) disulfide), and ethylmercaptopyruvate [19]. However, MST has much smaller Michaelis's constant (Km) for 3-merkaptopyruvate. The recombinant form of enzyme purified from rat liver has been characterized by overexpression in *E. coli* [6], and MST has been demonstrated to have 66 % similarity with TST in terms of amino acid sequence [20]. 3-MST is found in various subcellular compartments including cytosol, nucleus, mitochondria, and endoplasmic reticulum and even in erythrocytes [21].

The exact physiological roles of the STSs have not been elucidated, although it is known that STSs including TST and MST are involved in the detoxification of cyanide and oxygen radicals in the mitochondria by acting like thioredoxin oxidase [22]. There are role of STSs in restructuring of Fe-S clusters with an unexplained mechanism of intracellular formation [23] in Fe-S proteins (succinate dehydrogenase, NADH dehydrogenase, spinach- and clostridial-ferredoxin, and nitrogenase of *Klebsiella pneumoniae*) [24–26].

We planned to examine the activity of TST and MST and their relationship with type 1 diabetes mellitus in liver, heart, kidney, and lung tissue homogenates and mitochondrial parts of these tissues in rats with type 1 diabetes mellitus induced by the chemical agent STZ.

Material and methods

Development of experimental type 1 diabetes mellitus

In this study, six male *Wistar albino* rats, 250–300 g, reproduced at the Experimental Animals Laboratory, Faculty of Medicine, Cumhuriyet University, were used. Rats were classified into two groups as control and study groups and were provided with free access to food and water. An approval document of 11/01/2007 no B.30.2.CUM.0.01.00.00-50/214 was obtained from the Experimental Animals Ethics Committee, Cumhuriyet University.

Blood glucose levels of rats were measured following an overnight fasting (Lever Check TD-4222). Rats with blood glucose levels 80–110 mg/dl were considered normal. A single dose STZ (Sigma Chemical Co., St. Louis Missouri, USA (60 mg/kg)) was resolved in 0.1 M citrate buffer with 4.5 pH. This solution was used to develop type 1 diabetes mellitus in rats without sex distinction.

The intravenous blood samples are obtained from the tails of rats. Blood glucose levels were measured in the intravenous blood samples obtained from the tails of rats at 48 h of streptozotocin injection. Rats with blood glucose levels of 250 mg/dl were considered type 1 diabetes mellitus.

Surgical procedure

All rats were anesthetized at 14th day with an intramuscular injection of 90 mg/kg ketamine hydrochloride, 3 mg/kg of xylazine hydrochloride into left foreleg muscle. Thoracic and abdominal cavities were incised open; liver, lung, kidney, and heart tissues were removed, and this tissue stored at −70 °C.

Mitochondria extract

All defrosted tissues were homogenized in ice shower containing 4 ml of 0.2 M phosphate buffer at pH 7.4. Homogenates were centrifuged at 3000*g* for 15 min at 4 °C to remove tissue remnants. Enzyme activities were determined in the supernatant. Mitochondria were extracted according to the method defined by Max et al. [27] and modified by Mousa [28]. Samples of 1 g were obtained from each tissue and homogenized in 5 ml of 0.25 M sucrose solution. Homogenates were centrifuged at 800*g* for 15 min at 4 °C; supernatant was later recentrifuged at 20,000*g* for 10 min. Following the removal of the supernatant, the mildly swollen layer on top of the mitochondrial pellet was removed with the gentle addition of sucrose solution. Pellet was later prepared for analyses by suspension with 1.2 ml of 0.02 M phosphate buffer at pH 7.4.

TST and MST assays

TST and MST activities were assayed spectrophotometrically by the method of Sorbo [4, 7, 10]. The enzyme activity was expressed as units/mg protein.

Protein determinations

Protein content of tissue extracts was determined by the method of biuret using bovine serum albumin (BSA) as the standard [29].

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 14 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Nonparametric data are expressed as median, minimum and maximum, mean±standard deviation, and categorical data as percentages. The activity levels of MST and TST were compared by tissue types using Kruskal-Wallis test. The Mann-Whitney *U* test was used in intergroup comparisons. The Mann-Whitney *U* test was used to compare the activity levels of MST and TST by fraction types. $p \leq 0.05$ was considered significant.

Results

The distribution of the activity levels of homogenate MST ($MST_{Homo.}$) in type 1 diabetes mellitus group by all tissue homogenates was found as follows from the largest to the smallest: liver>lung>heart>kidney. The distribution of the activity levels of $MST_{Homo.}$ in control group by all tissue homogenates was found as follows from the largest to the smallest: liver>lung>kidney>heart (Fig. 1). The $MST_{Homo.}$ activity of the type 1 diabetes mellitus group was compared with the control group and −30, −18.4, −44 % decrease was seen in the lung, liver, and kidney, respectively, and a 7 % increase was seen in the heart (Fig. 1).

The MST_{Mito} levels were compared between type 1 diabetes mellitus with control group and decreases were observed in lung, liver, kidney, and heart tissues (−20.7, −27.1, −29.3, and −13.4 %, respectively) (Fig. 2).

Based on the data in Figs. 1 and 2, it was found that the decrease in MST enzyme activities in type 1 diabetes mellitus rats was highest in both the homogenate and mitochondrial fractions of the kidney. Despite an increased cardiac $MST_{Homo.}$ enzyme activity (7 %) in the type 1 diabetes mellitus group compared with the control group, there was a decrease in the $MST_{Mito.}$ level (−13.4 %) (Figs. 1 and 2). These increases and decreases in both fractions are not statistically significant ($P>0.05$).

The mitochondrial rhodanese ($TST_{Mito.}$) enzyme activities in the control group were found as follows from the largest to the smallest: liver>kidney>lung>heart. While the $TST_{Mito.}$ activity in type 1 diabetes mellitus group was found −11.6, −6.1, and −30.5 % in the lung, liver, and kidney, respectively, there was 1.8 % increase in the heart tissue. The highest decrease in the $TST_{Mito.}$ level in type 1 diabetes mellitus rats was seen in the kidney (Fig. 3). These increases and decreases in the type 1 diabetes mellitus and control groups were not considered to be statistically significant ($P>0.05$).

Discussion

In this study, we investigated the effects of type 1 diabetes mellitus on the TST, MST activities of homogenate and

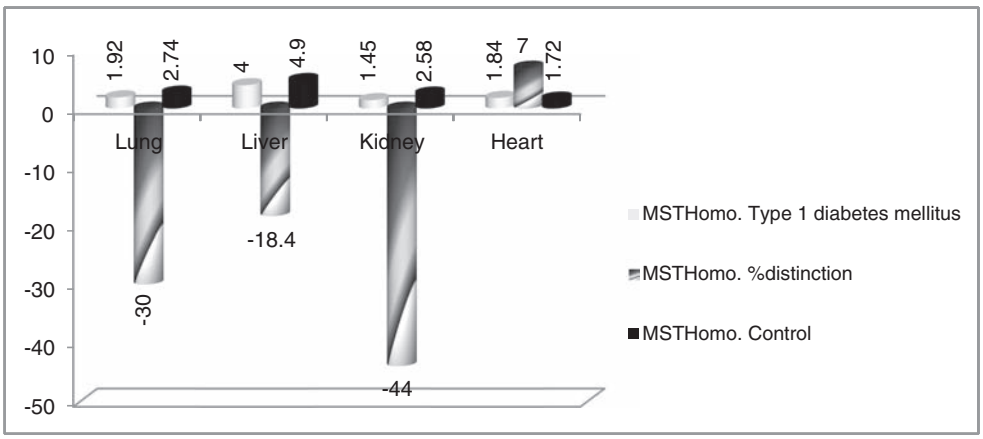

Fig. 1 Comparison of MSTHomo. levels in tissue homogenates

Homogenates $MST_{Homo.}$ Levels (U/mg.protein)

Median(Min-Max) $LungHomo._{Type\ 1}$ 1,92(1,23-2,78), $LungHomo._{Cont.}$ 2,74(1,48-4,64), distinction -30% and P value 0,513

Median(Min-Max) $LiverHomo._{Type\ 1}$ 4,00(3,70-4,27), $LiverHomo._{Cont.}$ 4,90(2,75-7,44), distinction -18,4% and P value 0,512

Median(Min-Max) $KidneyHomo._{Type\ 1}$ 1,45(0,96-1,93), $KidneyHomo._{Cont.}$ 2,58(0,97-5,27), distinction -44% and P value 0,515

Median(Min-Max) $HeartHomo._{Type\ 1}$ 1,84(1,50-2,46), $HeartHomo._{Cont.}$ 1,77(1,41-2,02), distinction 7% and P value 0,507

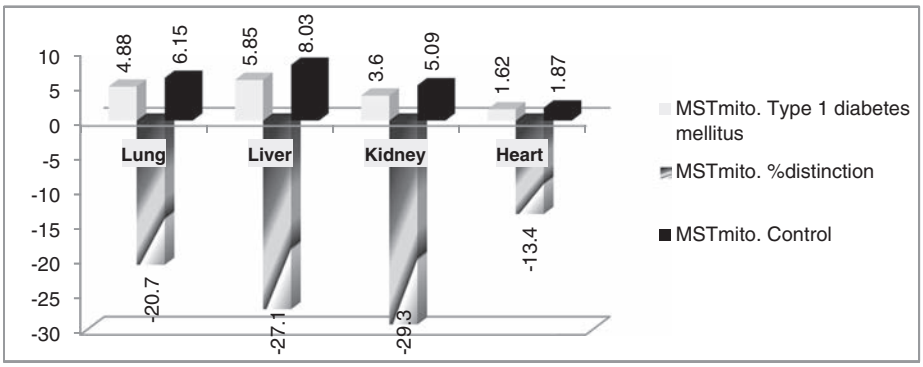

Fig. 2 Comparison of MSTMito. levels in tissue homogenates

Mitochondrial $MST_{Mito.}$ (U/mg.protein)

Median(Min-Max) LungMito.$_{Type\ 1}$ 4,88 (1,86-6,76), LungHomo.$_{Cont.}$ 6,15 (1,35-11,59), distinction -20,7% and P value 0,827

Median(Min-Max) LiverMito.$_{Type\ 1}$ 5,85 (2,27-8,30), LiverMito.$_{Cont.}$ 8,03(5,01-13,74), distinction -27,1% and P value 0,827

Median(Min-Max) KidneyMito.$_{Type\ 1}$ 3,60 (2,18-5,58), KidneyMito.$_{Cont.}$ 5,09 (3,89-7,22), distinction -29,3% and P value 0,275

Median(Min-Max) HeartMito.$_{Type\ 1}$ 1,62 (1,47-1,86), HeartMito.$_{Cont.}$ 1,87(1,60-2,25), distinction -13,4% and P value 0,275

mitochondrial fractions of lung, liver, kidney, and heart tissues of rats with type 1 diabetes mellitus induced by STZ. A comparison of the MST_{Homo}. enzyme activity in the study group with the control group showed a decrease in the lung, liver, and kidney in the homogenates, and an increase (7 %) in the heart tissue. The MST_{Homo}. enzyme activity was found most decreased (−44 %) in the kidney of rats with induced type 1 diabetes mellitus (Fig. 1). There were decreased MST_{Mito}. enzyme activities in all tissues compared with the control group, and the largest decrease (−29.3 %) was found in the kidney tissues (Fig. 2).

The TST_{Mito}. enzyme activity was found minimally decreased in lung (11.6 %), liver (6.1 %), and maximum decreased in kidney tissue (30.5 %) of rats with induced type 1 diabetes mellitus. At the same time, minimally increased in the heart tissue (1.8 %) compared with the control group (Fig. 3). The lowest TST_{Mito}. enzyme activity level was found in the kidney tissue (−30.5 %) of rats with induced type 1 diabetes mellitus (Fig. 3). In the present study, the largest reduction in MST_{Homo}., MST_{Mito}., and TST_{Mito}. enzyme activity was detected in kidney tissue of rats with type 1 diabetes mellitus (Figs. 1, 2, and 3).

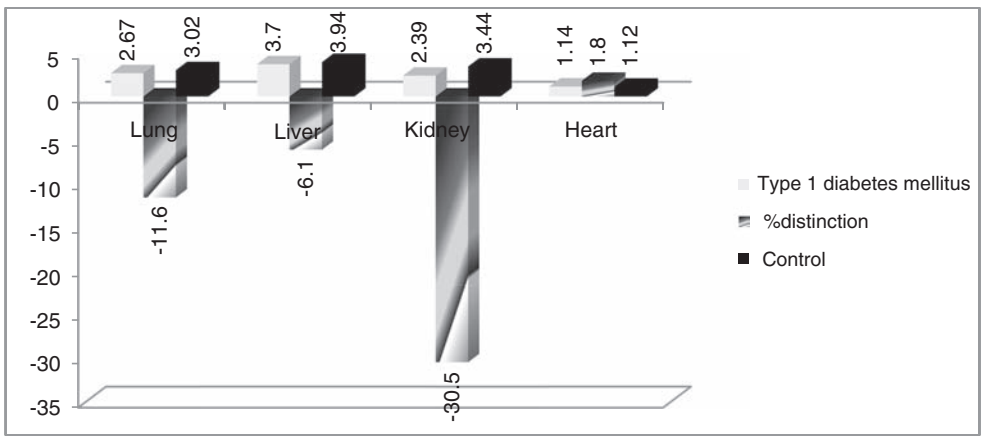

Fig. 3 Comparison of RhodanaseMito. levels in tissue homogenates

Mitochondrial $Rhodanase_{Mito.}$ (U/mg.protein)

Median(Min-Max) LungMito.$_{Type\ 1}$ 2,67 (1,26-4,66), LungHomo.$_{Cont.}$ 3,01(0,73-4,85), distinction -11,6% and P value 0,825

Median(Min-Max) LiverMito.$_{Type\ 1}$ 3,70 (1,62-4,77), LiverMito.$_{Cont.}$ 3,94 (3,31-4,80), distinction -6,1% and P value 0,826

Median(Min-Max) KidneyMito.$_{Type\ 1}$ 2,39 (1,68-3,70), KidneyMito.$_{Cont.}$ 3,44 (2,27-5,04), distinction -30,5% and P value 0,275

Median(Min-Max) HeartMito.$_{Type\ 1}$ 1,14 (1,02-1,24), HeartMito.$_{Cont.}$ 1,12 (1,07-1,16), distinction 1,8% and P value 0,827

Cyanogenic glycosides (e.g., amygdalin, dhurrin, ısolinamarin, linamarin, lotoustralin, prunasin) are found in many plants consumed by humans and animals. Hydrogen cyanide (HCN) is produced as a result of their hydrolysis. Cyanide which can get into the blood, through the respiratory and digestive system, as well as absorption through the skin, forms cyanomethemoglobin after binding methemoglobin [30].

The toxic effect of CN^- can be eliminated by transforming it into thiocyanate (SCN) by MST or TST. On the other hand, CN^- is most effectively inhibiting compound which causes inhibition of mitochondrial cytochrome oxidase system in the electron transport chain (ETC). The CN^- binding to the Fe^{+3} of cytochrome oxidase inhibits the transfer of electrons to molecular oxygen; thus, oxidative metabolism and phosphorylation is disrupted.

STSs participating in the detoxification of the CN^- and creating vital Fe-S groups in the cytochromes acting as an invertase further increases the importance of these enzymes for the organism [24, 25, 30, 31]. Reduced STS activities do not protect the organism against CN^- poisoning and impair energy production. In addition, considering that it acts to detoxify oxygen radicals [22], it is necessary in sulfate assimilation [32], and in many cofactor and vitamin syntheses where sulfur transfer is available by acting as a thioredoxin in the mitochondria. Thus, it can be seen that STSs have important regulatory functions in the organism [31–33].

The decrease in both in the MST and TST activities of the cytoplasmic and mitochondrial fractions of the type 1 diabetes mellitus group as found by our results shows that these tissues will be exposed to CN^- toxicity or shall have reduced defensive abilities. On the other hand, reduced ATP production will result in the production of energy in the tissues. Obviously, this will affect the kidneys most. A previous study has suggested that glycolysis increases TST activity when suppressed by the Pasteur effect. Mendel et al. suggest that TST failure can result in CN^- accumulation in the partial or complete absence of the Pasteur effect. It has been reported that TST takes roles in the activation of mitochondrial respiration, and it did so by interacting with Fe-sulfur centers and by contributing to the formation of such centers [34, 35]. From here, the organism will be poorly regulated in the chronic and prolonged periods of type 1 diabetes mellitus as a result of decreasing enzyme activities in the homogenates of the type 1 diabetes mellitus group, which will affect primarily the kidney, liver, and lung.

Up to the present, the lack of studies on diabetes and STSs do not provide us any opportunities to compare our findings. However, we obtained that some evidence about decreased activities of STSs may lead to reduced energy production in tissues; hence, cells may remain vulnerable to powerful poisons such as CN^-.

Metabolic diseases such as chronic renal failure, nephropathy [36], and heart failure [37] associated with differentiation of the energy production are major problems in patients with diabetes. Comprehensive research which will be done on STSs could contribute to the reduction of metabolic damage of diabetes mellitus.

Conclusion

In conclusion, this study has found reduced levels of enzyme both in tissue homogenates and in mitochondria of rats with induced type 1 diabetes mellitus. However, these results did not turn out to be statistically significant. The study reveals low levels of TST and MST activities—except for the activities MST_{Homo} and TST_{Mito} in hearth tissue—for all tissues compared to the control group. This result suggests that reduced STS activity contributes to pathogenesis of metabolic organ damage caused by diabetes. In our study, the greatest decrease in TST and MST activities of diabetic rats was observed in kidney tissue. This result implies that the metabolic damage caused by diabetes may first occur in kidneys, and treatments that increase STSs activities may prove effective in prevention of the kidney damage caused by diabetes. It is further suggested that long-term effects of type 1 diabetes are most likely to affect kidney tissues, while it is also likely that liver, lung, and heart tissues may be affected. We think that further studies should be conducted in different disease groups to further understand the biological roles of STSs.

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ORIGINAL ARTICLE

Acute phase proteins and diabetes microvascular complications

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Abstract The association of serum high-sensitivity C-reactive protein and ferritin with diabetes microvascular complications has not been examined concurrently in people with type 2 diabetes. So, we carried out this study in order to investigate this association in a group of type 2 diabetic patients. In a prospective cross-sectional study, 242 people with type 2 diabetes were enrolled. All of the participants were evaluated for diabetes microvascular complications. Retinal status was evaluated by retinal color photography and indirect ophthalmoscopy exam with dilated pupils. Michigan neuropathy screening instrument was used for detection of peripheral neuropathy, and albumin/creatinine ratio in a spot urine sample was considered to diagnose diabetic nephropathy. High-sensitivity C-reactive protein and ferritin were measured as indicators of acute phase proteins. The mean for high-sensitivity C-reactive protein was 5.3±13.02 mg/L, and for ferritin was 126.9±114.4 ng/mL. Statistically significant difference was found between the high-sensitivity C-reactive protein levels and diabetic nephropathy. Spearman's correlation coefficients test revealed that high-sensitivity C-reactive protein was positively correlated with diabetic nephropathy (P=0.05, r=0.14). However, such a correlation was not found for diabetic neuropathy and retinopathy. Using binary logistic regression analysis, a significant odds ratio was defined for nephropathy and high-sensitivity C-reactive protein level (OR=2.62; CI=1.13–6.06; P=0.025). Our findings suggest that low-grade inflammation is an independent predictor of diabetic nephropathy and measurement of high-sensitivity C-reactive protein can be useful for early detection of high-risk individuals.

Keywords Diabetes mellitus · Neuropathy · Nephropathy · Retinopathy · C-reactive protein · Ferritin

Abbreviations

Alb	Albumin
ETDRS	Early treatment diabetic retinopathy
HbA1C	Glycosylated hemoglobin
hs-CRP	High-sensitivity C-reactive protein
MNSI	Michigan neuropathy screening instrument
OGLDs	Oral glucose-lowering drugs

Introduction

Diabetes mellitus (DM) is one of the most common non-communicable diseases worldwide and is a growing concern. The increase of diabetic population worldwide is escalating public health problem globally. Its incidence and prevalence is increasing rapidly and is expected to grow further that represents a significant threat to human health because of its numerous and often serious micro- and macrovascular complications. Many factors are accused of contribution to the development of diabetes and its complications. These include genetics, diet, lifestyle, perinatal factors, aging, and obesity [1, 2]. Nevertheless, an inflammatory basis for diabetes and its complications has attracted interest. Several studies suggest that inflammation plays an important role in the pathogenesis of DM, and acute phase reactants have been proposed to monitor the process [3].

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High-sensitivity C-reactive protein (hs-CRP) is a protein found in the blood, and its levels rise in response to inflammation (i.e., hs-CRP is an acute phase reactants) [4]. hs-CRP, a sensitive marker of systemic inflammation, has been indicated to be increased in patients with type 2 DM [5, 6]. Recent prospective studies have recommended that an elevated level of hs-CRP is associated with an increased risk of developing type 2 diabetes [7–10]. hs-CRP levels are also elevated in individuals with features of metabolic syndrome [5, 6, 11] and cardiovascular disease [5, 6, 12]. On the other hand, the inflammatory response may make ferritin migrate from the interstitial space to the plasma, elevating the serum ferritin level [13]. The concentration of ferritin has been proved to increase in response to stress, infection, and inflammation [14]; this implies that it is an acute phase protein [15, 16]. According to previous studies [5, 17], among microvascular complications, only diabetic nephropathy is associated with high serum hs-CRP level. It has been suggested that the inflammatory process might play a role in diabetic nephropathy, and higher serum hs-CRP concentrations can predict the occurrence of diabetic nephropathy in type 2 diabetes [5, 17].

Similar correlations have been reported between ferritin level and diabetic retinopathy and nephropathy. So, diabetic microangiopathy might be associated with abnormally high levels of acute phase proteins [18–20]. Investigations into the role of inflammatory mechanisms in diabetes and its complications are expected to provide insight into the processes underlying the onset and progression of the disease. Such improved understanding of the inflammatory basis for diabetes may prove valuable for introducing novel approaches to treatment [2].

To our best knowledge, the association of hs-CRP and ferritin and diabetes microvascular complications has not been examined concurrently in people with type 2 diabetes. So, we carried out this study in order to investigate the association of serum hs-CRP level and ferritin with diabetes microvascular complications.

Methods

From January 2012 to February 2013, a total of 242 subjects, with previous documented diagnosis of type 2 diabetes, were enrolled in this prospective cross-sectional study.

The ethics committee of the university approved the protocol, and written informed consent was obtained from all of the participants.

Detailed history and physical examination were performed for all of the participants by an expert physician. Demographic and clinical parameters including sex, age, occupation, level of education, type of treatment for diabetes, duration of diabetes as well as history of concurrent systemic diseases and use of medications were obtained by a face to face interview.

The anthropometric measures were undertaken by a trained nurse. Standing height was measured using a stadiometer (Seca Gmbh & Co. Kg. Germany) calibrated before each measurement, and weight was measured using a calibrated digital scale (Seca Gmbh& Co. Kg. Germany). Body mass index (BMI) defined as weight in kg/height (meters) squared. Blood pressure was measured in a standard condition (sitting position, after 5 min of resting, and ceasing smoking, drinking tea or coffee, and eating food for at least half an hour).

The exclusion criteria were history of recent surgery, cancer, connective tissue disease, cardiovascular disease (recent myocardial infarction or cerebrovascular accident), any acute or chronic infection, and/or inflammatory process including SLE, inflammatory bowel disease, lupus, pneumonia, rheumatoid arthritis, giant call arteritis, rheumatic fever, tuberculosis, gout, bronchial asthma, osteoarthritis, and osteomyelitis, as well as use of oral contraceptive pills, hormone replacement therapy, pregnancy, and cigarette smoking. Furthermore, patients with a white blood cell count ≥10,000/μL, leukocytes in a spot urine sample, or a creatinine ≥1.4 mg/dL were also excluded.

Retinal status was evaluated by retinal color photography and indirect ophthalmoscopy exam with dilated pupils following the administration of tropicamide ophthalmic drop (Mydrax 1 %) by an expert ophthalmologist. Diabetic retinopathy was classified according to the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria [21]. The Michigan Neuropathy Screening Instrument (MNSI) was used for detecting peripheral neuropathy [22]. A total score greater than 2 points on a 10-point scale was considered to define a neuropathy. A urine sample was sent for measurement of urine albumin and creatinine. An albumin to creatinine ratio greater than 30 μg/mg was considered to identify microalbuminuria. For diagnosis of macroalbuminuria, a ratio greater than 300 μg/mg was considered as the cutoff value.

Definitions of risk factors

The diagnosis of diabetes was based on the criteria explained by the American Diabetes Association [23]. The participants were considered to be hypertensive when the measured blood pressure was ≥140/90 mmHg, or they had a history of treatment for hypertension. Based on the manufacturer's instructions, serum hs-CRP level higher than 6.3 mg/L (coefficients of variation: intra-assay=5.0, inter-assay=7.8), and serum ferritin level higher than 270 ng/mL (male) and 73 ng/mL (female) (coefficients of variation: intra-assay=6.3, inter-assay=5.8) were considered to be high.

Laboratory measurements

After an overnight fasting period of 12 h, venous blood sample were taken from all of the participants for measurement of fasting glucose, glycosylated hemoglobin (HbA1C), serum creatinine, ferritin, and serum hs-CRP.

Blood glucose was measured by enzymatic calorimeter method using a standard kit (EliTech kit) supplied by EliTech Group (France) and HbA1C by chromatography method using a standard kit (BioCode Hycel kit) supplied by BioCode Hycel Co. (USA). Creatinine was measured by the enzymatic calorimeter, Jaffe method, using a standard kit (Pars Azmoon kit) supplied by Azma Teb Sahand Co. (Iran). High-sensitivity C-reactive protein (hs-CRP) was measured by Eliza method using a standard kit (LDN kit) supplied by Labor Diagnostika Nord GmbH & Co. KG (Germany). Serum samples for hs-CRP levels were stored at −80 °C until assayed. Ferritin was measured by IRMA method using a standard kit (ImmunoTech kit) supplied by ImmunoTech Co. (Czech Republic). Urine albumin was measured by Eliza method using a standard kit (ORGENTEC kit) supplied by ORGENTEC Diagnostika GmbH (Germany). Urine creatinine was measured by the enzymatic calorimeter, Jaffe method, using a standard kit (Pars Azmoon kit) supplied by Azma Teb Sahand Co. (Iran).

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS version 18.0, Chicago IL). Descriptive statistics methods were used for baseline characteristics (mean±SD and proportions). Chi square test was used to evaluate the differences in proportions between the two groups. For comparison of quantitative variables, independent sample *t* test and Mann-Whitney *U* test were utilized. Pearson correlation coefficients test was used for assessment of correlation between quantitative variables, and Spearman's correlation coefficients test was used to compare qualitative or ordinal variables. A $P<0.05$ showed the level of statistical significance. Results were given with their 95 % CIs.

Results

Two hundred forty two people with type 2 diabetes, 140 (57.9 %) female and 102 (42.1 %) male, enrolled in this study. The mean age of the participants was 55.79±10.33, and the mean duration of diabetes was 9.1±7.9 years. The mean for BMI was 29.2±4.86 kg/m^2. The mean for hs-CRP was 5.3± 13.02 mg/L, and for ferritin was 126.9±114.4 ng/mL.

Table 1 illustrates demographic and clinical characteristics of the participants, according to the presence of diabetes microvascular complications; i.e., retinopathy, neuropathy, and nephropathy.

In a Spearman's correlation coefficients analysis, high hs-CRP levels were associated with a higher Alb/Cr ratio ($P=0.00$, $r=0.21$).

Statistically significant difference was found between the hs-CRP levels and diabetic nephropathy. Spearman's correlation coefficients test revealed that hs-CRP was positively correlated with diabetic nephropathy ($P=0.05$, $r=0.14$). However, such a correlation was not found for diabetic neuropathy and retinopathy. The frequency of diabetes microvascular complications in two groups of high and normal values of ferritin and acute phase proteins are demonstrated in Table 2.

Using binary logistic regression analysis, we found a significant odds ratio for nephropathy (dependent variable) and hs-CRP level as independent variable (OR=2.62; CI=1.13–6.06; $P=0.02$) (Table 3).

We also found that higher HbA1C was associated with higher hs-CRP levels ($P=0.02$), and Pearson correlation coefficient showed a positive correlation between HbA1C and hs-CRP ($r=0.16$).

Table 1 Demographic and clinical characteristics of the participants, according to the presence of diabetes microvascular complications

Characteristic	Diabetic retinopathy	Diabetic nephropathy	Diabetic neuropathy
Age (yrs.)	56.52±9.73	60.27±11.07	58.66±10.23
Gender (female) *n* (%)	26 (53 %)	25 (45 %)	24 (58 %)
BMI (kg/m^2)	29.41±5.38	28.73±4.88	29.23±4.68
Diabetes duration (yrs.)	13.68±9.28	11.53±8.71	11.66±9.20
FBS (mg/dl)	161.28±77.88	178.64±74.78	162.9±76.98
HbA1C (%)	8.28±2.09	8.27±2.02	7.79±2.17
hs-CRP (mg/L)	6.92±13.53	8.63±19.93	3.45±3.32
Ferritin (ng/ml)	146.23±112.56	147.62±118.95	126.9±76.98

Data are mean±SD unless otherwise indicated

BMI body mass index, *FBS* fasting blood sugar, *HbA1C* hemoglobin A1C, *hs-CRP* high-sensitivity C-reactive protein

Table 2 Diabetes microvascular complications and levels of ferritin and acute phase proteins

	Ferritin High	Normal	*P* value	hs-CRP High	Normal	*P* value
Retinopathy *n* (%)	28/47 (60 %)	19/47 (40 %)	0.2	12/ 40 (30 %)	28/40 (70 %)	0.33
Neuropathy *n* (%)	18/40 (45 %)	22/40 (55 %)	0.2	7/37 (19 %)	30/37 (81 %)	0.65
Nephropathy *n* (%)	23/56 (41 %)	33/56 (59 %)	0.09	17/ 53 (32 %)	36/53 (68 %)	0.05

Data are shown as *n* (%). Statistical method: $\chi 2$ test

hs-CRP high-sensitivity C-reactive protein

Forty two percent of the participants had a history of hypertension with significant correlation to the ferritin level (P= 0.04). Moreover, high blood pressure was significantly correlated to the ferritin level (P=0.02).

On the other hand, there was no statistically significant difference between the type of diabetes treatment and hs-CRP and ferritin (Table 4).

Discussion

Acute phase proteins are a class of proteins; their plasma concentrations increase (positive acute phase proteins) or decrease (negative acute phase proteins) in response to inflammation [24]. Previous studies demonstrated that inflammation plays an important role in the pathogenesis of diabetes mellitus [3, 5–7, 25]. Serum hs-CRP levels in people with diabetes are known to be higher than in the normal population [5, 26, 27].

In our study, we found a positive correlation between hs-CRP level and diabetic nephropathy. However, no correlation was found for the other diabetic microvascular complications. In addition, no correlation was found between ferritin level and diabetes microvascular complications.

According to the Third National Health and Nutrition Examination Survey (NHANES) III, the mean serum hs-CRP concentration in adults over 20 years is 4.14 mg/L [28], and in diabetic subjects is 4.8 mg/L [29]. In the present study, the mean hs-CRP concentration was 5.3±13.02 mg/L.

Sitzer et al. [30] reported that the median hs-CRP concentration is 1.135 mg/L in Germany. In the other study [Hashimoto et al. [31]], the median value of serum hs-CRP concentration was around 0.7 mg/L which is not compatible with our result. Therefore, there might be ethnical differences in the serum hs-CRP level. In addition, there are several factors that affect serum hs-CRP levels such as smoking [32], heavy exercise [33], hormone replacement therapy [32], and various medications [34, 35].

hs-CRP induced impaired self-regulation of glomerular pressure and/ or dysfunction of glomerular endothelium [36]. As a result, there is increased filtration of albumin via the damaged glomeruli and hence, an increased albumin loss in the urine [37]. These observations suggest that low-grade inflammation, reflected by hs-CRP levels, may play a role in the induction of microalbuminuria [38].

Currently, hs-CRP and microalbuminuria are considered to be markers of arterial wall inflammation, preclinical atherosclerosis, and systemic endothelial dysfunction [5, 25, 39–47]. Endothelial dysfunction, subclinical inflammation, and impaired fibrinolysis might contribute to the progression of macrovascular as well as microvascular complications

Table 3 Binary logistic regression analysis between nephropathy (dependent variable) and demographic, clinical characteristics and hs-CRP level

Variables	*B*	S.E.	*P* value	Odds ratio	95 % CI for odds ratio	
					Lower	Upper
Age	0.85	0.38	0.025	2.34	1.11	4.93
Sex	1.22	0.40	0.003	3.37	1.53	7.42
Diabetes duration	−0.68	0.41	0.09	0.50	0.23	1.12
BMI	0.002	0.04	0.96	1.002	0.93	1.08
HbA1C	−0.47	0.38	0.22	0.63	0.29	1.32
hs-CRP level	0.96	0.43	0.025	2.62	1.13	6.06
HTN	−0.25	0.38	0.51	0.78	0.37	1.62
Constant	0.65	1.45	0.65	1.92		

Statistical method: Logistic regression

BMI body mass index, *HbA1C* hemoglobin A1C, *hs-CRP* high-sensitivity C-reactive protein, *HTN* hypertension

Table 4 Types of diabetes treatment and acute phase proteins

	OGLDs	Insulin	OGLDs+insulin	*P* value (χ^2 test)
High hs-CRP	31 (22 %)	5 (25 %)	11 (32 %)	0.4
Normal hs-CRP	109 (78 %)	15 (75 %)	23 (68 %)	
High ferritin	79 (50 %)	13 (54 %)	23 (64 %)	0.3
Normal ferritin	80 (50 %)	11 (46 %)	13 (36 %)	

Data are shown as *n* (%). Statistical method: $\chi 2$ test

hs-CRP high-sensitivity C-reactive protein, *OGLDs* oral glucose-lowering drugs

[48–54]. However, the mechanisms responsible for development of microvascular and macrovascular complications are different [48–54].

Higher serum hs-CRP concentration in patients with type 2 diabetes may be a risk factor that gives rise to diabetic nephropathy [5, 17, 36]. According to the previous studies, hypertension, triglyceride, and obesity are the major sources of variation in the hs-CRP levels. However, hs-CRP can predict the occurrence and progression of diabetic nephropathy in type 1 and 2 diabetic patients [42, 55–58].

The EURODIAB study [59], and Spijkerman et al. [60], reported an association between hs-CRP and diabetic retinopathy but this association was not independent of HbA1C levels and BMI; the difference might be due to the difference in baseline level of hs-CRP, type of diabetes, and the larger sample size of their study.

Multiple studies [5, 61–64] reported that hs-CRP levels were not associated with diabetic retinopathy progression and severity that may be due to low prevalence of any diabetic retinopathy in the mentioned studies.

As a point in our study, however, most subjects with diabetic retinopathy had non-proliferative diabetic retinopathy. The level of hs-CRP in our study differed from other studies. But the strength of our study in this part include standardized assessment of serum biochemistry, evaluation of diabetic retinopathy by retinal color photography, and complementary physical examination by an expert ophthalmologist in a large group of patients with a relatively high prevalence of diabetic retinopathy. However, we found no correlation between hs-CRP and diabetic retinopathy.

In addition, we found no correlation between hs-CRP and diabetic neuropathy in parallel with the findings from another study [5]. It might be due to low frequency of diabetic neuropathy and the method used for detection of the neuropathy.

The inflammatory response may cause ferritin to migrate from the interstitial space to the plasma, elevating the serum ferritin level. The concentration of ferritin has been shown to increase in response to stresses [14]; this implies that it is an acute phase protein [15], since its levels are increased in the blood by infection or any type of chronic inflammation [65].

Some studies suggested that plasma ferritin concentration is positively correlated with insulin resistance and with the risk of development of type 2 DM, but ferritin does not have a major role in the development of diabetes microvascular complications [66, 67]. However, another study reported [19] a correlation between ferritin level and diabetic retinopathy which was significantly correlated to poor glycemic control. Furthermore, serum concentration of ferritin is reported to closely correlate to diabetic nephropathy [20].

In our study, similar to the other study [55], higher HbA1C was associated with higher hs-CRP level. Inflammation is a likely link among diabetes, obesity, and insulin; hence, a failure to improve insulin resistance and/or weight may have translated into a similar inability to decrease hs-CRP levels [68].

On the other hand, in the present study, there was no correlation between glycemic control and ferritin level. This correlation is still a controversial issue; some studies reported that there is a correlation [19, 69, 70] while the others [18, 66, 67] found no correlation between ferritin and glycemic control. This difference might be due to the different small sample size as well as study design in different studies.

Conclusions

Our findings suggest that low-grade inflammation is an independent predictor of diabetic nephropathy. Therefore, inflammatory markers, like hs-CRP, can be useful for early detection of high-risk individuals.

Further studies will be needed to reveal the function of hs-CRP in the progression of diabetic nephropathy. Further insight into the mechanisms involved in the etiology of diabetes and the specific effects of acute phase proteins will be essential for the development of new preventive strategies for diabetes microvascular complications.

Limitations

We have some limitations in this study. The cross-sectional study design lacked the specific information on the time sequence of the parameters. We used MNSI for detection of

diabetic peripheral neuropathy. Other objective methods such as nerve conduction velocity are more accurate for diagnosis of peripheral neuropathy. In addition, the diagnosis of diabetic nephropathy was based on a single measurement of albumin and creatinine in spot urine sample, although we excluded other causes of a positive albumin/ creatinine test. Several patients were not certain about the type of medications that they were on, and some of the patients were on different medications. The effects of these medications are unknown.

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Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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ORIGINAL ARTICLE

Clinical and genetic features of permanent neonatal diabetes mellitus

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Abstract Neonatal diabetes mellitus (NDM) is one of the most unusual and exceptional type of diabetes that occurs in infants before the age of 6 months. Transient neonatal diabetes mellitus (TNDM) and permanent neonatal diabetes mellitus (PNDM) are identified clinically. The study conducted was a retrospective cohort study by selecting eight children with neonatal diabetes mellitus between March 2009 and February 2012. The study was presented to King Abdul Aziz University Hospital, in Jeddah, Saudi Arabia. Mutational analysis was performed retrospectively to identify phenotype and genotype characteristics. All patients had NDM and the first symptoms were observed during 1 to 17 weeks of birth, with five males and three females. None of them showed dysmorphic features, seizures, or developmental delay. The timespan of symptoms reported by parents before diagnosis ranged from 3 to 10 days with mean duration of 5.6 days. In two patients (25 %), genetic studies revealed positive mutations, with one patient depicting KCNJ11 mutation and the other had an INS mutation additional screening for ABCC8 and FOXP3 mutations were negative. All patients showed permanent NDM and no transient NDM or the remission at any stage of the disease was observed. Neonatal diabetes is a rare medical condition which needs to be differentiated from transient neonatal hyperglycemia. The medical practitioners should look for molecular basis of neonatal diabetes in order to treat it as it will lead to proper treatment with an appropriate therapy.

Keywords Neonatal diabetes · Transient NDM · KCNJ11 mutation · Genetic analysis · Sulfonylurea

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Introduction

Neonatal diabetes mellitus (NDM) is the type of diabetes that occurs before the age of 6 months in infants. It is a very rare and unique form of diabetes and occurs only in one out of 400,000–50,000 newborns all across the world with no local registry of neonatal diabetes. Neonatal diabetes mellitus is characterized by intra uterine growth retardation. Moreover, the auto-antibodies of the pancreas are rarely found in this disease and human leukocyte antigen is found to be protective in nature. Neonatal diabetes mellitus has been classified into two clinically identified forms. One is the transient NDM which is found to be prevalent in 50–60 % of the NDM patients, and the other one is the permanent NDM [1, 2].

In transient NDM, infants with growth retardation develop diabetes during the first few weeks of their development. Later on, these infants develop permanent NDM, usually during the time period of adolescence. In transient NDM, the spontaneous onset of insulin secretion occurs and transient NDM can be resolved within a median time of 3 months. It is believed that in transient NDM, the pancreatic dysfunctions persists throughout the lifetime and become heightened during the times of metabolic stress like puberty and pregnancy. The mechanisms and systems involved in transient NDM might affect the development of the fetal pancreatic development and physiology of islet cells. Moreover, these mechanisms also lead to the development of type 2 diabetes. On the other hand, in permanent neonatal diabetes mellitus, the failure of

insulin secretion occurs during the early post natal period and this condition requires lifetime insulin treatment. A lot of conditions are associated with permanent NDM and some of these conditions have been demonstrated and described at the molecular level. For the treatment of permanent NDM, insulin pumps are utilized, since insulin therapy might be difficult to perform during the neonatal period [3].

A genetic study has demonstrated that mutations in the homozygous and the heterozygous genes are among the most common causes of the neonatal diabetes mellitus. During the first 3 months of the neonatal diabetes, the clinical type could not be identified since, minor hyperglycemia is persistent requiring an insulin treatment throughout. The study stated that the heterozygous activating mutations in the KCNJ11, the encoding gene for pore forming subunits of the KATP channels, and the mutations at the 6q24 band of the chromosomal gene can cause neonatal diabetes mellitus. Other genetic causes of permanent NDM include the insulin promoter factor-1 mutations, forehead box-P3, and glucokinase. Seventy percent of the transient neonatal diabetes mellitus occurs because of the over expression of chromosome at the band 6q24 [4]. Another study stated that ABCC8 who encodes the genes for two subunits of proteins namely the kir6.2 and SUR1of the ATP-sensitive potassium channel is the most common cause of both transient and permanent neonatal diabetes mellitus. The study also suggested that the mutations of the KCNJ11 and the INS are also the causes of permanent neonatal diabetes mellitus in 50 % of the patients [5]. Pancreatic cell ATP-sensitive potassium channel (KATP) is made up of kir6.2 and SUR1. The inward rectifier kir6.2 is encoded by KCNJ11 and the ABCC8 encodes the sulfonylurea receptor SUR1. These two are fundamental for the regulation and maintenance of the glucose-initiated secretion of insulin [6]. Recent studies have shown that the mutations of the KCNJ11 and ABCC8 genes that encode the pancreatic beta cells specifically the KATP channel are found to be the most common causes of the neonatal diabetes mellitus. Moreover, it has also been reported that 30–58 % of the permanent neonatal diabetes mellitus cases are found to have these two mutations. Moreover, the KCNJ11 mutations are found to be more commonly present in the transient neonatal diabetes mellitus or the neonatal diabetes associated with developmental delay. Patients who have KATP mutation have KATP channels with decreased response to ATP inhibition. Mutations in the KCNJ11 and the ABCC8 can be controlled with sulfonylurea in most of the patients. Sulfonylureas are a particular group of drugs that are used for the treatment of type 2 diabetes mellitus. The sulfonylurea can close the ATP-sensitive potassium channels by creating an independent ATP route [7, 8]. Because of this reason, the determination of the clinical etiology of this patient's neonatal diabetes mellitus carries critical and crucial importance. Therefore, this study aims at identifying the genetic and clinical characteristics of the neonatal diabetes mellitus cases in Saud Arabia. Moreover, the study also aims at initiating the sulfonylurea (SU) therapy where applicable.

Materials and methods

Subjects

A retrospective study was conducted on eight patients for the detection of genetic mutations in neonatal diabetes mellitus. The study was conducted in King Abdul Aziz University Hospital during the period of February 2009 to March 2012. Inclusion criteria for the study included that the subjects should have been diagnosed with diabetes within the first 6 months of their birth. Moreover, the clinical data, full medical history, anthropometric measurement, physical examination, and neurological findings were conducted. The signs and symptoms during the study time, blood glucose levels, and biochemical testing were also done. A legal consent was taken from all the selected patients of the study and their parents as well. The research study maintained all the ethical codes and regulations and followed the legal procedure of research mentioned in the Helsinki declaration.

Genetic studies

DNA extraction For the purpose of study, genomic DNA was taken out and extracted from the peripheral blood leucocytes of all the patients. Moreover, genetic data was also extracted from the parents of the eight patients. The genomic DNA samples which were extracted from the patients were screened and checked for the KCNJ11 [9] mutations. The DNA samples were also screened and monitored for the presence of FOXP3 [10] and the IPF1 [11] by the method of direct sequencing. Direct sequencing was done by the help of sequences and conditions published in the PCR primer design. The analysis of ABC88 was carried out by the help of a modified method demonstrated by Nestorowicz et al. [12].

Results

Clinical

The patients selected for the study were all presented with neonatal diabetes during the time period of birth till 6 months of age. The study group consisted of three males and five females, and all of them were full-term diabetes mellitus patients. It was observed in the study that the first signs of the onset of diabetes in these patients were seen during the time range of 1 to 17 weeks. None of the study subjects depicted dysmorphic facial appearance or features. Moreover, none of

the patients ever had seizures nor did any of them face developmental delay. The parents reported that the duration of appearance of symptoms before diagnosis occurred within 3 to 10 days. The mean value for the occurrence of symptoms was 5.6 days at the time of diagnosis. The symptoms that were present at the time of study were lethargy, fever, dehydration, polyuria, tachypnea, and poor feeding. The occurrence rate of symptoms varied among the study subjects. Six out of eight patients showed symptoms of poor feeding and lethargy. Fever was diagnosed in four patients whereas the polyuria was present in seven out of the total patients. Moreover, six patients showed depicted the symptoms of tachypnea and dehydration was present in all of the eight patients.

The weight at the time of birth for the all the patients had an average range of 140 to 2600 g. All the patients selected for the study had IUGR except only one patient. All the study subjects had severe and acute hyperglycemia and their median blood glucose level was 560 mg/dl (range 310–740 mg/dl). Ketonuria was also present in the patients and its severity level ranged from mild to moderate. Mild to moderate ketonuria was found to be present in four out of the total eight selected subjects. The family history was positive for consanguinity in three cases. Moreover, no clinically significant and related history of viral disease was found in the mothers during the time of pregnancy.

Genetic

Molecular basis for the neonatal development of diabetes was found in two out of the total eight subjects. KCNJ11 and p.E227K mutations were found in one patient and INS mutation was also found in another patient. But, none of the patients had ABCC8 mutation or FOXP3 mutation (Table 1).

All the patients depicted permanent NDM and none of the patients showed transient NDM, since not a single patient showed remission at any stage of the disease.

Discussion

Recent developments have been made for the treatment and diagnosis of the neonatal diabetes which is present during the first 6 months of the infant's life. Neonatal diabetes is a special form of diabetes that occurs because of a single mutation in the single gene, i.e., monogenic, and it is likely to be autoantibody-mediated (type 1 diabetes). Monogenic diabetes can be inherited from either the recessive gene or the dominant gene. It may also be retained from a de novo mutation [2]. Changes that occur during the neonatal diabetes are caused by the specific mutations in the KCNJ11 and ABC88 genes. These two genes encode the SUR1 and the inward rectifier Kir6.2. Patients diagnosed with the transient NDM, permanent NDM, and (developmental delay epilepsy neonatal diabetes) DEND syndrome were identified for KCNJ11 activating mutation in their genomic DNA. A definite and perfect phenotype-genotype relationship was studied and examined in a large cohort study [10]. But, at the same time, exceptional cases have also been reported. It has also been illustrated in many studies that the similar mutation in KCNJ11 does not always account for the same phenotype. Moreover, many studies suggest that the patients with KCNJ11 mutation might also have neurological dysfunction, i.e., the DEND syndrome. But, in this study, no developmental delay was observed in any of the patients. In our study, we had one patient with mutation in KCNJ11 who was diagnosed at the age of 3 months and she

Table 1 Clinical data of NDM patients with diabetes manifestation before the sixth month of life

Patient no.	1	2	3	4	5	6	7	8
Mutation	*KCNJI*	No	No	No	No	INS	No	No
Gender	Female	F	M	Fe	M	Ma	Fe	Fe
Birth weight (g)	2450	2600	2450	2200	2350	2480	1450	2545
Gestation (weak)	40	40	40	39	42	39	38	40
DM onset (weak)	12	3	11	17	11	4	1	2
Dysmorphic features	No	No	No	No	No	No	No	No
Seizures	No	No	No	No	No	No	No	No
Developmental delay	No	No	No	No	No	No	No	No
Auto antibodies	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Therapy after onset	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin
Insulin (IU/kg/day)	0.9	1.7	0.9	1	1.2	.2	0.7	0.6
C-peptide (ng/ml)	0.02	0.03	0.42	0.04	0.11	0.10	0.13	0.02
Current status Current age (year)	10	4	12	1.5	2	6	0.3	3
Current therapy	SU	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin
Current HbA1c (%)	7.6	10.5	10.8	10.9	10.7	12.3	12.4	6.9

was given insulin till the age of 9 years when she was shifted to glibenclamide with great success.

For the clinical classification of neonatal diabetes, the characteristics and features of the patients with ABCC8 and KCNJ11 mutations are to be considered. Almost half of the patients depicted SGA which resulted from the insufficiency of insulin during the fetal lifetime. These results were consistent and similar to the previous studies [5, 6, 9]. This comes in concordance with our patients as seven of eight patients were SGA. Significant differences were present in the onset of KCNJ11 and ABCC8 mutations. The duration of symptoms was shorter in neonates as compared to the older children. This reflects either of the two conditions. One is that their beta cell function deteriorated at a faster rate and they required a higher concentration of insulin/kilogram. The second condition might be that they had an increased susceptibility to dehydration as compared to the older infants or children.

In KATP channel mutation, the KATP channel fails to close in response to ATP in patients with neonatal diabetes. Because of this mutation, the patients had to remain dependent on insulin for the rest of their lives. Moreover, the KATP mutation prevents the depolarization of the beta cell membrane and also prevents the influx of calcium +2 ions. This leads to failure of the beta cells to release insulin into the blood. But, in majority of the cases with this kind of condition, they are treated with sulfonylurea therapy, in which the KATP channel closes when sulfonylurea binds with THE KATP channel.

The sulfonylurea therapy also has some other notable benefits. The sulfonylurea therapy improves the glycemic control and also provides flexibility and maintains the insulin secretion. The maintenance of insulin secretion is very important for a healthy life that is why the sulfonylurea therapy plays an important role in maintaining a healthy normal life. But, in addition to these, the sulfonylurea treatment also has some side effects like abdominal pain and diarrhea which are almost negligible, temporary, and mild [13].

Conclusion

Neonatal diabetes mellitus is one of the rarest diseases that occur in one out of four to five million infants worldwide. There are two clinical types of neonatal diabetes mellitus, i.e., the transient NDM which can be cured and the permanent NDM which persists throughout the life. In this study, successful results were obtained when the patients were treated with the sulfonylurea therapy. Moreover, the study also found molecular evidence for the presence of neonatal diabetes mellitus. These findings suggest that the medical practitioners have to consider molecular testing when looking for monogenic causes of diabetes for all patients who present ≤6 months of age. Establishing an accurate diagnosis in case of permanent neonatal diabetes is important as this may lead to the treatment with sulfonylurea therapy. This might be an appropriate therapy because of the transition and betterment in the results. The therapy would change the treatment modality from multiple daily insulin injections to sulfonylurea therapy with the potential for improved efficacy and convenience.

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Conflicts of Interest None

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ORIGINAL ARTICLE

Association of fatty acid profile in plasma lipid fractions with HbA1c in type 2 diabetic patients

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Abstract The aim of this pilot study has been the comparison of fatty acid profiles of diabetic and healthy subjects in order to evaluate the relationship between fatty acid profiles in plasma lipid fractions and glycated haemoglobin (HbA1c) in type 2 diabetes (T2D) patients. The fatty acid composition of fasting plasma lipid subfractions has been analyzed in patients (n=26) diagnosed with T2D and in corresponding control group (n=26) of healthy voluntary blood donors. Five subfractions containing phospholipids (PLs), diglycerides (DGs), free fatty acids (FFAs), triglycerides (TGs), and cholesterol esters (CEs) were isolated from plasma samples and separated by thin-layer chromatography. Fatty acid composition of these subfractions was analyzed by GC/FID. Significant changes in fatty acid profiles were found in all lipid fractions from T2D patients in comparison with the control group. HbA1c correlated negatively with delta 9 desaturation (9D) index. Significantly positive correlation of palmitic acid levels and negative correlation of oleic acid levels with HbA1c concentration were found in PL and TG fractions with higher significance in TGs. This pilot study has shown possible associations of HbA1c, common parameter measured in routine laboratories, with lipid metabolism. The strongest correlation was found in plasma TGs, especially in case of palmitic and oleic acids. This is the first report showing that metabolic control assessed by HbA1c is negatively associated with delta 9D index.

Keywords Type 2 diabetes mellitus · Fatty acid profile · Lipid fractions · Glycated haemoglobin · Desaturation index

Abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
CE	Cholesterol ester
DG	Diglycerides
DHA	Docosahexaenoic acid (22:6n-3)
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acids
FFA	Free fatty acid
GC	Gas chromatography
LA	Linoleic acid
MUFA	Monounsaturated fatty acid
PG	Prostaglandin
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
TG	Triglyceride
TLC	Thin-layer chromatography
T2D	Type 2 diabetes mellitus
5D	5 desaturation
6D	6 desaturation
9D	9 desaturation

Introduction

Type 2 diabetes mellitus (T2D) is a metabolic disease [1, 2]; its occurrence is increasing in Central European population. This disease is characterized not only by elevated glucose and glycated haemoglobin contents but also by impairment of

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lipid metabolism with all its inevitable consequences [3, 4]. Several studies showed close association of being overweight, adipose tissue, insulin resistance, dyslipidaemia and inflammatory markers in T2D patients [5–7].

Dietary fatty acids (FAs) are partly reflected by the FA profile of various biological media including adipose tissue (which reflects the intake of past months to years), erythrocyte membranes, and plasma or serum (which indicates the intake during several weeks). However, the FA profile in biological tissues is also strongly dependent on the endogenous metabolism of FAs. It has been shown that the suppression of the regulatory enzyme activity (desaturases and elongases) by experimental diabetes provoked an alteration in the FA composition of phospholipids in different tissues [8, 9].

Dyslipidaemia of T2D patients and patients with metabolic syndrome may be characterized by a decrease of the content of plasma n-3 and n-6 polyunsaturated fatty acids (PUFAs) and an increase of the content of saturated fatty acids (SFAs) that enhance insulin resistance, causing disappearance of pancreatic β-cells, and having also proinflammatory effects [10–15]. N-3 PUFAs help to prevent glucose intolerance and have anti-inflammatory properties [16, 17].

All these findings indicate a close relationship between diabetes and lipid metabolism. It is clear that it is necessary to study the mechanisms of the development of both dyslipidaemia and the pathogenesis of type 2 diabetes as well as to have a more detailed monitoring of lipid profiles in patients with diabetes in practice. However, analysis of fatty acid composition of plasma lipids is not available for routine laboratories. Therefore, we tried to address the question whether HbA1c is associated with dysregulation of lipids in T2D patients. Several studies showed an association between HbA1c and serum lipid profile in T2D patients [18–22]. HbA1c can provide valuable supplementary information about the level of circulating lipids—triglyceride (TG), LDL, HDL, and their ratios [4, 23, 24].

There is a possibility to utilize HbA1c for screening high-risk diabetic patients for early diagnosis of dyslipidaemia and timely intervention with lipid-lowering drugs [25]. An understanding of relations between HbA1c and lipid metabolism requires to study in detail relations between HbA1c and individual fatty acids in plasma lipid fractions—in phospholipids (PLs), diglycerides (DG), free fatty acid (FFA), TG, and cholesterol esters (CEs).

Subjects and methods

Study subjects

This case control study has involved 26 T2D patients and 26 healthy blood donors as the control group. The inclusion criteria of the study were all patients (1) above 30 years of age, (2) either gender, (3) with diagnosed type 2 diabetes mellitus for at least 3 years, (4) who keep an anti-diabetic low-calorie diet plus per-oral anti-diabetic drugs.

The exclusion criteria of the study were (1) type 1 diabetes; (2) renal failure, hepatic, oncologic or thyroid disease; (3) regularly consumption of alcohol and (4) insulin treatment. None of the study participants suffered from apparent cardiovascular disease, diabetic nephropathy and retinopathy. None of control persons was aware of metabolic disorder such as diabetes mellitus or hyperlipidaemia. They did not follow any specific dietary recommendation. They had not been taking any long-term medication. A written informed consent was obtained from all the participants. The study was approved by the Hospital Ethical Committee on Human Research (Germany).

Methods

Venous blood was obtained under standard conditions, from 7 to 8 a.m. after fasting for at least 12 h. Blood was collected in tubes with EDTA; plasma was obtained by centrifugation of the blood samples at 1500*g* for 20 min and immediately stored at −80 °C in 1.5-ml polypropylene tubes. Plasma lipids were separated by thin-layer chromatography to five subclasses (phospholipids, diglycerides, free fatty acids, triglycerides and cholesterol esters). Fatty acid methyl esters were prepared at University Hospital Tübingen, and then, the methyl esters of fatty acids were transported to the University of Pardubice.

Fatty acids of these lipid classes were after derivatization to the corresponding methyl esters quantified by gas chromatograph Agilent Technologies 7890A with an autosampler and a flame ionization detector, using a chromatographic fused column HP-88, 100 m × 0.25 mm × 0.2 μm. Helium was used as a carrier gas at a flow rate of 3 ml/min. The gas chromatograph oven temperature was initially held at 130 °C for 1 min; then, the temperature was programmed up to 176 °C at 2 °C/min and held for 2 min; then, the temperature was programmed to 186 °C at 1 °C/min and held for 1 min, then at 0.1 °C/min to 190 °C and held for 1 min and then at 1 °C/min to 220 °C and held for 4 min. The samples were injected in split mode (split ratio 10:1). The temperature of the injector was 250 °C and the temperature of the detector was set to 280 °C [26]. Total TGs were measured by ADVIA 1800 (Siemens AG, Munich, Germany) and HbA1c by HLC-723G8 (Tosoh Bioscience GmbH, Stuttgart, Germany).

Calculation of enzyme activity indices

The 16:1n7/16:0 and 18:1/18:0 ratios were calculated as indices of stearoyl-CoA desaturase 1, i.e. 9 desaturase activity. Furthermore, the 20:4n6/20:3n6 and 20:3n6/18:2n6 ratios

were calculated as indices of 5 and 6 desaturases activities, respectively. The ratio of 18:0 to 16:0 was calculated as an elongation index [26]. All enzyme ratios were calculated from concentrations of fatty acids (μmol/l).

Statistical analysis

FAs were presented as relative percentages of FAs analyzed. All statistical analyses were computed using STATISTICA 10 (StatSoft CR s.r.o., Prague, Czech Republic) and SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA). Differences in variables between the groups were evaluated using Mann-Whitney test. p value less than 0.05 was considered statistically significant. For evaluation of correlations, Pearson's correlations were used.

Results

From the two-factor analysis of variance (ANOVA) evaluating the changes in percentages of individual fatty acids, sums of plasma fatty acids and enzyme indices in the control group and type 2 diabetic patients as a function of gender and age showed no significant changes in the PL fraction and changes of certain fatty acid levels by age in TG fraction (see Tables 1 and 2).

The FA composition differed significantly in all lipid fractions of T2D patients when compared with the control group (Tables 3, 4, 5 and 6).

Polyunsaturated fatty acids

Total PUFAs were markedly decreased both in PL (p=0.033) and CE (p=0.038) fractions of the T2D group compared to the control group, predominantly due to down-regulation of n-6 PUFA content.

Significantly lower sum of n-6 PUFA was found in T2D patients in comparison with the control group in PL (p=0.006) and CE (p=0.030) fractions. The difference was caused mainly by linoleic acid, which was significantly lower in PL (p=0.016) and CE (p=0.029) fractions in the diabetic group. Index n6/n3 was lower in the PL fraction (p=0.022) of the T2D group. No correlations were found between PUFAs and HbA1c.

Saturated fatty acids

Lower value of the SFA sum was found in the FFA fraction in T2D patients (p<0.001) compared to the controls. The most significant decrease was observed in the case of stearic acid (p<0.001) and lignoceric acid (p<0.001).

The HbA1c positively correlated with palmitic acid (r=0.68, p<0.001) in TGs and (r=0.40, p=0.040) in PLs.

Monounsaturated fatty acids

The sum of monounsaturated fatty acids (MUFAs) was markedly higher in the diabetic group in the FFA fraction (p<0.001), mainly due to oleic acid (p<0.001) and vaccenic acid (p=0.010).

In the case of MUFAs (Fig. 2f), our results revealed a negative correlation of oleic acid (r=−0.46, p=0.020) with HbA1c in the TG fraction.

Desaturase and elongase indices

The activity of 9 desaturation (9D) index, characterized by the ratio of 16:1 n7 to 16:0, was markedly lower in T2D patients (p=0.005) in comparison with the control group in the CE fraction; 6 desaturation (6D) index, characterized by the ratio of 20:3 n6 to 18:2 n6, was markedly higher in T2D patients (p=0.014) compared to the control subjects. Elongation index (characterized by the ratio of 18:0 to 16:0) was in the FFA fraction significantly lower in T2D patients (p<0.001) compared to the control group. In the PL fraction, desaturation and elongation indices were higher in T2D patients, but the difference did not reach the statistical significance.

Further, we focused on relationships between enzyme activities and glycated haemoglobin as a marker of compensation of diabetes. Negative correlation was found in the case of the 9D index in the PL fraction (r=−0.45, p=0.020) as well as in the TG fraction (r=−0.54, p=0.004).

Discussion

The presented study has been focused on the evaluation of the differences in fatty acid profile between T2D patients and the control group. The aim of the study has been elucidation of the relationship between the amount of glycated haemoglobin (HbA1c), as a marker of compensation of diabetes, and fatty acid profile, desaturation and elongation indices in T2D patients.

The T2D is known to be associated with disintegration of the FA profile. The FA composition is affected by activities of lipid enzymes reflected by desaturation and elongation indices. The FA composition was analyzed in lipid fractions (PL, FFA, TG and CE) of plasma samples of 26 healthy donors and 26 T2D patients. We have found that the 9 D index was markedly lower in the plasma CE fraction of T2D patients compared to the control group. Similarly, the elongation index was lower in the FFA fraction.

Our results support the hypothesis that formation of FA from their precursors by desaturation is impaired under insulin resistance conditions and that reduced desaturation activities may be involved [27]. It has been described that hepatic stearoyl-CoA desaturase activity and de novo lipogenesis are

Table 1 Summary table for the two-factor analysis of variance (ANOVA) investigating the changes in percentages of individual fatty acids, lipidic enzyme indices, and sums of fatty acid groups in controls and type 2 diabetics in the phospholipid fraction as a function of gender and age

Controls						Diabetics			
Set	Factor	*F*	*p*	Power (1-β)	Conclusion	*F*	*p*	Power (1-β)	Conclusion
Palmitic acid (C16:0)	Sex	0.000	0.998	0.050	–	0.103	0.753	0.050	–
	Age	0.813	0.535	0.050	–	0.757	0.595	0.050	–
	Sex ∗ Age	2.743	0.065	0.410	–	0.340	0.880	0.050	–
Stearic acid (C18:0)	Sex	0.335	0.571	0.050	–	0.673	0.426	0.050	–
	Age	1.142	0.372	0.072	–	1.064	0.421	0.060	–
	Sex ∗ Age	0.830	0.526	0.050	–	0.404	0.838	0.050	–
Oleic acid (C18:1-N9)	Sex	0.149	0.705	0.050	–	2.337	0.149	0.180	–
	Age	1.258	0.327	0.092	–	2.723	0.064	0.427	–
	Sex ∗ Age	1.633	0.215	0.163	–	0.911	0.502	0.050	–
Linoleic acid (C18:2-N6)	Sex	0.058	0.813	0.050	–	0.394	0.540	0.050	–
	Age	0.504	0.733	0.050	–	1.074	0.416	0.061	–
	Sex ∗ Age	1.841	0.170	0.207	–	0.498	0.773	0.050	–
9D (16:1N7/16:0)[a]	Sex	0.041	0.842	0.050	–	0.019	0.892	0.050	–
	Age	0.401	0.805	0.050	–	2.594	0.073	0.396	–
	Sex ∗ Age	0.304	0.871	0.050	–	0.902	0.506	0.050	–
Elongase (18:0/16:0)[a]	Sex	0.167	0.688	0.050	–	0.361	0.558	0.050	–
	Age	1.221	0.341	0.085	–	0.914	0.500	0.050	–
	Sex ∗ Age	1.829	0.173	0.205	–	0.293	0.909	0.050	–
5D (20:4N6/20:3N6)[a]	Sex	0.468	0.504	0.050	–	0.126	0.728	0.050	–
	Age	0.180	0.946	0.050	–	1.006	0.450	0.051	–
	Sex ∗Age	0.249	0.906	0.050	–	1.224	0.349	0.087	–
6D (20:3N6/18:2N6)[a]	Sex	0.633	0.438	0.050	–	0.426	0.524	0.050	–
	Age	0.428	0.787	0.050	–	1.358	0.289	0.117	–
	Sex ∗ Age	1.444	0.265	0.126	–	0.337	0.882	0.050	–
Total SFA	Sex	0.948	0.345	0.050	–	0.673	0.426	0.050	–
	Age	0.760	0.760	0.050	–	1.179	0.368	0.079	–
	Sex ∗ Age	1.288	1.288	0.097	–	0.801	0.567	0.050	–
Total MUFA	Sex	0.016	0.901	0.050	–	2.088	0.170	0.154	–
	Age	1.130	0.376	0.070	–	1.845	0.169	0.216	–
	Sex ∗ Age	1.544	0.237	0.145	–	1.457	0.265	0.132	–
Total PUFA	Sex	0.436	0.518	0.050	–	4.546	0.051	0.408	–
	Age	0.375	0.823	0.050	–	1.197	0.360	0.082	–
	Sex ∗ Age	2.293	0.104	0.308	–	0.805	0.564	0.050	–
Total n-3 PUFA	Sex	0.070	0.794	0.050	–	3.410	0.086	0.291	–
	Age	0.026	0.999	0.050	–	1.511	0.249	0.143	–
	Sex ∗ Age	1.273	0.321	0.094	–	3.158	0.041	0.527	–
Total n-6 PUFA	Sex	0.451	0.512	0.050	–	0.653	0.432	0.050	–
	Age	0.264	0.897	0.050	–	1.156	0.378	0.075	–
	Sex ∗ Age	2.151	0.121	0.276	–	0.805	0.564	0.050	–
Index n6/n3	Sex	0.149	0.705	0.050	–	0.369	0.553	0.050	–
	Age	0.034	0.997	0.050	–	0.309	0.899	0.050	–
	Sex ∗ Age	2.855	0.058	0.435	–	0.996	0.455	0.050	–

The *F* test statistic is provided for comparisons within each factor and between the factors. The *p* value is the probability of being wrong in concluding that there is a true difference between the groups. There are significant differences if $p<0.05$. The power or sensitivity is the probability that the test will detect the observed difference among the groups if there really is a difference

9D 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Calculated by the concentration in μmol/l

Table 2 Summary table for the two factor analysis of variance (ANOVA) investigating the changes in percentages of individual fatty acids, lipidic enzyme indices, and sums of fatty acid groups in controls and type 2 diabetics in triglycerides fraction as a function of gender and age

Controls						Diabetics			
Set	Factor	*F*	*p*	Power (1-β)	Conclusion	*F*	*p*	Power (1-β)	Conclusion
Palmitic acid (C16:0)	Sex	0.054	0.820	0.050	–	0.169	0.687	0.050	–
	Age	0.352	0.839	0.050	–	2.099	0.126	0.276	–
	Sex ∗ Age	0.507	0.732	0.050	–	2.875	0.054	0.463	–
Stearic acid (C18:0)	Sex	0.724	0.407	0.050	–	1.230	0.286	0.070	–
	Age	0.097	0.982	0.050	–	0.868	0.527	0.050	–
	Sex ∗ Age	1.466	0.259	0.130	–	0.948	0.481	0.050	–
Oleic acid (C18:1-N9)	Sex	1.246	0.281	0.071	–	7.736	0.015	0.679	–
	Age	1.310	0.308	0.101	–	6.164	0.003	0.923	Significant
	Sex ∗ Age	1.358	0.292	0.110	–	4.957	0.008	0.826	Significant
Linoleic acid (C18:2-N6)	Sex	0.009	0.924	0.050	–	0.961	0.344	0.050	–
	Age	0.033	0.998	0.050	–	0.587	0.004	0.905	Significant
	Sex ∗ Age	0.456	0.767	0.050	–	2.668	0.068	0.414	–
9D index (16:1N7/16:0)[a]	Sex	0.389	0.541	0.050	–	0.035	0.853	0.050	–
	Age	1.097	0.391	0.064	–	0.621	0.686	0.050	–
	Sex ∗ Age	2.674	0.07	0.394	–	0.564	0.726	0.050	–
Elongase (18:0/16:0)[a]	Sex	0.973	0.339	0.050	–	0.406	0.534	0.050	–
	Age	0.071	0.990	0.050	–	1.032	0.437	0.055	–
	Sex ∗ Age	0.981	0.445	0.050	–	1.628	0.217	0.168	–
5D index (20:4N6/20:3N6)[a]	Sex	0.351	0.562	0.050	–	0.035	0.854	0.050	–
	Age	1.298	0.312	0.098	–	0.573	0.720	0.050	–
	Sex ∗ Age	0.312	0.866	0.050	–	0.633	0.678	0.050	–
6D index (20:3N6/18:2N6)[a]	Sex	0.031	0.863	0.050	–	1.001	0.334	0.050	–
	Age	0.899	0.488	0.050	–	3.251	0.037	0.547	–
	Sex ∗ Age	0.262	0.898	0.050	–	1.861	0.165	0.220	–
Total SFA	Sex	0.013	0.910	0.050	–	0.403	0.536	0.050	–
	Age	0.204	0.932	0.050	–	2.308	0.100	0.327	–
	Sex ∗ Age	0.710	0.597	0.050	–	2.649	0.069	0.409	–
Total MUFA	Sex	0.654	0.431	0.050	–	6.603	0.022	0.595	–
	Age	2.294	0.104	0.308	–	4.918	0.008	0.822	Significant
	Sex ∗ Age	2.105	0.128	0.265	–	3.912	0.020	0.677	–
Total PUFA	Sex	0.129	0.724	0.050	–	2.962	0.107	0.244	–
	Age	0.040	0.997	0.050	–	7.997	<0.001	0.981	Significant
	Sex ∗ Age	0.715	0.593	0.050	–	3.857	0.021	0.668	–
Total n-3 PUFA	Sex	1.117	0.306	0.059	–	2.140	0.166	0.160	–
	Age	0.472	0.755	0.050	–	4.270	0.014	0.736	–
	Sex ∗Age	0.722	0.590	0.050	–	2.737	0.063	0.430	–
Total n-6 PUFA	Sex	0.035	0.852	0.050	–	1.444	0.249	0.091	–
	Age	0.070	0.990	0.050	–	6.069	0.003	0.918	Significant
	Sex ∗ Age	0.579	0.682	0.050	–	2.824	0.057	0.057	–
Index n6/n3	Sex	6.736	0.020	0.614	–	0.041	0.842	0.050	–
	Age	2.908	0.055	0.447	–	1.115	0.396	0.068	–
	Sex ∗ Age	1.939	0.153	0.228	–	1.058	0.424	0.058	–

The *F* test statistic is provided for comparisons within each factor and between the factors. The *p* value is the probability of being wrong in concluding that there is a true difference between the groups. There are significant differences if $p<0.05$. The power or sensitivity is the probability that the test will detect the observed difference among the groups if there really is a difference

9D 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Calculated by the concentration in μmol/l

Table 3 The content of main FA and desaturation and elongation indices in PL subfraction

Parameter	Control group (n=26)	T2D patients (n=26)	p value[a]
Palmitic (C16:0)	33.03±2.85	31.84±2.82	0.083
Stearic (C18:0)	13.36±1.26	13.51±1.77	0.612
Oleic (C18:1-N9)	10.65±1.99	10.89±2.16	0.378
Linoleic (C18:2-N6)	18.20±3.41	16.82±3.79	0.016
α-Linolenic (C18:3-N3)	0.12±0.16	0.16±0.11	0.096
EPA (C20:5-N3)	0.72±0.37	0.88±0.39	0.369
DHA (C22:6-N3)	2.24±1.10	2.69±0.89	0.045
9D (16:1N7/16:0)[b]	0.010±0.008	0.012±0.004	0.350
Elongase (18:0/16:0)[b]	0.40±0.07	0.43±0.08	0.290
5D (20:4N6/20:3N6)[b]	4.41±3.24	5.15±2.80	0.429
6D (20:3N6/18:2N6)[b]	0.12±0.08	0.12±0.09	0.880
Total SFA	49.72±2.55	49.63±2.38	0.625
Total MUFA	13.74±2.46	14.61±2.38	0.128
Total PUFA	35.54±2.73	34.23±2.59	0.033
Total n-3 PUFA	3.91±1.19	4.77±1.46	0.089
Total n-6 PUFA	31.39±2.57	29.51±3.28	0.006
Index n6/n3	7.89±2.16	5.95±2.07	0.022

Data are presented as median of percentages±IQR

T2D non-insulin-dependent diabetes mellitus, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *9D* 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Mann-Whitney test

[b] Calculated by the concentration in μmol/l

Table 4 The content of main FA and desaturation, elongation indices in FFA subfraction

Parameter	Control group (n=26)	T2D patients (n=26)	p value[a]
Palmitic (C16:0)	30.83±4.80	29.28±3.93	0.069
Stearic (C18:0)	12.80±6.57	8.24±2.64	<0.001
Oleic (C18:1-N9)	29.51±10.75	35.08±7.15	<0.001
Linoleic (C18:2-N6)	9.89±2.83	10.55±3.46	0.167
α-Linolenic (C18:3-N3)	0.94±0.49	1.01±0.56	0.301
EPA (C20:5-N3)	0.27±0.32	0.11±0.05	0.005
DHA (C22:6-N3)	0.47±0.47	0.43±0.30	0.589
9D (16:1N7/16:0)[b]	0.049±0.035	0.041±0.033	0.310
Elongase (18:0/16:0)[b]	0.45±0.17	0.29±0.09	<0.001
5D (20:4N6/20:3N6)[b]	3.32±4.09	2.79±1.26	0.293
6D (20:3N6/18:2N6)[b]	0.036±0.049	0.024±0.020	0.058
Total SFA	46.88±10.37	40.81±5.04	<0.001
Total MUFA	32.98±10.43	39.53±6.82	<0.001
Total PUFA	13.78±4.26	14.10±4.33	0.504
Total n-3 PUFA	2.31±0.88	1.96±0.83	0.098
Total n-6 PUFA	11.83±3.51	11.95±3.09	0.749
Index n6/n3	5.16±2.90	5.74±1.76	0.197

Data are presented as median of percentages±IQR

T2D non-insulin-dependent diabetes mellitus, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *9D* 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Mann-Whitney test

[b] Calculated by the concentration in μmol/l

Table 5 The content of main FA and desaturation and elongation indices in TG subfraction

Parameter	Control group (n=26)	T2D patients (n=26)	p value[a]
Palmitic (C16:0)	31.75±4.38	29.50±4.79	0.146
Stearic (C18:0)	3.57±1.30	3.42±0.71	0.365
Oleic (C18:1-N9)	38.91±4.49	38.60±3.93	0.805
Linoleic (C18:2-N6)	12.28±4.86	12.92±4.86	0.964
α-Linolenic (C18:3-N3)	0.86±0.39	0.81±0.47	0.964
EPA (C20:5-N3)	0.20±0.18	0.20±0.12	0.905
DHA (C22:6-N3)	0.39±0.30	0.44±0.24	0.426
9D (16:1N7/16:0)[b]	0.053±0.054	0.036±0.020	0.094
Elongase (18:0/16:0)[b]	0.11±0.03	0.12±0.04	0.654
5D (20:4N6/20:3N6)[b]	4.93±5.10	4.13±2.43	0.268
6D (20:3N6/18:2N6)[b]	0.016±0.011	0.021±0.010	0.167
Total SFA	38.15±6.16	36.83±4.13	0.126
Total MUFA	42.34±4.95	42.31±4.36	0.920
Total PUFA	16.09±6.35	17.43±6.40	0.891
Total n-3 PUFA	2.02±0.77	1.84±0.77	0.993
Total n-6 PUFA	13.63±5.44	15.52±4.25	0.876
index n6/n3	7.96±3.94	6.91±2.86	0.654

Data are presented as median of percentages±IQR

T2D non-insulin-dependent diabetes mellitus, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *9D* 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Mann-Whitney test

[b] Calculated by the concentration in μmol/l

Table 6 The content of main FA and lipid desaturation and elongation indices in CE subfraction

Parameter	Control group (n=26)	T2D patients (n=26)	p value[a]
Palmitic (C16:0)	14.57±1.95	14.68±1.60	0.922
Stearic (C18:0)	1.12±0.43	1.08±0.70	0.650
Oleic (C18:1-N9)	20.07±3.57	20.77±3.57	0.244
Linoleic (C18:2-N6)	46.92±8.30	42.59±7.84	0.029
α-Linolenic (C18:3-N3)	0.45±0.27	0.45±0.20	0.676
EPA (C20:5-N3)	0.70±0.34	0.78±0.45	0.274
DHA (C22:6-N3)	0.39±0.37	0.38±0.20	0.259
9D (16:1N7/16:0)[b]	0.11±0.10	0.050±0.020	0.005
Elongase (18:0/16:0)[b]	0.08±0.03	0.073±0.057	0.783
5D (20:4N6/20:3N6)[b]	14.44±23.34	12.66±5.77	0.378
6D (20:3N6/18:2N6)[b]	0.008±0.009	0.013±0.008	0.014
Total SFA	17.32±2.08	17.83±2.13	0.415
Total MUFA	23.83±3.16	23.03±5.08	0.470
Total PUFA	56.21±6.31	53.36±7.73	0.038
Total n-3 PUFA	1.67±0.52	1.77±0.74	0.470
Total n-6 PUFA	54.40±7.18	51.32±8.03	0.030
index n6/n3	33.22±15.78	26.92±10.64	0.094

Data are presented as median of percentages±IQR

T2D non-insulin-dependent diabetes mellitus, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *9D* 9 desaturase, *5D* 5 desaturase, *6D* 6 desaturase, *SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

[a] Mann-Whitney test

[b] Calculated by the concentration in μmol/l

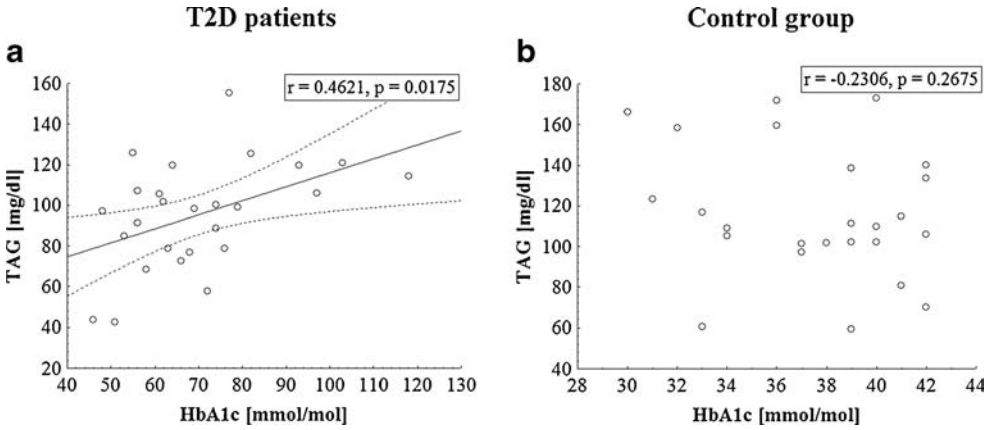

Fig. 1 Cross-sectional relationship of glycated haemoglobin with total plasma TGs **a** in T2D patients and **b** in the control group

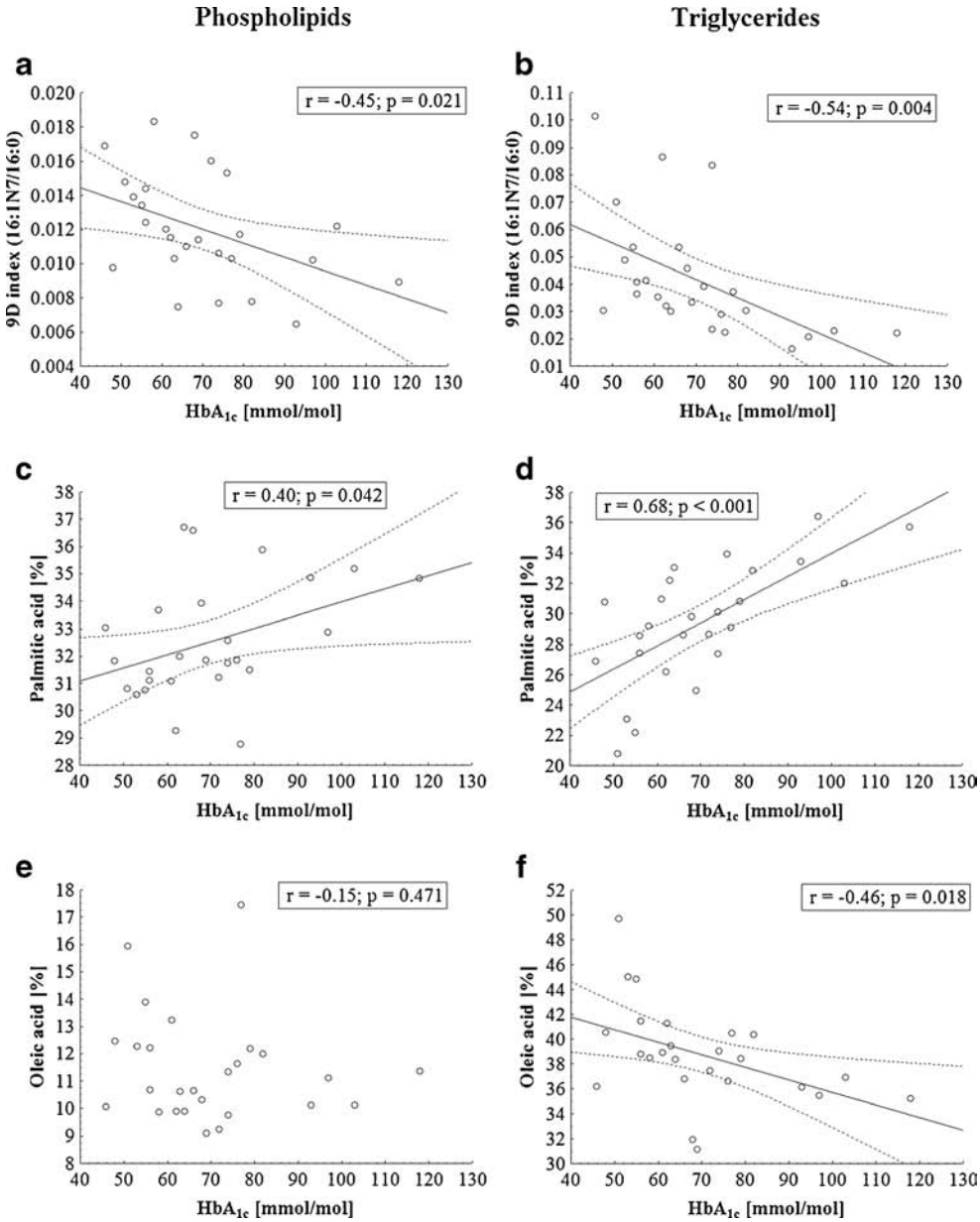

Fig. 2 Cross-sectional relationships of glycated haemoglobin values with **a** 9D index in the PL fraction, **b** 9D index in the TG fraction, **c** percentage of palmitic acid in the PL fraction, **d** percentage of palmitic acid in the TG fraction, **e** percentage of oleic acid in the PL fraction and **f** percentage of oleic acid in the TG fraction in T2D

not affected by 4-week sugar supplementation in healthy donors [28], but in T2D patients, we found an association between the 9 D index and glucose metabolism assessed by HbA1c (marker of variation in blood glucose) in PL and TG fractions.

The cytotoxic effect of most SFAs on pancreatic, liver and muscle cells is considered as a major cause of insulin resistance with subsequent development of diabetes mellitus type 2 and other complications, such as cardiovascular diseases, nephropathy and inflammation [29]]. It was described that SFA diet was accompanied by a significant decrease of HDL cholesterol [30]. These conclusions are in accordance with our results showing the positive correlation between palmitic acid and HbA1c values in the PL and TG fraction in T2D patients, unlike the situation in the healthy group. Comparing T2D patients and the control group, we found generally reduced sum of saturated fatty acids in PL, FFA and TG fractions of T2D patients, namely, of myristic acid and stearic acid. This could have been caused by reduced fat intake or by the quality of dietary intake.

Low levels of MUFAs can cause adverse effects. Palmitoleic acid can increase cell membrane fluidity and reduce inflammation associated with diabetes [17]. It was found that 16:1 can prevent insulin resistance in mice and predict insulin sensitivity in humans, independently of age, sex, and adiposity. There is a growing body of evidence that palmitoleic acid plays an important role in the pathophysiology of insulin resistance [26]. Palmitoleic acid stimulates muscle insulin action and suppresses haepatosteatosis serving as a lipokine [31]. On a cellular level, some studies indicate that

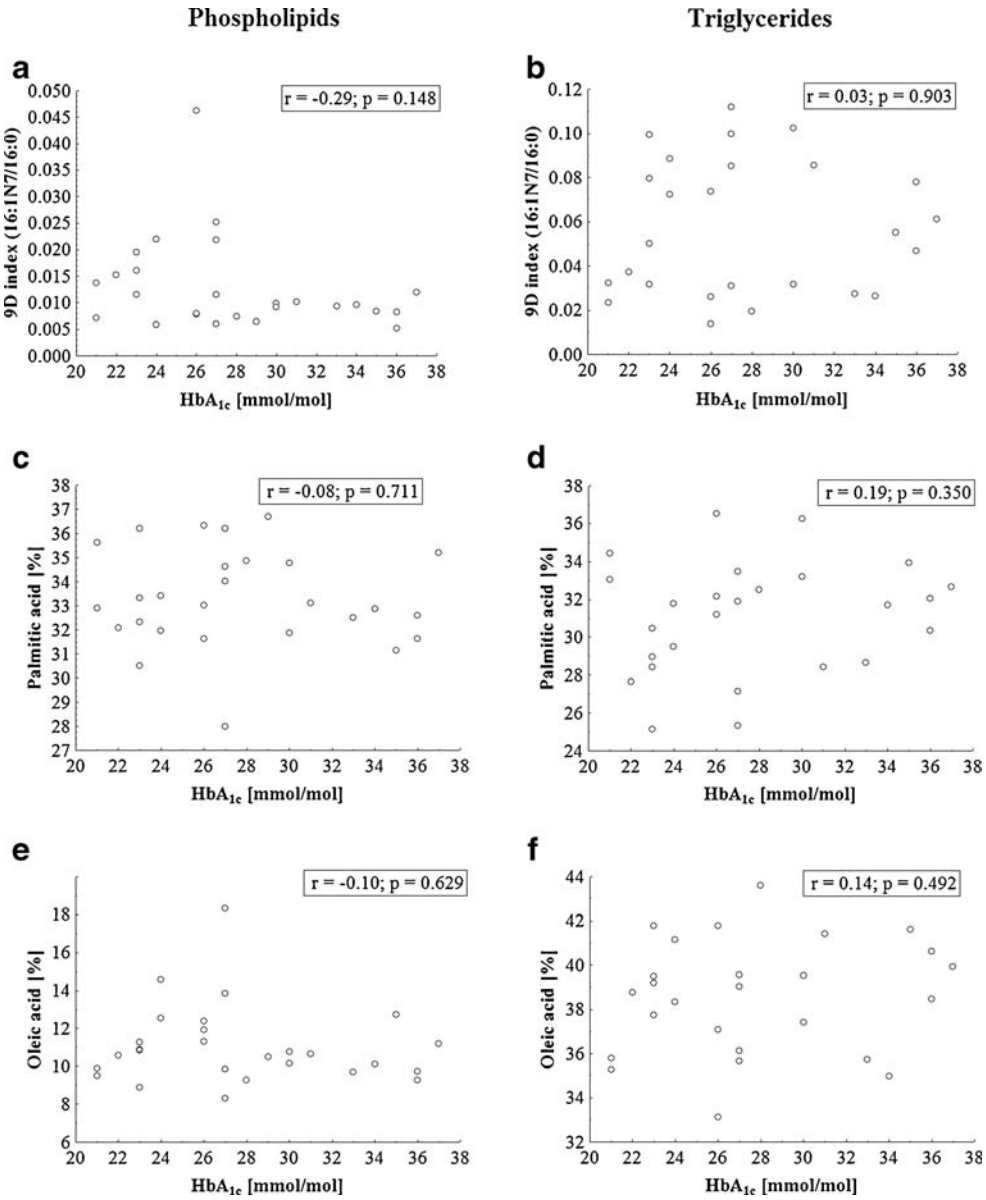

Fig. 3 Cross-sectional relationships of glycated haemoglobin values with **a** 9D index in the PL fraction, **b** 9D index in the TG fraction, **c** percentage of palmitic acid in the PL fraction, **d** percentage of palmitic acid in the TG fraction, **e** percentage of oleic acid in the PL fraction and **f** percentage of oleic acid in the TG fraction in the control group

palmitoleic acid can reduce cell death and endoplasmic reticulum stress in human and mouse cells and counteract the palmitate induction of these processes [17]. The proportion of MUFA was markedly higher in the FFA fraction in diabetic patients in comparison with the control group of blood donors that is in contradiction with above-discussed assertions. We expect that it is due to anti-diabetic diet and the fact that changes of FFA composition is most prone to fat intake.

In our study, relationship between MUFAs and HbA1c was investigated. Oleic acid showed negative correlation with HbA1c ($r=-0.52$, $p=0.006$) in the TG fraction (Fig. 2f). This is in accordance with the above-mentioned statements.

Type 2 diabetes is known to be associated with a metabolic disorder of n-3 and n-6 PUFAs, which has a great impact on the pathogenesis of inflammatory diseases. N-3 PUFAs help to prevent glucose intolerance and have anti-inflammatory properties; they give rise to anti-aggregatory and vasodilatory substances [16, 17, 32]. Furthermore, the eicosanoid metabolic products derived from arachidonic acid (AA), specifically prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids and lipoxins, cause a shift of the physiological state to the prothrombotic and proaggregatory one. Prostaglandins (PGs) derived from n-3 PUFA are created more slowly. Their task is to dampen the effects of elevated levels of PG derived from n-6 PUFA. Suppression of eicosanoid production from n-6 PUFA by n-3 PUFA is probably caused by competition among PUFA n-3 and n-6 for the common enzymes involved in elongation and desaturation of linoleic acid to AA and α-linolenic acid to EPA and DHA [33, 34]. We expected higher concentrations of n-3 PUFA and lower concentrations of n-6 PUFA in the control group, but no differences in n-3 PUFA were detected. Significant difference in n-6 PUFA levels was found in PL and CE fractions where lower levels were detected in T2D patients ($p=0.006$ in PLs, $p=0.03$ in CEs). We did not find any relationship between PUFAs and HbA1c. FAs that cannot be synthesized endogenously are the best biomarkers of FA intake [16, 35, 36]. Thus, there is a possibility that results in PUFAs were influenced by healthy diet and/or by treatment.

Our data showed that the fatty acid profile of T2D patients seems to be more positive than the fatty acid profile of blood donors without any severe diseases. Explanation is in treatment and good dietary habits of T2D patients that reduce disintegration of the fatty acid profile, despite of the occurrence of significantly lower 9 D indices. Anti-diabetic diet reduces the intake of food rich in saturated fatty acids and cholesterol and gives preference to vegetable fats rich on MUFAs and food rich on PUFAs or its supplements. Moreover, donors did not follow any specific dietary recommendation and lipid composition of this group. It reflects dietary fat intake and current poor dietary habits of the Central European population. Mild significant association was reported between HbA1c and serum lipid profile [18–22], which is in accordance with our findings of wide correlation of HbA1c with total TGs (Fig. 1). Our pilot study revealed that HbA1c is a good marker of lipid composition in plasma lipid fractions, especially TGs and PLs in T2D patients (Fig. 2). In the case of the control group (Fig. 3), significant correlations of HbA1c with lipids were not found, and therefore, it does not reflect the lipid metabolism in healthy individuals. Plasma TG seems to be the most suitable lipid fraction for interpretation of relation of HbA1c and lipid metabolism.

Conclusions

The study shows a positive effect of medical treatment and optimized diet on the composition of fatty acids in T2D. This pilot study investigated the relationship between HbA1c (common parameter of diabetes compensation measured in routine laboratories) and fatty acid composition of plasma lipid fractions and showed that there are significant associations with FA content especially in PL and TG fractions. There is a limiting factor of age, especially in the TG fraction (Table 2). Further, we have found a negative association of metabolic control assessed by HbA1c with the delta 9D index.

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Conflict of interest None.

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ORIGINAL ARTICLE

Seminal plasma zinc and magnesium levels and their relation to spermatozoa parameters in semen of diabetic men

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Abstract There is a growing body of evidence that diabetic conditions are associated with sexual problems. Metabolism of trace elements is altered in diabetic mellitus, and they may have a specific role in the pathogenesis and progression of the disease. The aims of this study are to investigate the levels of zinc and magnesium in semen of patients with diabetes mellitus and to find out if there is any association between trace elements and semen parameters in these diabetic subjects. Semen samples from 25 diabetic men and 25 nondiabetic were analyzed for physical and biochemical parameters. Zinc (Zn) and magnesium (Mg) levels were estimated by atomic absorption spectrophotometry. Zn and Mg concentrations in seminal plasma of nondiabetic men were more elevated than in diabetic groups. Zn showed positive and significant correlations with sperm motility ($p<0.05$, $r=0.52$) and morphology ($p<0.05$, $r=0.44$). Mg was significantly correlated with sperm motility ($p<0.05$, $r=0.51$) and morphology ($p<0.05$, $r=0.48$). Seminal plasma Zn and Mg showed a negative correlation with HbA1c, which was statistically significant ($r=-0.380$ and $r=-0.367$, $p<0.05$). Zn and Mg deficiencies become visible as an additional risk factor in the development of diabetes, and they are involved in the pathogenesis of diabetes mellitus and its complications. Adequate administration of these elements may be an effective therapeutic intervention in the prevention of the progression of the diabetes and its complications, along with a glycemic control and control of other risk factors.

Keywords Zinc · Magnesium · Spermatozoa · Diabetes

Introduction

Diabetes mellitus (DM) is one of the greatest threats to modern global health and based on epidemiological studies diabetes is a huge and growing problem. In 2013, more than 382 million people were affected by diabetes and every 6 s a person dies from diabetes [1]. Hyperglycemia in diabetes condition leads to be microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiac, cerebral) complications and early death [2].

There is evidence about effects of diabetic conditions on the male reproductive system at different levels [3, 4]. Studies on animal models revealed histological alteration on testes and damages on seminiferous tubules in hyperglycemic condition. Diabetic animals show diminished fertility, and their spermatogenesis was changed [5, 6, 4].

Many diabetic patients have problems in reproductive systems, but reports on the exact mechanism of hyperglycemia conditions on human male reproductive function are limited. Previous results on the relationship between diabetes and semen parameters are contradictory [7, 8]. Conventional semen parameter analysis only in severe abnormalities has value in the determination of fertility status [9].

Based on previous studies, there is a link between changes in trace elements metabolism and diabetes [10, 11]. Zinc (Zn) is an essential trace element for the function of more than 300 enzymes; however, abnormality of zinc homeostasis and its association with diabetes is not surprising [12]. Zinc

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administration can help to improve glycemic control and reduce oxidative stress in type 1 and 2 diabetes [12]. Zinc reduces blood glucose by inducing movement of the GLUT to the plasma membrane and rising entrance of glucose into the cells [13]. The human prostate secretes high levels of zinc and the concentration of Zn in seminal plasma is about 2 mM [14]. There is evidence that Zn in reproductive systems is important for stabilization of chromatin in sperm and acrosin [15, 16].

Magnesium (Mg) plays an important role in enzymatic reactions in many pathways of energy metabolism and nucleic acid biosynthesis. Mg acts as an intracellular calcium antagonist. Mg may be involved in sperm motility, and this element is a marker of seminal vesicle secretions [17].

Sperm count, motility, and morphology are parameters used to evaluate potential male fertility. Since Mg and Zn are believed to be important for spermatogenesis, we aimed to investigate the relation of these elements to male factor fertility and semen parameters by quantifying the concentrations in seminal plasma of diabetic and nondiabetic males.

Materials and methods

Sample collection and population

The project was studied and approved by the Research Ethics Committee of Hamadan University of Medical Sciences, and written consent was received from all participants. Diabetic men (type 1, $n=15$; type 2, $n=10$) aged 22–46 years attending the Diabetes Research Center (Hamadan University of Medical Sciences), who were diagnosed with diabetes 5 years prior to the study, was invited to participate in this project. The patients with diabetes were undertaking standard medication according to their diabetic situation. Twenty-five age-matched normospermic and healthy nondiabetic men were participated voluntarily in this study on a control group. The participants who were already using supplementary antioxidants or any other medication were not included within this study. The exclusion criteria included leukocyte concentration $>10^6$ per milliliter of ejaculate, medication or trauma, infection, history of smoking, varicocele, occupational exposure to chemicals or excessive heat. and specimens with hyperviscosity.

The semen samples were obtained during 4 days after abstinence by masturbation. Samples were collected in a clean, sterile, and wide-mouthed container made up of plastic that was confirmed as nontoxic for spermatozoa. The sample container was kept at a temperature of 37 °C. After collection, the specimen was labeled by the name to the patient, identification number, date, and time of collection. The semen container was placed in an incubator at 37 °C while the semen liquefied. The liquefied sample was taken for further analysis of semen parameters. The semen parameter analysis includes physical appearance, volume, viscosity, pH, and microscopic analysis. Conventional semen tests were examined in all samples based on WHO recommendations [18]. The seminal plasma samples were separated by centrifugation (Hermle, Z 230A, Wehingen, Germany) at 600*g* for 10 min and kept on −80 ° C.

Biochemical analysis on blood

Peripheral blood samples were taken after 12- to 14-h overnight fasting. Serum glucose was performed using a glucose assay kit (Pars Azmun, Tehran, Iran), and HbA1c was quantified using BioSystems kit (736CA, Barcelona, Spain) based on manufacturer's protocol.

Evaluation of trace elements in seminal fluid

All chemicals were of analytical reagent grade and were supplied by Merck (Germany). Seminal plasma (0.5 mL) were mineralized by placing the samples in mineralization tubes, adding 1 mL of a nitric and hydrochloric acid mixture (HNO3-HCl; 4:1), and heating at 120 °C for 65 min. The resulting solution was diluted to 10 mL with demineralized water. The digested samples were analyzed for zinc and magnesium by an atomic absorption spectrophotometer (Varian Spectron 220, Australia). The flame conditions were those recommended by the instrument manufacturer for zinc and magnesium (wavelength 231.9 and 285.2 nm, respectively). Concentrations are expressed as ppm.

Statistical analysis

Statistical analysis was performed using SPSS 11 software, and independent sample *t* test was used to compare mean differences between test and control samples. The association between variables was tested using Pearson's correlation coefficient and linear regression analysis. Data were represented as mean±SD, and $p<0.05$ were considered statistically significant.

Results

Semen analysis

The age (mean±SD) of diabetic and nondiabetic subjects was 35.84±8.89 and 32.58±5.68 years, respectively, and no significant difference was observed at the age of these two groups. Characteristics of semen quality are summarized in Table 1. These data show that there are no significant differences in volume of semen, sperm concentration, morphology, and motility of sperms between the two studied groups. All obtained data were in reference intervals that are recommended by the WHO.

Table 1 Semen characteristics and biochemical data of the diabetic and nondiabetic men

Parameters	Diabetics (n=25)	Nondiabetic (n=25)	p value
Semen parameters:			
Volume (ml)	3.22±1.47	3.64±1.30	0.226
Sperm concentration (10^6/ml)	98.28±54.76	87±37.59	0. 326
Total sperm number (10^6)	326.44±233.71	311.31±134.27	0.744
Motility grade a+b+c (%)	63.84±8.37	67.51±5.73	0.137
Normal morphology (%)	33.84±3.21	35.25±3.82	0.108
Biochemical parameters:			
Fasting blood glucose (mg/dl)	199.30±50.60	83.60±6.20	0.001
HbA1c (%)	7.60±1.17	4.1±0.42	0.001

Results are presented as mean values±standard deviation (SD)

The grades of sperm movement, according to the WHO criteria, are as follows: a=rapid progressive, b=slow progressive, and c=nonprogressive.

Trace element levels

In this study and as shown in Fig. 1, concentrations of seminal trace elements "Zn and Mg" were significantly (p<0.05) lower in the diabetic group in comparison with the nondiabetic group.

Correlation studies

Correlation analysis between semen parameters and seminal plasma levels of Zn and Mg in diabetic and nondiabetic groups were determined. As shown in Fig. 2, significant positive correlations were detected between seminal Zn concentrations and sperm motility (p<0.05, r=0.52) and sperm morphology (p<0.05, r=0.44) in the diabetic group. Seminal Mg also showed significant positive correlations with sperm motility (p<0.05, r=0.51) and sperm morphology (p<0.05, r=0.48) in the diabetic group. No significant relationship was found between Zn and Mg levels and semen parameters in the nondiabetic group.

In the diabetic group, the levels of seminal plasma Zn and Mg showed a negative correlation with HbA1c, which was statistically significant (r=−0.380 and r=−0.367, p<0.05, Table 2). No significant relationship was found between Zn and Mg levels and HbA1c in the nondiabetic group.

Discussion

The biochemical and clinical roles of trace elements in metabolism pathways of humans have been interesting. Trace elements have important physiological effects when present at concentrations other than those associated with classical toxicity or with extreme deficiency. There is accumulating report on alteration in trace elements metabolism in diabetic condition [19, 20]. Although there are studies on trace elements status in blood samples of diabetic men, the role and levels of trace elements in the semen of diabetic men are largely unknown.

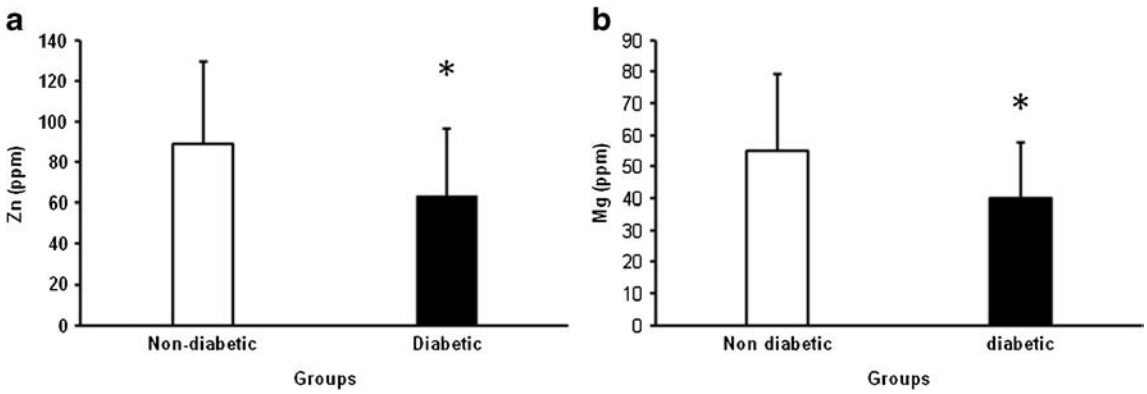

Fig. 1 Assessment of **a** Zn and **b** Mg concentrations between nondiabetic and diabetic groups. The levels of Zn and Mg in the diabetic group were significantly lower than in the nondiabetic group (*p<0.05). Results are represented as mean±SD

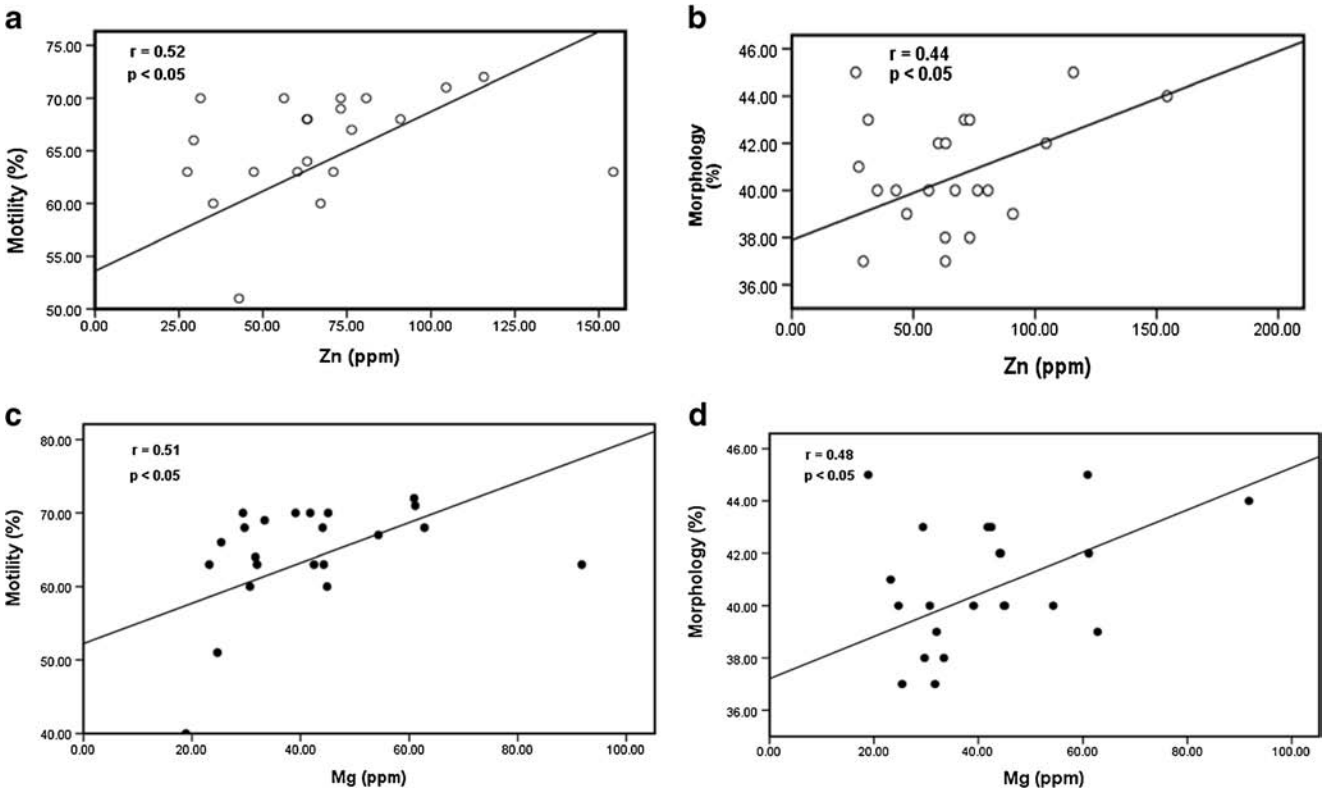

Fig. 2 Correlation between Zn levels and sperm motility (**a**) and sperm morphology (**b**) and correlation between Mg levels and sperm motility (**c**) and sperm morphology (**d**) in seminal plasma of diabetic men. Correlation coefficients between variables were statistically significant at the indicated *p* value

There are some reports showing decreased levels of Zn in the blood of diabetic subjects [21, 22]. In the present study, we demonstrated a significantly lower zinc level in seminal plasma of the diabetic group in comparison with the nondiabetic controls. Although we did not find data on the Zn status in seminal plasma of diabetic men, Turk et al. have reported low levels of Zn in seminal plasma of infertile men compared with control [23]. Other studies did not find significant difference in Zn level of semen between fertile and infertile men [24, 25]. Low concentrations of Zn in seminal plasma have been reflected as prostatic dysfunction, and it is regarded as a key factor in the regulation of fertility [26].

Our results revealed a significant positive correlation between increased seminal Zn levels and good motility and morphology of sperms in the diabetic group. In the nondiabetic group, no significant correlation was observed. This finding is evidence for a role of Zn in sperm quality in diabetic men and may be a useful explanation for the idiopathic sexual problem in diabetic subjects. Fuse et al. and Atig et al. found positive correlation between Zn content and sperm motility and sperm concentration [27, 28]. Baccetti et al. demonstrated zinc binding structures in human sperm and revealed the role of Zn in motility of sperm [29].

Based on our finding in this study, Mg level in seminal plasma of diabetic men was significantly lower than in nondiabetic men. Other studies showed low serum Mg content in diabetic patient [21, 30]. Mg plays a key role in glucose oxidation and release of insulin [21]. No reported concentration of Mg in seminal plasma of diabetic men could be found within the literature for comparison with our finding. A low level of Mg in seminal plasma of infertile men in comparison with that of fertile men have been shown by some researchers [31, 32].

We showed a significantly positive correlation between Mg levels in seminal plasma of diabetic men and sperm motility and normal morphology. Abdul-Rashid reported that seminal plasma magnesium correlated significantly and positively with sperm morphology of the oligozoospermic group

Table 2 Correlation of seminal plasma zinc and magnesium levels with HbA1c in diabetic patients

Pearson's correlation	*r* value	*p* value	Statistical significance
Seminal zinc v/s HbA1c	0.380	−0.019	Significant negative correlation
Seminal magnesium v/s HbA1c	0.367	−0.024	Significant negative correlation

compared with the control group. Wong et al. demonstrated a significant correlation between Mg in seminal plasma and sperm concentration, but not motility [14]. In the nondiabetic group, we did not find a correlation between Mg and semen parameters. Other studies did not find a correlation between Mg and semen parameters in normal condition that it supports our finding [33]. Although the exact role of magnesium in spermatozoa needs to be elucidated, magnesium is important for the maintenance of the structure of ribosomes, nucleic acids, and some proteins. Decreased Mg levels are known to affect glycolysis, protein synthesis, respiration, insulin resistance, carbohydrate intolerance, reproduction, and complications of diabetes [34].

HbA1c shows average plasma glucose during about the previous 10 weeks. HbA1c levels increased with the poor control of diabetes mellitus [35]. There was a significant negative correlation of seminal Zn and seminal Mg with HbA1c. It is believed that diabetic condition impairs kidney function. However, abnormal Zn and Mg metabolism has been suggested to play a role in the pathogenesis of diabetes and its complications. There were no published studies to compare seminal plasma trace element levels with HbA1c in the diabetic state.

Our study had several strengths and limitations. The major strengths are matching area of residence, similar aged distributions between cases and controls, and the simultaneous quantification of trace elements in the same laboratory and under the same quality control procedures. Some possible limitations to this study should be mentioned. Trace elements were measured in seminal plasma samples, and they may not be sensitive to spermatozoa or tissue-specific changes in trace element functioning. Our sample size of 25 men may be insufficient to detect small changes between groups.

In conclusion, although the majority of patients with diabetes have disorders in sexual function, associations between diabetes mellitus and sperm function at the molecular level are largely unknown. Zn and Mg deficiencies become visible as an additional risk factor in the development of diabetes, and they are involved in the pathogenesis of diabetes mellitus and its complications. Adequate administration of these elements may be an effective therapeutic intervention for the prevention of the progression of the diabetes and its complications, along with a glycemic control and control of other risk factors. Further studies are required to be done on other elements in the semen of diabetic men.

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

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ORIGINAL ARTICLE

MicroRNAs 103 and 107 link type 2 diabetes and post-menopausal breast cancer

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Abstract Type 2 diabetes mellitus (T2DM) increases the incidence of post-menopausal breast cancer (PMBC). This study is intended to determine whether microRNA-103/107 (miR-103/107) should be regarded as a potential molecular link between T2DM and PMBC. Samples of serum from 90 patients with T2DM and/or PMBC were collected. Samples of serum from 20 non-diabetic post-menopausal women were used as the control. The body mass index (BMI) of patients with T2DM and PMBC was lower than the BMI of patients with only T2DM or PMBC ($p<0.05$). The expression of miR-103/107 was higher in the serum of T2DM patients compared with that in control samples (2.80±0.46/36.29±3.41 vs 0.88±0.25/8.59±1.91, $p<0.05$). The expression of miR-103/107 in the serum of PMBC patients was higher than that in T2DM patients (5.06±0.92/49.59±6.99 vs 2.80±0.46/36.29±3.41, $p<0.05$) but lower than that in patients diagnosed with both T2DM and PMBC (7.67±0.87/63.24±8.58, $p<0.05$). miR-103/107 was positively correlated with the homeostasis model assessment-insulin resistance (HOMA-IR) index ($r=0.71$, 0.685, $p<0.01$). The expression of miR-103/107 was an independent factor of the HOMA-IR index ($\beta=0.638$, 0.073, $p=$ 0.02, 0.01). There were higher levels of estradiol (E2) in patients with T2DM and/or PMBC than that in the control group. High expression of miR-103/107 results in insulin resistance and is associated with overweight or obese patients with T2DM and PMBC through elevated levels of E2. miR-103/107 may be a potential molecular link between T2DM and PMBC.

Keywords miR-103/107 · Type 2 diabetes · Post-menopausal breast cancer

Introduction

Epidemiologic data have shown that compared with younger women, older women are at higher risk for breast cancer and diabetes. Females affected by type 2 diabetes mellitus (T2DM) and/or post-menopausal breast cancer (PMBC) are usually overweight or obese. This correlation may indicate age and obesity as a common pathogenesis. The molecular mechanism of the correlation between obesity and these conditions is complicated, and research shows that insulin resistance [1–4] may be one of the molecular mechanisms.

MicroRNAs are small, non-coding RNAs that regulate the expression of mRNA and are involved in a variety of diseases, such as T2DM and breast cancer. MicroRNAs 103 and 107 (miR-103/107), which belong to a family of microRNAs, are highly expressed in patients with obesity, T2DM, and breast cancer. In obese mice, high expression of miR-103/107 in liver or fat downregulates the expression of caveolin-1 (Cavelin-1, CAV-1), which contributes to insulin resistance and dysfunction of glucose transcription factor-4, leading to increased hepatic glucose output and decreased peripheral glucose uptake in muscles [5–7]. These responses, in turn, cause elevated blood glucose. When islet β cells are uncompensated, T2DM results. Relatively high levels of estrogen have been reported in post-menopausal obese women, and downregulated CAV-1 activates estrogen receptor-alpha (ER-α) [8]. The binding of estrogen with receptor-α is

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associated with the development and progression of breast cancer. miR-103/107 is stably expressed in serum, plasma, and blood.

miR-103/107 may be used as potential molecular markers of breast cancer [9–11]. Currently, there are no reports on miR-103/107 in overweight or obese patients with T2DM and PMBC. The purpose of this study is to investigate the relationship between miR-103/107 and insulin resistance and determine whether miR-103/107 should be regarded as a potential molecular link between T2DM and PMBC.

Research design and methods

Subjects

This study was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the institutional ethics review board at the Department of Endocrinology and Breast Cancer of the Second Affiliated Hospital of the Medical College of Qingdao University. Written informed consent for the collection of the samples and subsequent analysis was obtained from each patient.

A cohort of 90 patients with T2DM and/or PMBC who had been treated at the Department of Endocrinology and Breast Cancer of the Second Affiliated Hospital of the Medical College of Qingdao University from November 2011 to April 2013 was collected in the present study. Twenty age-matched, non-diabetic, post-menopausal women were selected as controls (with no T2DM and PMBC). The blood pressure and electrocardiogram of all patients in both the study and control groups were normal. The patients with diabetes were newly diagnosed and were not taking medication for diabetes. The diagnosis of diabetes mellitus was confirmed according to the WHO guidelines by the presence of fasting glucose levels greater than 7.0 mmol/L in routine laboratory evaluation [12]. All patients with breast cancer were diagnosed by pathological examination. The definition or criteria for overweight or obesity was previously described by St-Onge, M. P. et al. [13]. Body mass index (BMI)=weight/height^2 [14].

Measurements

The fasting glucose and insulin, hemoglobin A1c (HbA1c), triglyceride (TG), estradiol (E2), and miR-103/107 levels were measured. Blood samples were collected in an 8-h fasting state. Fasting plasma insulin was measured by RIA (RuiQi Biotechnology Corporation, Shanghai, China) with a sensitivity of 2 mU/L (normal range 0.5–25 mU/L) and the homeostasis model assessment of insulin sensitivity. Homeostasis model assessment (HOMA) was estimated using these values [15]. Serum concentrations of E2 were identified using enzyme-linked immunosorbent assay kits (Heng Yuan Biotechnology Co., Ltd, Shanghai, China). The expression of miR-103/107 was detected using quantitative polymerase chain reaction (qPCR).

Microarray profiling of serum miRNAs

Total RNA was extracted from healthy patients and patients with T2DM and/or post-menopausal breast cancer (Shanghai Fu Sheng Industrial Co., Ltd., China). miRNA microarray analysis, including labeling, hybridization, scanning, normalization, and data analysis, was performed by Biological Technology Co., Ltd., Chengdu Norn in China using a miRCURY LNA™ microRNA Array Kit v.16.0 (Exiqon, Vedbaek, Denmark).

Quantitative polymerase chain reaction

Total RNA was isolated with a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA). All specific primers for miRNA expression were designed and synthesized by Guangzhou RiboBio Co. Ltd., Guangzhou, China, using the mirVana™ qRT-PCR Primer Sets. The differential expression levels for miRNA were validated using the SYBR Green® Quantitative PCR Protocol on an Applied Biosystems 7500® Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The levels of an endogenous control, U6 (RiboBio, Guangzhou, China), were used to normalize the expression levels of each miRNA. The fold change in miRNA expression was calculated using the comparative CT method.

Statistical analysis

All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Normally distributed data are expressed as the means±SD. One-way ANOVA was used in the study. Pearson correlation analysis and stepwise regression analysis were used for the simple correlation and multivariate analysis. A p value of less than 0.05 was considered statistically significant.

Results

General characteristics

A total of 110 subjects were included in this study (NC=20, T2DM=30, PMBC=30, DM with PMBC=30). Compared to non-diabetic post-menopausal controls, the BMI, homeostasis model assessment-insulin resistance (HOMA-IR) index, HbA1c, and TG of patients with T2DM and/or PMBC were higher ($p<0.05$). The BMI of patients with T2DM and PMBC was lower than that of patients with only T2DM or PMBC

($p<0.05$). The HOMA-IR index of patients with T2DM and PMBC was higher than that of patients with only T2DM or PMBC ($p<0.05$). The levels of E2 and fasting plasma insulin in the serum in patients with T2DM and/or PMBC were higher than that in non-diabetic post-menopausal controls ($p<0.05$) (Table 1).

Differential expression of miR-103/107 in the serum in patients with T2DM and/or PMBC and in non-diabetic post-menopausal controls

When comparing differences between groups by univariate analysis of variance, if the difference was statistically significant, the LST-*t* test was used to make pairwise comparisons; the test of miR-103/107 showed homogeneity of variance ($p=0.358$, 0.176). The expression of miR-103/107 in the four groups was different ($F=432.64$, 344.06, $p=0.00$), and in pairwise comparisons, we identified statistical significance in each group ($p<0.05$). The levels of expressed miR-103/107 in the serum in PMBC patients were higher than that in T2DM patients (5.06±0.92/49.59±6.99 vs 2.80±0.46/36.29±3.41, $p<0.05$) but were lower than that in patients diagnosed with both T2DM and PMBC (7.67±0.87/63.24±8.58, $p<0.05$). The levels of expressed miR-103/107 in the serum in T2DM patients were higher than that in the non-diabetic post-menopausal control group (2.80±0.46/36.29±3.41 vs 0.88±0.25/8.59±1.91, $p<0.05$) (Table 2).

Table 2 Differential expression of miR-103/107 levels in serum in patients with T2DM and/or PMBC and healthy controls ($n=110$)

	Control ($n=20$)	T2DM ($n=30$)	PMBC ($n=30$)	DM with PMBC ($n=30$)
miR-103	0.88±0.25	2.80±0.46[a]	5.06±0.92[ab]	7.67±0.87[abc]
miR-107	8.59±1.91	36.29±3.41[a]	49.59±6.99[ab]	63.24±8.58[abc]

Normally distributed data were expressed as means±SD

Control non-diabetic post-menopausal woman, *T2DM* type 2 diabetes mellitus, *PMBC* post-menopausal breast cancer, *miR103* microRNA-103, *miR107* microRNA-107

[a] $P<0.05$, comparison with control

[b] $P<0.05$, comparison with T2DM

[c] $P<0.05$, comparison with PMBC

Associations between miR-103/107 and BMI, HOMA-IR index, HbA1c, and TG in patients with T2DM

The Pearson correlation between miR-103/107 and other parameters for the three groups (T2DM, T2DM and post-menopausal breast cancer, post-menopausal breast cancer only) is shown in Table 3. We identified a positive correlation between miR-103/107 and BMI ($r=0.454/0.633$, $p<0.01$); miR-103/107 and HOMA-IR index ($r=0.696/0.831$, $p<0.01$); miR-103/107 and HbA1c ($r=0.467/0.558$, $p<0.01$); and miR-103/107 and TG ($r=0.640/0.770$, $p<0.01$). The HOMA-IR index was a dependent variable, and miR-103, miR107, TG, HbA1c, and BMI were independent variables. Multivariate linear regression analyses showed that miR-103/107 were independent factors of the HOMA-IR index (Table 4).

Discussion

Insulin resistance initiates T2DM etiology. T2DM is characterized by deficient glucose uptake in metabolic tissues and is manifested when insulin secretion fails to cope with

Table 1 Clinical characteristics of the subjects

	Control ($n=20$)	T2DM ($n=30$)	PMBC ($n=30$)	DM with PMBC ($n=30$)
Age (years)	59.91±11.32	60.90±10.21	62.02±9.51	61.32±10.85
BMI (Kg/m^2)	20.13±1.71	29.01±1.28[a]	29.06±1.34[a]	27.05±1.47[abc]
HOMA-IR index	1.72±0.35	7.62±0.38[a]	7.57±0.41[a]	8.17±0.60 [abc]
HbA1c (%)	4.68±0.43	7.83±0.35[a]	5.42±0.36[ab]	7.74±0.33[ac]
E2 (pg/ml)	15.57±3.35	23.61±3.24[a]	27.52±3.40[ab]	22.78±3.45[ac]
Fasting plasma insulin (IU/ml)	7.80±2.31	12.67±3.12[a]	17.72±2.98[ab]	13.74±3.01[ac]
TG (mmol/L)	0.91±0.31	2.63±0.43[a]	2.58±0.37[a]	2.67±0.39[a]

Normally distributed data were expressed as means±SD

Control non-diabetic post-menopausal woman, *T2DM*, type 2 diabetes mellitus, *PMBC* post-menopausal breast cancer, *BMI* body mass index, *HOMA-IR* homeostasis model assessment-insulin resistance, *HbA1c* hemoglobin A1c, *TG* triglyceride, *E2* estradiol

[a] $P<0.05$, comparison with non-diabetic post-menopausal control

[b] $P<0.05$, comparison with T2DM

[c] $P<0.05$, comparison with PMBC

Table 3 Pearson correlation analysis of miR-103/107 and BMI, HOMA-IR, HbA1c, and TG

	BMI	HOMA-IR	HbA1c	TG
r (miR-103)	0.454[a]	0.696[a]	0.467[a]	0.640[a]
r (miR-107)	0.633[a]	0.831[a]	0.558[a]	0.770[a]

r correlation coefficient, *BMI* body mass index, *HOMA-IR* homeostasis model assessment-insulin resistance, *HbA1c* hemoglobin A1c, *TG* triglyceride

[a] $p<0.01$

worsening insulin resistance. Therefore, investigating the molecular mechanism of T2DM is of primary importance in preventing and controlling this disorder.

The molecular mechanism of insulin resistance is complicated. Insulin resistance is described as a subnormal response to both endogenous and exogenous insulin. It is characterized by a decreasing sensitivity of the target tissues to the action of insulin, by elevated blood glucose concentrations, and by increased hepatic production of atherogenic lipids. The accumulation of central abdominal fat in post-menopausal women is associated with a decline in the production of the protein adiponectin. Low serum adiponectin levels are associated with insulin resistance. In addition to adiponectin [16], high serum resistin, inflammatory cytokines [17], and miR-103/107 are also involved in insulin resistance [7] by affecting insulin signaling transduction.

miR-103/107 results in insulin resistance. Trajkovski M. et al.[7] reported that silencing of miR-103/107 leads to improved glucose homeostasis and insulin sensitivity. In contrast, an increase in miR-103/107 function in either liver or fatty tissue is sufficient to induce impaired glucose homeostasis and insulin resistance. Furthermore, there is a positive correlation between the HOMA-IR index and miR-103/107 expression levels, indicating an association of these miRNAs with insulin resistance, which contributes to the etiology of T2DM. Further studies confirmed that high expression of miR-103/107 leads to insulin resistance by downregulating CAV-1, which is the direct target gene of miR-103/107 and also a critical regulator of insulin receptors [7]. In our study, we found that miR-103/107 were positively correlated with the HOMA-IR index and were independent factors in overweight or obese patients with T2DM.

Table 4 Linear regression analysis of affecting HOMA-IR index

	Independent regression coefficient	SE	Standard regression	*t* value	*p* value
BMI	0.052	0.047	0.182	1.120	0.269
miR-103	0.638	0.198	0.569	3.228	0.002
HbA1c	−0.213	0.162	−0.117	−1.314	0.192
TG	1.236	0.459	0.372	2.692	0.08
miR-107	0.073	0.020	0.473	3.724	0.001

BMI body mass index, *miR-103* microRNA-103, *HbA1c* hemoglobin A1c, *TG* triglyceride, *miR-107* microRNA-107, *HOMA-IR* homeostasis model assessment-insulin resistance, *SE* standard error

Epidemiologic studies showed that the development of post-menopausal breast cancer was linked to several factors related to estrogen production in women and to an increased risk of breast cancer, such as early menarche, late menopause, obesity, use of post-menopausal hormone therapy, and plasma estradiol levels [18]. Several studies have shown that the ER pathway [19] and DNA damage [20] contribute to breast cancer. Estrogen contributes significantly to breast tumor formation and growth. In estrogen-dependent tumors, estrogen promotes cell proliferation and suppresses apoptosis. Circulating estrogen binds to ER-α in breast cancer cells and stimulates cell division and growth. Kim et al. [21] reported that higher preoperative serum E2 levels had a negative prognostic effect in post-menopausal women with breast cancer. Insulin resistance is involved in T2DM and PMBC. Insulin resistance results in hyperinsulinemia, which leads to high insulin-like growth factors (IGF-1) and induces estrogen receptor-α (+). Moreover, insulin resistance impacts pancreatic β cells [22]. Hyperglycemia and insulin resistance increase breast cancer risk [23]. An epidemiological study demonstrated a positive correlation between increased insulin resistance and increased risk of all cancers, even in non-obese individuals. Hyperinsulinemia may affect breast cancer etiology rather than progression [24]. In the present study, there may be less incidence of diabetes in the women with PMBC, despite their having a higher BMI. Incidentally, the E2 levels of women with PMBC are also significantly higher in the absence of diabetes. Obesity is a risk factor for breast cancer in women with PMBC. High BMI is significantly associated with larger size breast tumors in both pre- and post-menopausal women [25]. We noted that there were higher levels of fasting insulin, HbA1c, and E2 in patients with post-menopausal breast cancer without T2DM than in non-diabetic post-menopausal controls. This finding suggests that insulin resistance and E2 are associated with the development of T2DM with PMBC.

Although insulin resistance contributes to breast cancer etiology, miR-103/107 is also involved. Downregulation of caveolin-1 by miR-103/107 is susceptible to tumorigenesis. Mutations in the gene encoding caveolin-1 are associated with the development and progression of breast cancers because a caveolin-1-deficient tumor microenvironment provides favorable conditions for breast cancer tumor growth [8]. Downregulated CAV-1 activates ER-α, which is involved in breast cancer. Our study showed that the levels of expressed miR-103/107 and E2 in serum in PMBC and T2DM patients were higher than those in non-diabetic post-menopausal controls. This result indicates that miR-103/107 may be involved in the development of T2DM with PMBC through elevated levels of E2.

This study did not examine the estrogen receptor status for all samples, and the number of samples was limited. In addition, the expression of miR-103/107 between breast cancer tissue and adjacent normal tissue was not detected. Further studies will collect more samples and investigate whether miR-103/107 is a biomarker for breast cancer in postmenopausal diabetic populations. In summary, high expression of miR-103/107 is involved in insulin resistance, which activates the development of T2DM and PMBC by inhibiting the expression of cavelin-1 and elevated levels of E2. Therefore, miR-103/107 may be linked to T2DM and PMBC.

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M.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Author contributions Q.X., Y.S., and Y.L. participated in the conception and design of the study and the critical revision of the manuscript for important intellectual content. Q.X., Y.S., F.Z., S.G., and M.Y. performed the data collection and analysis. Q.X. interpreted the data and produced the draft of the manuscript. M.Y. obtained funding for the study. All authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

CTLA-4 +49A/G gene polymorphism and type 1 diabetes mellitus in the Chinese population: a meta-analysis of 2238 subjects

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Abstract Previous studies reported that *cytotoxic T lymphocyte-associated antigen 4* (*CTLA-4*) +49A/G gene polymorphism is correlated with type 1 diabetes mellitus (T1DM) risk. However, their results remain disputable. This study aims to discuss the relationship between *CTLA-4* +49A/G gene polymorphism and T1DM in a Chinese population. The current meta-analysis involved 2238 participants from seven individual studies. The pooled odds ratio (OR) and its corresponding 95 % confidence interval (95 % CI) were assessed by the random- or fixed-effects model. A significant relationship between *CTLA-4* +49A/G gene polymorphism and T1DM was detected under allelic (OR: 1.84, 95 % CI: 1.62–2.10, $P<0.00001$), dominant (OR: 1.152, 95 % CI: 1.062–1.249, $P=0.001$), recessive (OR: 1.631, 95 % CI: 1.443–1.844, $P<0.00001$), and additive (OR: 1.292, 95 % CI: 1.224–1.363, $P<0.00001$) genetic models. A significant relationship exists between *CTLA-4* +49A/G gene polymorphism and increased T1DM risk in the Chinese population. Individuals having the G allele of *CTLA-4* +49A/G gene polymorphism have a higher risk for T1DM in the Chinese population.

Keywords Cytotoxic T lymphocyte-associated antigen 4 · +49A/G · Polymorphism · Type 1 diabetes mellitus

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Introduction

Type 1 diabetes mellitus (T1DM) has become a hot research point because of its increasing incidence worldwide [1]. T1DM is an organ-specific disorder characterized by the autoimmune destruction of the β cells of pancreatic islets. T1DM has a polygenic background. This disorder is a result of the interaction between multigenic predisposition and environmental factors. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a membrane protein expressed by the activated T lymphocyte, plays a negative regulation role in the proliferation of T cell. Only activated T cells could express the CTLA-4 molecule, which could be binded to the B7 molecule on antigen-presenting cells and deliver negative regulatory signals. Termination of T cell activation and immunoreaction leads to T cell apoptosis [2]. Hence, CTLA-4 genetic mutations might be involved in the genetic susceptibility to autoimmune diseases such as T1DM by modifying the inhibitory effect on T cell activity [3].

Studies on the Belgian population first reported that the +49A/G (rs231775) polymorphism in the first exon of the *CTLA-4* gene is correlated with T1DM risk; the same finding was subsequently observed in Polish, Japanese, Chinese, and West African populations [4–9]. The *CTLA-4* gene, which is located on chromosome 2q33, spans 6.175 kb and contains four exons. Substitution of the 49th base adenine (A) with guanine (G) yields alanine (Ala) in place of wild type threonine (Thr) at the 17th codon in the signal peptide.

Several reports on the association between *CTLA-4* +49A/G gene polymorphism and T1DM are available. However, the individual results are still inconsistent. In 2011, Philip et al. found a significant association between *CTLA-4* +49A/G polymorphism and T1DM in the Madurai population of Southern India [10]. In 2012, Mosaad et al. also found that the *CTLA-4* +49GG homozygous genotype was associated

with T1DM in Egyptian children [11]. By contrast, Osei-Hyiaman et al. found in 2001 that *CTLA-4* +49A/G gene polymorphism was significantly associated with T1DM in the Chinese but not in the West African population [9]. In 2005, Kavvoura et al. performed a meta-analysis on the association between *CTLA-4* +49A/G gene polymorphism and T1DM in different countries and found that the G genotype was associated with T1DM [2]. In 2009, Jung et al. found that CTLA4 +49A/G polymorphisms did not directly confer any susceptibility to T1DM in a Korean population [12]. Given that allele distribution differs between different populations, many studies on the same issue have different results in China [8, 9, 13–17]. Consequently, a recognized conclusion has yet to be established.

Data from 1096 T1DM patients and 1142 controls were included in the present meta-analysis to draw a valuable conclusion on the relationship between *CTLA4* +49A/G gene polymorphism and T1DM in the Chinese population.

Materials and methods

Publication search and inclusion criteria

The following keywords were searched in electronic databases Web of Science, PubMed, Embase, China National Knowledge Infrastructure, and China Biological Medicine Database: "type 1 diabetes mellitus," "*cytotoxic T lymphocyte-associated antigen 4*," "+49A/G," and "polymorphism." The selected studies were retrieved within the publication year before 2012 (last research updated on May 14, 2015).

The studies were selected based on the following inclusion criteria: studies evaluating *CTLA-4* +49A/G gene polymorphism and T1DM, studies with diagnosis and classification criteria of T1DM based on the American Diabetes Association and modified by the World Health Organization in 1997, case-control or cohort studies published in official journals, and studies conforming to the Hardy-Weinberg equilibrium (HWE).

Data extraction

In the current meta-analysis, repeated publications and studies violating the inclusion criteria or providing negligible data were excluded. If the same information appeared in multiple studies, the datum was only once used. The drawn data included the following items: first author's name, publication year, study region, number of genotypes, genotyping method, study design, matching criteria, and total number of T1DM cases and controls.

Statistical analysis

Allelic (G allele distribution frequency), recessive (GG vs. AA +AG), dominant (GG +AG vs. AA) and additive (G vs. A) genetic models were used. The relationship between *CTLA-4* +49A/G gene polymorphism and T1DM was compared by using odds ratio (OR) corresponding to its 95 % confidence interval (CI). The chi-square-based Q test was used to calculate the between-study heterogeneity, with the significance level set at $P<0.05$ [18]. If heterogeneity emerged among the studies, the pooled OR was estimated by the random-effects model (the DerSimonian and Laird method) [19]. Otherwise, the fixed-effects model was used (the Mantel-Haenszel method) [20]. The pooled OR was determined by Z test, with the significance level set at $P<0.05$.

HWE was assessed by the Fisher's exact test, with the significance level set at $P<0.05$. The funnel plot was used to estimate the potential publication bias. The Egger's linear regression test on the natural logarithm scale of the OR was used to assess the funnel plot asymmetry, with the significance level set at $P<0.05$ [21]. Statistical analyses were carried out using the STATA 11.0 software (StataCorp, College Station, TX).

Results

Studies and populations

Through the literature search, 14 papers were retrieved, of which seven met the inclusion criteria. The seven excluded studies comprised one repeated publication [22], two reviews [2, 23], and three studies that report the association out of China [24–26]. One study was excluded for deviating from the HWE [27]. The total data were drawn out from 1096 T1DM patients and 1142 controls (Table 1) [8, 9, 13–17]. The included study regions were Shanxi, Shandong, Liaoning, Hunan, Ningxia, Henan, and Taiwan.

Pooled analyses

A significant relationship between *CTLA-4* +49A/G gene polymorphism and T1DM was detected under allelic (OR: 1.84, 95 % CI: 1.62–2.10, $P<0.00001$), dominant (OR: 1.152, 95 % CI: 1.062–1.249, $P=0.001$), recessive (OR: 1.631, 95 % CI: 1.443–1.844, $P<0.00001$), and additive (OR: 1.292, 95 % CI: 1.224–1.363, $P<0.00001$) genetic models (Fig. 1, Table 2).

Significant heterogeneity existed between the individual studies under the dominant genetic models ($P_{\text{heterogeneity}}<0.00001$, $I^2=75.3$ %). A meta-regression was performed to explore the heterogeneity source. Under the dominant genetic model, the heterogeneity could be explained by the study

Table 1 Characteristics of the investigated studies of the association of the *CTLA-4* +49A/G gene polymorphism and type 1 diabetes mellitus in the Chinese population

Author	Year	T1DM			Control			Matching criteria	Sample size (T1DM/control)
		AA	AG	GG	AA	AG	GG		
Lee [8]	2000	18	85	150	9	45	37	Ethnicity	253/91
Osei-Hyiaman [9]	2004	110	166	74	201	177	42	Ethnicity	350/420
Wang [13]	2001	13	54	23	32	42	10	Ethnicity	90/84
Zhang [14]	2005	4	42	36	18	48	20	Sex, ethnicity	82/86
Xiang [15]	2006	14	86	79	50	153	87	Sex, ethnicity	179/290
Yang [16]	2006	3	8	23	11	28	32	Sex, ethnicity	34/71
Song [17]	2012	10	25	73	16	39	45	Sex, ethnicity	108/100

The ethnicity is Han in all of the above studies. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping method and case-control study design were adopted in all of the above studies

T1DM type 1 diabetes mellitus

region (P=0.004) and the AA (AA1, P=0.004), AG (AG1, P=0.002), and GG (GG1, P=0.012) genotype sample sizes of the T1DM group (Tables 2 and 3, Fig. 2).

According to AG1, the entire population was divided into the AG1<60 and AG1>60 subgroups under the dominant genetic model. In the AG1<60 subgroup, heterogeneity was not detected ($P_{\text{heterogeneity}}$=0.072), and even significantly strengthened association between *CTLA-4* +49A/G gene polymorphism and T1DM was observed (P<0.00001). In the AG1>60 subgroup, heterogeneity still existed ($P_{\text{heterogeneity}}$< 0.00001), and not any significant association between *CTLA-4* +49A/G gene polymorphism and T1DM was detected yet (P= 0.069) (Tables 2 and 3).

Bias diagnostics

The funnel plot and Egger's test were used to assess the publication bias of the individual studies. No visual publication bias was observed in the funnel plot (Fig. 3), and no significant difference was found in the Egger's test. These results indicate that a low publication bias exists in the current meta-analysis using the allelic genetic model.

Discussion

In the present meta-analysis, a significant relationship was found between *CTLA-4* +49A/G gene polymorphism and T1DM under the allelic (OR: 1.84), dominant (OR: 1.152), recessive (OR: 1.631), and additive (OR: 1.292) genetic models. Patients with the G allele of *CTLA-4* +49A/G gene polymorphism have a higher risk for T1DM in the Chinese population.

Significant heterogeneity was found between the individual studies under the dominant genetic models ($P_{\text{heterogeneity}}$< 0.05). Meta-regression analysis showed that the study region (P=0.004), AA1 (P=0.004), AG1 (P=0.002), and GG1 (P=

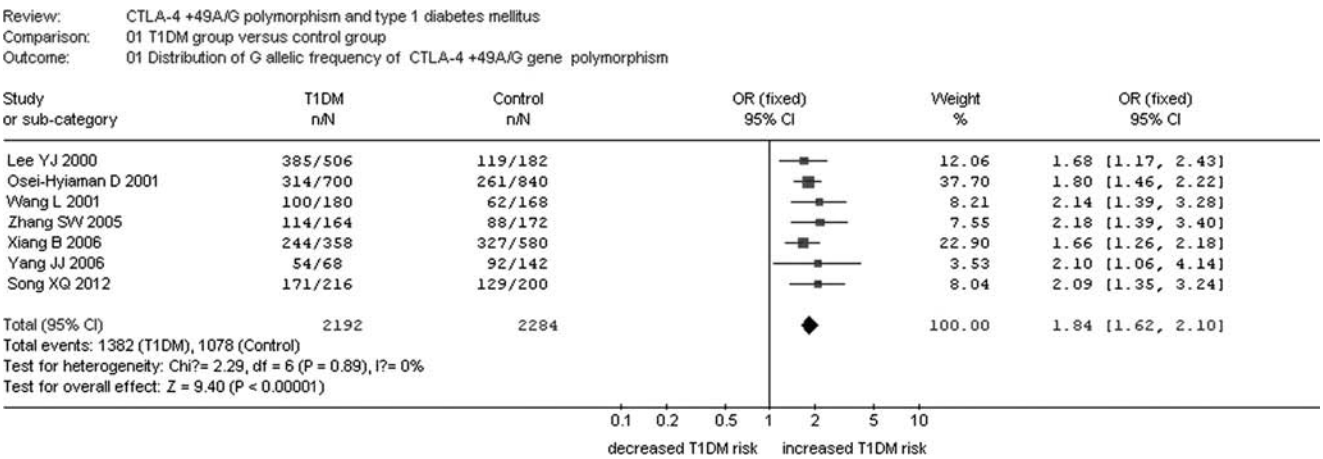

Fig. 1 Forest plot of T1DM associated with *CTLA-4* +49A/G gene polymorphism under an allelic genetic model (distribution of G allelic frequency of *CTLA-4* gene)

Table 2 Summary of meta-analysis of association of *CTLA-4* +49A/G gene polymorphism and type 1 diabetes mellitus in the Chinese population

Genetic model	Group analysis	Pooled OR (95 % CI)	*P* value	Literature number	T1DM size	Control size	$P_{heterogeneity}$
Allelic genetic model	Whole population	1. 84(1.62–2.10)	<0.00001*	7	1096	1142	0.89
Recessive genetic model	Whole population	1.631(1.443–1.844)	<0.00001*	7	1096	1142	0.528
Dominant genetic model	Whole population	1.152(1.062–1.249)	0.001*	7	1096	1142	<0.00001*
	Subgroup 1: AG1<60	1.179(1.100–1.263)	<0.00001*	4	314	341	0.072
	Subgroup 2: AG1>60	1.141(0.990–1.315)	0.069	3	782	801	<0.00001*
Additive genetic model	Whole population	1.292(1.224–1.363)	<0.00001*	7	1096	1142	0.106

T1DM type 1 diabetes mellitus, *CI* confidence interval, *OR* odds ratio, *T1DM size* the total number of T1DM cases, *control size* the total number of control group

*$P<0.05$

0.012) could partially explain the heterogeneity under the dominant genetic model.

In the subgroup analysis stratified by AG1 under the dominant genetic model, the heterogeneity still existed in the AG1>60 subgroup ($P_{heterogeneity}<0.00001$) but disappeared in the AG1<60 subgroup ($P_{heterogeneity}=0.072$). The association strength between *CTLA-4* +49A/G gene polymorphism and T1DM was obviously weakened in the AG1>60 subgroup ($P=0.069$) but not in the AG1<60 subgroup ($P<0.00001$). These findings suggest that the individual studies with smaller AG genotype sample size have less heterogeneity and could be used to explain more clearly the association between *CTLA-4* +49A/G gene polymorphism and T1DM.

As shown through OR in Fig. 1, all the studies that have been included for meta-analysis seem to show significant association with T1DM individually. However, the association strength between *CTLA-4* +49A/G gene polymorphism and T1DM was different among the individual studies. Under the allelic genetic model, the OR value ranged from 1.68 to 2.18. Compared with the different ORs, the eventual OR value is 1.84 after the current meta-analysis. Hence, the association power between *CTLA-4* +49A/G gene polymorphism and T1DM obtained in the current meta-analysis is moderate.

As a candidate gene of T1DM, the costimulatory molecule *CTLA-4* is named as IDDM12 besides the human leukocyte antigen (HLA) and insulin genes. CTLA-4 could negatively regulate T cell proliferation. In 1998, Colucci et al. found deficiency in CTLA-4 expression in non-obese diabetic (NOD) mice, suggesting that CTLA-4 might be involved in the pathogenesis of T1DM [28]. In 2004, Vijayakrishnan et al. found that the expression of ligand-independent CTLA-4 (liCTLA-4) lacking B7-binding domain signals was higher in memory/regulatory T cells from diabetes-resistant NOD congenic mice compared with susceptible NOD mice. Their results suggested that the increased expression and negative signaling delivered by liCTLA-4 might regulate the development of T cell-mediated autoimmune diseases [29]. In 2011, Qureshi et al. reported that CTLA-4 might act as an effector molecule to inhibit CD28 costimulation by the cell-extrinsic depletion of ligands [30]. In 2011, Gerold et al. found that soluble CTLA-4 (sCTLA-4) participates in immune regulation by potentiating the function of regulatory T cells. The functional outcome of silencing this splice variant in the NOD model explains the association of CTLA4 variation with autoimmunity. Lower sCTLA-4 expression from the susceptibility allele might directly affect the suppressive capacity of regulatory T cells and thereby modulate disease risk [31].

Hence, *CTLA-4* gene mutation might confer patient susceptibility to T1DM. *CTLA-4* +49A/G gene polymorphism causes Thr to be replaced with Ala in the signal peptide region. However, the mechanism by which *CTLA-4* gene mutation contributes to the functional change remains unclear. In

Table 3 The meta-regression results among seven studies under the dominant genetic model

Item	Coefficient	SE	*T* value	*P* value	95 % Confidence interval
Study region	1.786081	0.1098028	16.27	0.004*	1.313638~2.258524
AA1	0.034631	0.0023286	14.87	0.004*	0.0246117~0.0446501
AG1	−0.03709	0.0016511	−22.47	0.002*	−0.0441987~−0.0299902
GG1	0.006893	0.0007716	8.93	0.012*	0.0035731~0.0102128
Cons	−2.46907	0.0759909	−32.49	0.001*	−2.796034~−2.142109

SE standard error, *AA1* AA genotype sample size of T1DM group, *AG1* AG genotype sample size of T1DM group, *GG1* genotype sample size of T1DM group, *cons* constant item

*$P<0.05$

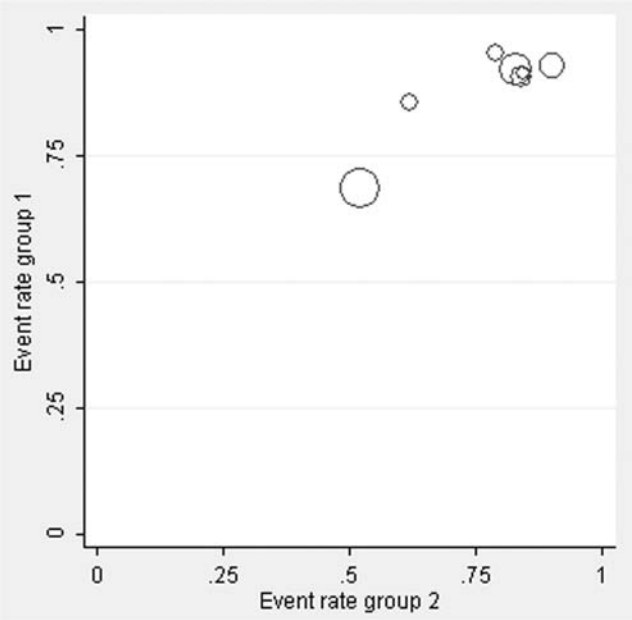

Fig. 2 L'Abbe plot for the meta-*regression* analysis on the association of *CTLA-4* +49A/G gene polymorphism and T1DM under a dominant genetic model (AG+GG vs. AA)

2002, Anjos et al. revealed that the signal peptide could determine the efficiency of post-translational modifications other than cleavage [32]. Their findings suggested that the genetic effect on T1DM might be attributed to the inefficient processing of the autoimmunity pre-disposing the Ala allele. They also concluded that the association between T1DM and +49A/G gene polymorphism was correlated with increased T cell proliferation through a mechanism that might involve inefficient N-linked glycosylation contributing to less mature CTLA-4 at the cell surface.

In 2005, Kavvoura conducted a meta-analysis on the association between *CTLA-4* +49A/G gene polymorphism and T1DM [2]. However, the allele distribution was different among different ethnic populations. For example, a significant difference in *CTLA-4* +49 genotype distribution was found among Chinese, Tunisian, and Caucasian populations [17, 33, 34]. Given that Kavvoura's work was conducted 7 years ago, a new meta-analysis in the Chinese population should be performed.

The self-tolerance disruption of the immune system is involved in T1DM. CTLA-4 might also play a central role in the progression of T1DM. To prove this hypothesis, the correlation between soluble/membrane-attached CTLA-4 and *CTLA-4* +49A/G gene polymorphism as well as the importance of *CTLA-4* +49A/G gene polymorphism, HLA, and insulin genes should be further investigated [35].

Some limitations were encountered in the present meta-analysis. First, large-scale studies on the relationship between T1DM and *CTLA-4* +49A/G gene polymorphism are lacking. Second, the plasma CTLA-4 level is influenced not only by *CTLA-4* +49A/G gene polymorphism but also by other genetic and environmental factors such as inflammatory and other potential immune system diseases. Previous studies revealed that other polymorphisms in the 3′ region of *CTLA-4* (rs3087243, CT60, A/G) are more strongly associated than +49A/G gene polymorphism in the UK and USA [23, 36]. However, only one study on the association between rs3087243 gene polymorphism and T1DM in China could be performed as a meta-analysis [22]. As there are more studies on the association between *CTLA-4* +49A/G gene polymorphism and T1DM than other *CTLA-4* gene polymorphisms in China, *CTLA-4* +49A/G gene polymorphism was chosen to do the meta-analysis. After more studies have been performed on the association between *CTLA-4* other gene polymorphism and T1DM in the Chinese population, a new meta-analysis might be conducted to see which genetic factors that influence the plasma CTLA-4 levels are stronger than others on earth.

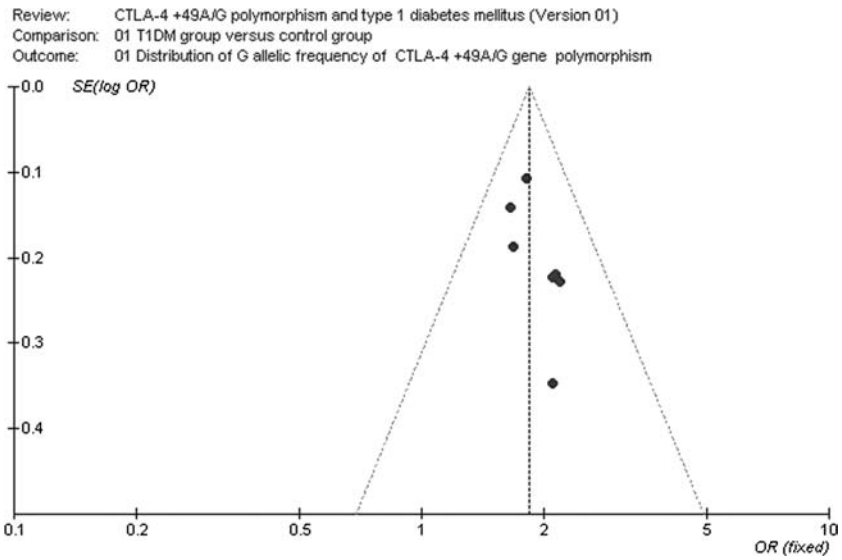

Fig. 3 Funnel plot for studies of the association of T1DM associated and *CTLA-4* +49A/G gene polymorphism under an allelic genetic model (distribution of G allelic frequency of *CTLA-4* gene). The *horizontal and vertical axis* correspond to the OR and confidence limits. *OR* odds ratio, *SE* standard error

A significant association was found between *CTLA-4* + 49A/G gene polymorphism and increased T1DM risk in the Chinese population. In the Chinese population, individuals with the G allele have a higher risk for T1DM. If the conclusion was also supported by large-scale studies on CTLA-4 + 49A/G gene polymorphism and T1DM, the current conclusion might serve as a foundation to establish T1DM individual therapy in the Chinese population. However, given the aforementioned limitations, more studies should be performed to support this conclusion.

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Author contributions YL conceived and designed the experiments. YL and QY performed the experiments. YL, CZ, and JY analyzed the data. YL contributed reagents/material/analysis tools, wrote the manuscript, and made the reference collection and data management, statistical analyses and paper writing, and study design.

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

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ORIGINAL ARTICLE

Omentin and apelin concentrations in relation to obesity, diabetes mellitus type two, and cardiovascular diseases in Egyptian population

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Abstract Dysregulation of omentin, a beneficial adipokine, and apelin, an inflammatory adipokine, is thought to play a role in the development of type 2 diabetes mellitus and cardiovascular disease. The objective of this study was to evaluate the relationship between circulating omentin and apelin concentrations and components of the metabolic syndrome in adults with and without type 2 diabetes mellitus or cardiovascular disease. A total of 240 adults, sex- and age-matched, were included in the current case–control study, including 80 healthy, non-obese controls, 80 obese patients with T2DM without cardiovascular disease, and 80 obese patients with T2DM with cardiovascular disease. A fasting blood sample was collected to determine biochemical indicators and insulin resistance index (HOMA-IR). Omentin, apelin, interleukin-1β (IL-1β), troponin-T, and oxidized LDL (Ox-LDL) plasma level was assessed by ELISA. Associations of adipokines with biochemical parameters of the patients were determined. Serum omentin levels were significantly lower and serum apelin and IL-1β concentrations were significantly higher in obese diabetic groups compared to non-obese controls. In correlation analyses, omentin negatively associated with the HOMA-IR index, apelin, and troponin-T, whereas apelin was positively associated with IL-1β, BMI, and troponin-T. Our study supports the hypothesis that abnormal production of omentin and apelin can contribute to the pathogenesis of obesity-related complications including T2DM and cardiovascular disease.

Keywords Cardiovascular disease · Omentin · Apelin · IL-1β · Troponin-T · Ox-LDL · Obesity · Type 2 diabetes mellitus

Introduction

Obesity is a chronic multifactorial disease that is associated with numerous metabolic disorders, including type 2 diabetes mellitus (T2DM) [1, 2]. The major link between obesity and T2DM is insulin resistance (IR). Adipose tissue depots are the most vulnerable target to mediate significant immune cell infiltration and inflammation contributing to systemic inflammation and IR in obese humans [3].

Diabetes has become an epidemic and remains a major public health issue. In 2010, it was estimated that 4.787 million Egyptians (10.4 % of the Egyptian population) had diabetes and that diabetes will increase to 8.615 million Egyptians by the year 2030 [4]. Diabetes mellitus increases the incidence of coronary heart disease, being the most common and clinically important complication in DM [5].

Adipose tissue represents an active endocrine organ by releasing the large number of bioactive mediators (adipokines) that plays an important role in modulating glucose metabolism and inflammation [6]. The adipokine secretion pattern reflects adipose tissue function and seems to be important for determining the individual risk to develop metabolic and cardiovascular comorbidities of obesity [7]. When adipose tissue inflammation and dysfunction have developed,

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adipokine secretion is significantly changed towards a diabetogenic, pro-inflammatory, and atherogenic pattern [8].

Omentin, apelin, and IL-1β are adipokines that play a key role in the cardiovascular disease (CVD) pathophysiology [9]. Omentin is a newly identified secretory protein that is relative to subcutaneous adipose tissue and is highly and selectively expressed in visceral adipose tissue. Low omentin expression was observed in obesity, IR, and T2DM [10]. It was shown that omentin levels correlate inversely with troponin-T and total cholesterol in obese patients with heart disease. Recent studies underscore an anti-inflammatory, anti-atherogenic, and anti-diabetic properties of omentin [11].

The other newly discovered adipokine, apelin-12, is a novel 12-amino peptide expressed in adipocytes of humans; it is encoded by the APLN gene [12]. The synthesis of apelin in adipocytes is triggered by insulin and its plasma levels are reported to increase in association with insulin resistance, hyperinsulinemia, and diabetes mellitus [13]. Our previous studies indicated that in T2DM patients, with or without CVD, the concentration of apelin was significantly increased [14] and positively correlated with concentration of pro-inflammatory cytokine, IL-1β, as well as negatively correlated with triacylglycerol (TAG) and BMI [15]. Apelin was up-regulated in the atherosclerotic coronary artery, and this peptide localized to the plaque with markers for macrophages and smooth muscle cells [16].

Epidemiological studies showed that IL-1β as a pro-inflammatory cytokine was significantly increased and correlated with troponin-T and Ox-LDL in obese diabetic patients [17]. IL-1β has been reported to contribute to β-cell failure and has also been implicated in the progression of atherosclerosis and heart failure [18]. The present work aimed to study the association between novel adipokines and obesity and its diabetic and cardiovascular complications in Egyptian population.

Subjects and methods

Study design

A total of 240 Egyptian adult men were included in this case–control study. Subjects were selected according to our defined inclusion criterion, which was age 35–45 years. Eighty served as healthy, non-obese controls. Patients enrolled in the study were classified into the following groups: 80 type 2 diabetic obese subjects without CVD (T2DM group) selected from patients attending the Endocrinology Department of Suez Canal University hospitals and 80 type 2 diabetic obese subjects with CVD (T2DM+CVD group) admitted to the Intensive Care Unit–Cardiology Department. A patient was considered to have CVD if he had a history of myocardial infarction or the diagnosis was based on the result of coronary angiography. Exclusion criteria were defined as having the history of any condition that affects inflammatory markers such as known thyroid diseases, malignancies, current smoking, acute or chronic infections, acute or chronic inflammatory disease, hepatic or renal diseases, and alcohol or drug abuse. We limited our study to nonsmokers. The study was approved by the Committee on Medical Ethics of Suez Canal University. The study was carried out in accordance with the regulations and recommendations of the Declaration of Helsinki. All subjects gave their written informed consent prior to participation. A detailed medical history and drug treatment(s) were collected for all subjects. Body mass index (BMI) of all subjects was calculated as weight (kg)/height (m^2) and subjects with a BMI equal or more than 30 kg/m^2 were considered as obese subjects and placed in the obese diabetic group. The control group was those with a BMI lower than 30 kg/m^2.

Biochemical assay

Participants were weighed in a gown and without shoes. Blood pressure was measured with an automated monitor two times over a 5-min rest period, and the average of the two blood pressures was used in study analyses. The peripheral blood samples were obtained following 10–12 h overnight fasting. Serum was separated, aliquoted, and stored at −80 °C. All samples were analyzed by means of a single assay. Standard enzymatic techniques were used for the measurement of fasting serum glucose (FSG) [19] and lipids [total cholesterol (TC) [20] and TAG [21]]. High-density lipoprotein (HDL) was determined after precipitation of APO lipoprotein B-containing lipoproteins [22]. The reference values for the lipid profile were according to established guidelines [23]. Serum insulin concentrations were measured by ELISA method (human insulin ELISA kit; Monobind, Inc., USA) with a minimum detectable concentration of 1.76 mIU/mL.

HOMA calculation

Insulin resistance was calculated by homeostasis model assessment (HOMA). The HOMA-IR was calculated according to HOMA-IR equation=[fasting plasma glucose (mg/dL)× fasting plasma insulin (mIU/mL)]/405 [24].

Cardiovascular marker determination

Troponin-T as well as Ox-LDL was measured in serum aliquots kept frozen at −80 °C using an ELISA kit (MyoBioSource, Inc., USA) according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany).

Adipokine determination

Inflammatory cytokine, serum IL-1β, was measured by ELISA kit (Boaster Biological Technology, Inc., USA) with a sensitivity of 6.5 pg/mL [25]. As for novel adipokines, serum omentin levels were measured by ELISA kit (Alpco Diagnostics, Inc., USA) with a sensitivity of 0.4 ng/mL [26]. Serum apelin-12 levels were measured by ELISA kit (MyoBioSource, Inc., USA). The sensitivity of the assay was 0.2 ng/mL and the inter-assay error was below 5 % [27].

Statistical analysis

The data are presented as mean±standard deviation (SD). Differences between variables were calculated using Student's *t* test. To determine differences between groups, analysis of variance (ANOVA) was used for multiple comparisons between different groups. Pearson's correlations were computed to assess the relationship between variables. All statistical analyses were performed with SPSS, version 17.0 (SPSS Inc.). *P* values less than 0.05 were considered to be statistically significant.

A multiple linear regression analysis was performed to investigate the independent association between serum apelin and omentin levels (dependent variable) and selected variables that had *P* values <0.05 in univariate analysis (sex and age were also included). *P* values <0.05 were considered statistically significant with a confidence interval of 95 %

Results

A total of 240 subjects were included in this study, and their clinical characteristics are given in Table 1. Compared to controls, patients had significantly changed all conventional risk factors for obesity complications, including BMI, hypertension (defined as a systolic blood pressure (BP) >140 mmHg, a diastolic BP >90 mmHg, or both), FSG, insulin, HDL, TAG, and TC ($P<0.05$). In diabetic groups, a significantly higher serum FSG and insulin levels as well as the HOMA-IR values were observed. In addition, there were no significant differences in the baseline characteristics between diabetic patients and controls in terms of age and sex. Regarding cardiovascular markers in Table 2, there was a significant increase in troponin-T levels in the T2DM group (0.69±0.05 ng/mL) and T2DM+CVD group (4.71±1.02 ng/mL) compared with the control group (0.008±0.01 ng/mL). The Ox-LDL levels were increased 2.9-fold in the diabetic groups compared to controls ($P<0.05$). The T2DM+CVD group also showed significantly higher serum troponin-T and Ox-LDL levels compared to the T2DM group ($P<0.05$). Regarding serum adipokine levels in Table 2, IL-1β concentrations were increased in the T2DM and T2DM+CVD groups compared to the control group (28.8±2.34 and 29.7±2.1 pg/mL vs. 19.17±1.76 pg/mL, respectively; $P<0.05$). For omentin, there was a significant decrease in its serum levels in both T2DM and T2DM+CVD groups compared to controls (23±4.9 and 20.49±5.4 pg/mL vs. 58.8±8 pg/mL, respectively; $P<0.05$). Regarding apelin, its serum levels were increased 2.5-fold in the T2DM+CVD group compared to controls ($P<0.05$).

In Pearson's correlation analyses, omentin levels were negatively correlated with insulin ($r=-0.92$, $P=0.001$) in Table 3, HOMA-IR ($r=-0.89$, $P=0.001$), troponin-T ($r=-0.6$, $P=0.0001$), and TC levels ($r=-0.87$, $P=0.0001$) in Fig. 1. However, apelin levels were negatively correlated with omentin ($r=-0.82$, $P=0.001$) in Fig. 2 and positively with IL-1β ($r=0.8$, $P=0.001$) in Fig. 3, troponin-T ($r=0.86$, $P=0.001$), BMI ($r=0.84$, $P=0.0001$), and TAG ($r=0.83$, $P=0.0001$).

Multiple regression analysis with all the significant variables confirmed that BMI, TAG, troponin-T, and IL-1β were all determinants of serum apelin levels independently of age, FSG, insulin, and TC in Table 3, while serum omentin levels were dependent on insulin, TC, and troponin-T as well as independent from age, BMI, FSG, and IL-1β.

Discussion

Obesity is a chronic pathological condition and a risk factor for metabolic syndrome development, T2DM, and CVD [28]. Several studies have shown that visceral obesity is strongly associated with IR, hyperglycemia, dyslipidemia, and hypertension [29]. Moreover, DM is one of the most common chronic diseases in nearly all countries; it is estimated that Egypt will be listed in the top ten countries with the highest numbers of people with diabetes in 2030, reflecting anticipated changes in the population size and structure in Egypt [4].

Type 2 diabetes mellitus and its associated complications have become a public health problem of considerable magnitude. CVD causes most of the excess morbidity and mortality in DM [30]. The cardiovascular risk factors hypertension, dyslipidemia, obesity, IR, and hyperinsulinemia cluster in the metabolic syndrome [31]. All of these mentioned factors, being observed well in the current study, create a state of constant and progressive damage to the vascular wall (increased troponin-T and Ox-LDL), manifested by a low-grade inflammatory process (increased IL-1β).

Oxidative stress and the oxidation of low-density lipoprotein (LDL) play a role in atherosclerosis and associated risk factors [32]. It is worthy to state that Ox-LDL was significantly increased in the diabetic groups as compared to the control ones in our study. Our results revealed that troponin-T and Ox-LDL were significantly higher in the T2DM+CVD group as compared to the T2DM and control groups. This was also in

Table 1 General characteristics of the study population

Groups *n*	Control 80	T2DM 80	T2DM+CVD 80	*P* value
Age (years)	38.6±4.2	42±3	40.3±2.5	NS
BMI (kg/m^2)	21±1.7	32.4±1.4a	32.6±1.6a	0.01
DM duration (years)	–	3.5±1	4.2±0.8	
Systolic blood pressure (mmHg)	114±10.5	133±16.9a	182.6±12.3ab	0.01
Diastolic blood pressure (mmHg)	73.4±4.9	85.2±8.8a	94.7±10.6ab	0.01
FSG (mg/dL)	100±3.7	180±24.6a	182±21.9a	0.01
Insulin (μIU/mL)	7.6±1.3	17.7±3.2a	18.12±3a	0.01
HOMA-IR index	1.9±0.3	11±1.55a	11.07±1.8a	0.01
TAG (mg/dL)	129±29	280±19a	284±20.4a	0.01
TC (mg/dL)	172±16	285±32a	290±23a	0.01
HDL (mg/dL)	38±1.7	25.6±2a	27.4±2.1a	0.01

Data are given as mean±SD, range. Different letters indicate significant difference from control and T2DM without CVD groups, respectively. *P* values are for the comparison between the control and the study groups

T2DM type 2 diabetes mellitus without cardiovascular diseases, *T2DM+CVD* type 2 diabetes mellitus with cardiovascular diseases, *BMI* body mass index, *FSG* fasting serum glucose, *TAG* triacylglycerol, *TC* total cholesterol, *HDL* high density lipoprotein, *NS* not significant

support of a previous study [33] where it has been stated that there is a strong, clear association between cardiovascular abnormalities and troponin-T level.

We sought to test the usefulness of IL-1β in our population of diabetic patients. A recent study [15] has described a positive association between IL-1β and obesity, suggesting functional effects on fat mass, fat metabolism, and body mass. This is supported by the positive correlation found between IL-1β and BMI in our study. However, it is known that adipose tissue can synthesize and release the main pro-inflammatory cytokine, IL-1β, which also impairs insulin secretion and induces β-cell apoptosis leading to T2DM [34].

Accumulating evidence indicates that the diseases related to metabolic syndrome are characterized by the abnormal cytokine production, including elevated circulating IL-1β. This was also in support of a previous study [17] where it has been shown that IL-1β plays a role in diseases associated with metabolic syndrome such as atherosclerosis and T2DM. In our present study, IL-1β was positively correlated with troponin-T and Ox-LDL in our diabetic groups.

According to a previous study [35], in addition to the effective pro-inflammatory adipokine described above, adipose tissues also secrete a smaller number of anti-inflammatory factors, such as omentin. Omentin is a novel visceral fat

Table 2 Troponin-T, Ox-LDL, IL-1β, omentin, and apelin in studied groups

Groups *n*	Control 80	T2DM CVD 80	T2DM+ 80	*P* value
Troponin-T (ng/mL)	0.008±0.01	0.69±0.05a	4.71±1.02ab	0.01
Ox-LDL (mg/dL)	1.25±0.35	3.1±0.53a	3.73±0.59ab	0.01
IL-1β (pg/mL)	19.2±1.6	28.8±2.3a	29.7±2.1ab	0.01
Omentin (pg/mL)	58.8±8	23±4.9a	20.5±5.4ab	0.01
Apelin (ng/mL)	0.79±0.07	1.79±0.17a	1.99±0.49ab	0.01

Data are presented as mean±SD, range. Different letters indicate significant difference from control and T2DM without CVD groups, respectively. *P* values are for the comparison between the control and the study groups at significance level ≤0.05

IL-1β interleukin-1β, *Ox-LDL* oxidized LDL

Table 3 Multiple linear regression analysis using either apelin or omentin as dependent variable

Variable	Omentin		Apelin	
	β	*P*	*β*	*P*
Age	−0.235	NS	0.168	NS
BMI	−0.449	NS	0.44	<0.001
D.M duration	−0.148	NS	0.258	NS
FSG	−0.117	NS	0.224	NS
TC	−0.62	0.01	0.183	NS
TAG	−0.18	NS	0.45	<0.001
HDL-C	0.464	NS	−0.229	NS
Insulin	−0.42	0.01	0.689	NS
HOMA-IR	−0.44	0.01	0.69	NS
Troponin-T	−0.66	<0.0001	0.67	<0.0001
Ox-LDL	−0.431	NS	0.51	NS
IL-1β	−0.534	NS	0.46	<0.01
Omentin			−0.64	0.01
Apelin	−0.64	0.01		

Evaluated by multiple linear regression models with several levels of adjustment

β standardized coefficients, *NS* not significant

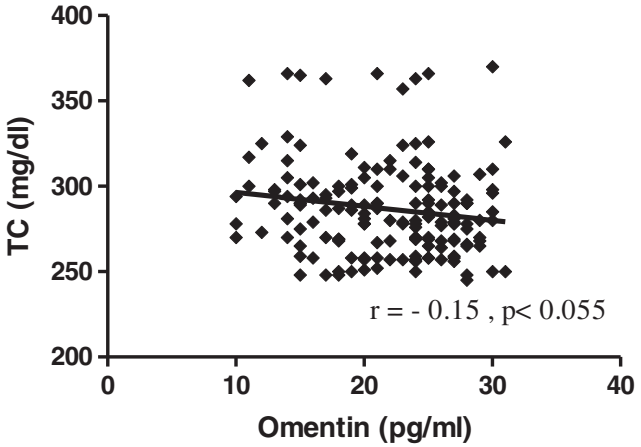

Fig. 1 Correlation between omentin and total cholesterol (TC) in diabetic patients (groups T2DM and T2DM+CVD) (n=160). Each individual value is represented by a *filled square*. r=Pearson's correlation coefficients

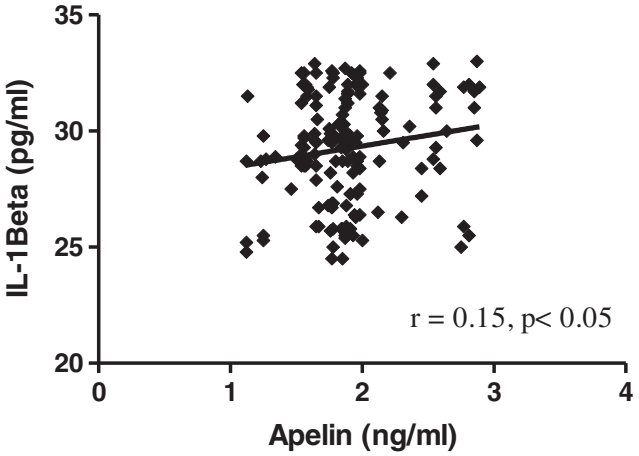

Fig. 3 Correlation between apelin and IL-1β in diabetic patients (groups T2DM and T2DM+CVD) (n=160). Each individual value is represented by a *filled square*. r=Pearson's correlation coefficients

depot-specific adipokine which is considered to be linked to T2DM in various populations. Omentin has been reported to have an association with visceral obesity, IR, and glucose metabolism [36]. In the present study, we demonstrated that circulating levels of omentin were inversely correlated with a number of metabolic risk factors (TC and troponin-T). Individuals in our study with an excess of visceral fat accumulation (diabetic groups) have a high risk of the development of metabolic syndrome in comparison with non-obese control group.

Our results showed that omentin level was significantly reduced in the diabetic patients with and without CVD as compared to the healthy controls. Moreover, the negative correlation of troponin-T with omentin in our diabetic groups is consistent with the study [37] on Chinese patients that showed that low levels of circulating omentin are also associated with the prevalence of coronary artery disease. These data suggest that omentin may represent a biomarker for not only metabolic disorders but also CVD.

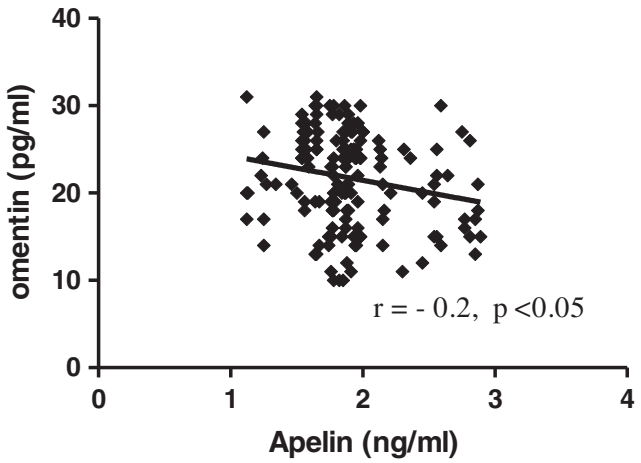

Fig. 2 Correlation between apelin and omentin in diabetic patients (groups T2DM and T2DM+CVD) (n=160). Each individual value is represented by a *filled square*. r=Pearson's correlation coefficients

In another study [38] on an obese Caucasian population, omentin levels were found to be correlated with some markers of lipid metabolism such as TC which indicates that omentin may play a role in lipid metabolism or diabetic dyslipidemia as a compensatory mechanism; this is consistent with our results which showed a negative significant correlation between omentin levels and TC levels in our obese diabetic groups.

A previous study [39] showed that decreased serum omentin levels observed in obese humans might cause a reduction of insulin-stimulated glucose uptake in visceral and subcutaneous adipocytes or other insulin-sensitive tissues and contributing, at least partially, to insulin resistance, and this was supported in our study by the negative correlation between omentin levels and insulin levels as well as HOMA-IR as an indicator of insulin resistance in our obese diabetic groups (T2DM and T2DM+CVD).

According to a recent study [35], obesity leads to the down-regulation of anti-inflammatory factors, such as omentin, and the up-regulation of IL-1β and apelin that activate endothelial cells and promote a dysfunctional phenotype. Apelin is another short peptide released from adipocytes originating from a 77-amino-acid precursor and its synthesis is stimulated by insulin.

Collected data from both the clinical and basic research settings showed that apelin correlates with states of IR and obesity and decreases insulin secretion [40]. Recently, studies [41] have disclosed a markedly increased plasma apelin level in obese T2DM subjects; this result was supported by the significant positive correlation between apelin and BMI as an indicator of obesity in our obese diabetic groups (T2DM and T2DM+CVD). The connection between apelin and T2DM has been postulated.

Meanwhile, we also found that apelin was significantly correlated with IL-1β in our obese diabetic groups. Therefore, we speculated that apelin might be involved in the pathophysiologic process in obese T2DM patients, taking into account the role of IL-1β in the development of IR and atherosclerosis.

Although apelin has been viewed as a beneficial adipokine up-regulated in obesity as confirmed by a previous study [42], our results revealed that apelin has a positive and negative significant correlation between troponin-T and omentin in our diabetic groups, respectively.

As new adipokines, apelin was likely to be involved in the pathophysiology of T2DM and CVD, and this could be explained by different mechanisms such as the level of apelin in our obese T2DM patients that correlated closely with BMI and the elevated levels that may be a result of IR compensatory reaction. However, as the other side of the coin, apelin may also inhibit the release of insulin, aggravating the disorders of glucose metabolism which was also proved by a previous study [43]. Moreover, by coordinating with other factors associated with increased circulating free fatty acids, apelin may cause the occurrence of IR [44].

Another explanation was shown by another study [45] where it has been reported that apelin correlated with oxidative stress and inflammation markers (Ox-LDL and IL-1β). As important inflammatory factors, they could be involved in the development of atherosclerosis. Thus, understanding the contribution of such an adipokine in obesity-associated disorders appears to be of major importance.

In conclusion, in the face of the current obesity epidemic, the nature of the relationship between obesity and T2DM is of great importance. However, it seems that in obese patients, such as those suffering from diabetes or CVD, in addition to obesity, the type of illness also affects inflammatory or anti-inflammation mediators' levels. The present study indicates that lower concentrations of circulating omentin together with higher concentrations of apelin are linked with an increase in multiplicity of metabolic risk factors, suggesting that omentin and apelin serve as beneficial biomarkers for assessment of metabolic risk factors.

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Conflicts of interest There are no conflicts of interest.

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Ethical approval The study was approved by the Committee on Medical Ethics of Suez Canal University. The study was carried out in accordance with the regulations and recommendations of the Declaration of Helsinki (REC number GH2008H).

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ORIGINAL ARTICLE

Fatigue and oxidative stress response to physical activity in type 2 diabetic patients

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Abstract Physical activity (PA) and exercise is known to have a positive impact on a variety of variables pertinent to diabetes and cardiovascular disease. The aims of this study were to investigate the effects of physical activity on fatigue scores, oxidative stress, and glycemic control variables of individuals with type 2 diabetes mellitus (T2DM). Seventy-five subjects diagnosed with T2DM for more than 5 years aged 18–65 years participated in this study. The participants classified according to energy expenditure into, physically inactive [≤500 metabolic equivalents (METs)-min/week, n=25], moderate PA (500–2500 METs-min/week, n=25), and PA (≥2500 METs-min/week, n=25). The Global Physical Activity Questionnaire (GPAQ) version 2.0 was used to classify physical activity. The multidimensional checklist individual strength questionnaire (CIS20r) was used to measure chronic fatigue. Blood glucose was measured using a glucose oxidase and peroxidase (GOD-POD) colorimetric method. HbA1c was measured using a commercial kit. Serum insulin level was determined using an ELISA. Analysis of oxidative stress parameters including malonaldehyde (MDA) and total antioxidant capacity (TAC) was done. To test differences between severely fatigued and healthy subjects, an independent t test was performed. Spearman correlations were used to assess correlations between fatigue severity score and disease-related and psychosocial factors. A level of significance was set at $p<0.05$. The results showed a significant reduction of fasting blood sugar, glycosylated hemoglobin, fasting insulin, and MDA along with significant increase in TAC activity in the participants with moderate PA ($P<0.05$) and PA ($P<0.01$), respectively. In relation to CIS-fatigue measurements, about 33 % of the study population (n=25) had a CIS score above the cutoff score of 37 with 59.5 mean CIS score, and 67 % of the study population (n=50) had CIS score below the cutoff 37; they were classified into heightened fatigue (score 27–35) and healthy (score ≤27). There was a significant correlation between the reduction of diabetic related variables, BMI, PA status, and CIS-fatigue score analyses in T2DM patients. CIS-fatigue scores correlated positively with diabetic related variables and negatively with PA, BMI, and TAC activity. PA plays a vital role in improving CIS-fatigue score in type 2 diabetic patients via reducing oxidative stress and diabetic related variables.

Keywords Physical activity · Diabetes · Oxidative stress · Fatigue · Insulin

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Introduction

Physical activity (PA) and exercise is known to have a positive impact on a variety of variables pertinent to diabetes and cardiovascular disease, including blood pressure, lipid profiles, insulin sensitivity, endothelial function, and body composition [1]. The worldwide prevalence of type 2 diabetes mellitus

(T2DM) is increasing at an alarming rate [2]. The increase in obesity and physical inactivity is associated with the epidemic of T2DM; hence, exercise and physical activity would be a key strategy to reverse this pattern. The risk of developing T2DM in high-risk groups have been shown to reduce following interventions involving physical activity by 47 to 58 % [3, 4], and this reduction in incidence was maintained for 10 years following the initial intervention [5].

In addition to smoking habits, arterial hypertension, and dyslipidemia, the low level of physical activity has been considered a risk factor for early mortality. The interventions involving moderate intensity physical training indicated ability to reduce body weight, enhance insulin sensitivity, improve circulating levels of high-density lipoprotein (HDL), diminish triglyceride levels, and normalize blood pressure [6, 7]. Besides, a growing body of evidence suggests that lifestyle interventions that incorporate regular physical activity and healthier dietary habits are valuable for diabetic patients [4, 8].

Oxidative stress has often been involved in the pathogenesis of microvascular and macrovascular ailments seen in diabetic people. The role of regular exercise in reducing lipid peroxidation is evident. Undoubtedly, if regular exercise can demonstrate a preventive effect against oxidative stress in people with diabetes mellitus, the use of exercise as a non-pharmacological therapeutic approach for T2DM, get to be considerably more appealing [9]. The regular practice of physical activity has shown to reduce the risk of cardiovascular disease, cardiovascular death, and total mortality in subjects with T2DM. Previous studies have shown positive effects of PA on glycemic control and blood pressure than other therapies alone [10, 11].

Fatigue is a commonest clinical complaint among adults with T2DM [12, 13], has been associated with poor self-reported health [14], and is likely to affect self-management of diabetes [15]. Overweight and physical inactivity have been strongly associated with fatigue in the general population [16] and individuals with T2DM [17, 18]. The level of physical activity is found to be low in individuals with diabetes [19–21]. The commonest cause of reduced physical activity in T2DM include poor health status and physical functioning [19–24], obesity [19, 22, 24, 25], and fatigue complaints [18, 19, 23].

Therefore, better understanding of fatigue and oxidative response to physical activity in T2DM is vital. The aim of present study was to investigate the effects of physical activity (PA) on fatigue scores, oxidative stress, and glycemic control variables of individuals with type 2 diabetes.

Materials and methods

Subjects

Seventy-five subjects diagnosed with T2DM for more than 5 years aged 18–65 years participated in this study. The diagnosis of T2DM was based on the American Diabetes Association criteria (fasting plasma glucose level higher than 126 mg/dL and/or a glucose level exceeding 200 mg/dL at 2 h in a 75 g oral glucose tolerance test) [26]. All were non-smokers and had no history of abnormal alcohol intake. Standardized measurements of weight and height were taken in light clothing without shoes. Exclusion criteria included type 1 diabetes, smokers, anemia, overt complications of diabetes like nephropathy, neuropathy, retinopathy, obvious ischemic heart disease (angina, myocardial infarction, and lead electrocardiogram abnormalities), chronic liver disease, hypothyroidism, and drugs (diuretics, oral contraceptives). This study was approved by the ethics committee of the Rehabilitation Research Chair (RRC), King Saud University, Saudi Arabia. All participants completed and signed an informed consent form before starting the study. All procedures followed were in accordance with the ethical standards of the local Institutional Ethics Review Board and with the Helsinki Declaration of 1975, as revised in 2008.

Assessment of physical activity

The Global Physical Activity Questionnaire (GPAQ) version 2.0 was used for data collection and accompanied with the GPAQ. It assesses the frequency (days) and time (min/h) spent in doing moderate and vigorous intensity. Previous studies have shown that GPAQ has good reproducibility and relative validity [27, 28]. The duration, intensity, and frequency of PA performed in a typical week were used to estimate energy expenditure. The metabolic equivalent (MET) derived from activity variables of the GPAQ was used as a unit of measurement for energy expenditure. The participants were classified according to energy expenditure into physically inactive (METs-min/week of ≤500, n=25), moderate PA (METs-min/week of 500–2500, n=25), and PA (≥2500 METs-min/week; n=25).

Assessment of fatigue

The chronic fatigue was assessed using the multidimensional checklist individual strength questionnaire (CIS20r). The CIS was developed to measure various aspects of fatigue and consists of four dimensions: the subjective experience of fatigue and reduction in motivation, reduction in activity, and reduction in concentration. Previous studies have shown that CIS has good test-retest repeatability and relative validity [29, 30]. The CIS had a good internal consistency (Chronbach's α 0.90, range 0.83 to 0.92). Higher scores on the CIS-fatigue indicate a higher level of experienced fatigue. A score of ≥35 indicates severe fatigue and is 2 SD above the mean score of healthy adults [31]. A score between 27 (mean score of healthy adults plus 1 SD) and 35 indicates a heightened level of fatigue [32].

Table 1 General characteristics of subjects with type 2 diabetes based on level of physical activity

Parameters	Physically inactive (≤500 METs-min/week)	Moderate PA (500–2500 METs-min/week)	Physically active (≥2500 METs-min/week)
N	25	25	25
Male/female	17/8	14/11	18/7
Age (years)	58.32±1.84	56.48±7.85	58±4.3
BMI (kg/m2)	24.5±1.74	23.1±1.75*	22.4±1.46*
Waist (cm)	76.1±27.9	69.7±18.5*	64.6±16.5*
Hips (cm)	85.9±24.3	85.7±22.3	85.4±21.7
Systolic BP (mmHg)	111.2±9.5	108.9±10.3	109.5±10.5
Diastolic BP (mmHg)	72.5±13.7	72.5±11.3	72.8±8.5
VO2 max (ml/kg min)	32.44±4.22	35.4±3.9*	38.3±2.6*
Disease duration (years)	5.65±3.8	8.5±4.2	8.3±2.9

Values are expressed as mean±SD

*$P<0.05$

Blood sampling and analysis

Blood samples were obtained from all subjects at 8:00 am after an overnight fast. Venous blood samples (5 mL) were collected into plain tubes, and the samples were allowed to clot for half an hour following which samples were centrifuged for 15 min at 2000 rpm. Samples were given a coded study identification number and were stored frozen at −80 °C until analysis.

Analysis of blood sugar, glycated hemoglobin (HbA1c), and insulin

Blood glucose was measured using a glucose oxidase and peroxidase (GOD-POD) colorimetric method (Quanti Chrom Glucose Assay Kit, DIGL-100, BioAssay Systems, Hayward, CA, USA). HbA1c was measured using a commercial kit (Bio-Rad, Richmond, CA, USA), normal range from 3.5 to 5.5 % (coefficient of variation 5 %). The assays were performed according to the instructions provided by the manufacturers. Serum insulin level was determined using an ELISA (human insulin ELISA kit, KAQ1251, Invitrogen Corporation, Camarillo, CA, USA).

Analysis of oxidative stress parameters

Determination of malonaldehyde

Malonaldehyde (MDA) was determined by the thiobarbituric acid method according to Devi et al. [33], and the absorbance of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. The data of TBARS were expressed in MDA, using a molar extinction coefficient for MDA of 1.56 9 105/cm/m, and the results were expressed in nmol/ml.

Total antioxidant capacity

Serum total antioxidant capacity (TAC) was measured Colorimetric Assay Kit (catalog #K274-100; BioVision Incorporated; CA 95035 USA). The antioxidant equivalent concentrations were measured at 570 nm as a function of Trolox concentration according to the manufacturer's instructions.

$$Sa \,/\, Sv = \text{nmol} \,/\, \mu\text{l or mM Trolox equivalent}$$

Table 2 Mean, standard deviation (SD), and statistical comparison of the studied variables (n= 75) based on level of physical activity

Parameters	Physically inactive (≤500 METs-min/week)	Moderate PA (500–2500 METs-min/week)	Physically active (≥2500 METs-min/week)
Fasting blood sugar	198±29.3	156.4±12.2*	140.1±14.1**
Glycosylated hemoglobin (HbA1c)	8.9±1.1	6.95±0.74*	6.46±0.69**
Fasting insulin	37.12±5.64	14.9±2.54*	11.92±1.16**
MDA (μmol/l)	8.12±1.53	5.44±0.96*	4.64±0.97**
TAC (nmol/μl)	8.1±2.0	17.2±3.6*	23.22±4.01**

Values are expressed as mean±SD

TAC total antioxidant capacity (nmol/μl), *MDA* malonaldehyde

*$P<0.05$; **$P<0.01$

Table 3 CIS-fatigue scores in type 2 diabetic patients based on the level of physical activity [means (SD)]

Parameters	Physically inactive (≤500 METs-min/week)	Moderate PA (500–2500 METs-min/week)	Physically active (≥2500 METs-min/week)
Subjective fatigue (8 items)	22.95±2.71	12.53±2.95*	8.7±3.02**
Activity (3 items)	12.14±4.25	7.2±2.13*	6.1±1.95**
Motivation (4 items)	13.3±4.7	6.15±3.46*	5.0±2.8**
Concentration (5 items)	11.03±1.71	7.12±1.6*	6.2±1.54**
CIS total (20 items)	59.5±7.9	33±6.21*	26.0±4.32**
Fatigue diagnosis	Severe fatigue (score ≥35)	Heightened fatigue (score 27–35)	Healthy subjects (score ≤27)

Values are expressed as mean±SD

*P<0.01; **P<0.001

where Sa is the sample amount (in nmol) read from the standard curve, and Sv is the undiluted sample volume added to the wells.

Statistical analysis

Statistical analyses were done using Statistical Package for Social Sciences (SPSS) 16.0 for Windows. A single imputation method was used on the condition of the data being missing at random to replace missing values. The values were imputed using group mean or median, when only one or two observations of a variable were missing. An independent t tests, Mann–Whitney U tests and χ^2 tests were performed as appropriate to test univariately for differences between severely fatigued and not-severely fatigued RA patients. Spearman correlations were used to assess correlations between fatigue severity score and disease-related and psychosocial factors. Statistical significance was considered at p<0.05.

Results

A total of 75 patients aged 18–65 years and diagnosed with T2DM for more than 5 years were recruited for this study. Based on the level of physical activity, subjects were divided into three groups: inactive, moderately active, and active. The demographics and descriptive characteristics of recruited subjects are given in Table 1. The subjects who were physically inactive were approximately 33.3 % of the total participants. A significant increase (p<0.05) in body mass index (BMI) and waist circumference (WC) were reported in physically inactive patients compared to other PA groups. The results showed a significant reduction of fasting blood sugar, glycosylated hemoglobin, fasting insulin, and MDA along with significant increase in TAC activity in the participants with moderate PA (P<0.05) and PA (P<0.01), respectively (Table 2). In relation to CIS-fatigue measurements, about 33 % of the study population (n=25) had a CIS score above the cutoff score of 37 with 59.5 mean CIS score, and 67 % of the study population (n=50) had CIS score below the cutoff 37; they were classified into heightened fatigue (score 27–35) and healthy (score ≤27) (Table 3). Correlation coefficients between the independent variables under study were calculated; there was significant correlation between the reduction of diabetic related variables, BMI, PA status, and CIS-fatigue score analyses in

Table 4 Results of stepwise multiple regression analysis, fatigue predicted by diabetes related variables

Parameters	Severe fatigue (score ≥35)		Heightened fatigue (score 27–35)		Healthy subjects (score ≤27)	
	β	R^2	β	R^2	β	R^2
Fasting blood sugar	0.640*	0.435	0.42**	0.411	0.51**	0.382
HbA1c	0.087*		0.234**		0.45**	
Fasting insulin	0.352*		0.782**		0.65**	
MDA (μmol/l)	0.541*		0.038**		0.78**	
TAC (nmol /μl)	−0.387*		−0.094**		−0.32**	
Physical activity	−0.321*		−0.374**		−0.59**	
BMI (kg/m2)	−0.023*		−0.012**		−0.035**	
Disease duration (years)	0.78*		0.045**		0.067**	

Estimated standardized regression coefficients (β) and variance explained (R^2) are presented

*Significance at <0.05; **significance at <0.01

type 2 diabetic patients. CIS-fatigue scores correlated positively with diabetic related variables and negatively with PA, BMI, and TAC activity as shown in Table 4.

Discussion

The aim of present study was to investigate the effects of physical activity (PA) on fatigue scores, oxidative stress, and glycemic control variables of individuals with T2DM. There was significant increase in BMI and WC found in physically inactive patients compared to other physically active patients. There was significant reduction of fasting blood sugar, glycosylated hemoglobin, fasting insulin, and MDA in participants with moderate PA and PA. In addition, there was significant increase in TAC activity in participants with moderate PA and PA. Previous studies reported positive effects of PA on glycemic control and blood pressure than other therapies alone [11, 34]. Other studies reported improved insulin sensitivity, reduced glycated hemoglobin (HbA1c), and increased peak oxygen consumption following physical activity and exercise [6, 7]. Several studies have reported increased oxidative stress following exercise [35, 36]. In a review, Ribeiro et al. reported improved antioxidant defences and reduced oxidative stress following exercise training [37].

In the present study, about 33 % of participants had a CIS score above the cutoff score of 37 and 67 % of participants had CIS score below the cutoff 37; they were classified into heightened fatigue (score 27–35) and healthy (score, ≤ 27). There was significant correlation between the reductions of diabetic related variables, BMI, PA status, and CIS-fatigue score in type 2 diabetic patients. CIS-fatigue scores correlated positively with diabetic related variables and negatively with PA, BMI, and TAC activity. Fritschi et al. [38] reported significant relationship between fatigue and diabetes symptoms, diabetes emotional distress, depressive symptoms, BMI, and reduced physical activity. In addition, Stewart et al. [39] and Thomas et al. [40] reported inverse relationship between PA and fatigue levels in patient with diabetes and hypertension. Previous study suggests that regular PA can improve aerobic capacity and muscle mass, enhance metabolic substrate use for energy, and mood [41].

Conclusions

The present study concluded that the PA plays a pivotal role in improving CIS-fatigue score among type 2 diabetic patients via reducing oxidative stress and diabetic related variables.

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

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ORIGINAL ARTICLE

Psychosocial correlates of life satisfaction among patients diagnosed with type-II diabetes mellitus

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Abstract The comorbidity between chronic physical conditions and psychosocial health became common interest for health professional and researchers. The purpose of this study was to investigate the relationship among health locus of control, self-efficacy, and life satisfaction in patients with type-II diabetes mellitus. Data were collected using cross-sectional, correlational design from 793 patients with type-II diabetes in regard to health locus of control, self-efficacy, biomarkers, and satisfaction with life scale. Mean of HbA1c was 7.5 (SD=2.6). Patients had moderate to high perception of powerful others health locus of control, self-efficacy, and life satisfaction. Results also showed that self-efficacy (Beta=.29, $p<.001$) and powerful others health locus of control (HLOC) (Beta=.10, $p=.012$) were significant predictors of patients' life satisfaction, while type of treatment (Beta=−.084, $p=.054$) was marginally significant predictor. HbA1c and other demographic factors were not associated with life satisfaction ($p>.05$). Health care professionals need to enhance patient's self-efficacy and internal power of their patients for better health outcomes. Nurses and other health professionals need to emphasize the psychosocial health aspects of patients with chronic illnesses and, in particular, those diagnosed with type-II diabetes mellitus.

Keywords Health locus of control · Self-efficacy · Life satisfaction · HbA1c · Type-II DM · Jordan

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Introduction

Diabetes mellitus (DM) is a global health problem and has been described as the epidemic of the twenty-first century [1]. In Jordan, the prevalence of type-II diabetes of age 25 years and above was 13.2 %, and when combined with impaired fasting blood glucose, the rate increased to 30.5 % [2]. According to Doumit and Nasser [3], patients with chronic illnesses are overwhelmed with psychological stressors due to the requirement related to management of their illnesses. On the other hand, patients' psychosocial status interferes with their ability to manage their needs independently that may exacerbate their health condition [4]. The impact of chronic illnesses such as diabetes mellitus on the bio-psycho-social aspects of individual's health and wellbeing cannot be interpreted solely in terms of disease process, but also relates to difficulties of individuals' adjustment to their illnesses, and the evolved changes of their lifestyle [5, 6]. Factors such as health locus of control, self-efficacy, and life satisfaction are consider significant ones for health care professionals, in particular, with the nature of treatment plans for patients with type-II diabetes mellitus and the high level of nonadherence [7]. In general, patients in different health care settings want to assume more control and involvement in decision-making [8, 9]. The holistic management of diabetes requires adequate

attention focusing not only on the prevalence of psychosocial health problems but also on the subclinical indicators of distress such as patients' level of life satisfaction. This study came to address this issue and attempts to explore the interrelationships between the three psychological factors that are assumed influencing health outcome of patients with type-II diabetes mellitus. Therefore, the purpose of this study was to investigate the relationship among health locus of control, self-efficacy, and life satisfaction in patients with type-II diabetes mellitus.

Research questions:

1. What is the prediction power of health locus of control, self-efficacy, and selected biomarkers on life satisfaction among patients with type-II diabetes mellitus?
2. What is the difference in the health locus of control, self-efficacy, and life satisfaction among patients diagnosed with type-II diabetes mellitus related to selected demographic and personal characteristics?

Methods

This is a quantitative study using cross-sectional correlational design to collect data from a convenience sample of 791 patients with type-II DM in regard to health locus of control, self-efficacy, and life satisfaction from patients diagnosed with type-II diabetes mellitus. Data were collected using structured interview format from Jordanians patients diagnosed with type-II diabetes attended the National Center for Diabetes, Endocrinology and Genetics (NCDEG) in Jordan. Inclusion criteria were (1) at aged of 18 years or older with type-II diabetes mellitus and (2) able to read and write Arabic. Exclusion criteria included (1) no history of diagnosed mental or cognitive disorders that may interfere with their ability to answer the survey questions.

Data collection: Prior to data collection, ethical approval was obtained from NCDEG. A package of three self-report questionnaires and an author-developed demographic survey were used in this study. The package had a cover letter that includes information about the purpose of the study, its significance, what is expected from them, and contact information of the principal investigator for any further information and for answering the questions related to the study only. Patients had the chance to have all their questions answered.

Instruments

The data were collected using an Arabic version of health locus of control scale and life satisfaction scale, while the self-efficacy scale has been translated as follow: We have followed the WHO guideline for translation and tool adaptation. Initially, the tool was translated by a diabetes consultant who has the knowledge and proficiency in English-speaking culture and whose mother tongue is Arabic. Then, a bilingual (in English and Arabic languages) expert identifies and resolves the inadequate expressions/concepts of the translation, as well as any discrepancies between the forward translation and the existing or comparable previous versions of the questions. Then, the instrument translated back to English by an independent translator, whose mother tongue is English and who has no knowledge of the questionnaire. Emphasis in the back translation was on conceptual and cultural equivalence and not linguistic equivalence. Discrepancies discussed and no further work needed. Then, the tool was pretest on the target population (20 patients diagnosed with type-II diabetes mellitus and met all illegible criteria of the study) given full test of the modules.

Pretest respondents should have a minimum number of ten for each section. They should represent males and females from all age groups (18 years of age and older) and different socioeconomic groups. Pretesting followed by a debriefing to ask respondents what they thought the question was asking, whether they could repeat the question in their own words, and what came to their mind when they heard a particular phrase or term. It should also ask them to explain how they choose their answer. These questions should be repeated for each item. Then, the final version of the tool in Arabic reached. The instruments were as follows:

Health locus of control was measured using the Multidimensional Health Locus of Control scale (MHLC Form C) [10]. The Arabic version of this scale has been used [11]. The scale is formed of three main subscales: internal subscale, chance, and powerful others. Each subscale contained six items with Likert responses, ranging from 1 (strongly disagree) to 6 (strongly agree) with a possible subscale score range of 6–36. Higher scores reflected stronger beliefs. The subscales were reportedly internally consistent with Cronbach's α ranging from .67 (external) to .77 (internal) [10]. The scale showed good reliability with Cronbach's α ranging from .72 (external subscale) to .64 (internal subscale). In this study, Cronbach's α was good ranging from .79 (external subscale) to .73 (chance).

Self-efficacy was measured using the Self-Efficacy for Diabetes scale [12]. Self-Efficacy for Diabetes (SED) scale is an eight-item scale used to measure patients' feeling of confidence in managing their illness. Patients are asked to rate their responses on a visual analogue scale ranging from 1 (not at all confident) to 10 (totally confident). Higher score on the scale indicates higher self-efficacy, and lower score indicates low self-efficacy. The scale has good internal consistency with Chronbach's alpha of .76. In this study, Chronbach's alpha was .81.

Life satisfaction was measured using the Satisfaction with Life Scale [13]. The Arabic version of this scale has been used [6]. This is a general measure of life satisfaction, which consisted of five statements with responses ranging from (1) strongly disagree to (7) strongly agree. The scores of the total scale ranges from 5 to 35 and interpreted as follow: from 31 to 35 (extremely satisfied), from 26 to 30 (satisfied), from 21 to 25 (slightly satisfied), 20 (neutral), from 15 to 19 (slightly dissatisfied), from 10 to 14 (dissatisfied), and 5 to 9 (extremely dissatisfied). The test–retest reliability was estimated to be .87 [12]. In this study, Cronbach's Alpha was .78.

Potential covariates are as follows: gender, age, marital status, type of DM, duration of DM, smoking status, education level, work status, and biomarkers such as HbA1c.

Results

Descriptive characteristics

A total of 860 patients approached and 793 of them completed and returned the questionnaire forming 92.2 % response rate. The analysis showed that the age of the patients was ranged from 19 to 85 years with mean age of 54.8 years (SD=11.5). There were 359 (45.3 %) male patients and 434 (54.7 %) female patients. About 82.0 % (n=650) were married, 5.5 % (n=44) were single, 11 % (n=88) were widowed, and 1.4 % (n=11) were divorced. Among the patients, 35.9 % (n=285) reported that they are not working, while 22.8 % (n=238) were working full time, 5.5 % (n=44) were working part time, and 28.7 % (n=228) were retired. Regarding the type of treatment that the patient receive, the majority of them (59.5 %, n=472) reported that they were receiving oral medication, while 23.7 % (n=188) reported that they receive oral medication and insulin and 14.1 % (n=112) received only insulin therapy. Also, the analysis showed that 52 % (n=412) of the patients reported controlled HbA1c, 14.2 % (n=113) fairly controlled, and 33.8 % (n=268) poor glycemic control.

Patients' psychosocial health

In regard to patients' level of satisfaction, the analysis showed that the mean score for patients was 26.1 (SD=5.2) with scores ranging from 5.0 to 35.0. About 50 % of the patients had a score of 27.0 or above and 50 % of them had a score between 23.0 and 29.0 indicating that patients in general had moderate to high level of life satisfaction.

Regarding patients' health locus of control, the analysis showed that the mean score for the internal subscale was 27.2 (SD=6.32), for the powerful others subscale was 30.9 (SD=5.7), and for the chance subscale was 18.6 (SD=6.8). The mean total score of locus of control scale was 76.6 (SD=13.1) ranging from 19 to 108. The results indicate that patients believed that their health outcomes were dependent on powerful others more than themselves or due to chance.

Regarding patients' perception of self-efficacy, the analysis showed that patients had moderate level of perception of efficacy that they are able to manage their health need related to their illness. The analysis showed that the mean score was 52.4 (SD=15.5) with scores ranging from 8 to 80. Fifty percent of the patients had a score between 41 and 64, and 50 % of them had a score of 54 or above.

Bivariate analysis

In regard to the relationship between life satisfaction, self-efficacy, health locus of control, and demographic variables, the analysis using Pearson r showed that life satisfaction has significant and positive correlation with health locus of control (r=.16, $p\geq$.01), self-efficacy (r=.32, p>.01), and negative relationship with HbA1c (r=−.12, p>.01). While self-efficacy has positive and significant correlation with health locus of control (r=.18, p>.01) and negative relationship with HbA1c (r=−.11, p>.01). On the other hand, age has no significant correlation with life satisfaction, self-efficacy, and health locus of control.

In relation to differences in life satisfaction, self-efficacy, health locus of control, and demographic variables, the analysis using t test showed that there were no significant differences between male and female patients in life satisfaction, self-efficacy, and health locus of control. Using ANOVA test, the analysis also showed no differences found in regard to marital status and smoking status (p>.05). There was a significant difference in self-efficacy in relation to working status ($F_{4,\ 792}$=4.2, p=.002). Moreover, the analysis showed that there was a significant difference in health locus of control in regard to the level of education ($F_{6,\ 792}$=2.3, $p\geq$.04) and type of complications ($F_{5,\ 792}$=4.7, p=>.001). Regarding the life satisfaction, the analysis showed that there is a difference in life satisfaction in regard to the type of treatment ($F_{3,\ 792}$=3.2, p=.024).

Predictors of life satisfaction

Two-step multiple hierarchical regression analysis was performed to examine whether locus of control and self-efficacy are significant predictors of life satisfaction, controlling for the selected demographic characteristics (age, gender, level of education, duration of diagnosis of diabetes, working status, HbA1c, type of complication, and type of treatment). The results showed that model 1 that contained demographics and life styles explained 3.5 % (R^2=.035) of the variance in life satisfaction (see Table 1) and the model was not significant ($F_{14,\ 792}$=2.0, p=.16). In this model, HbA1c (p=.041) was a significant predictor of life satisfaction. After entry of health subscales of locus of control and self-efficacy at step 2, the total

Table 1 Two-step multiple hierarchal regressing life satisfaction on self-efficacy and health locus of control subscales controlling for demographic (N=793)

Variable	Model 1		Model 2	
	Beta	p value	Beta	p value
Age	.074	.079	.068	.086
Gender	.010	.792	−.004	.917
Marital status	.035	.363	.030	.403
Work	−.017	.682	−.055	.159
Education	.076	.066	.068	.087
Duration of diabetes	.058	.231	.043	.351
Type of treatment	−.070	.132	*−.086*	*.054*
Duration of insulin therapy	.001	.978	.009	.830
Diabetic complication	−.051	.187	−.029	.427
Smoking	.005	.930	.023	.658
Number of cigarettes per day	.020	.709	.017	.736
HbA1c	−.084	*.041*	−.048	.226
Number of coffee per day	−.076	*.048*	−.039	.284
Number of tea per day	.013	.726	.010	.779
Self-efficacy	.018		*.285*	*<.001*
Internality HLOC	.155		.034	.386
Chance HLOC	.271		.009	.784
Powerful others HLOC	.165		*.100*	*.012*
R^2	.035		.140	
Adjusted R^2	.017		.120	
R^2 change	–		.106	
F	2.0		7.0	
p value	.160		<.001	

variance explained by the model as a whole was 14 % (R^2=.14) and was significant ($F_{18,\ 792}$=7.0, p<.001*)*. The variables in step 2 explained an additional 10 % of variance in life satisfaction. In model 2, self-efficacy ($\hat{a}$=.29, p<.001) and powerful others ($\hat{a}$=.10, p=.012) were significant predictors of life satisfaction. That is, there was a positive association between self-efficacy and life satisfaction indicating that patients who reported higher level of self-efficacy are more likely to report higher level of life satisfaction. Also, there was a positive association between perception of powerful others and life satisfaction indicating that patients who reported higher level of perception of powerful others are more likely to report higher level of life satisfaction. It is noted also that HbA1c are appeared to be nonsignificant in model 2 while the type of treatment had marginal nonsignificant prediction power (p=.054).

Discussion

The purpose of the present study was to investigate the relationship among health locus of control, self-efficacy, and life satisfaction of patients with type-II diabetes mellitus. The findings showed that patients reported that their health outcomes mainly influenced by the powerful others rather than themselves or by chances. Also, the study found that patients had moderate level of perception of self-efficacy and moderate to high level of life satisfaction. The findings in this study agree with previous ones who found that patients diagnosed with chronic illnesses believed that their health is more influenced by health professionals and friends and family members rather than themselves or chance [14]. One explanation is that long experience of illness may influenced patients' perception of their health locus of control (HLOC) that may contribute to higher level of perception of external HLOC than other forms [15]. However, patients' perception of high external HLOC was associated with patients' adherence to treatment plans than those with internal HLOC [16]. Regarding patients' perceptions of self-efficacy, the present findings showed that patients had moderate level of perception of self-efficacy that corresponds with previous international studies that found moderate to high level of self-efficacy among patients diagnosed with type-II DM [17]. The moderate and high levels of self-efficacy, according to the literature, have been associated with positive outcome of quality of life and social support among people with type-II diabetes [18].

This study also found that patients had moderate to high level of life satisfaction. The results agree with previous ones that patients with type-II DM have moderate level of life satisfaction [7]. However, the results have to be interpreted cautiously in terms of the health condition of the patients. Several studies reported that complications and treatment approach have negative impact on patients' perception of their life satisfaction [7]. In the current study, and by using post hoc comparison, the analysis showed that patients who received oral medication only reported higher life satisfaction than those who received oral medication and insulin therapy.

The study also found that self-efficacy and powerful others HLOC were strong predictors of patients' life satisfaction. While the type of treatment was marginally significant predictor of life satisfaction, demographic factors and health biomarkers such as HbA1c were not. This study is first to address this issue among patients with diabetes mellitus; however, in comparison with other studies targeting patients with chronic illness, the results agree to those reports. These reports [8, 18, 19] found that that self-efficacy and locus of control have significant association with life satisfaction. One limitation for this study is that data collected from patients referred to the NCDEG, while multisite convenience sample may produce alternative reports.

Conclusion

This study has showed that patients with type-II diabetes mellitus in Jordan had moderate to high levels of life

satisfaction, powerful others health locus of control, and self-efficacy. Also, the study found that powerful others, self-efficacy, and type of treatment were significant predictors of patients' life satisfaction, and life satisfaction and glycemic control (HbA1c readings) were weakly associated. It is recommended that health care professionals enhance patient's self-efficacy and internal power of their patients for better health outcomes.

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ORIGINAL ARTICLE

Do the human umbilical cord blood CD34+ progenitor cells home in the pancreas and kidney of diabetic mice?

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Abstract Type 1 diabetes is a chronic metabolic disorder in which pancreatic islet β cells are irreversibly destroyed by autoimmunity. Many studies suggest great promise for the utility of human umbilical cord blood (HUCB) stem cells as a cure for diabetes; however, the mechanism for their effect requires further elucidation. This study investigated the presence of human DNA in the pancreas and kidney of diabetic mice treated with HUCB CD34+ cells. Eighteen albino male mice were equally and randomly divided into three groups: normal control group, diabetic untreated streptozotocin (STZ) group, and diabetic STZ-treated group. Diabetes was induced by intraperitoneal (IP) injection of STZ (180 mg/kg). CD34+ progenitor cells were separated from HUCB and injected intravenously in dose of 10^6 cells/mouse. Blood glucose was measured every 2 days for 3 weeks. Mice were sacrificed after 3 weeks, and real-time PCR analysis was conducted for the presence of human-specific ALU sequence in the pancreata and kidneys from all animals. Injection of CD34+ cells caused significant improvement in blood glucose level (230±50 mg/dl in treated group vs. 590±24 mg/dl in untreated group, p= 0.001). Real-time PCR analysis showed negative results in the control and untreated groups, while in the treated group, engraftment of the HUCB CD34 cells was positive in 100 % of the kidneys with a mean transplanted cell percentage 1.8± 0.98 % and 66 % of the pancreata with a mean 0.41± 0.42 %. A significant negative correlation was found between the concentration of the ALU sequence in the pancreata and the change of glucose level in the treated group (p value=0.03 and r=−0.6). HUCB CD34 cells engraft in the pancreas of the diabetic mice and improve hyperglycemia. Based on our results, HUCB CD34 cell transplantation may provide a potential therapy for human diabetes mellitus.

Keywords Homing of CD34 cells · Human umbilical cord blood · Streptozotocin-induced diabetic mice

Introduction

Type 1 diabetes mellitus (T1DM) is an insulin-dependent, autoimmune disorder resulting from T-cell-mediated destruction of insulin-producing pancreatic β cells [1]. The real cure of T1DM is the replacement of pancreatic β cells. While pancreatic islet transplantation may offer a cure for diabetes, the limited supply of donors and the immunological rejection restricts the availability of this treatment [2]. To reduce the need for such organ transplantation, investigators have been trying to identify stem/progenitor cells that can physiologically generate insulin in response to glucose. In a xenogeneic model of stem cell transplantation, human mononuclear umbilical cord blood (UCB) cells were able to reduce blood glucose levels and increase survival in mouse models of type 1 and type 2 diabetes [3]. In another animal model of type 2 diabetes, UCB cell infusion also improved renal abnormalities and neuropathy caused by diabetes, suggesting a regenerative action in renal parenchyma and nerves [4, 5]. However, a major limitation for UCB transplantation is the high rates of poor engraftment which is related to the limited number of stem cells in the cord blood [6, 7]. Also, studies that looked for homing of systemically infused cells in the affected organs showed controversial results. Some studies reported homing of human stem cells in the tissues of the diabetic mice treated by the stem cells [4, 8, 9]. Others reported that the majority of cell

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types exhibit poor homing to disease sites [10]. Thus this research aimed to verify homing of HUCB CD34+ cells in the diabetic mice by detecting the presence of human DNA in the pancreata and kidneys after intravenous injection of the cells.

Materials and methods

Eighteen albino male mice, of 6-weeks age, were acclimatized for 1 week and kept with free access to standard pellet animal diet and tap water. Mice were equally divided into the following three groups: group 1 served as a normal control group: this group was injected by the solvent of the HUCB (phosphate buffer saline); untreated group 2 (diabetic control): this group was subjected to induction of diabetes mellitus by intraperitoneal (IP) injection of streptozotocin (STZ; 180 mg/kg) without any type of treatment; and group 3 (the treated group) was subjected to induction of diabetes mellitus with STZ (180 mg/kg, IP) and received HUCB CD34+ cells in a dose of 1×10^6 cells/mouse by intravenous (IV) injection in the tail vein after 5 days of diabetes induction.

All groups were followed up by measuring blood glucose by glucometer (Medismart, Switzerland), by snipping the mouse tail under non-fasting condition every 2 days for 3 weeks. Then, all mice were sacrificed under anesthesia. Samples from pancreata and kidneys from all animals were taken and put in a freezer of −20 °C to be analyzed by the real-time PCR for the presence of human-specific ALU sequence.

Collection, preparation, and transplantation of HUCB CD34+ cells:

(1) Cell source: HUCB samples were obtained from the umbilical cord while the placenta was still in utero. HUCB samples were obtained from healthy full-term neonates. Each HUCB sample was collected into 50-ml sterile polypropylene test tube containing 5 ml of citrate phosphate dextrose adenine-1 as anticoagulant. Each sample volume was about 50 ml. Samples were stored at 4 °C in a refrigerator and processed within 24 h [11].
(2) Separation and purification of CD34+ cells: separation of UCB CD34+ stem cells was carried out according to the method described before by immune-magnetic separation technique [12]. Briefly, by mixing and incubation, CD34+ cells were bound to Dynabeads M-450 CD34. The formed rosettes were isolated from the suspension using a DYNAL Magnetic Particle Concentrator (Dynal MPC). With subsequent incubation with DETACHaBEAD, CD34 gently detach isolated cells from the beads. A DYNAL MPC was then used to separate the purified, positively selected CD34+ cells from the released Dynabeads M-450 CD34. The quantity of the isolated CD34+ cells was assessed by automated cell counter, and the viability was determined by trypan blue exclusion test [13].
(3) Transplantation of HUCB CD34+ cells: after separation, 0.2 ml of PBS solution was added to the CD34+ cell pellet for final dilution and IV injection in the tail vein in a dose of 10^6 cells/mouse.

Detection of human DNA from HUCB CD34+ cells in pancreata and kidneys of the diabetic mice DNA was extracted using Wizard Genomic DNA Purification Kit. Amplification and on-line monitoring of the DNA was achieved by a combined procedure on the lightCycler® Instruments. Light Cycler-DNA Amplification kit SYBR Green I (Cat.No.2015137) was used. The kit is for one-step RT-PCR using the light Cycler 2.0 System (Roche, Germany).

Results

Table 1 demonstrates the blood glucose level in all groups. At the beginning of the study (day 0), the blood glucose levels were normal in all groups.

Detection of human DNA from HUCB cells in the pancreata and kidneys of diabetic mice Tissues from all groups were assayed for engraftment by real-time PCR for human-specific ALU sequence. PCR assays were positive in four samples of six pancreata of the treated group. PCR assays were positive in all kidney samples in the treated group and were negative in the control group and untreated group in both pancreas and kidney tissues (please see Table 2, Figs. 1, 2, 3, and 4).

Discussion

In the current study, HUCB CD34+ cells in a dose of 10^6 cells/mouse by intravenous injection in the tail vein succeeded in improving hyperglycemia in the treated group from 588.33± 20 to 230±50 mg/dl after 2 weeks ($p<0.001$). In agreement with our results, a study [3] demonstrated a dose-dependent decrease in blood glucose levels in non-obese diabetic (NOD) mice that received HUCB mononuclear cells. The mice that received the highest dosage (200×10^6) of cells had greater reduction in blood glucose levels than the mice that received a lower dosage ($100–150\times10^6$) of cells. Similarly, another study [9] demonstrated significant lowering of blood glucose levels in STZ diabetic mice after intracardiac injection of (2.5×10^6) multipotent stromal cells from human bone marrow (hMSCs). Also, others [14] reported significant reduction of blood glucose levels reaching nearly euglycemic values in STZ-induced type 1 diabetes C57BL/6 mice a month after a

Table 1 Comparison of blood glucose level among the studied groups

Study groups Blood glucose level (mg/dl)	Control group	Untreated group	Treated group	*p* value
Day 0	106.6±28	100.3±14.7	110±37.2	NS
Day 5	102±28	590±24	588.33±20	0.001
Day 21	116.6±37	590±24	230±50	0.001

NS non-significant

single intravenous dose of multipotent mesenchymal stromal cells. One study [15] reported normalization of blood glucose levels within a week after implanting one million mouse embryonic stem cell-derived insulin-secreting cells into the spleen of diabetic mice.

In this study, we used the local strain albino mice without induction of immune suppression as the incidence of graft-versus-host disease (GVHD) is lower in HUCB transplantation compared to bone marrow transplantation; this may be due to immaturity of UCB lymphocytes and reduced T-cell dose infused with UCB grafts [16].

In this study, real-time PCR analysis for human-specific ALU sequence demonstrated that 0.29 to 1.45 % of the injected cells engrafted into the pancreatic tissues of four out of six animals in the treated group. While 1.7 to 2.1 % of the injected cells engrafted into the kidney tissues obtained from all animals in the treated group. Negative results were obtained in both the control and untreated groups. In agreement with our results, others [9] used also real-time PCR for assaying ALU sequence, where they detected up to 3 % of the infused hMSCs engrafted into pancreas and up to 11 % of the infused hMSCs engrafted into kidneys in diabetic mice. In their study, no ALU sequence was detected in the lung, liver, or spleen of diabetic mice. The percentage of engrafted cells is relatively higher in the other studies and may be explained by the higher number of injected stem cells and also because intracardiac infusion was used instead of an intravenous one which probably decreased trapping of the cells in the capillary beds of the lung. The kidneys have a higher percentage of engrafted cells; this may be due to the high blood supply that reaches the kidneys. Also, another study [17] investigated the effect of bone-marrow-derived mesenchymal stem cells on the cardiovascular complications of the STZ diabetic rats. They obtained mesenchymal stem cells from male albino rats and injected them into female diabetic rats, and the SRY gene as a marker of the Y chromosome was detected using PCR in the pancreas and heart of the diabetic treated group. Conversely, a previous study suggested that hematopoietic stem cells do not engraft with absolute efficiencies, and they adhere in low number within the local microcirculation of injured organs [18]. Therefore, following systemic infusion of hematopoietic stem cells, poor homing and a subsequent low efficiency of tissue engraftment occurs, processes that are essential for stem cells to promote tissue repair [10, 19]. To improve engraftment, various methods have been investigated, such as ex vivo expansion of hematopoietic stem cells (HSCs) [20] and use of double cord blood transplant instead of single unit transplant [21]. Also, co-transplantation of placental mesenchymal stem cells (MSCs) enhanced cord blood engraftment in NOD/SCID mice [22].

In this study, there was a significant negative correlation between the concentration of the ALU sequence in the pancreata and the change of glucose level in the treated group. This finding shows that amelioration of hyperglycemia is most probably due to engraftment of HUCB CD34+ cells. The possible explanation may include the generation of HUCB-derived insulin-producing cells (IPCs). A study [8] demonstrated that, following the intravenous transplantation of HUCB into non-obese diabetic/severe combined immune deficient (NOD/SCID) mice, IPCs of human origin were found in mouse islets. IPCs containing human and mouse chromosomes, as well as only human chromosomes, were identified using species-specific probes. Based on these results, the

Table 2 Concentrations of human-specific ALU sequence in the treated group

Sample no.	Pancreas	Kidney
1	0.31 %	1.80 %
2	1.46 %	2.1 %
3	0.29 %	1.8 %
4	0.39 %	1.7 %
5	0	1.6 %
6	0	2 %
Mean±SD	0.41±0.05	1.8±0.018

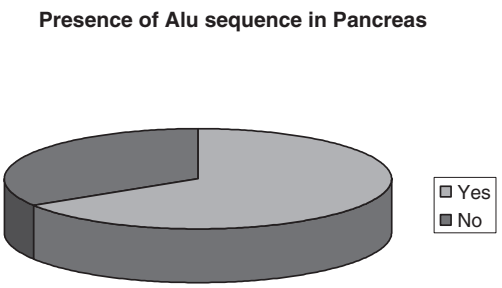

Fig. 1 The figure shows that 66 % of pancreatic samples in the treated group were positive, and 34 % were negative for the presence of human-specific ALU sequence

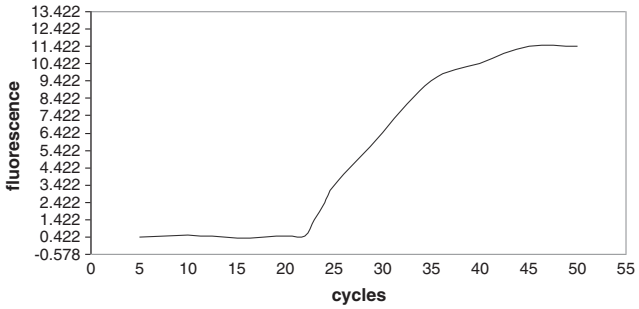

Fig. 2 Amplification curve of a positive sample where ct=22.88

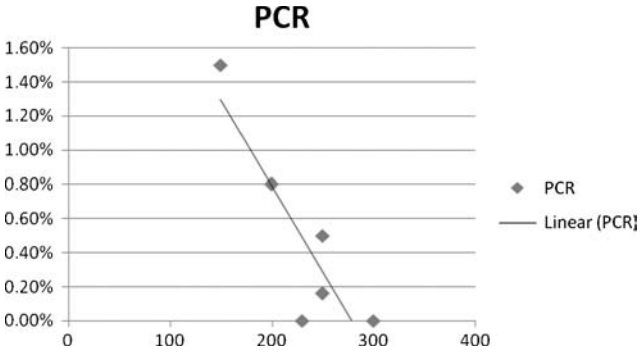

Fig. 4 The correlation between the concentration of the human-specific ALU sequence in the pancreata and the change of glucose level in the treated group. It shows a significant negative correlation where p value=0.03 and $r=-0.6$

authors suggested that the generation of HUCB-derived IPCs can be mediated through both fusion-dependent and fusion-independent mechanisms. Similarly, another study [23] transfused via the tail vein, bone marrow cells marked with enhanced green fluorescent protein (EGFP) into irradiated NOD/SCID mice, and demonstrated that these cells were capable of differentiating into IPCs with features similar to ß cells. Another explanation is supposed to be mediated by the indirect participation of HUCB cells in the regenerating process. HUCB cells are able to secrete mediators critical for the migration, proliferation, and homing of endogenous progenitors and participate in a neovascularization of injured or ischemic tissue [24]. Similarly, others [25] investigated the capability of bone marrow (BM)-derived cells to initiate pancreatic regeneration. They transplanted BM-derived cells from green fluorescent protein transgenic mice into diabetic mice. It was found that BM-derived cells especially CD34+ cells can promote repair of pancreatic islets. Moreover, both proliferation of beta cells and differentiation of pancreatic stem cells contribute to the regeneration of beta cells. Another possible explanation of the amelioration of hyperglycemia is the paracrine mechanisms; researchers have found that MSCs naturally produce a variety of cytokines and growth factors, promoting the survival of surrounding cells, called paracrine mechanisms. Paracrine effects have been proved to play an important role in tissue regeneration and repair in recent researches. MSC transplantation into diabetic animals may prevent apoptosis of injured pancreatic beta cells and enhance regeneration of endogenous progenitor cells through paracrine actions

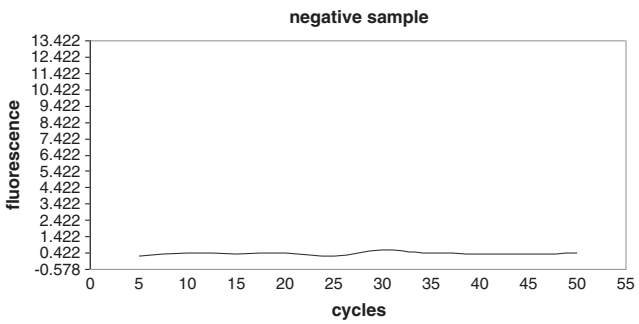

Fig. 3 Amplification curve of a negative sample

such as angiogenic, cytoprotective, anti-inflammatory, mitogenic, and anti-apoptotic effects [26].

In a previous study [27], our research group used immunohistochemical techniques to detect human insulin in the pancreatic islets by using primary antibody against human insulin. They detected human insulin in the pancreatic islets in 33 % of the treated mice, so the number of human insulin positive cells was too small to have a significant effect on blood glucose level, while in our study we detected homing of stem cells in 66 % of the pancreata of treated mice. This observation supports the possibility that generation of HUCB-derived IPCs may not be the only mechanism involved in improvement of hyperglycemia but also the potential capacity of the transplanted cells to support the recovery of pancreatic islets through paracrine mechanisms or enhancement of the proliferation of tissue endogenous stem cells.

HUCB have been used to treat T1D patients in clinical trials. One trial has demonstrated that autologous hematopoietic stem cell transplantation had preserved β-cell function in Chinese patients with new onset type 1 diabetes and diabetic ketoacidosis [28]. In a non-randomized, controlled, open-label intervention trial, seven children with newly diagnosed type 1 diabetes were infused with a single autologous cord blood (CB) and ten children were enrolled as natural controls; it was found that an autologous CB infusion did not change the natural course of metabolic and immune parameters after disease onset [29].

In conclusion, this study showed a significant metabolic improvement after injection of HUCB CD34+ cells in a diabetic mouse model and provided an evidence of engraftment in 66 % of the pancreatic tissue and 100 % of the kidney tissue of the treated animals.

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

Compliance with Ethical Standards This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Suez Canal University. All procedures performed in studies involving animals were in accordance with the ethical standards of the Suez Canal Faculty of Medicine. Informed consent was obtained from all women before obtaining the umbilical cord blood.

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ORIGINAL ARTICLE

Molecular investigation of *WFS1* gene exon 8 in Iranian patients with Wolfram syndrome

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Abstract Most patients with Wolfram syndrome carry mutations in *WFS1*, while a lower percentage present a mutation in *CISD2* (also known as *WFS2*). The aim of this study was to investigate the presence of mutations in exon 8 of *WFS1* gene in two Iranian patients with Wolfram syndrome (WFS). In this study using polymerase chain reaction (PCR) followed by direct sequencing, we screened the entire length of WSF1 gene exon 8 for presence of mutations. Patients included were two male subjects who developed diabetes mellitus earlier than the age of 8 years old, showing early-onset diabetes, followed by reduced visual acuity, deafness, and diabetes insipidus. The presence of two missense mutations G736D and R629W were confirmed. These mutations have been previously reported in patients with WFS in other populations. Identification of pathogenic mutations in patients with Wolfram syndrome will be helpful in earlier diagnosis of the disease and in understanding the frequency of mutations in various populations and their relation with clinical features of Wolfram syndrome.

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Introduction

Wolfram syndrome (WFS: OMIM 222300) is a rare monogenic and progressive neurodegenerative disorder with a prevalence ranging from 1/100,000 to 1/770,000 worldwide [1, 2]. The primary diagnostic symptoms for Wolfram syndrome are the combination of juvenile-onset insulin-dependent DM1 at a median age of 6 years and optic atrophy at 11 years of age [3]. Wolfram syndrome usually presents with a spectrum of clinical features which encompasses DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) [4]. Additional clinical features may include neurological and endocrinological abnormalities such as atonic bladder, renal abnormalities, ataxia, dementia or mental retardation, peripheral neuropathy, hypogonadism, and a relatively high incidence of depression and psychotic behavior. Most patients with Wolfram syndrome carry mutations in the *WFS1* located on chromosome 4p16.1 encoding a 890 amino acid transmembrane glycoprotein being localized in the endoplasmic reticulum named wolframin. *WFS1* comprises 8 exons, and the last exon contains the majority of mutations reported in *WFS1*. Exon 8 covers almost half of the coding region of the gene [4]. Numerous mutations have been found in *WFS1* largely in exon 8 including missense, insertion, deletion, and splice site mutations. Compound heterozygote for two missense mutations has also been reported and leads to a relatively mild phenotype [5, 6]. More than 90 % of WS patients carry mutations that result in a loss of function of the *WFS1* [7–9]. A second causative gene in Wolfram syndrome is *CISD2* which encodes a mitochondrial protein of ERIS and has been reported in some sporadic cases [10, 11]. Dysfunction of *CISD2* gene can cause

WFS2 [12]. Moreover, a further nuclear gene locus (chromosome 4q22-24) associated with Wolfram syndrome is suggested [13]. Life expectancy of patients with Wolfram syndrome is about 30 years [14].

Material and methods

Blood samples were collected from two males (coded as WF023 and WF025), and genomic DNA was extracted by phenol chloroform technique. The entire length of exon 8 of *WFS1* was amplified in seven fragments (8a–8g). Table 1 shows the sequence of primers used for polymerase chain reaction (PCR).

PCR analysis was carried out in a total volume of 25 μl containing 0.5 μl of each forward and reverse primers (10 Pmol), 10 μl of PCR Master mix $Mgcl_2$ 1.5 Mm and 1 μl DNA (about 100 ng). The reaction was adjusted to the total volume of 25 μl by ddH2O.

The PCR was performed in touchdown mode by using an initial denaturation step at 94 °C for 5 min, followed by 14 cycles of denaturation at 94 °C for 30 s, annealing at 70 to 60.5 °C for 45 s (after each cycle, the temperature was reduced by 0.5 °C), and elongation at 72 °C for 1 min. Twenty additional PCR cycles at a constant annealing temperature of 60.5 °C were performed prior to the final elongation step at 72 °C for 5 min. PCR products were examined by 2 % agarose gel electrophoresis for the presence and sizes of amplicons.

Consequently, DNA sequencing of the PCR products was performed on 3130 ABI capillary electrophoresis using the same primers.

Sequencing chromatograms were analyzed by using Chromas v2.4 software and BLAST database.

Table 1 Oligo nucleotide sequences used for PCR amplification and direct sequencing

Primer name	Primer sequence
8a	F: $^{5'}$GTCAGAGGGAGGCGTGAGAT$^{3'}$ R: $^{5'}$GCCTGCTCCACATCCAGGTT$^{3'}$
8b	F: $^{5'}$GGTGTTCCAGGACAGCAAGG$^{3'}$ R: $^{5'}$CCGACAGGCACGGTGATGAA$^{3'}$
8c	F: $^{5'}$GCTATCGCTGCTGCCCTCCA$^{3'}$ R: $^{5'}$AGCTCCAGAGACGTGAACCAC$^{3'}$
8d	F: $^{5'}$GTGAGCTCTCCGTGGTCATC$^{3'}$ R: $^{5'}$TGTAGACCTTCATGCCCTCTG$^{3'}$
8e	F: $^{5'}$CTCCATGGTCAAGCTCATCCT$^{3'}$ R:$^{5'}$TGTAGCGGTCGAACTTCTTGAT$^{3'}$
8f	F: $^{5'}$CAGCCCTGGCAACACCTCCA$^{3'}$ R: $^{5'}$CCAGTCGTGCTCGATCTTCAC$^{3'}$
8g	F: $^{5'}$CTTCGAGCTCAAGGCCATCAG$^{3'}$ R: $^{5'}$AGAGCTACACAGCAGCCTTCC$^{3'}$

F forward primer, *R* reverse primer

Secondary structure prediction of wolframin (*WFS1*) was made using a previously reported method [15], and the scheme was prepared with TMRPres2D [16].

Clinical presentation

Patient 1

Patient 1 is a 21-year-old male (WF023) with diabetes diagnosed at the age of 6 years, followed by reduced visual acuity at 13 years old with history of nocturia and polyuria, excretion of extraordinarily large volumes of very dilute urine about 12 l passed in a day, urinary incontinence, and extreme thirst. The patient was managed with self-intermittent catheterization four times a day since 2 years ago. He admitted to ophthalmology clinic complaining about decreased visual acuity. Ophthalmological examination showed a progressive loss of vision and visual acuity owing to bilateral optic atrophy. There was no evidence of diabetic retinopathy. At the age of 16, during follow-up visits pure tone audiometry showed loss of auditory acuity in both ears. Renal sonography showed pelvicalyceal dilatation in both kidneys equal to hydronephrose grade IV with dilation in proximal of urethra and bladder enlargement secondary to high urine output, and urethral pressure profile shows an atonic bladder. He was found to have profound dilation of the bladder and was admitted for bladder decompression. Diabetes insipidus was diagnosed based on a series of tests including urine analysis and a fluid deprivation test. Administration of vasopressin improved the volumes of urine. The dose is adjusted to maintain the body's water balance and a normal urine output.

Patient 2

A 16-year-old male (WF025) with the history of diabetes more than 8 years ago with reduced visual acuity has been referred to the outpatient clinic, and optic atrophy was observed in fundoscopic examination. He also was using a hearing aid as a result of loss of auditory acuity since 1 year before being admitted. Decreased urine osmolality was observed in laboratory test, and diabetes insipidus was diagnosed by dehydration test.

Results

Two mutations, G736D (Fig. 1) and R629W (Fig. 2), were identified in exon 8 of WF023 and WF025, respectively. V333I polymorphism in fragment 8a of both patients was also observed (Table 2).

From a biochemical point of view, both R to W and G to D mutations could result into considerable change in the microenvironment of the affected residues. The side chain of the basic residue arginine has the ability to make electrostatic

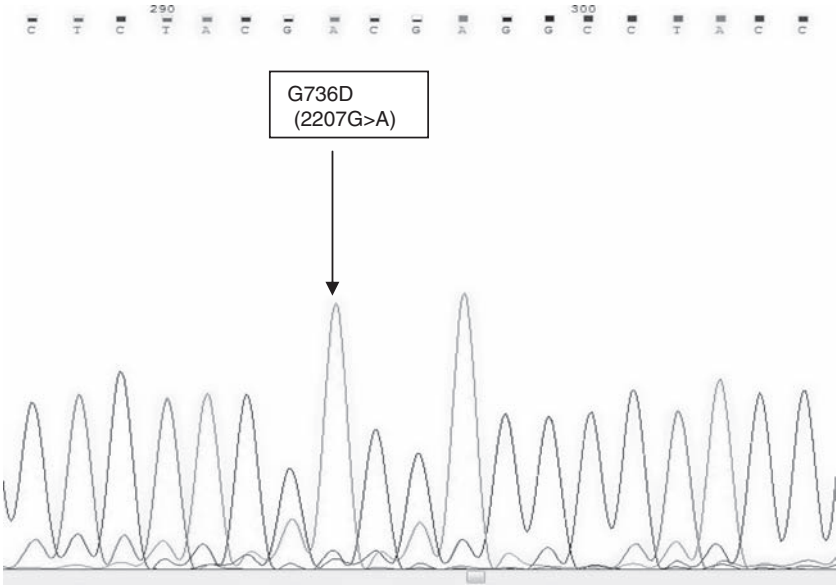

Fig. 1 Chromatogram indicating G736D mutation

interactions, while tryptophan is an aromatic and hydrophobic residue. Glycine is devoid of side chain, while aspartate may form electrostatic and polar interactions (including hydrogen bond). The predicted location of these residues is indicated in Fig. 3. R629 is positioned on a connecting loop between the last two helices of the transmembrane segment of wolframin, while G736 is located on the C-terminal part.

Discussion

Wolfram syndrome which was first described by Wolfram and Wagener in 1938 [17] is an autosomal recessive disorder caused by a defect in the *WSF1* gene and codes for a transmembrane protein (wolframin) located in the ER which is expressed in different tissues, such as the brain, heart, and pancreas. A ubiquitous expression of the *WFS1* with the highest transcript levels in the brain, pancreas, heart, and insulinoma β-cell lines shows the importance of this protein [18, 19]. A wide range of mutations including stop, frameshift, deletion, and insertion mutations mostly in exon 8 in *WFS1* have been described. Therefore, loss of function mutations in *WFS1* is the cause of the Wolfram syndrome [9, 20–23]. The majority of mutated transcripts are rapidly degraded so the absence of truncated wolframin proteins is caused by instability of the mutated transcripts [24]. The function of wolframin is unknown; altogether, membrane proteins are often involved in processing and folding of proteins and calcium homeostasis. Disrupting these processes can lead to cellular apoptosis [25, 26]. Studies in mouse models showed that ER stress has a

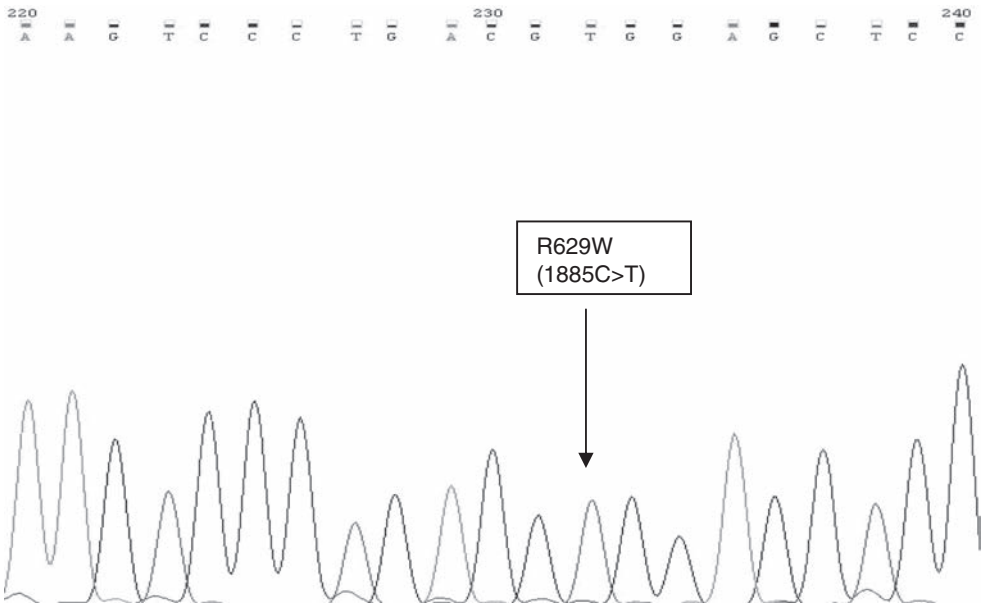

Fig. 2 Chromatogram indicating R629W mutation

Table 2 Mutations in the WFS1 gene

Patient code		Fragment	Nucleotide change	Amino acid change	Type of mutation
WF023	rs1801212	8a	G/A	V333I	Missense
	Rs71530912	8e	A/G	G736D	Missense
WF025	rs1801212	8a	G/A	V333I	Missense
	rs71530910	8d	C/T	R629W	Missense

key role in death of beta cells and neurons [27–29]. The protein model used in the current study is based on a previous report and contains nine transmembrane segments which span from 314 to 652 [15].

Many of the missense mutations are located in the C-terminal domain of the protein underlining the functional importance of the C-terminus of wolframin [9, 23]. Wolfram syndrome in Iran was first described in two siblings that presented with diabetes mellitus followed by bilateral optic atrophy in the first decade of life. Additional symptoms included diabetes insipidus and sensorineural deafness, dilated renal outflow tracts, and multiple neurological abnormalities in the second, third, and fourth decade, respectively [30]. Few studies have been done on Wolfram syndrome in Iranian population. Previous studies in Iranian patients described some mutations in *WFS1*. Alimadadi et al. reported five mutations in exon 8 including three novel mutations, W588X (c.1763 G > A, a non-sense mutation), A684G (c.2051 C > G, a missense mutation), and E752K (c.2254G > A). Q486X (c.1456C > T) and E717K (c.2149 G > A) have been reported before [15]. Sobhani et al. found one novel pathogenic mutation, which causes frameshift alteration c.2177_2178insTCTTC (or c.2173_2177dupTCTTC) in exon eight [31]. They also defined three homozygous mutations including c.1885 C > T, c.2205C > A both in exon 8, and c.460 + 1G > A in intron 4 later on. The mutation c.2205C > A was found to be novel [32]. Haghighi et al. identified two homozygous mutations previously reported with apparently milder phenotypes: c.631G4A (p.Asp211Asn) in exon 5 and c.1456C4T (p.Gln486) in exon 8[33]. In this study, two mutations including R629W and G736D have been identified in the *WFS1* in patients with Wolfram syndrome and also a polymorphism V333I has been detected. R629W mutation was first reported in a French population [34]. It has been revealed that this mutation shows a significantly reduced half-time of the wolframin protein suggesting that protein instability is responsible for the low wolframin levels. Therefore, missense mutation R629W causes rapid degradation of protein [35–37]. G736D mutation was first reported as a novel mutation in Japanese people [38], and we first detected this mutation in Iranian population. These mutations are located within *WFS1* exon 8, which hosts 80 % of the mutations found in Wolfram syndrome patients. V333I polymorphism which was found in our patients has also been reported as polymorphism in previous studies [9, 39–41]. However, in some studies V333I has been reported as a pathogenic mutation [7, 42, 43]. Currently, no data is available regarding the prevalence of *WFS1* mutations or carrier frequency in Iran. However, in

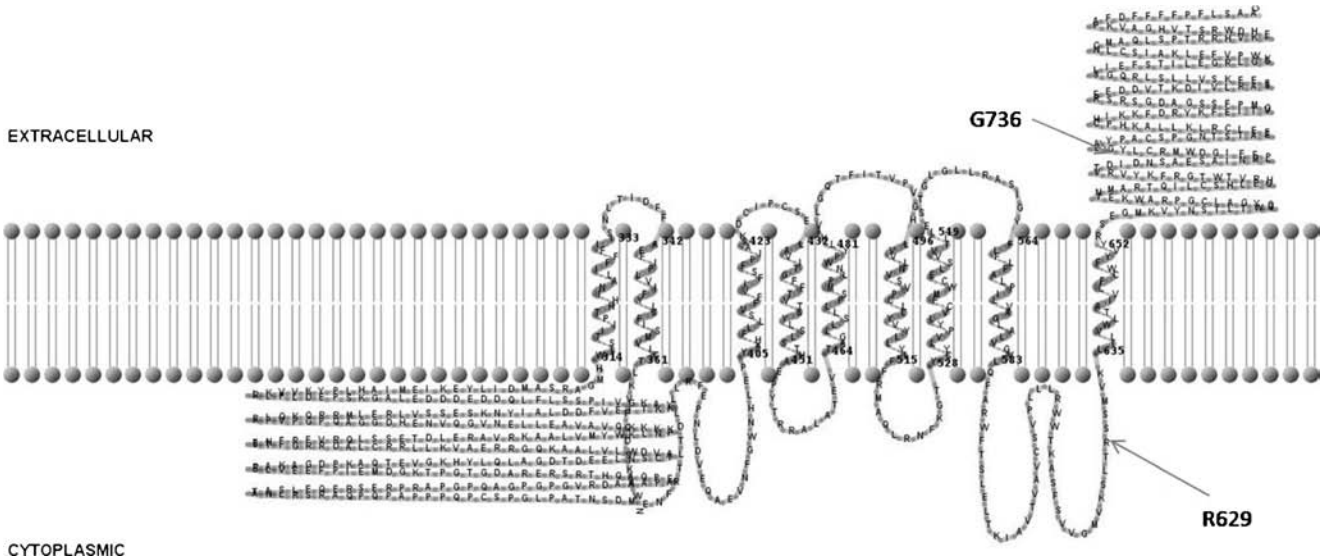

Fig. 3 Theoretical model of wolframin. The N-terminal domain is located in the cytoplasm, while the C-terminal section is in the endoplasmic reticulum. The nine predicted transmembrane helices are formed by residues 314–652. Positions of R629 and G736 are indicated by *arrows*

view of high consanguineous marriages in Iranian population, the frequency is expected to be higher than the world average. More than 200 different variations in *WFS1* have been described in patients with Wolfram syndrome which complicates the correlation between genotype and phenotype [44] although genotype prevalence differences may exist among countries. Genetic screening for *WFS1* mutations in Iranian patients for detection of common mutations has implications for genetic counseling and family planning advice for other affected families. Early diagnosis can be helpful in relieving the symptoms and preventing future complications.

Conclusion

In this study, two mutations G736D and R629W in Iranian patients with Wolfram syndrome were identified. Few studies have been done on *WFS1* mutation in Iranian population. It seems that more investigations are needed to exactly identify mutations related to Wolfram syndrome in Iran.

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ORIGINAL ARTICLE

Clinical, molecular, and therapeutic aspects of NDM in ten cases with diabetes in 1st 6 months of life

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Abstract Neonatal diabetes mellitus (NDM) is defined as hyperglycemia occurring within the first 6 months of life; it may be permanent or transient. Diagnosis of NDM is vital for prognosis, genetic counseling, and treatment. The aim of this study was to study clinical, biochemical, molecular, and therapeutic aspects of diabetes presenting in the first 6 months of life. Ten cases with NDM were studied regarding perinatal and family history, clinical features and biochemical tests on admission and follow-up, HLA typing and molecular studies, and mode of therapy. Molecular studies for the common mutations associated with NDM (*KCNJ11*, *INS*, *ABCC8* and methylation defects) were done using PCR amplification and gene sequencing. Ten cases developed diabetes in the first 6 months of life (three in the first 8 weeks, seven between 8 and 24 weeks). All presented with ketoacidosis, one had developmental delay with convulsions. Molecular studies revealed methylation defect in two cases, *KCNJ11* in three cases, *INS* mutations in two cases, and no detectable defect in three cases. Insulin was stopped on follow-up in three cases (TND), successfully substituted with sulfonylurea (SU) in three cases, and continued in four cases (PND). Molecular genetic testing is essential in cases with NDM for guiding mode of therapy as SU receptor defects can be successfully treated with oral SU instead of insulin injections. SU was more effective in achieving diabetes control in cases with *KCNJ11* mutations.

Keywords Neonatal diabetes · Molecular diagnosis · Sulfonylurea · Insulin

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Introduction

Neonatal diabetes mellitus (NDM) has been defined as insulin-sensitive hyperglycemia diagnosed within the first 6 months of life. Two main groups have been recognized, transient NDM (TND) and permanent NDM (PND), which differ in the duration of insulin dependence in the disease course [1–3]. PND is less common than TND. By definition, diabetes develops in the neonatal period and never goes into remission [4]. Anomalies at the 6q24 locus, spanning two candidate genes *PLAGL1* and *HYMAI*, are the single most common cause of neonatal diabetes and always result in TNDM [5]. The three mechanisms that cause TND in 90 % of cases are paternal uniparental disomy (UPD) of chromosome 6, duplication of 6q24 on the paternal allele, and 6q24 methylation defect. The cardinal features of 6q24-related TND are presence of severe intrauterine growth retardation, dehydration, hyperglycemia and absence of ketoacidosis. Macroglossia and umbilical hernia are often present. Infants usually require insulin. Diabetes lasts from 2 weeks to over 1 year of age; the need for insulin gradually declines. Recurrence in adolescence is more akin to type 2 diabetes mellitus. In PND, diabetes develops within days to months after birth and persists throughout life. Other genetic forms causing NDM include *KCNJ11*, *ABCC8*, *INS*, and *GCK* mutations [6]. Gloyn et al. (2004) first reported NDM to be caused by activating dominantly inherited mutations in *KCNJ11*, which encodes the Kir6.2 subunit of the ATP-

sensitive potassium (KATP) channel [7]. Activating mutations in *ABCC8* and encoding the SUR1 subunit of the KATP channel were also reported to cause both PND and TND, similar to *KCNJ11* [8]. Later studies have shown that misfolding mutations in proinsulin can also cause PND [9]. Syndromic forms of neonatal diabetes can be caused by mutations in *FOXP3*, *PTF1A*, *GLIS3*, *NEUROD1*, *RFX6*, *NEUROG3*, *EIF2AK3*, *GATA6*, *SLC19A2*, *HNF1B*, *PAX6*, and *WFS1* [10].

Genetic testing will allow diagnosis of a specific type of monogenic diabetes in over 80 % of patients whose diabetes is diagnosed before the age of 6 months [10]. All patients with NDM should have immediate molecular genetic testing to define their subtype of NDM, as type 1 diabetes (T1D) is extremely rare in this subgroup [11]. In patients diagnosed between 6 and 12 months of age, testing for NDM should be limited to those without islet antibodies as the majority of patients in this age group have T1D. The molecular genetic diagnosis of NDM will give information on which patients have a potassium channel mutation and can be treated with high-dose sulfonylureas [10].

The aim of our work was to study clinical, biochemical, molecular, and therapeutic aspects of diabetes presenting in the first 6 months of life.

Materials and methods

Ten Egyptian Caucasian patients with diabetes presenting in the 1st 6 months of life, diagnosed and managed at Diabetes, Endocrine and Metabolism Pediatric Unit, Cairo University were studied regarding perinatal and family history, clinical features, biochemical tests on admission and follow-up, HLA typing and molecular studies, and mode of therapy. Written informed consent was obtained from legal guardians of participants included in the study, and the study protocol was approved by the Ethics Committee of Cairo University and following the 1964 Helsinki declaration.

Regarding biochemical tests, 8 ml venous blood was withdrawn from the ten cases and divided into 2 ml on EDTA used for HLA DQA1 and DQB1, *KCNJ11*, *ABCC8* and *INS* genetic studies, 2 ml on EDTA used for HbA1c measurement, and 4 ml on plain tubes sera separated were used for cytomegalovirus (CMV) and Rubella IgM antibodies, C-peptide, and pancreatic antibodies. *KCNJ11*, *ABCC8*, and *INS* genetic studies were performed by DNA gene analyzer 310 as they were amplified according to the published protocol [12], followed by purification of amplified products using the QIA quick PCR Purification Kit (Qiagen, Germany). Cycle sequencing of the amplified products was performed using a BigDye terminator cycle sequencing kit, version 3.1(Applied Biosystems, USA), in both directions. Removal of excess DyeDeoxy™ terminators from reactions was performed using Centri-Sep columns (Princeton separations, USA). Sequencing analysis was done for each reaction with a 310 Genetic Analyser (Applied Biosystems, USA). All sequencing results were compared to the published reference sequence of the *Homo sapiens* genes from the NCBI (National Centre of Biotechnology Information): analysis was performed using BLAST (Basic Local Alignment Search Tool) and the CLC-BIO sequence viewer 6 program. Ch.6q24 methylation defect was determined by real-time PCR after bisulfide conversion used SYBR green based allele PCR on step one [13]. HLA DQA1 and DQB1 genotype assay was done using Luminex (Luminex Corporation, Austin). HbA1c was assayed on dimension RXL chemistry analyzer by turbidimetric inhibition immune assay technique (TINIA) (Siemens Health Care Diagnostic Inc. USA). CMV and Rubella IgM were assayed by immunochemiluminescence on LIAISON (Diasorin Saluggia Vercelli, Italy). C-peptide was assayed by enzymatic immunochemiluminescence on Immulite 2000 (Siemens Health Care Diagnostic Inc. USA). Antiglutamic acid decarboxylase antibodies (GAD65), islet cell antibodies (ICA), insulin autoantibodies (IAA), and tissue transglutaminase (TTG) IgA were assayed by ELISA using kits from DRG (DRG instruments GmbH).

Results

Our study included 10 cases with neonatal diabetes, 3 cases had TND (disappeared during follow-up) and 7 cases had PND.

Regarding perinatal and family history, results are shown in Table 1. Regarding onset of diabetes, 3 cases (30 %) presented in the 1st 8 weeks of life, while 7 cases (70 %) presented between 8 and 24 weeks of life [mean onset 0.2 ± 0.1 years]. As for the presenting symptoms at the onset of diabetes, results are shown in Table 2. Diabetic keto acidosis (DKA) was the main presentation in all cases (100 %), 6 cases (60 %) had polyuria, 6 cases (60 %) showed weight loss, 7 cases (70 %) had neurological manifestations; 1 of them had developmental delay, epilepsy in addition to neonatal diabetes described as DEND syndrome, and 1 case had associated umbilical hernia, macroglossia, and hyperlipidemia. Initial estimation of blood glucose and urinary ketones at presentation are shown in Table 2. Regarding biochemical tests, all cases with NDM had low C-peptide at the onset of diagnosis [mean C-peptide 0.4 ± 0.04 ng/ml], 8 cases were negative for pancreatic antibodies, and only 2 cases had 2 positive antibodies.

HLA typing for HLA DQA1 and DQB1 was done as well as molecular diagnosis, and results are shown in Table 3.

Molecular studies shown in Fig. 1 revealed that 2/3 cases with TND had methylation defect, 3 cases with PND had *KCNJ11* mutations, and 2 cases with PND had *INS* mutations. However, 2 cases with PND were negative for those common mutations (early onset T1D). Overexpression of pleiomorphic

Table 1 Detailed perinatal history, family history, and therapy in cases with NDM

Cases		Sex	Onset of DM	DM in the family	Gestational age	Birth weight (g)	Mode of delivery	NICU admission	Molecular diagnosis	Insulin therapy	Insulin dose	Insulin regimen	Basal insulin
Transient NDM	Case 1	M	0.04	No	Full term	2250	NVD	Yes	Methylation defect	Stopped (gradually withdrawn)			
	Case 2	M	0.34	2nd degree	Full term	4000	CS	No	Methylation defect	Stopped (gradually withdrawn)			
	Case 3	F	0.08	No	Preterm (30 weeks)	1060	CS	Yes	Normal	Stopped (gradually withdrawn)			
Permanent NDM	Case 4	F	0.17	No	Full term	1800	CS	Yes	*KCNJ11*	Switched to sulfonylurea			
	Case 5	F	0.15	No	Full term	1500	CS	Yes	*KCNJ11*	Switched to sulfonylurea			
	Case 6	M	0.18	No	Full term	3200	NVD	No	*KCNJ11*	Switched to sulfonylurea			
	Case 7	M	0.23	No	Full term	2200	CS	Yes	*INS*	Insulin	1.2 IU/kg/day	Basal and bolus	3 times/day
	Case 8	M	0.27	No	Full term	2100	CS	Yes	*INS*	Insulin	1.3 IU/kg/day	Basal and bolus	3 times/day
	Case 9	M	0.25	2nd degree	Full term	3500	NVD	No	Normal	Insulin	0.8 IU/kg/day	Basal	Twice/day
	Case 10	F	0.46	1st degree T1D	Preterm (35 weeks)	1750	CS	No	Normal	Insulin	0.8 IU/kg/day	Basal and bolus	3 times/day

DM diabetes mellitus, *NDM* neonatal diabetes mellitus, *CS* caesarian section, *NVD* normal vaginal delivery, *NICU* neonatal intensive care unit, *T1D* type 1 diabetes

adenoma gene-like 1; ZAC (*PLAGL1*) on human chromosome 6q24 due to methylation problem caused by mutations in the *ZFP57* gene was detected in two of the patients with TND. While in PND, two patients had proinsulin mutations (*G32R* and *Y108C*). Two patients had *KCNJ11* mutations due to single amino acid substitution at position 201, one had arginine-to-histidine substitution (*Arg201H*) and one had arginine-to-cysteine substitution (*Arg201Cys*) resulting in cytosine to thymine (*C>T*) and cytosine to guanine (*C>G*) DNA nucleotide change, respectively.

Regarding viral serology, 9 cases out of 10 were tested for Rubella and CMV infections; all of them were negative for Rubella IgM, and 1 case was positive for CMV IgM (suggesting congenital infection). Regarding other associated autoimmune diseases, thyroid functions (free thyroxine and thyroid stimulating hormone) were normal in all cases and only 1 case was positive for celiac antibody TTG IgA (10 %) [all had normal serum IgA], this patient was also positive for 2 pancreatic antibodies (IAA and Anti GAD).

Insulin therapy in cases was shown in Table 1. Follow-up of therapy revealed that 3 cases (30 %) stopped insulin completely (TND) as described in Table 1; 3 cases (30 %) were switched successfully to oral sulfonylurea, and 4 cases (40 %) continued on insulin therapy (3 were on basal-bolus regimen and 1 was on basal insulin only). Follow-up HbA1c was estimated every 3 months (for 1-year period). For cases with TND, all 3 cases had good control (mean HbA1c 5.9 %) after stopping insulin (Table 4). For cases on sulfonylurea, HbA1c results improved after switching to sulfonylurea (Table 5). While for cases continuing on insulin therapy, 2 cases had good control (50 %) and 2 cases (50 %) had fair glycemic control.

Discussion

Neonatal diabetes defined as uncontrolled hyperglycemia during the first 6 months of life arises from mutations in genes that play critical roles in the development of the pancreas, of β-cell apoptosis and insulin processing, as well as regulation of insulin secretion [3]. NDM is a monogenic form of diabetes, and identifying the causative gene through molecular genetic testing is very important as some cases (SUR defects) can be treated by oral drugs, without insulin, hence the value of our study.

In the present study, we reported 10 cases of NDM accounting for 2 % (10/496) of all new cases of pediatric diabetes presented during the period of the study. Their median age at diagnosis was 0.2 ± 0.1 years with 30 % diagnosed in the first 8 weeks. Similarly, Slingerland et al. (2009) studied the prevalence of neonatal diabetes (1 in 260,000 live births) in the UK, The Netherlands, and Poland and showed that the age of diagnosis was skewed to a median (IQR) of 6 (1–13) weeks, with

Table 2 Onset and presenting symptoms in cases with NDM

		No. (10)	Percent
Onset of DM	1st 2 weeks	2	20
	2–8 weeks	1	10
	8–24 weeks	7	70
Presenting symptoms	Polyuria	6	60
	Diarrhea	1	10
	Neurological manifestations	7	70
	Weight loss	6	60
	Other features (macroglossia and umbilical hernia	1	10
BG at onset (mg/dl)	<300	0	0
	300–500	3	30
	>500	7	70
DKA	Yes	10	100
	No	0	0

DM diabetes mellitus, *NDM* neonatal diabetes mellitus, *BG* blood glucose, *DKA* diabetic ketoacidosis

62 % in the first 8 weeks [14]. In addition, Tran et al. (2011), when studied the prevalence of diabetes in Vietnamese children, the incidence of neonatal diabetes was 11 % (12 cases of 108) with mean age of onset of 6 weeks (4–24 weeks) [15].

In our series with NDM, IUGR and low birth weight were common findings (7 patients; 70 %). Two patients (20 %) were preterms. Other clinical presentations included neurological findings (7 patients; 70 %), polyuria (6 patients; 60 %), loss of weight (6 patients; 60 %), diarrhea (1 patient; 10 %), and macroglossia with umbilical hernia (1 patient; 10 %). All patients presented with marked hyperglycemia and ketoacidosis. Similarly, in the 39 case series studied by Li et al. (2011), hyperglycemia and glycosuria were found in all cases with NDM, with intrauterine growth retardation in 47.5 %, polyuria in 30 %, dehydration in 52.2 %, and ketoacidosis in 47.5 % of cases [16].

In the present study, *KCNJ11* mutations were reported in three of the seven patients with PND (42 %). It has been reported that mutation in *KCNJ11* gene encoding Kir6.2 is the most common cause of NDM. Different mutations in *KCNJ11* have been reported in association with NDM [17–20]. All patients with *KCNJ11* mutations in our series were due to single amino acid substitution at position 201 denoting that this position is a hot spot for mutations.Two of them had arginine-to-histidine substitution (*Arg201H*) and one had arginine-to-cysteine substitution (*Arg201Cys*) resulting in cytosine to thymine (*C>T*) and cytosine to guanine (*C>G*) DNA nucleotide change, respectively. Similarly, previous studies have reported that the two most common hot spots for recurrent mutations in *KCNJ11* are at amino acid residues *Val59* and *Arg201* [21]. HLA typing in our 3 cases with *KCNJ11* mutation showed that despite the absence of pancreatic antibodies in all 3 cases, 2 of them had both predisposing and protective haplotypes [DQA1* 0201-DQB1* 0602/DQAl*0301-DQB1*0302 and DQA 1*0301 - DQB 1*0401] and 1 of them had only protective haplotype [DQA1*0102-DQB1*0203], so follow-up for their antibodies may show seroconversion later on. One of our patients with *KCNJ11* mutations had DEND syndrome as described in other studies in cases with NDM [22–25].

Table 3 HLA typing and molecular study in cases with NDM

Cases		HLA	HLA susceptibility		Molecular diagnosis
			Protective haplotype	Susceptible haplotype	
			Protective haplotype	Susceptible haplotype	
Transient NDM	Case 1	DQA1*0102-DQB1*0203	2	0	Methylation defect
	Case 2	DQA1*0102-DQB1*0602	4	0	Methylation defect
	Case 3	DQA1*0102-DQB1*0602	4	0	Normal
Permanent NDM	Case 4	DQA1*0201-DQB1*0602/ DQAl*0301-DQB1*0302	2	2	*KCNJ 11*
	Case 5	DQA 1*0301 - DQB 1*0401	0	2	*KCNJ 11*
	Case 6	DQA1*0102-DQB1*0203	2	0	*KCNJ 11*
	Case 7	DQA 1*0301 -DQB 1*0401	0	2	*INS*
	Case 8	DQA1*0102-DQB1*0602	4	0	*INS*
	Case 9	DQA1* 0201-DQB1* 0602/ DQAl*0301-DQB1*0302	2	2	Normal
	Case 10	DQAl*0501-DQBl*0201/ DQAl*0301-DQB1*0302	0	4	Normal

HLA human leucocyte antigen, *NDM* neonatal diabetes mellitus

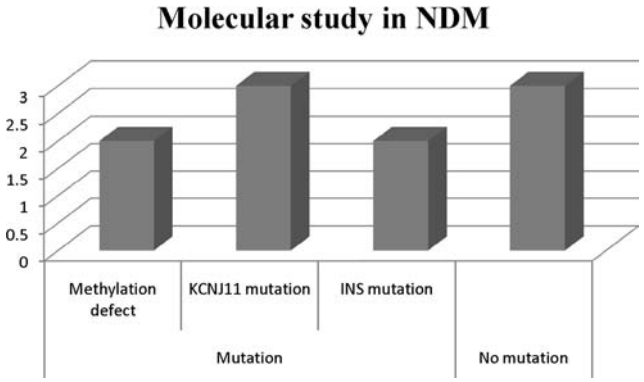

Fig. 1 Molecular study for cases with NDM

In the present study, two of the patients with PND were reported to have insulin gene defect representing 2/7 (28.6 %) of this group. The presentation of these *2* patients was relatively later than other forms of monogenic diabetes reported (at 12 and 14 weeks). Molecular testing revealed that both patients have the proinsulin mutations (*G32R* and *Y108C*). These mutations were previously reported and apparently are hotspots for insulin gene mutations [26]. HLA typing revealed protective haplotype [DQA1*0102-DQB1*0602] in 1 case and a predisposing haplotype [DQA 1*0301 – DQB1 *0401] in the other case, though both cases were negative for pancreatic antibodies. In the Italian cohort with PND, mutations of the *INS* gene were the second leading cause (9 of 37, or 24.3 %) following activating mutations of *KCNJ11* (19 of 37, or 51.3 %) [27]. Whereas in French cohort, the *INS* mutations represent approximately 10 % of all PND cases with later age at onset of diabetes [28].

So regarding molecular diagnosis in our PND cases (7), mutations were detected in 5 of them (*KCNJ11* in 3 cases and *INS* in 2 cases). Similarly, Jahnavi et al. (2013) detected 8 mutations in a total of 22 PND cases, 4 *ABCC8* (50 %) with one novel mutation (Asp212Tyr), and two novel intronic variants (IVS22 + 71C>A and IVS28 + 46A>C), 3 *KCNJ11* (37.5 %), and 1 *INS* (12.5 %) [20].

In our study, TND was reported in 3 patients. As previously noticed by other studies, the diagnosis of our patients with TND was relatively earlier than PND (2–16 weeks vs 8–22 weeks). Their birth weight was variable, low in two cases (1060 and 2250 g) and high in one case (4050 g). As with PND, they presented with DKA necessitating insulin infusion. Neurological manifestations in the form of convulsions and disturbed consciousness were observed in 2 patients. Coarse facial features with macroglossia and large umbilical hernia was present in one patient. Molecular genetic testing in those patients revealed mutations in the *ZFP57* gene in two of them. HLA typing showed that all of them had protective haplotypes explaining the absence of pancreatic antibodies in all 3 cases. Similarly, clinical data for 50 patients who had a history of NDM in a study done by Metz et al. (2002) showed that 29 patients had TND, and 21 patients had PND. Median age of infants at diagnosis of diabetes mellitus was significantly ($P < 0.01$) younger in the TND group (6 days [range, 1–81]) than the PND group (27 days [range, 1–127]). Birth defects were reported for 2 patients; one patient had isolated macroglossia, and another had a congenital heart defect [4]. Temple et al. (2010) reported that TND is usually diagnosed in the first week of life due to genetic or epigenetic aberrations at an imprinted locus on chromosome 6q24 and can be sporadic or inherited. Affected infants are typically born with lower birth weight (mean 2000 g) than those with PND but require less insulin, and doses can be tapered so that they are no longer insulin-treated by a median of 12 weeks [29]. In a cohort of 30 patients with TND reported by Temple et al. (2000), 23 were sporadic cases and 7 were familial. One of the three mechanisms was identified as the cause of TND in 24/30 cases studied (80 %). Of the sporadic cases 11/23 (»50 %) had UPD, 4/23 (»20 %) had a paternal duplication of 6q24, and 2/24 had a methylation defect (8 %) [30].

Two of our patients with neonatal diabetes (20 %) were categorized as having very early onset T1D as suggested by their HLA predisposing alleles [1 had DQA1* 0201-DQB1* 0602/DQAl*0301-DQB1*0302 haplotype with 2 predisposing alleles and the other had DQAl*0501-DQBl*0201/ DQAl*0301-DQB1*0302 haplotype with 4 predisposing alleles], positive pancreatic antibodies (ICA and GAD), and low C-peptide. Moreover, both patients had positive family history of diabetes and they were negative for the most common mutations (*KCNJ11*, *ABCC8*, *INS*, and methylation defect).

Table 4 Transient neonatal diabetes cases who stopped insulin therapy

	Age at onset of diabetes	Period of insulin therapy (days)	Period without insulin therapy (months)	Last HbA1c (%)	Molecular diagnosis
Case 1	0.04 (2 weeks)	45	14	5.4	Methylation defect (TND)
Case 2	0.08 (4 weeks)	60	18	5.7	Methylation defect (TND)
Case 3	0.34 (16 weeks)	30	11	5.5	Normal

Table 5 HbA1c values in cases with *KCNJ11* mutation

	Initial HbA1c (%)	Mean HbA1c		Glycemic control
		On insulin (%)	On sulfonylurea (%)	
Case 4	9.2	8.8	7.8	Good control (<8 %)
Case 5	10.3	8.4	7.3	Good control (<8 %)
Case 6	10	10.8	7.6	Good control (<8 %)

Interestingly, one of these patients was also positive for celiac antibodies which enforce the autoimmune etiology in this patient.

Regarding therapy in our cases, patients with TND (3) started insulin therapy in hospital then were discharged on a very low insulin dose (0.02, 0.1, and 0.8 IU/kg/day). Six weeks later, the patients stopped insulin therapy completely. Follow-up showed normal HbA1c with no need for insulin therapy. All 3 patients with *KCNJ11* mutations were switched from insulin to oral sulfonylurea therapy over a period of 3–4 weeks, until insulin was totally withdrawn and the patients were maintained on oral sulfonylurea with doses ranging between 0.5 and 0.8 mg/kg/day. Follow-up of these patients showed improved glycemic control. Three months later, on re-measuring C-peptide in 2 of them, it was completely normalized. Our results were similar to Stanik et al. (2007) who studied eight patients with PND in Slovakia; 3 of them had *KCNJ11* mutations (*R201H* and *H46Y*) and were switched from insulin to sulfonylurea, decreasing their glycosylated hemoglobin from 9.3–11.0 % on insulin to 5.7–6.6 % on sulfonylurea [31]. Successful switch to sulfonylurea in cases with *KCNJ11* mutations was elaborated in many studies [20, 32–38]. Four patients in our series continued on insulin therapy (*INS* mutations were identified in 2 of them; no mutations were identified in the other 2). Insulin dose ranged 0.8–1.3 IU/kg/day. Three of them used basal-bolus regimen while one of them received only basal insulin. NPH was the basal insulin used as insulin glargine was not approved in this age group despite being used in cases with NDM [39, 40], and insulin aspart was the bolus insulin used. NPH tend to have a shorter duration of action in infants, possibly because of smaller dose size or higher subcutaneous blood flow [5]. This explains the fact that 3 doses of NPH gave better glycemic control than twice daily regimen in this age group (2 cases had fair control and 2 had good control).

Thus, genetic diagnosis in NDM affected the therapeutic modality.

Conclusion

Infants presenting with diabetes in the first 6 months of life are mostly monogenic but T1D is also encountered. Neonatal diabetes can be transient or permanent. Molecular genetic testing is essential in all cases with NDM for guiding mode of therapy. NDM due to potassium channel mutations (*KCNJ11* and *ABCC8*) can be successfully treated with oral sulfonylurea instead of insulin. However, NDM due to *INS* mutations or methylation defects still require insulin therapy. Basal insulin was more safe in achieving glycemic control in this age group.

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ORIGINAL ARTICLE

Impact of sub-gastrectomy on glucose regulation in gastric cancer patients with T2DM: a follow-up study

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Abstract This study aims to investigate the changing characteristics of glucose regulation after gastric surgery for normal gastric cancer patients with type 2 diabetes mellitus (T2DM) (T2DM group) and without T2DM (normal group) in a tertiary hospital located in western China. We conducted a case-control study which retrospectively collected the data of 73 patients: (1) diagnosed as gastric cancer, (2) underwent sub-gastrectomy and Billroth II gastrointestinal anastomosis between October 2007 and December 2012, and (3) did not suffer a recurrent cancer and with sufficient clinical data. Fasting blood glucose (FBG), glycated hemoglobin (HbA1c), insulin sensitive index (HOMA-IS), insulin resistance index (HOMA-IR), and beta cell function index (HOMA-BCF) were compared at baseline and postoperative 7, 14, 28, 56, 120, and 356 days. Baseline FBG, HbA1c, HOMA-IS, HOMA-IR, and HOMA-BCF in the T2DM group were significantly higher than those in the normal group. Immediate postoperative glucose changed significantly, and it became stable and normal during follow-up period in both the T2DM and normal group that there was no significance anymore after postoperative 28 days between them. Normal patients with T2DM would benefit from gastric surgery in glucose control, and it may involve not only decreased food intake and weight loss but also gastrointestinal reconstruction.

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Keywords Gastric surgery · Sub-gastrectomy · Impaired glucose regulation · T2DM

Introduction

More than 221 million people around the world are suffering from type 2 diabetes mellitus (T2DM) [1], which has a really high threat to public health. The prevalence of diabetes in China is about 4.6 % of the whole population in western region and 8 % in eastern region, and the prevalence of diabetes was sharply increasing during past decades [2, 3]. Also, diabetes conveys a higher risk of gastric cancer mortality [4]. The continued medical care including moderate diet, intensified exercise, oral drugs, and insulin injection could hardly cure T2DM, especially obesity-related T2DM [5, 6].

Surgery has been applied regularly in obese patients with a body mass index (BMI) >40 kg/m^2 or >35 kg/m^2 when serious co-morbidities are associated with them [7]. It was reported that 85.4 % of 485 patients undergoing bariatric surgery experienced resolution or improvement in T2DM, and the follow-up results also revealed a significant weight loss and an effective controlment of the co-morbidities such as high blood pressure, insulin resistance, and dyslipidemia [8]. Meanwhile, a cohort study found that the patients who adopted bariatric surgery achieved a significant reduction of 40 % in the overall mortality, 56 % in specific mortality due to coronary heart disease [9]. Besides bariatric surgery, gastric surgery including sub-gastrectomy and Billroth II reconstruction was also demonstrated to have positive effects on T2DM improvement [10–12]. Due to unclear mechanisms responsible for the improvement in DM, the clinical outcome could not be clearly explained. It was certain that decreased food intake because of gastric restriction or intestinal malabsorption played an important role [13]. Meanwhile, some studies

mentioned that postoperative weight loss may be another important factor as it can to some extent reduce insulin resistance; however, T2DM resolution achieved by surgery happens mostly in 2–3 weeks when a high proportion of cases still did not acquire a significant weight loss yet.

Recently, more and more evidence revealed that for patients with normal or lower BMI, who would not acquire a significant weight loss compared with obese patients, surgery also achieved a significant improvement in T2DM remission. It was supposed that weight loss may not be important as considered for both normal and low-weight patients. As glucose regulation which mainly includes insulin release and sensitive was systematically impaired in T2DM, in order to investigate the changing characteristic of glucose regulation after gastric surgery, we performed this retrospective study to compare the relevant index before and after operation in T2DM patients with normal BMI patients.

Methods

Patients

We retrospectively included patients with normal BMI who had curative intent for primary gastric cancers (TNM stage, $T_{1\text{-}2}N_{1\text{-}2}M_0$) in our hospital between October 2007 and December 2012 and finally underwent sub-gastrectomy and Billroth II gastrointestinal anastomosis, and the postoperative survival time was more than 1 year in the study. Patients with (1) severe plasma insulin deficiency, (2) autoimmune antibody positive, (3) unresectable stomach carcinoma, (4) age >70 years, and (5) heart, lung, or kidney failure were excluded. Also, patient with insufficient follow-up data and/or a recurrence were also excluded. A total of 79 cases underwent gastric surgery, in which 73 patients were included and 6 of them were excluded (4 of them was lost to follow-up, and 2 of them suffered a recurrent gastric cancer). A fasting blood glucose (FBG) reduction of at least 1.5 mmol/L with a maximum standard deviation of 3 mmol/L when α=0.05 and β=0.8 is performed. According to the T2DM history and check result in admission, there were 33 patients in the T2DM group and 40 patients in the normal group. The study was approved by Lanzhou University Second Hospital ethics committee. All patients provided written informed consort of potential risks before operation and made verbal agreement of collecting their information for scientific study.

Surgery procedures

Gastric surgery was mainly recommended by a doctor according to pathology and imaging data and determined by the patients. Four experienced surgeons participated in this study, and each of them had performed more than 50 cases of sub-gastrectomy and Billroth II reconstruction. In all cases, distal subtotal gastric resection is indicated to the tumor located in the lower one third of the stomach. The standard lymphadenectomy (LAD) is the D2-LAD without distal pancreatectomy or splenectomy. The extended organ resection is only indicated in cases where R0 resection is possible. In China, the main goal of surgical intervention is the complete removal of the tumor. An indication for performing subtotal gastrectomy is based on tumor location, histological type, and trial for achieving surgical tumor-free margins.

The reconstruction of the digestive tract is performed by adopting Billroth II reconstruction after subtotal gastrectomy. For Billroth II reconstruction, 8–10 cm midline incision is made 2–3 cm below the xiphoid process, and wound is protected with wound protector. Then, the stomach is resected with adequate margins proximal to the tumor using tube-type staplers. An end-to-side anatomist is undertaken after the small bowel, and 15–20 cm distal to the Treitz ligament has been pulled up antecolically. The anastomosis is performed by hand sewing or by using staplers. Stapler inlet site is hand-sewn or closed with double clipping of staplers.

Data collection

Basic information of the patients including age, sex, BMI, pre- and postoperative treatment approaches to diabetes, preoperative fasting plasma glucose, glycated hemoglobin, and fasting plasma insulin were recorded. During the time of operation completion to discharge, fasting plasma glucose and fasting plasma insulin were measured twice. Sensitivity index of insulin (SEN) was calculated as: SEN = -In(FBG×FINS) [14]. Homeostasis model assessment-insulin resistance (HOMA-IR) index and homeostasis model assessment-derived beta cell function (HOMA-B) index [15] were calculated as follows: HOMA-IR = FIRI ×FBG/22.5,HOMA-B = 20 ×FIRI/(FBG - 3.5), where FIRI represents fasting plasma insulin level (μU/mL) and FBG represents fasting blood glucose level (mmol/L).

Follow-up

A follow-up at postoperative 28, 56, 120, and 356 days were then performed, and the data were collected according to the most recent blood check results through outpatient service. To ensure the comparability between the T2DM and control group, radiographical check was also conducted at postoperative 3, 6, and 12 months.

Statistical analysis

Data were collected and represented as mean±standard deviation for continuous variables and number of patients (percentage) for dichotomous variables. Levene's test was

adopted for equality of variances, and then, the difference of continuous variables between groups was analyzed by Student *t* test. Also, the difference of dichotomous variables between groups was analyzed by χ^2 test or Fisher exact test. Both are considered with statistical significance when $p<0.05$. For statistical analysis, the SPSS for Windows 17.0 (SPSS, Chicago, IL, USA) was used.

Results

A total of 73 gastric cancer patients were analyzed in the study, 33 cases in the T2DM group and 40 cases in the normal group. There were 41 males and 32 females, and the average age was 65.48 years (range, 45–69). There were no significant differences between the two groups in terms of age, sex, BMI, TNM stage, weight loss, and fast plasma insulin level (FPI). In the T2DM group, the level of FBG, the level of glycated hemoglobin (HbA1c), and homeostasis model assessment-insulin resistance (HOMA-IR) were significantly higher than those in the normal group ($p<0.01$), while homeostasis model assessment-insulin sensitivity (HOMA-IS) and homeostasis model assessment-derived beta cell function (HOMA-BCF) were significantly lower than those in the normal group. All the patients in the T2DM group have diabetes with a medical history ranged from 3 months to 13 years and received medical treatment including oral drugs and/or insulin injection and after operation had a response rate of 76.5 % (26/33) at postoperative 14 days and 93.9 % (31/33) at postoperative 28 days, as presented in Table 1.

Table 1 Baseline characteristic of participants

	T2DM (n=33)	Normal (n=40)	p value
Age (y)	65.18±12.17	65.72±12.01	0.85
Sex (M/F)	19/14	22/18	0.83
BMI	23.02±1.76	21.21±1.34	<0.01
TNM stage			
I a	6	8	0.84
I b	3	4	0.89
II a	14	16	0.83
II b	10	12	0.98
FPG (mmol/L)	9.52±2.30	6.63±0.85	<0.01
FPI (μU/ml)	3.16±0.31	3.23±0.15	0.21
HbA1c (%)	7.36±1.11	5.75±0.45	<0.01
Home-IS	0.30±0.01	0.33±0.01	<0.01
Home-IR	1.31±0.22	0.95±0.10	<0.01
Home-BCF	12.03±4.47	23.17±10.28	<0.01
Remission rate (n, %)			
Postoperative 14 days	26 (76.5 %)	N/A	N/A
Postoperative 28 days	31 (93.9 %)	N/A	N/A
Weight loss (kg)	9.28±1.12	9.86±1.34	0.26

T2DM type 2 diabetes mellitus, *FPG* fasting plasma glucose, *FPI* fasting plasma insulin, *HbA1c* glycated hemoglobin, *Home-IS* homeostasis model assessment-insulin sensitivity, *Home-IR* homeostasis model assessment-insulin resistance, *Home-BCF* homeostasis model assessment-derived beta cell function

Fasting blood glucose

The FBG level in T2DM was significantly higher than that in the normal group at baseline (9.74±2.13 vs. 6.63±0.85, t=7.38, $p<0.01$), while this trend changed until postoperative 28 days (6.05±0.96 vs. 5.41±0.68, t=−0.46, p=0.65). From the 4th week to the 12th month postoperatively, there was no significant difference between the groups (Fig. 1).

Glycated hemoglobin

The HbA1c level in the T2DM group was higher than that in the normal group at baseline (7.36±1.11 vs. 5.75±0.45, t=7.16, $p<0.01$). It decreased after operation, and 28 days later, the level tended to be stable with non-significant difference between the groups (5.53±1.32 vs. 5.12±1.03, t=1.49, p=0.14) (Fig. 2).

Homeostasis model assessment-insulin resistance

The Home-IR was higher in the T2DM group than that in the normal group at baseline (1.31±0.22 vs. 0.95±0.10, t=8.73, $p<0.01$), and it gradually decreased until postoperative 28 days when the difference between the groups was not statistically significant (0.78±0.11 vs. 0.73±0.10, t=1.93, p=0.06) (Fig. 3).

Homeostasis model assessment-insulin sensitivity

At baseline, the Home-IS in the T2DM group was lower (0.30±0.01 vs. 0.36±0.02, t=−10.73, $p<0.01$). After surgery, it

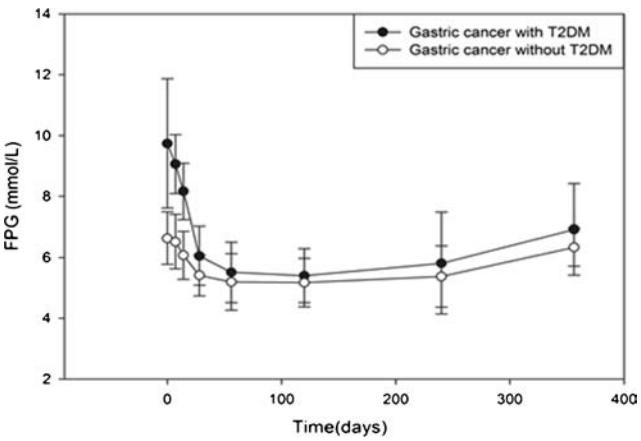

Fig. 1 Comparison of fasting plasma glucose from baseline to postoperation in the two groups (mean and standard deviation)

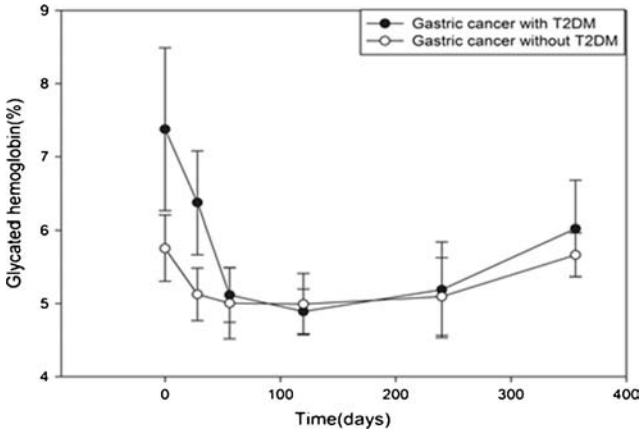

Fig. 2 Comparison of glycated hemoglobin from baseline to postoperation in the two groups (mean and standard deviation)

was not significantly influenced, while it persistently increased in the normal group. From postoperative 28 days, there was no significant difference between the groups (0.35 ±0.02 vs. 0.36±0.02, t=−1.84, p=0.07) (Fig. 4).

Homeostasis model assessment-derived beta cell function

The Home-BCF was obviously changed in both groups, and no stable or routine trends were found. At baseline, it was lower in the T2DM group than that in the normal group (12.03±4.47 vs. 23.17±10.48, t=−6.09, p<0.01); then it increased and there was no significant difference between the groups at postoperative 28 days (42.02±19.29 vs. 37.23± 18.49, t=1.08, p=0.28) (Fig. 5).

Complications

Gastric surgery was a safe procedure for gastric cancer patients, and there was no significant difference between the groups in aspects of complication incidence. There were four patients in the T2DM group and three patients in the normal

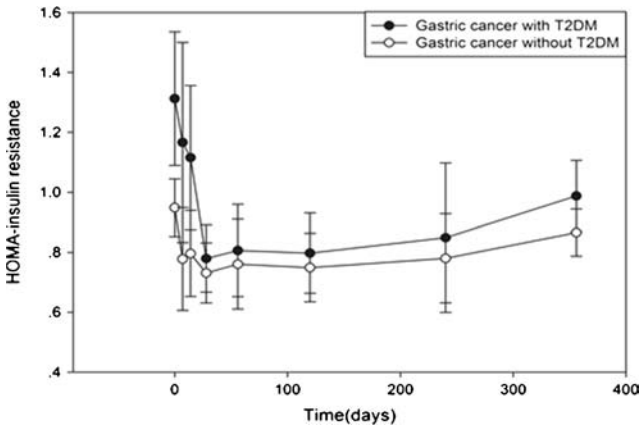

Fig. 3 Comparison of homeostasis model assessment-insulin resistance from baseline to postoperation in the two groups (mean and standard deviation)

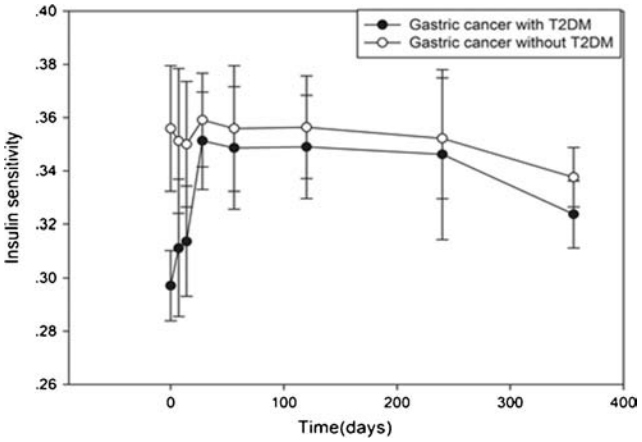

Fig. 4 Comparison of homeostasis model assessment-insulin sensitivity from baseline to postoperation in the two groups (mean and standard deviation)

group who had delayed healing (3/34 vs. 4/40, p=0.81), and five patients in the T2DM group and six patients in the normal group had postoperative wound bleeding (5/34 vs. 6/40, p= 0.97). Besides, long-term radiographical follow-up result showed that there was no evidence and incidence of recurrence and metastasis in primary gastric cancer.

Discussion

There is a strong relationship between obesity and T2DM, and approximately half of those patients diagnosed with T2DM are obese [3]. Surgical procedures were demonstrated to be able to provide durable weight loss in obese people effectively [16]. Also, it was reported that reducing body weight through operations involving intestinal diversions improved glucose homeostasis in obesity-related T2DM. Meanwhile, few studies are currently available on how certain surgery influences the glucose regulation in non-obese T2DM patients.

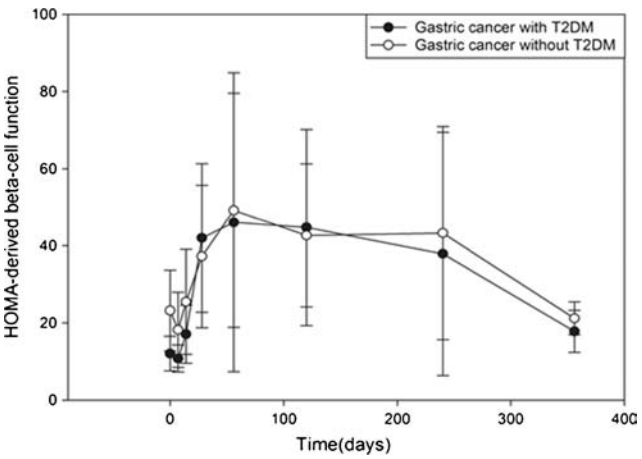

Fig. 5 Comparison of homeostasis model assessment-derived beta cell function from baseline to postoperation in the two groups (mean and standard deviation)

Our hospital is located in a western province of China, which is one of the highest incidence areas of gastric cancer. Due to the specific area and the disease, all the patients in this study were not obesity-related T2DM, with normal BMI 18.5 to 24.99 kg/m^2. Except for FPI, FPG, HbA1c, home-IS, home-IR, and home-BCF were all significantly differentiated between T2DM group and normal group at baseline. After surgery, all the indexes began to change and eventually achieved responses that there was no significant difference in T2DM compared with normal patients at postoperative 28 days. And all the patients in the T2DM group took insulin and oral drugs before surgery, while only 6.1 % of them need continued medical treatment at postoperative 28 days to maintain an adaptable glucose level. Therefore, the results suggested that glucose status was significantly improved in non-obesity-related T2DM group after gastric surgery.

Some studies found that T2DM was typically resolved within a few days to weeks following malabsorptive procedures before significant weight loss was achieved [17]. Compared with obese patients, current patients in this study with normal BMI lost less weight in the follow-up period (approximately 1/5 vs. 1/3) [18, 19]. Although patients in the T2DM group achieved significant response and diabetes remission in the follow-up periods, the study showed that no significant difference existed between the groups in terms of weight loss. Thus, surgery-induced weight loss may also to some extent influence glucose, but does not play important roles in controlling the glucose level in normal-BMI patients who underwent gastric surgery.

Nowadays, bariatric surgery was performed in many different procedures, which mainly included conventional surgical procedures (e.g., roux-en-y gastric bypass, laparoscopic adjustable gastric band, sleeve gastrectomy, and biliopancreatic diversion) and novel surgical procedures (duodenal-jejunal bypass and ileal interposition) [20]. In this study, because the patients were also diagnosed as gastric cancer, we failed to performed bariatric surgery in any of them. As the procedure of Billroth II surgery was similar to biliopancreatic diversion, compared with Mingrone et al. [21], patients undergoing biliopancreatic diversion achieved better, fast plasma glucose and glycated hemoglobin control at postoperative 1 month. Despite that the food did not pass through the duodenum anymore in both studies, compared with biliopancreatic diversion, Billroth II surgery had different lengths of proximal bowel loop and distal bowel loop and different cavities of remnant stomach. So, it was indicated that the mechanism of different surgical procedures may be the same that both remnant stomach and bowel loop contributed to the bariatric remission. Restrictive and malabsorptive procedures involving rerouting of food and vacant intestine might improve T2DM by enhancing insulin sensitivity and/or by improving beta cell function [22, 23].

And obviously, it was different from operation completion to postoperative 28 days of the changing trend of relevant index in the T2DM group and normal group. Except for diabetes diagnosis, the two groups had completely the same interventions. Gastric surgery as one of the most invasive gastric procedure put an enormous burden on physical condition. And it led to wasting condition, and glucose metabolism was changed and complicated. With limitation of food intake, there were many kinds of potential factors to influence glucose regulation and relevant indexes. When discharged from the hospital, patients began to adopt usual activities, diets, and life, and only gastrointestinal reconstruction was still existed. Compared with the normal group, patients in T2DM group had a similar trend but showed a slower speed and lower ability to go back to the baseline and relative high glucose level. Thus, it may be supposed that weakened absorption function as well as improved insulin sensitivity [24, 25]. In our study, home model index was improved much more quickly than the level of plasma glucose and glycated hemoglobin.

Otherwise, in this study, no mortality was observed. Major and minor surgery-related complication rate was also low. For the treatment of T2DM, prevention of diabetes-related complications such as macro- and microvascular diseases are as important as glycemic control. To prevent these diabetes-related complications, blood pressure needs to be controlled below 130/80 mmHg, cholesterol level below 200 mg/dL, and HbA1C level below 7 % [26, 27]. Unfortunately, only 7.3 % of adults with diabetes achieved all three recommended goals with conventional medical treatment [28, 29]. In contrast, most of the patients could control cholesterol levels below 200 mg/dL and HbA1C levels below 7 %.

Conclusions

Immediate postoperative glucose changed significantly, and it became stable and normal during follow-up period in both the T2DM and normal groups. Patients with non-obese T2DM benefited from gastric surgery in glucose controlment may involve decreased food intake, weight loss, and gastrointestinal reconstruction.

Compliance with ethical standards

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

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

Authors' contributions Zhang YC, Wang HL, Zhang YW, and Xu XD were involved in the study conception and design. Wei FX, Han W, and Zhang HH participated in the acquisition of data. Wei ZG, Zhang YW, Wang MC, and Xu XD were involved in the analysis and interpretation of data. Wei FX and Han W participated in the drafting of manuscript. Zhang YC and Wang HL were involved in the critical revision of manuscript.

Conflict of interest You Cheng Zhang, Feng Xian Wei, Wei Han, Hui Lin Wang, Man Cai Wang, Ya Wu Zhang, Xiao Dong Xu, and Hui Han Zhang declare that they have no conflict of interest.

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HSD11B1 gene polymorphisms in type 2 diabetes and metabolic syndrome—Do we have evidence for the association?

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Abstract Diabetes mellitus (DM) is a major health problem, and its prevalence has been rapidly increasing in the last century. Being polygenic in nature, multiple genes are involved in developing type 2 diabetes (T2DM). Genes involved in the cortisol pathway interact to develop metabolic syndrome (metS) and T2DM, and these conditions resemble Cushing's syndrome caused by excess cortisol activity in the visceral adipose tissue. Overexpression of 11β-hydroxysteroid dehydrogenase type 1 enzyme (11β-HSD1), engaged in the interconversion of inert cortisone and active cortisol in metabolically active tissues, is associated with insulin resistance (IR), T2DM, hypertension, and metS. Studies have reported the role of single-nucleotide polymorphisms (SNPs) of the HSD11B1 gene in the susceptibility to metS and T2DM. This review offers an overview of the contribution of common HSD11B1 single-nucleotide variants in the development of T2DM and metS. We conducted a literature survey in PubMed, Medline, Google, and Embrase databases with the belief that they may provide a starting point for further dialog or need to conduct further studies in this area. From this review study, the frequently studied SNPs rs12086634 and rs846910 were found to be associated with T2DM and rs12086634, rs1000283, and rs846910 associates with metS. The SNPs rs12086634 and rs846910 show conflicting association with T2DM and metS in various ethnic populations. Further studies with adequate sample size may be needed to confirm the association of HSD11B1 gene polymorphisms in different populations.

Keywords Type 2 diabetes · Metabolic syndrome · 11 beta hydroxysteroid dehydrogenase · Single-nucleotide polymorphism

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Introduction

Diabetes has emerged to be a major chronic disease prevailing in 347 million populations worldwide (www.who.int). The WHO projects that diabetes deaths will double between 2005 and 2030. Type 2 diabetes (T2DM), being the majority of diabetes (~90 %) is a complex polygenic disorder. Scientists have identified several single-nucleotide polymorphisms (SNPs) in certain genes that have been linked to a higher diabetes risk. To date, more than 50 genes have been studied for their T2DM susceptibility in various populations by the application of the most advanced (GWAS) genome-wide association studies [1]. Results have been conflicting in different populations, and reasons for these contrasting findings include inadequate sample sizes, variation in disease susceptibility across ethnic groups, role of environmental causes, and their influence on genes.

Metabolic syndrome (metS) is a prediabetic condition. An average of 35 % of the world's adult population has been estimated to have metS (www.idf.org). Abdominal obesity, physical inactivity, genetic factors, aging, and endocrine

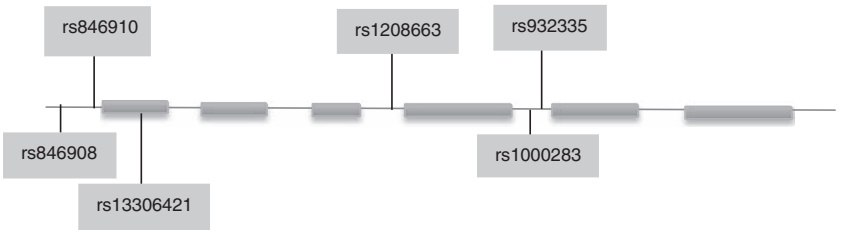

Fig. 1 Diagrammatic representation of HSD11B1 gene depicting exons(▬), introns(—), and location of common SNPs

factors such as insulin resistance and visceral cortisol excess are considered as major causes for metS.

T2DM and metS patients with Cushingoid features have been a target to explore gene polymorphisms in enzymes responsible for cortisol metabolism as the possible target for genetic mutations [2]. The distinct phenotypical resemblance of T2DM and metS with Cushing's syndrome is caused due to excess cortisol activity in the visceral adipose tissue [3–12] and these two conditions are thus linked to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). The 11β-HSD1 enzyme converts inert cortisone to active cortisol in metabolically active tissues for local glucocorticoid action [13]. Increased expression of 11β-HSD1 in mouse is found to be associated with insulin resistance (IR), increased glucocorticoids in adipose tissue, central obesity, and diabetes [14]. The association of overexpression of 11β-HSD1 with increased cortisol concentrations in adipose tissue may prove the possible relation of HSD11B1 gene polymorphisms with metS [15–18]. Polymorphisms in the HSD11B1 gene encoding this enzyme influences glucose and lipid levels to develop metS and T2DM [19]. This review offers an overview of the contribution of common HSD11B1 single-nucleotide variants to the development of T2DM and metS. Future studies should concentrate on understanding the exact role played by each of these risk variants in the development of T2DM. Here, we present studies of SNPs of HSD11B1 in diabetes and metabolic syndrome and its frequency in cases and controls.

11 beta hydroxysteroid dehydrogenase type 1 gene (HSD11B1)

The HSD11B1 gene is located on chromosome 1q32-q41. The gene has six exons and five introns with 4194 SNPs across. Figure 1 is the diagrammatic representation of the HSD11B1 gene depicting common SNPs whose association studies have been done in type 2 diabetes and metabolic syndrome. rs12086634, an intronic variant acts as an intronic enhancer [19]. rs932335 and rs1000283 are also intronic variants. rs846910 and rs701950, located in the promoter region of the HSD11B1 gene, are 5' UTR variants and is known to affect transcription [19]. The gene encodes the 11β-HSD1 enzyme that catalyzes conversion of cortisone to cortisol in mature adipocytes and hepatocytes for local glucocorticoid action [13]. Another isozyme 11β-HSD2, (encoded by HSD11B2 gene) oxidizes cortisol to cortisone and prevents illicit activation of the mineralocorticoid receptor in aldosterone-selective tissues [20].

Methods

All the studies in English literature were accessed using PubMed, Google, Embrase, and Medline databases using the search words -11Beta hydroxysteroid dehydrogenase type 1, HSD11B1 gene polymorphism, diabetes, blood glucose, cholesterol, and metabolic syndrome. Observational studies of the HSD11B1 gene polymorphism in type 2 diabetes and metabolic syndrome adults irrespective of the sample size, with or without controls were included in the study and strengths and weaknesses of the study were identified. All articles published on the topic "HSD11B1 gene polymorphisms in type 2 diabetes and metabolic syndrome" for the period between years 2004–2015 were included. Exclusion criteria were as follows: HSD11B1 gene polymorphism studied in subjects with Alzeimers disease, Cushing's disease, type 1 diabetes, osteoporosis, studies in children, and animal studies.

HSD11B1 and type 2 diabetes

Polymorphisms in the HSD11B1 gene-encoding 11β-HSD1 enzyme alter glucose and lipid metabolism due to alteration in glucocorticoid levels ultimately developing IR and T2DM. Eight studies on the association of HSD11B1 gene polymorphisms with T2DM were done. Among these, only one study showed positive association [19], and seven studies showed no association with T2DM [21–27]. Expression study by Uckaya et al. showed the increased 11β-HSD1 mRNA levels in visceral and subcutaneous adipose tissue of T2DM than control subjects [28]. An association study of the HSD11B2 gene polymorphism showed weak association with type 1 diabetes [29]. Five association studies of SNP rs12086634 with T2DM were done [19, 23, 25–27]. rs12086634 positively were associated with T2DM in Pima Indians but showed no association with T2DM in Koreans, Eurobrazilians, American Indians, and Southern European Caucasians of northern Italy. Table 1 mentions the common SNPs studied in association

Table 1 Association of HSD11B1 single-nucleotide polymorphisms with type 2 diabetes

Participants, authors, references	SNPs	*P* value	Association (OR/CI)	Strengths	Limitations
Pima Indians	Recessive model		Positive association	-Adequate sample size	All SNPs of HSD11B1 gene are not studied
	rs12086634	0.01	1.72/(1.14–2.56)	-Robust methodology	
rs12086634 (*n*=706)	rs846910	0.01	1.79/(1.14–2.8)	-Six SNPs studied	
rs846910 (*n*=839)	Additive model	0.01	1.64/(1.11–2.38)		
Nair et al. [19]	rs12086634	0.02	1.34/(1.05–1.72)		
	rs846910				
American Indians	rs846910	0.24	No association	-Adequate sample size	-All SNPs of HSD11B1 gene is not studied
T2DM (*n*=512)	rs12086634	0.42	No association	-Association of SNPs with hypertension is studied	
Non diabetic control (*n*=406)					-Insufficient data to test in utero factors that could modify expression of the HSD11B1 gene
Franks et al. [26]				-Robust methodology	
					-Confounding by population stratification
French Canadians	g.4478TT (*n*=138)	>0.05	No association	-Robust methodology	-No control subjects used
metS males	g.4478TG (*n*=78)	>0.05		-Two SNPs studied	-All the functional polymorphisms in HSD11B1 gene not studied
(*n*=217)	g.10733GG (*n*=128)				
Robitaille et al. [21]	g.10733GC (*n*=88)				-SNPs studied are different from those studied by Nair et al.
					-Female subjects are not studied
Koreans	rs846908	>0.05	No association	-Adequate sample size	-Only three SNPs of HSD11B1 gene studied
T2DM (n=757)	rs932335			-Polymorphism in the promoter region is studied.	
Non diabetic control (n=644)	rs13306422				-SNPs studied are different from those studied by Nair et al.
Ku YH et al. [22]					-other possible functional SNPs are not studied
North Italians	rs13306421	>0.05	No association	-Studied the effect of rs846910 on promoter activity	-Association study of one SNP done
Women with polycystic ovary syndrome (*n*=300)				-Tested the effect of rs13306421 on enzyme activity of 11β-HSD1 in	-All SNPs of HSD11B1 gene not studied
Control (*n*=300)					-Association of rs846910 with diabetes not studied
Malavasi et al. [24]				mammalian cells	
Koreans	rs12086634	>0.05	No association	-Adequate sample size	-Selection bias: Significant differences in the gender ratios and the distribution of ages between the control and diabetic subjects
T2DM (*n*=427)	rs1000283	>0.05		-Association of gene polymorphism with metS done in both cases and controls	
Non diabetic Control (*n*=358)					
Seong-Su Moon et al. [25]					-Entire gene was not sequenced
					-Only four SNPs studied
					-Functional analyses of the polymorphisms were not performed
Southern European Caucasians of northern Italy	Wild type rs12086634 TT and rs846910GG:	0.932 0.795	No association 1	-Evaluated adipose 11β-HSD1 expression and activity	-All SNPs of HSD11B1 gene not studied
Women with polycystic ovary syndrome (*n*=300)	4.5 % (*n*=9/199)		0.01 (0.00–0.02)	-Association of urinary and plasma cortisol with genotypes were studied	-Cases with metS had PCOD also

Table 1 (continued)

Participants, authors, references	SNPs	*P* value	Association (OR/CI)	Strengths	Limitations
	heterozygous & homozygous carriers				-Mean values of anthropometric and biochemical parameters between cases and controls are not explained
Control (n=300)	rs12086634TG and				
Gambineri et al. [23]	rs846910AG (n=0)				
Eurobrazilians	rs12086634	>0.05	No association	-Robust methodology	-Inadequate sample size
n=215	rs846910				-All SNPs of HSD11B1 gene not studied
Turek et al. [27]					

studies of HSD11B1 gene polymorphisms with type 2 diabetes.

HSD11B1 and metabolic syndrome

Increased cortisol produced by overexpression of 11β-HSD1 can lead to central obesity, and variations in the HSD11B1 gene have been associated with obesity, IR, and metS [30]. Nine studies on the association of HSD11B1 gene polymorphisms with metS were done, among which three studies [23, 25, 31] showed positive association and six studies showed no association with metS [21, 22, 26, 27, 32, 33]. The common SNP rs12086634 showed positive association with metS in South Indians, Italians, and Caucasians of northern Italy [23, 25, 31] but showed no association with metS in American Indians and Eurobrazilians [26, 27]. rs12086634 polymorphism contributed to hypertension, DM, and higher total cholesterol in South Indians. This SNP did not show high glucose risk among Caucasians as well. rs846910, an SNP in the 5' promoter region of the HSD11B1 gene is also studied where they were associated positively with metS in Caucasians [23, 24] but had no association with metS in American Indians, Eurobrazilians, Bosnian, and Herzegovina populations [26, 27, 33]. This SNP contributed to lower systolic and diastolic blood pressure, higher LDL, and lower HOMA-IR in the Bosnian population but contributed to hypertension and low HDL in Caucasians. rs846910 had no association with high glucose and triglycerides in Caucasians. Table 2 mentions the common SNPs studied in association studies of HSD11B1 gene polymorphisms with metabolic syndrome.

Summary and conclusions drawn from the association studies of HSD11B1 single-nucleotide polymorphisms with T2DM and metS

Among many SNPs of the HSD11B1 gene, rs12086634 and rs846910 are frequently studied. SNPs, rs12086634 and rs846910 associates with T2DM and rs12086634, rs1000283, and rs846910 associates with metS. It is evident from literature that the common SNPs rs12086634 and rs846910 show a conflicting association with T2DM and metS in different populations. rs12086634 showed a positive association with T2DM in Pima Indians but showed no association with T2DM in Koreans, Eurobrazilians, American Indians, and Southern European Caucasians of north Italy. rs846910 showed positive association with T2DM in Pima Indians but showed no association with T2DM in American Indians, Eurobrazilians, and Southern European Caucacians of northern Italy. rs12086634 showed a positive association with metS in South Indians, Italians and Caucasians of

Table 2 Association of HSD11B1 single-nucleotide polymorphisms with metabolic syndrome

Participants, authors, references	SNPs	*P* value	Association (OR/CI)	Strengths	Limitations
French Canadians metS males (*n*=217) Robitaille et al. [21]	g.4478TT g.10733GG	>0.05	No association	-Robust methodology -Two SNPs and one insertion polymorphism is studied	-No control subjects used -All the functional polymorphisms in HSD11B1 gene not studied -Female metS subjects are not studied
American Indians T2DM (*n*=512) Non diabetic control (*n*=406) Franks et al. [26]	rs846910 rs12086634	>0.05	No association	-Adequate sample size -Association of SNPs with hypertension is studied -Robust methodology	-All SNPs of HSD11B1 gene is not studied -Insufficient data to test in utero factors that could modify expression of the HSD11B1 gene -Confounding by population stratification
Koreans T2DM (*n*=757) Non diabetic control (*n*=644) Ku YH et al. [22]	rs846908 rs932335 rs13306422	>0.05	No association	-Adequate sample size -Polymorphism in the promoter region is studied.	-Only three SNPs of HSD11B1 gene is studied -other possible functional SNPs are not studied -SNPs studied are different from those studied by Nair et al. -NCEPAT III criteria used for metS
Koreans T2DM (*n*=427) Non diabetic control (*n*=358) Seong-Su Moon et al. [25]	rs12086634 rs1000283	0.016 0.006	Positive association	-Adequate sample size -Association of gene polymorphism with metS done in both cases and controls	-Selection bias: Significant differences in the gender ratios and the distribution of ages between the control and diabetic subjects -Entire gene was not sequenced -Only four SNPs studied -Functional analyses of the polymorphisms were not performed
Urban Japanese residents metS (*n*=431) Control (*n*=777) Miyamoto et al. [32]	+9410T>A Both, 14.2 % (*n*=61/370) Men, 13.6 % (*n*=45/286) Women, 16 % (*n*=16/84) +17925C>T, 57.8 % (*n*=249/182) +27447G>C 27.4 % (n=118/313)	0.041 0.029 0.683 0.683 -	No association 1.5 (1.0–2.2) 1.9 (1.1–3.5) 1.1 (0.6–2.1) 1.0 (0.7–1.2) -	-Adequate sample size -Found seven SNPs and insertion polymorphism	-The Japanese criteria used for metS differ from those of the NCEP ATP III & IDF criteria -Control subjects were not healthy (had high total cholesterol) -Cases and control subjects were not age matched
Southern European Caucasians of northern Italy. Women with polycystic ovary syndrome (*n*=300) Control (*n*=300) Alessandra Gambineri et al. [23]	Wild type rs12086634TT and rs846910GG: 19.5 % (*n*=79/405) rs12086634 TT and rs846910GA, 42.3 % (*n*=11/26) rs12086634TG/GG and rs846910GA,	1 0.023 0.606	Positive association - 2.77 (1.16–6.67) 0.07 (0.00–15.82)	-Evaluated adipose 11b-HSD1 expression and activity -Association of urinary and plasma cortisol with genotypes were studied	-All SNPs of HSD11B1 gene not studied -Cases with metS had PCOD also -Mean values of anthropometric and biochemical parameters between cases and controls are not explained -Control subjects excluded PCOD conditions but not features of metS

Table 2 (continued)

Participants, authors, references	SNPs	*P* value	Association (OR/CI)	Strengths	Limitations
	10 % (*n*=2/20)				
	rs12086634TT,GG and rs846910GG	0.011	0.43 (0.23–0.82)		
	(protective)				
	10.7 % (*n*=16/149)				
Bosnian and Herzegovina population	rs846910 Wild type,	0.615	No association	-Twenty percent of all samples were double-genotyped.	-Limited power to detect potentially small effects of the variants on metS
	79.1 % (*n*=34)				
metS (*n*=43)	Heterozygous carrier,	>0.05		-Insertion polymorphism is also studied.	-Not all SNPs in HSD11B1 gene studied
Control (*n*=43)					-Control subjects had atleast one feature of metS
Tanja Dujic et al. [33]	20.9 % (*n*=9)			-Effects of both haplotypes and diplotypes were studied	-Gender distribution was not uniform between cases and controls
					-Control subjects had higher total and LDL cholesterol
South Indian subjects metS (*n*=105)	rs12086634 Cases,	<0.001	Positive association OR, 6.64	-First study performed in population from South India	-Selection bias: Differences in the distribution of ages between controls and metS patients
control (*n*=100)	Wild type, 33 %		Chi-square value, 21.803	-Control subjects had no family history of diabetes	
Kunal et al. [31]	Heterozygous carriers, 67 %				-Entire gene was not sequenced
	Controls, Wild type, 93 %				- Sample size was small and subjects were chosen from various communities residing in Mangalore
	Heterozygous carriers, 7 %				
Eurobrazilians	rs12086634	>0.05	No association	-Robust methodology	-Inadequate sample size
n=215	rs846910				-All SNPs of HSD11B1 gene not studied
Turek et al. [27]					

Northern Italy but showed no association with metS in American Indians and Eurobrazilians. rs846910 showed a positive association with metS in Caucasians but had no association with metS in American Indians, Eurobrazilians, Bosnian and Herzegovina populations.

SNPs not associated with T2DM are rs846908, g.4478, g.10733, rs12086634, rs846910, rs932335, rs13306422, rs1000283, and rs13306421. SNPs not associated with MetS are rs846908, rs932335, rs13306422, rs846910, rs12086634, g.4478, g.10733, +9410, +17925, and +27447. However, this lack of association is restricted to American Indians, French Canadians, north Italians, Koreans, Eurobrazilians, Bosnian and Herzegovina subjects, and the urban Japanese population [21, 22, 24–27, 32, 33]. Since SNPs rs12086634 and rs846910 gave confusing results in various ethnic groups, future association studies must include these SNPs for T2DM and metS. Based on previous studies, adequate sample size needs to be calculated for further studies. There is scarcity of studies conducted on Indian and Chinese populations, though both these countries harbor the largest number of T2DM and metS subjects. It is worthwhile studying the frequency of these SNPs in the light of other genetic polymorphisms that are associated with T2DM and other environmental factors which may affect the expressions of these polymorphisms.

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Authors' contributions Mrs Nayana Devang carried out the literature survey of HSD11B1 gene association studies and drafted the manuscript. Dr. Prabha Adhikari guided in designing the manuscript, tables, and conclusions. Dr. Nandini M. and Dr. Sathish Rao participated in writing the introduction. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

The association between urodynamic findings and micro-vascular complications in type 2 diabetic patients with or without voiding symptoms

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Abstract The aim of the present study was to evaluate the association between diabetic micro-vascular complications with the varied urodynamic manifestations of diabetic cystopathy in both asymptomatic and symptomatic subgroup of patients as shown in previous studies. A total of 63 type 2 diabetic patients are stratified into those with and without voiding dysfunction according to International Prostate Symptom Score (IPSS) score. Urine for albumin/creatinine ratio, direct ophthalmoscopy, and nerve conduction study (NCS) along with multichannel urodynamic study (UDS) were performed to detect diabetic micro-vascular complications. Correlation between urodynamic and micro-vascular complications was evaluated in patients with and without voiding symptoms and compared. Among the 63 patients (34 patients asymptomatic and 29 patients symptomatic), diabetic nephropathy, diabetic retinopathy, motor conduction study (MCS) abnormality, sensory conduction study (SCS) abnormality, and combined NCS abnormality were seen in 74.6, 49.2, 66.7, 65.1, and 65.1 % patients, respectively. On urodynamic study, diabetic cystopathy motor (DCM), diabetic cystopathy sensory (DCS), detrusor overactivity (DO), and bladder outlet obstruction (BOO) were found in 58.7, 54, 34.9, and 36.5 % cases, respectively. Among the micro-vascular complications, sensory nerve conduction studies (SCS), motor nerve conduction studies (MCS), and combined NCS abnormality had significant association with UDS abnormalities in diabetic patients. The association was stronger in symptomatic patients. A large proportion of type 2 diabetic patients have shown clinical and electrophysiologic evidence of neurologic dysfunction which can predict the presence or absence of DCS and DCM even in the asymptomatic stage. The correlation is stronger in the symptomatic group.

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Keywords Type 2 diabetes · Microvascular complications · Urodynamic study · Diabetic cystopathy · Nerve conduction studies

Introduction

The term diabetic cystopathy (DC) to describe the spectrum of voiding dysfunction caused by diabetes mellitus (DM) was first coined by Cai Frimodt-Moller (1976) [1]. Since then, it had been an area of active research. About 5 to 59 % of DM patients report symptoms of voiding dysfunction, when specifically questioned [2]. The classical DC, which is considered a spectrum of peripheral and autonomic neuropathy first affecting sensory afferent pathways, causing impaired bladder sensation and finally decreased detrusor contractility in the end-stage diabetic bladder, occurs in only 35 % of cases [3, 4]. Whereas the prevalence of urodynamic abnormalities has been reported to be as high as 75–100 % even in the absence of lower urinary tract symptoms (LUTS) especially in patients with peripheral neuropathy [1, 5]. Apart from the typical urodynamic findings in classic DC, i.e., impaired bladder sensation, increased cystometric capacity, decreased bladder contractility, impaired urinary flow, and increased residual urine volume, other findings such as detrusor overactivity (DO), poor bladder compliance, and detrusor areflexia have been reported by several other investigators [3].

After 5–10 years of DM, most of the patients show electrophysiologic evidence of diabetic neuropathy (DN) and other micro-vascular complications (nephropathy, retinopathy) in variable degree [6, 7]. As both DC and DN have high prevalence, various investigators have sought a correlation between DC and DN [7–9] and other micro-vascular complications and have found that, in patients with DM, electrophysiologic evidence of neuropathy can predict the presence of DC in patient with LUTS or without LUTS in two different studies [10, 11].

In our study, we seek to find a correlation between urodynamic study (UDS) findings of DC and micro-vascular complications in DM patient in both the subgroup of patients with and without LUTS in a single study and compare the findings between the two groups.

Methods

Subjects

From May 2013 to April 2014, 63 patients with type 2 diabetes attending the diabetes clinic at a tertiary care hospital in Kolkata were enrolled. This study has been approved by our institutional ethics committee, and written informed consent was obtained from each participant. We included all type 2 diabetic patients with or without voiding symptoms of both sexes referred to our diabetic clinic between May 2013 and April 2014. The exclusion criteria included the following: type 1 diabetes, patients with bladder outlet obstruction due to benign prostatomegaly, genitourinary malignancies, urethral stricture, neurologic diseases affecting bladder, history of privous surgeries that might affect the vesicourethral junction, stress incontinence, and subjects who received pelvic radiotherapy. A thorough clinical history regarding duration of diabetes, presence of complications and clinical examination including dilated fundoscopy were performed. A fall in SBP by more than 20 mmHg and DBP by more than 10 mmHg, 3 min after standing from supine position, was considered orthostatic hypotension. LUTSs were evaluated by International Prostate Symptom Score (IPSS) Questionnaire (Table S1; Supplementary file) and stratified into two groups: (1) with voiding dysfunction according to IPSS score (moderate 8–19 or severe 20–35) and (2) without voiding dysfunction (IPSS score 0–7). To exclude benign prostatic enlargement as the cause of bladder outlet obstruction (BOO), digital rectal examination (DRE) and ultrasound were done.

Biochemical measurements

Investigations including blood for sugar (fasting and 2 h postprandial) and HbA1c were done to see glycemic control. Urea, creatinine, urine for routine and microscopic examination, and albumin/creatinine ratio to detect micro- (30–299 μg/mg creatinine) and macro-albuminuria (>300 μg/mg creatinine) were performed. Urine albumin/creatinine ratio was measured to detect the presence and extent of diabetic nephropathy. Blood sugar measurement was done by glucose oxidase method, urea measurement one by urease method, creatinine measured by Jaffe method, HbA1c measured by high-pressure liquid chromatography, and urine for albuminuria measured by nephelometry.

Urodynamic studies

The multichannel urodynamic study (UDS) was done using 6-Fr urethral catheter to measure detrusor pressure (P_{det}) and 8-Fr rectal catheter to measure abdominal pressure (P_{abd}). The urodynamic evaluation was done in the sitting position by filling cystometry infusing sterile normal saline via motor-driven and computerized infusion pump through another 6-Fr urethral catheter at a physiologic filling rate and pressure-flow analysis. The filling parameters used are first sensation, normal desire, strong desire, maximal cystometric capacity (MCC), end-filling detrusor pressure, and detrusor overactivity (DO). The voiding parameters were maximal flow rate, average flow rate, detrusor pressure at the maximal flow rate, voided volume, and postvoid residual urine volume (PVR). The definitions of UDS abnormalities are elaborated in Table S2 (supplementary file).

Nerve Conduction Studies

Both motor and sensory nerve conduction studies were performed in one of each of the upper and lower limbs sampling the median and ulnar nerves in the upper limb and the tibial and common peroneal nerve in the lower limb. The responses were considered normal or abnormal with respect to the standardized values. Combined neuropathy was defined as the presence of abnormalities in motor nerve conduction velocity (MCV) studies as well as sensory nerve conduction velocity (SCV) studies.

Statistical analysis

The data for the present study was collected on pre-designed format. Considering a statistical power of 90 %, the sample size calculation determined by the prevalence of DC and DN was calculated as 49 and 48, respectively. All the data was edited for completeness and consistency checks. The data was entered in MS Excel. The analysis included descriptive statistics and tests of significance. All continuous variables are expressed as the mean SD and the median and categorical variables in percentages. The Student's *t* test was used for continuous variables and proportion test for categorical variables. The Spearman rank correlation was also used to know the correlations between demographic factors (such as age, sex, duration of diabetes, and HbA1c) and diabetic micro-vascular complications with finding of urodynamic study. A "*p* value" less than 0.05 was considered as significant with two-sided test. Statistical analysis was carried out using the Statistical Package for Social Sciences, version 20, for Windows (SPSS, Chicago, IL, USA) software with binary logistic regression analysis using a forward logistic regression analysis method. The correlation between urodynamic and micro-vascular complications was seen separately in patients with and without voiding symptoms and compared.

Results

A total of 63 subjects were enrolled and all of them completed the study protocol. Among them 44 patients (69.8 %) were male and 19 patients (30.2 %) were female. The mean age group involved was 58.05±9.33 (range 40–76) and mean duration of diabetes was 10.11±2.5 years (range 6–18 years). Mean HbA1c was 8.27±1.58 (Table S3, supplementary file). Of the 63 patients, 44 patients were taking oral hypoglycemic agent, 17 patients were on insulin, and 2 patients were on dietary control alone. Out of 63 patients 34 (54 %) were asymptomatic or mildly symptomatic and 29 (46 %) were moderately or severely symptomatic according to IPSS scores. Orthostatic hypotension was found in 19 patients (30.2 %).

Among the micro-vascular complications, 19 (30.2 %) patients and 28 (44.4 %) patients had micro- and macro-

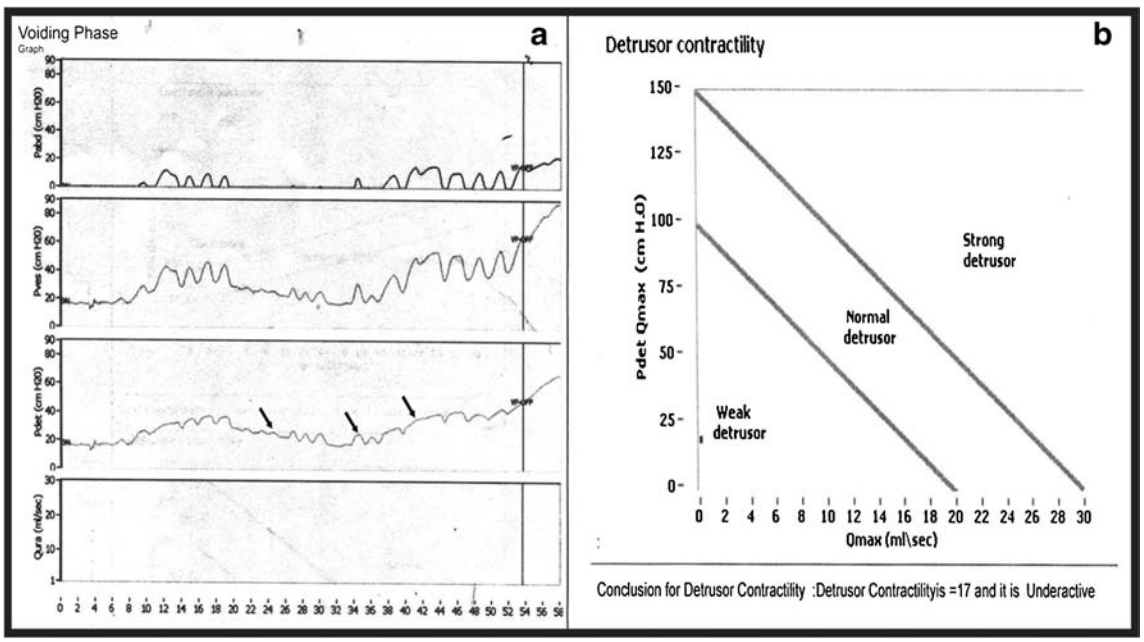

Fig. 1 **a** Horizontal axis shows time, and in vertical axis Pdet denotes detrusor pressure, Pabd denotes abdominal pressure, Pves denotes vesical pressure, and Qura denotes filling rate. The *arrows* denote weak detrusor contractions during filling of bladder. The figure denotes detrusor underactivity as a there is contraction of reduced strength and duration, resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying within a normal time span. **b** Abrams-Griffiths nomogram (in this nomogram, horizontal axis denotes filling rate and vertical axis denotes detrusor pressure at maximal flow rate; detrusor contractility index <100 is weak detrusor contraction; 100–150, normal detrusor contraction; and >150 is strong detrusor). This figure is showing detrusor contractility index of 17 suggestive of weak detrusor contraction. Detrusor underactivity (DUA) is defined by detrusor contractility index [detrusor pressure at maximum flow rate+(5 × maximum flow rate)]<100

albuminuria, respectively. Diabetic retinopathy was found in 31 (49.2 %) patients. On neurophysiologic testing, motor conduction study (MCS) abnormality was seen in 42 (66.7 %), sensory conduction study (SCS) abnormality in 41 (65.1 %), and combined NCS abnormality in 41 (65.1 %) patients.

On urodynamic study, impaired first sensation (IFS), increased maximum cystometric capacity (MCC), and diabetic cystopathy sensory (DCS) were found in 35 patients (55.6 %) whereas detrusor underactivity (DUA) (Fig. 1a, b, weak detrusor; Fig. 2, detrusor areflexia), postresidual volume (PVR) more than one third of MCC, and diabetic cystopathy motor (DCM) were found in 32 patients (50.8 %), 39 patients (61.9 %), and 32 patients (50.8 %), respectively.

Additional findings such as bladder outlet obstruction (Fig. 3a, b) and detrusor overactivity (Fig. 4) were seen in 23 patients (36.5 %) and 22 patients (34.9 %), respectively. High, low, and normal bladder compliance was seen in 33 patients (52.4 %), 16 patients (25.4 %), and 14 patients (22.2 %), respectively (Table S4, supplementary information). Comparative demographic and clinical data, micro-vascular complications, and UDS findings in both groups are summarized in Table 1 which shows that the symptomatic patients had significantly (p value <0.05) higher mean age and mean duration of diabetes, higher percentage of orthostatic hypotension, DCS, and high compliance bladder compared to asymptomatic patients.

The following significant associations were found among urodynamic findings and diabetic micro-vascular complications: Diabetic cystopathy (DCS and DCM) had significant association with sensorimotor somatic neuropathy (SCS, MCS, and combined NCS abnormality) after binary logistic regression for age, sex, DM duration, HbA1c, DO, BOO, orthostatic hypotension ($p<0.001$, Table 2). No such association was found with other diabetic micro-vascular complications (nephropathy and retinopathy). No significant association was found between glycemic control and age with urodynamic abnormality in total patient population and symptomatic subgroup (except with DUA), but a significant association was found in asymptomatic subgroup (Table 3). Among the additional urodynamic findings, a significant association was also seen between age with DO and high compliance bladder.

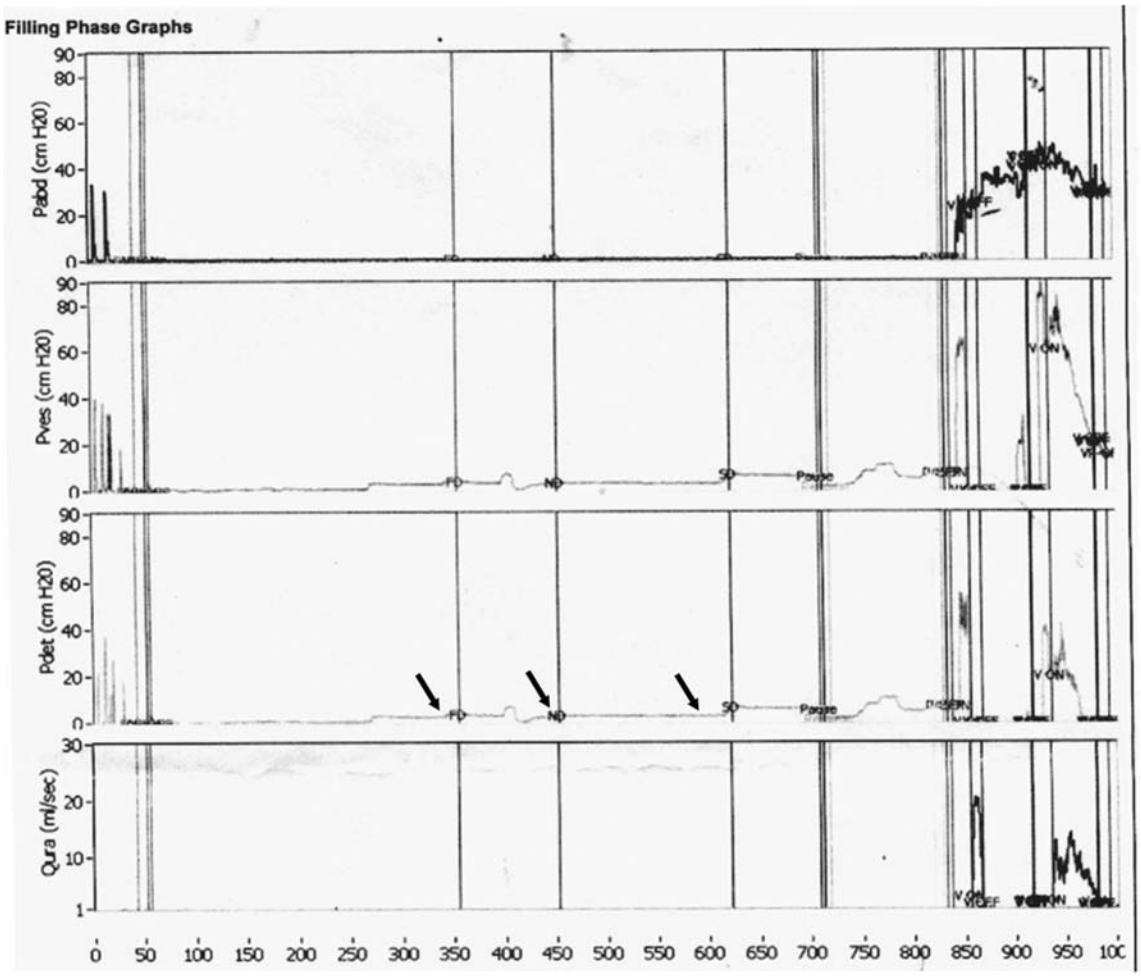

Fig. 2 Horizontal axis shows time, and in vertical axis Pdet denotes detrusor pressure, P_{abd} denotes abdominal pressure, P_{ves} denotes vesical pressure, and Q_{ura} denotes filling rate. The *arrow* denotes minimal contractility of detrusor is when there is no demonstrable contraction during urodynamic study suggestive of detrusor areflexia (flat filling curve)

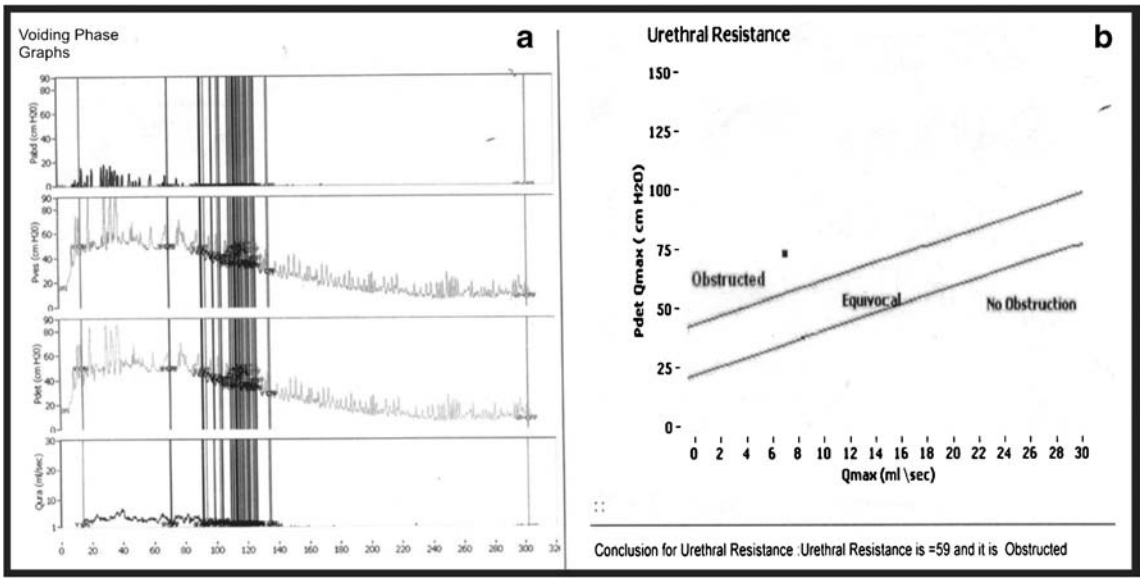

Fig. 3 **a** Urodynamic study: Horizontal axis showing time, and in vertical axis Pdet denotes detrusor pressure, Pabd denotes abdominal pressure Pves denotes vesical pressure, and Qura denotes voiding rate. An interrupted or straining pattern seen in the figure is suggestive of bladder outlet obstruction. **b** Urethral resistance nomogram (in this nomogram, horizontal axis denotes voiding rate and vertical axis denotes detrusor pressure at maximal voiding rate). Bladder outlet obstruction is said to be present if urethral resistance factor is greater than 40, unobstructed if it is less than 20, and equivocal if it is 20 to 40)

Combined NCS abnormality was associated with high compliance bladder (Table S5, supplementary file).

In subgroup analysis, in asymptomatic and symptomatic subgroup, diabetic cystopathy (DCS and DCM) had significant association with sensorimotor somatic neuropathy (SCS, MCS, and combined NCS abnormality) after binary logistic regression for age, sex, DM duration, HbA1c, DO, BOO, and orthostatic hypotension. In symptomatic patients, DUA in isolation did not correlate with neurophysiologic abnormalities (Table 4) but along with increased PVR (i.e., DCM) had significant association. No such significant association was found between urodynamic abnormalities and retinopathy or nephropathy in both the subgroups.

Among additional findings, increased age had significant association with high bladder compliance and combined NCS abnormality had association with DO in asymptomatic and mildly symptomatic patients (Table S6, supplementary file) whereas age had significant association with BOO, and male sex was associated with high compliance bladder in moderately and severely symptomatic patients (Table S7, supplementary file).

Discussion

Diabetic cystopathy frequently go unnoticed by patients as well as physicians until decompensation occurs due to its insidious onset and progression. The prevalence of DC is associated with duration of diabetes as seen in our study and not gender. In our study, male sex had an association with high PVR and DUA (i.e., DCM), but it was not significant. A large majority had neurophysiologic evidence of diabetic neuropathy (65.1 %) even in the asymptomatic patients (61.8 %), although higher incidence was found in symptomatic patients (72.4 %) indicating advanced stage of DM.

In this study, we looked for association between diabetic micro-vascular complications and various UDS findings in diabetic patients with or without voiding symptoms .The purpose of our study was to predict UDS abnormalities in a diabetic patient with diabetic micro-vascular complications so that DC can be predicted in diabetic patients without invasive urodynamic studies and appropriate measures can be undertaken. The prevalence of DCS was 41.2 and 72.4 %, and DCM was 50 and 69 %, in the asymptomatic and symptomatic groups, respectively. On the other hand, the prevalence of neurophysiologic abnormality was 61.8 and 72.4 % in the asymptomatic and symptomatic groups, respectively. Moreover, DCS and DCM had a significant association with SCS, MCS, and combined NCS abnormality in both the subgroups.

We evaluated 63 patients for the possible association between diabetic micro-vascular complications and UDS findings. We combined both pre-symptomatic and symptomatic group patients for the possible association between diabetic micro-vascular complications and UDS findings. In the age group of patients studied, cases of genitourinary malignancies, urethral stricture, neurologic diseases affecting bladder and patients who got pelvic radiotherapy, and stress incontinence in females were excluded to eliminate independent factors which can alter UDS findings other than diabetes.

The most common urodynamic findings classically described in DC are impairment of bladder sensation, increased

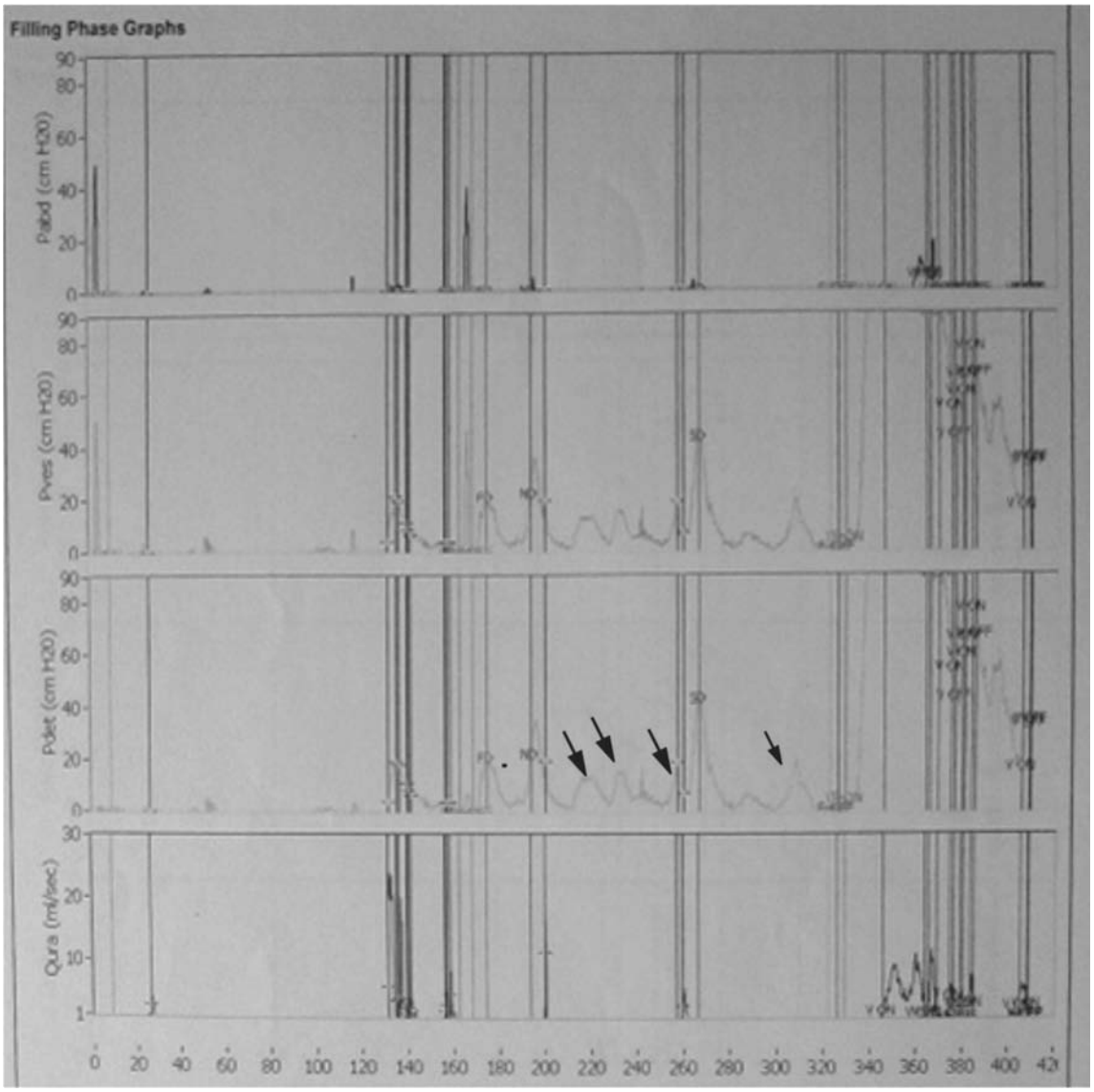

Fig. 4 Horizontal axis shows time, and in vertical axis Pdet denotes detrusor pressure, Pabd denotes abdominal pressure Pves denotes vesical pressure, and Qura denotes filling rate. The *arrows* denote involuntary detrusor contractions; there is a rise in Pves with no associated rise in Pabd, and therefore the subtracted Pdet looks identical to the Pves—this suggests detrusor overactivity, i.e., involuntary detrusor contractions (IDCs) during the filling phase, which may be spontaneous or provoked (*arrows*)

postvoid residual volume, decreased detrusor contractility that may progress to detrusor areflexia, and diminished urinary flow. The most common urodynamic findings in our study were PVR more than one third of MCC (61.9 %), impaired first sensation (55.6 %), increased MCC (55.6 %), high compliance (52.4 %), and DUA (50.8 %). Other less common abnormalities in UDS were DO (34.9 %) and low compliance bladder (25.4 %) [7, 10, 12, 13]. In a study by Kaplan et al. in 115 male and 67 female, UDS abnormalities were impaired detrusor contractility (UDS findings of classic DC) 23 %, detrusor hyper-reflexia 55 %, detrusor areflexia 10 %, indeterminate 11 % (non-classic UDS findings), and normal 1 % [7]. In another large study by Kitami et al. in 173 patients with DM and LUTS, classic pattern of DC (i.e., high first sensation and low end-filling detrusor pressure) was found in 66.9 %, and non-classic patterns of DC were overactive bladder (14.5 %), low compliance bladder (11.0 %), and loss of detrusor-external sphincter coordination (31.7 %) [14]. Chancellor and Blaivas investigated 43 patients with DM in whom 32 % had impaired contractility (UDS findings of classic DC), 56 % had detrusor overactivity, 23 % had detrusor areflexia (non-classic UDS findings), and only 12 % had normal UDS [12].

Compared to the study by Esteghamati et al. [11], who evaluated the possible association between micro-vascular complications of diabetes and abnormalities in UDS in asymptomatic patients, a higher incidence of high PVR was found (50 % compared to 45.5 %), whereas a lower incidence

Table 1 Comparison of demographic and UDS findings in asymptomatic and mildly symptomatic group with moderate to severely symptomatic group

Demographic data comparison	No LUTS or mild (IPSS ≤7) (34 patients)	Moderate (IPSS 8–19) and severe (IPSS 20–35) (29 patients)	*p* value
Age			**0.003**
Mean±SD	54.94±9.924	61.69±7.177	
Median	59	62	
Sex			0.680
Male:female	23:11	21: 8	
DM duration (years)			**0.001**
Mean±SD	8.85±1.708	11.59±2.500	
Median	9.00	12	
HbA1c (%)			0.07
Mean±SD	8.72±1.740	7.748±1.2235	
Median	8.40	7.5	
Orthostatic hypotension	5 (14.7 %)	16 (55.2 %)	**0.001**
Impaired first sensation	14 (41.2 %)	21 (72.4 %)	**0.008**
Increased MCC	14 (41.2 %)	21 (72.4 %)	**0.008**
DUA	17 (35.3 %)	20 (69 %)	0.118
PVR ≥1/3 of MCC	17 (50 %)	22 (75.9 %)	**0.027**
UDS findings comparison	No LUTS or mild (IPSS≤7)	Moderate (IPSS 8–19) and severe (IPSS 20–35)	*p* value
BOO (AG >40)	14 (41.2 %)	10 (34.5 %)	0.584
DO	15 (44.1 %)	7 (24.1 %)	0.086
High compliance	16 (47.1 %)	22 (58.6 %)	**0.014**
Low compliance	10 (29.4 %)	6 (20.7 %)	0.421
DCS	14 (41.2 %)	21 (72.4 %)	**0.008**
DCM	17 (50 %)	20 (69 %)	0.118
Micro-albuminuria	9 (26.5 %)	10 (34.5 %)	0.491
Macro-albuminuria	12 (35.2 %)	16 (55.1 %)	0.107
Retinopathy	16 (47.05 %)	15 (51.72 %)	0.712
MCS abnormality	21 (61.8 %)	21 (72.4 %)	0.365
SCS abnormality	20 (58.8 %)	21 (72.4 %)	0.251
Combined NCS abnormality	20 (58.8 %)	21 (72.4 %)	0.251

We have used Student's *t* test for comparing the continuous variables and proportion test for categorical variable. Significant values marked bold

LUTS lower urinary tract symptom, *IPSS* International Prostate Symptom Score, *DM* diabetes mellitus, *MCC* maximal cystometric capacity, *DUA* detrusor underactivity, *PVR* postvoid residual urine volume, *UDS* urodynamic study, *BOO* bladder outlet obstruction, *DO* detrusor overactivity, *DCS* diabetic cystopathy sensory, *DCM* diabetic cystopathy motor, *MCS* motor conduction study, *SCS* sensory conduction study, *NCS* nerve conduction study

of high bladder capacity (41.2 % compared to 64.6 %) and bladder compliance (47.1 % compared to 48.5 %) was found in the asymptomatic subgroup of our study. The results were more or less comparable. But, compared to study in diabetic men with LUTS by Bansal et al. [10], who evaluated diabetic men with LUTS for association between diabetic autonomic and peripheral neuropathy and UDS abnormalities, a remarkably higher proportion of patients had DCS (increased MCC and impaired first sensation; 72.4 % compared to 23.1 %), although prevalence of UDS correlates of detrusor decompensation—DUA (69 % compared to 78.8 %) and high PVR (75.9 % compared to 65.4 %)—were comparable in symptomatic subgroup of our study. A higher incidence of BOO (34.55 compared to 28.8 %) but a lower incidence of DO (24.1 % compared to 38.5 %) and low detrusor compliance (20.9 % compared to 32.7 %) were found in symptomatic subgroup. As in our study, both DCS and DCM correlated with NCS studies [10]. Mitsui et al. performed urodynamic study and nerve conduction studies and completed (IPSS) of 29 patients (21 male 8 female) and found that asymptomatic patients (62 %) had no evidence of voiding dysfunction according to uroflowmetry findings and postresidual volume (PVR) and symptomatic patients (38 %) have higher incidence of delayed first sensations (48 %) and a large capacity (38 %) bladder. In the study, the mean IPSS was similar regardless of

Table 2 Spearman rank correlation between UDS and demographics, glycemic control, and micro-vascular complications with total patients (n=63)

	IFS	Increased MCC	DUA	PVR 1/3 of MCC	DCS	DCM
Age	−0.011	−0.08	−0.284	−0.118	−0.003	0.064
Sex (male compared to female)	0.045	0.045	−0.076	−0.017	0.024	**0.081**
Duration	−0.157	−0.157	0.104	−0.126	**−0.210**	−0.099
HbA1c	−0.179	−0.179	−0.052	−0.088	−0.133	−0.128
Micro-albuminuria	0.232	0.23	0.067	0.159	0.183	0.200
Macro-albuminuria	−0.078	−0.076	0.052	−0.088	−0.050	−0.094
Retinopathy	0.080	0.080	0.177	−0.012	−0.111	−0.013
SCS abnormality	**0.514****	**0.544****	**0.071**	**0.454****	**0.521****	**0.536****
MCS abnormality	**0.516****	**0.516****	**0.187**	**0.555****	**0.494****	**0.638****
Combined NCS abnormality	**0.516****	**0.506****	**0.187**	**0.555****	**0.494****	**0.638****

Significant values are marked bold

MCC maximal cystometric capacity, *DUA* detrusor underactivity, *PVR* postvoid residual urine volume, *DCS* diabetic cystopathy sensory, *DCM* diabetic cystopathy motor, *MCS* motor conduction study, *SCS* sensory conduction study, *NCS* nerve conduction study

**Significant at 0.001 level, two-sided

prevalence of voiding dysfunction, so they concluded that the voiding dysfunction could not be predicted from symptoms alone [6]. The incidence of DO in various studies was varied (25–55 %) [Ueda et al. (25 %), Bansal et al. (36 %), Kaplan et al. (55 %), Chancellor et al. (56 %)] [7, 10, 12, 13]. In our study, the incidence was 34.9 %.

Among the demographic factors, age was associated with DO, DUA (in symptomatic subgroup only), and increased bladder compliance; duration of diabetes was associated with DUA (in symptomatic subgroup). In symptomatic subgroup, age had a significant association with BOO and male sex was associated with high compliance bladder. In another study, old age predicted low flow rate and outlet obstruction, detrusor instability was associated with shorter diabetes duration, and male sex was associated with decreased bladder compliance, and BOO and female sex on the other hand was associated with increased risk of having an increased bladder capacity [11].

Association of micro-vascular complications of diabetes with UDS findings was as follows: IFS, increased MCC, high PVR, DCS, and DCM had a significant association with SCS, MCS, and combined NCS abnormality after binary logistic regression for age, sex, duration of diabetes, HbA1c, DO, BOO, and orthostatic hypotension in total subject analysis. All the urodynamic abnormalities had a significant association with SCS, MCS, and combined NCS abnormality in asymptomatic subgroup analysis. In the symptomatic subgroup, the above association remained significant except that DUA lost significant association with MCS and combined NCS abnormality; moreover, HbA1c had significant association with IFS, increased MCC, high PVR, DCS, and DCM. Duration of

Table 3 Spearman rank correlation between demographics, glycemic control, micro-vascular complications, and UDS findings with no LUTS or mild LUTS (IPSS ≤7) (n=34 patients)

	IFS	Increased MCC	DUA	PVR 1/3 of MCC	DCS	DCM
Age	−0.012	−0.012	−0.076	−0.146	0.022	0.022
Sex(male compared to female)	−0.060	−0.060	−0.019	0.063	−0.103	−0.103
Duration	0.082	0.082	0.266	0.171	- 0.016	−0.016
HbA1c	−0.437**	−0.437**	0.315	−0.386*	−0.365*	−0.365*
Micro-albuminuria	0.175	0.175	0.138	0.200	0.077	0.077
Macro-albuminuria	−0.007	−0.007	0.036	0.000	0.074	0.074
Retinopathy	0.190	0.190	0.007	0.118	0.257	0.257
SCS abnormality	0.579**	0.579**	0.382*	0.359*	0.535**	0.535**
MCS abnormality	0.535**	0.535**	0.577**	0.545**	0.495**	0.495**
Combined NCS abnormality	0.535**	0.535**	0.577**	0.545**	0.495**	0.495**

MCC maximal cystometric capacity, *DUA* detrusor underactivity, *PVR* postvoid residual urine volume, *DCS* diabetic cystopathy sensory, *DCM* diabetic cystopathy motor, *MCS* motor conduction study, *SCS* sensory conduction study, *NCS* nerve conduction study

*Significance at 0.05 level with two sided test; **significance at 0.001 level with two sided test

Table 4 Spearman rank correlation between demographics, glycemic control, micro-vascular complications, and UDS findings in patients with moderate (IPSS 8–19) and severe LUTS (IPSS 20–35) (n=29 patients)

	IFS	Increased MCC	DUA	PVR 1/3 of MCC	DCS	DCM
Age	0.115	0.115	−0.640	0.126	0.115	0.115
Sex (male compared to female)	0.154	0.154	−0.137	−0.168	0.154	0.154
Duration	−0.190	−0.190	−0.389*	−0.176	−0.190	−0.019
HbA1c	0.022	0.022	−0.420*	0.158	0.022	0.022
Micro-albuminuria	0.268	0.268	−0.285	0.070	0.268	0.268
Macro-albuminuria	0.048	0.048	−0.125	0.089	0.048	0.048
Retinopathy	−0.010	−0.010	0.374	−0.140	−0.010	−0.010
SCS abnormality	0.472**	0.472**	- 0.374*	−0.553**	0.472**	0.472**
MCS abnormality	0.472**	0.472**	- 0.310	0.553**	0.472**	0.472**
Combined NCS abnormality	0.472**	0.472**	−0.310	0.553**	0.472**	0.472**

MCC maximal cystometric capacity, *DUA* detrusor underactivity, *PVR* postvoid residual urine volume, *DCS* diabetic cystopathy sensory, *DCM* diabetic cystopathy motor, *MCS* motor conduction study, *SCS* sensory conduction study, *NCS* nerve conduction study

*Significance at 0.05 level with two sided test; **significance at 0.001 level with two sided test

diabetes and HbA1c had significant association with DUA in asymptomatic subgroup. Neither retinopathy nor albuminuria predicted occurrence of diabetic cystopathy.

Diabetic micro-vascular environment causes diabetic cystopathy by damaging vascular and neurological innervation of the bladder. The pathogenesis of diabetic cystopathy has been studied in alloxan-induced and streptozotocin-induced diabetic rats replicating the diabetic state and cystopathy in animal model [10]. Liu and Daneshgari (2005) concluded that bladders of diabetic and diuretic rats weighed more than control animals and that diabetes and diuresis caused a significant increase in overall fluid intake, urine output, and bladder size indicating the possibility of neurogenically mediated bladder contraction in the diabetic rat [15]. Decreased Na,K-ATPase activity [16], significantly lower level of nerve growth factor (NGF) [17], reduced mitochondrial counts in the urothelium and bladder muscle [18], impairment of nitric oxide–mediated urethral smooth muscle relaxation [19], translocation of protein kinase C isoforms, increased myosin light chain phosphorylation and decreased sensitivity to activator calcium [20], and autoimmune antibody-mediated bladder dysfunction [9] have been implicated in the pathogenesis of DC.

Diabetic cystopathy is thought to be a result of an alteration in the physiology of detrusor smooth muscle cell, the innervations, and function of the neuronal component. Diabetic neuropathy is characterized by demyelination, axonal degeneration, and nerve fiber loss in bladder. Previous investigators also tried to find a correlation between diabetic neuropathy and diabetic cystopathy, but results were conflicting. Moreover, the other diabetic micro-vascular complications like retinopathy and nephropathy have the same pathological basis and, commonly, they are found together in a patient with diabetes. Hence, diabetic micro-vascular complications are selected. We did not find a significant association between glycemic levels and UDS in total patients and symptomatic subgroup analysis but found correlation with most of the urodynamic abnormalities in asymptomatic patients indicating that glycemic control may have a role in preventing diabetic cystopathy especially in asymptomatic patients. However, to prove this hypothesis, it would have been better to have a group without diabetes.

Conclusions

To conclude, diabetic cystopathy have a variety of urodynamic abnormalities apart from the typical urodynamic findings such as detrusor overactivity, bladder outlet obstruction, and decreased compliance.

Diabetic neuropathy has a significant association with urodynamic study findings irrespective of the presence of voiding symptoms. The urodynamic abnormalities in diabetic patients with and without voiding symptoms were compared in a single study, which was first of its kind. Association of urodynamic correlates of diabetic cystopathy and diabetic neuropathy was stronger in symptomatic subgroup. Glycemic control is better correlated with urodynamic abnormalities in the asymptomatic patients. So the neurophysiologic abnormalities suggestive of diabetic neuropathy can be used as a means for early screening of diabetic cystopathy in both symptomatic and asymptomatic group.

The limitations of our study are that our study was a cross-sectional study, the relatively small sample size, and that there were no control groups due to ethical issues in performing invasive urodynamic studies in healthy patients. Moreover bladder autonomic neuropathy was not evaluated. Further prospective studies with larger sample sizes are needed in future to determine the existence of potential associations more accurately.

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Ethical standards This study was approved by the Institutional Ethics Committee of Calcutta National Medical College, Kolkata, India [Dated 3rd December, 2012].

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Conflict of interest Partha Pal, Sayantan Ray, Nabankur Ghosh, Sujoy Ghosh, Kaushik Biswas, Krishnendu Roy, Debabrata Mukherjee, and Dilip Karmakar declare that they have no conflict of interest.

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

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

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ORIGINAL ARTICLE

Polymorphic analysis of MC4R gene in ethnic Kashmiri population with type 2 diabetes

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Abstract Activation of MC4R gene has been shown to inhibit appetite and increase basal metabolic rate while deficiency leads to obesity, hyperphagia, severe hyperinsulinemia, increased linear growth and decrease in metabolic activity, facts that drew strong attention as a possible cause of obesity and diabetes. In this study, we aimed to investigate the sequence variations of MC4R gene in a diabetic population of the Kashmir region and work out association of such variations (if any) with the disease phenotype. No such study has ever been taken up in the Jammu and Kashmir State, despite the existence of a significant population of type 2 diabetic patients. A total of 420 samples (200 with type 2 diabetes and 220 controls) were taken. Genomic DNA was extracted from whole blood samples using standard protocols like salting out and proteinase k. The specific fragments of DNA were amplified and hence then purified. Purified amplicons were subjected to heteroduplex assay to screen for SNPs/mutations. Samples which showed heteroduplex bands were sent for sequencing. The genotype and allele frequencies were evaluated using the χ^2 tests or the Fisher exact tests. We here report one novel heterozygous mutation, i.e. C to T at codon 7 in diabetic patients. The results showed significant differences in the 7C/T genotype ($p < 0.001$) and allele ($p < 0.0001$) frequencies between type 2 diabetes mellitus and control subjects. The fasting blood sugar (FBS), postprandial blood sugar (PPBS) and random blood sugar (RBS) levels were higher with CT genotype in type 2 diabetes mellitus patients but difference was not found statistically significant. C to T substituting arginine with cysteine appeared to associate with type 2 diabetes in the Kashmiri population. Insilico predictions show that substitutions likely have an impact on structure and functional properties of protein making it imperative to understand their functional consequences in relation with diabetes and obesity.

Keywords Diabetes · Obesity · MC4R · CSGE · Genetic polymorphisms

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Abbreviations

PCR	Polymerase chain reaction
CSGE	Conformation sensitive gel electrophoresis
MC4R	Melanocortin 4 receptor
α-MSH	α-Melanocyte stimulating hormone

Introduction

Type 2 diabetes and the accompanying risk of common diseases such as obesity and premature cardiovascular morbidity and mortality are increasing global health burdens [1]. The incidence of type 2 diabetes mellitus is increasing at an alarming rate, increasing sharply with age and high prevalence has been reported in certain ethnic groups, like Hispanics,

Native Americans, African Americans and Asian/Pacific Islanders [2, 3]. The most powerful risk factor in the pathogenesis of diabetes mellitus is obesity. It is well established now that there is a direct co-relation between the emerging epidemic of diabetes mellitus and the obesity [4, 5]. The prevalence of overweight and obesity is increasing rapidly worldwide both in the developing as well as the developed countries [6]. Severe obesity in about 6 % of morbidly obese adults and children has been linked to autosomal dominant mutations in the gene for the MC4R [7–12]. This receptor integrates orexigenic and anorexigenic signals in the hypothalamus and elsewhere in the central nervous system to regulate food intake and energy expenditure [13]. MC4R gene has only a single exon and thus is amenable to mutation analysis. Disruption of the MC4R gene in mice and humans results in hyperphagia and obesity [9, 14–16], (including increased fat [17] and lean body mass [7, 9, 18–22] increased bone mineral density, [10] accelerated linear growth, [10, 19] and severe hyperinsulinemia [9]). Loss-of-function mutations in the MC4R are the commonest known form of monogenic obesity and are found in 4–5 % of subjects with severe obesity of onset in childhood [19–20, 22–23]. Much has been learned from studies on monogenic obesity; however, the role of variation at the MC4R locus in influencing inter-individual variation in body size and composition in the general population remains controversial [7, 20, 23]. Genome-wide association studies have shown that variants near the MC4R (rs17782313 and rs12970134) are associated with risk of obesity in Europeans. As obesity is associated with an increased risk of type 2 diabetes, many studies have investigated the association between polymorphisms near the MC4R gene and type 2 diabetes risk across different ethnic populations with inconsistent results [24]. To date, MC4R mutations have proven to be of large individual relevance but of small epidemiological relevance [18]. Therefore, further investigation, involving polymorphic variation and their co-relation with the measures of diabetes in the general population, would help elucidate the role of MC4R with regard to public health. Variants in MC4R gene have shown the strongest associations with diabetes and obesity in different populations [11, 14, 16, 24–29]. Because potential associations between type 2 diabetes mellitus and particular single nucleotide polymorphisms are often population dependent, the objectives of this study were to examine polymorphisms of this gene in a Kashmiri population and to assess their contributions to the development of type 2 diabetes in Kashmir.

Material and Methods

Subjects

A total of 420 subjects (200 patients with type 2 diabetes and 220 healthy control subjects) were recruited from the Endocrinology Department of the Sher-i-Kashmir Institute of Medical Sciences, Soura (SKIMS) Srinagar, Jammu and Kashmir. Informed consent was obtained from the study subjects after an explanation of the nature and possible consequences of the study. Criteria for selection included a history of onset of diabetes in all affected subjects. The study was approved by the research ethics committee. Blood samples of diabetic patients were collected after the complete clinical investigation. A careful history taken including age of the subject, gender, personal habits, dietary history, socioeconomic factors, history of onset of diabetes/obesity, any associated complications and information regarding close work. Venous blood samples were collected in EDTA for DNA extraction. Samples were kept at −70 °C until analyzed.

Polymerase chain reaction

Genomic DNA was extracted from whole blood samples using standard protocols like salting out [30] and proteinase k [31] methods. PCR reactions were carried out in a total volume of 50 μl, containing 50–100 ng genomic DNA, 2–6 pmole of each primer, 1× of Taq polymerase buffer and 0.5 units of Taq DNA polymerase (Sigma Aldrich). The following primer sequences were used for amplification: (1F) 5′-AATAACTGAGACGACTCCCTGAC-3′ and (1R) 5′-CTGATCCATTTGAAACGCTCACC-3′. (2F) 5′-GCAGCTTGGCTGTGGCTGATATGC-3′ and (2R) 5′-GTGAAGCCTGGCCATCAGGAAC-3′ and (3F) 5′-CTCATGGCTTCTCTCTATGTCCAC-3′ and (3R) 5′-CAGAAGTACAATATTCAGGTAGGG-3′. Expected PCR products of 369, 416 and 459 bp were generated successfully. The PCR cycling conditions involved one cycle of denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s and one final 7 min elongation cycle at 72 °C. PCR products were then purified using purification kit (Sigma) or NaI.

Conformation sensitive gel electrophoresis

Purified PCR products were subjected to denaturation and renaturation procedures for generation of potential heteroduplexes (Fig. 1) and analyzed by conformation sensitive gel electrophoresis (CSGE) strictly as described by Ganguly et al. [32]. Samples with unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations along with controls (Macrogen, Korea).

Sequencing

Samples that showed presence of heteroduplex bands were sent for sequencing to confirm the presence of sequence variations.

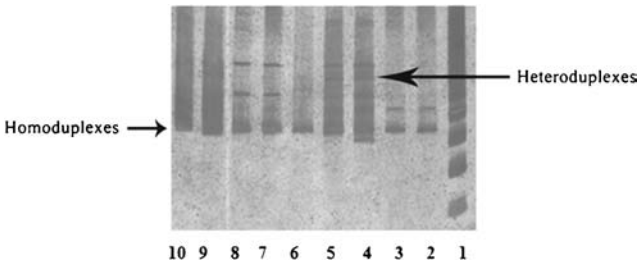

Fig. 1 Heteroduplex analysis of exon 1 amplicons (369 bp) by conformation sensitive gel electrophoresis. *Lane 1* shows separation pattern of 100-bp DNA marker. *Lanes 2–6* and *7–8* show (heteroduplexes shown by *arrow head*) analysis of affected samples and *lanes 9* and *10* (homoduplexes shown by *arrow head*) show analysis of normal samples

Sequence analysis

Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software [33, 34] and by Chromas Pro version 1.49 beta 2 software for the detailed inspection of individual chromatograms.

Statistical analysis

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the Woolf's approximation test and probability p values. A p value of <0.05 was considered significant.

Insilico analysis

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction [35, 36]. The server furnishes predicted 3D structure in a pdb format. Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures [37, 38].

Results

Anthropometric and biochemical characteristics of two groups

The anthropometric and biochemical characteristics of type 2 diabetes mellitus patients and healthy control subjects are presented in Table 1. Compared to healthy control subjects, type 2 diabetes mellitus patients had significantly higher concentrations of fasting blood sugar ($p = 0.000$), postprandial blood sugar ($p = 0.000$), random sugar ($p = 0.000$), total cholesterol ($p = 0.001$), triglycerides ($p = 0.000$) and low-density lipoproteins ($p = 0.000$) whereas these patients had significantly lower averages of high-density lipoproteins ($p = 0.415$) (Table 1).

Association of MC4R 7C/T polymorphism with T2DM

A total of one mutation was found in the exon of MC4R gene (Fig. 2). This missense allelic mutation was observed at codon 7 C/T (Arg → Cys). Genotype analysis of individual variant revealed the presence of only heterozygous genotypes. The genotypes distribution and allele's frequencies of the 7C/T MC4R polymorphism in type 2 diabetes patients and healthy control subjects are presented in Table 2. The genotype frequencies were as follows: 90 % (CC), 10 % (CT) and 0 % (TT), in T2DM patients, and 100 % (CC), 0 % (CT) and 0 % (GG) in healthy control subjects. In addition, the frequencies of alleles C and T were as follows: 94 and 6 % in type 2 diabetes patients and 100 and 0 % in healthy control subjects. The genotype and allele frequencies of the 7C/T MC4R polymorphism were different between healthy control subjects and type 2 diabetes patients. A subtle but statistically significant ($p \leq 0.05$; Table 2) difference in the allelic frequency was indicative of its possible association with diabetes.

Association of biochemical parameters and MC4R 7C/T mutation

No significant difference was found in type 2 diabetes patients having heterozygous variation in all the parameters including age, basal metabolic index, total cholesterol, triglycerides, low-density lipoproteins, high-density lipoproteins, fasting blood sugar, postprandial blood sugar and random blood sugar ($p > 0.05$) (Table 3).

Insilco prediction results

MC4R was modeled by I-TASSER to obtain its PDB structure and analysis (energy calculations) was done using PDB Viewer. Insilico prediction results show that calculated energy for wild-type protein is more (−14,217.289 kj/mol) compared to mutant protein (−14,498.063 kj/mol). This change in energy of mutant protein is suggestive of affecting the protein tertiary structure which may in turn have some impact on protein function. Therefore, further studies are needed to elucidate the actual role of this mutation on protein structure and function.

Discussion

Recent research has shown that several genetic factors play a role in regulating energy balance via the neuroendocrine system and several candidate genes have been identified [39]. The melanocortin system is important in the regulation of energy balance [40, 41]. In particular, a functional MC4R has been shown to be necessary to prevent adiposity [42]. The melanocortin-4 receptor is a part of the melanocortin

Table 1 Anthropometric and biochemical characteristics of T2DM and controls

Variables	Controls (n = 220)	T2DM (n = 200)	p value
Gender (M/F)	100/120	75/125	
Age (years)	50.32 ± 8.83	51.28 ± 13.05	
BMI (kg/m^2)	23.32 ± 1.70	23.22 ± 1.49	0.553
Total cholesterol (mg/dl)	151.55 ± 8.48	181.78 ± 9.56	0.000
Triglyceride (mg/dl)	127.21 ± 5.38	154.40 ± 6.0	0.000
LDL (mg/dl)	87.95 ± 8.80	105.08 ± 6.08	0.000
HDL (mg/dl)	33.18 ± 5.9	32.73 ± 5.3	0.415
Fasting blood glucose (mg/dl)	92.21 ± 10.71	189.14 ± 49.52	0.000
PPBS	109.65 ± 23.73	258.95 ± 77.06	0.000
Random	110.41 ± 18.76	270.13 ± 70.33	0.000

BMI body mass index, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins, *PPBS* postprandial blood sugar

pathway controlling food intake and energy homeostasis [43]. Transgenic knockout mice, lacking MC4R, show maturity-onset obesity, hyperphagia, hyperglycemia, increased linear growth [44] and hyperinsulimenia [3]. Heterozygous MC4R knockout mice show intermediate obesity, their average weight being between the homozygous knockouts and wild-type mice, suggesting that the quality of receptors is important for weight regulation [44]. The prevalence of type 2 diabetes and impaired glucose tolerance among humans with a defective MC4R gene are very similar to those in obese subjects [5]. Farooqi et al. [8] reported normal fasting plasma glucose levels despite hyperinsulinemia in a group of patients with a MC4R mutation. The MC4R knockout mouse also displays hyperinsulinemia, but not diabetes [44, 45]. Five melanocortin receptor subtypes with different expression patterns in the brain and elsewhere have been cloned [46]. Most MC4R variants are found at very low frequencies and have only been identified in obese patients [21] although a few variants have been seen with similar frequencies in patients and controls [20].

This is the first study to show an association between variations in the MC4R gene with diabetes in Kashmiri population. We screened the entire MC4R coding region in 420 subjects (200 diabetic and 220 controls, obtained from patients being treated for diabetes in SKIMS) from Kashmir valley and identified one heterozygous novel missense mutation (Arg7Cys). The heterozygous missense mutation found at codon 7 produces the substitution of arginine with cysteine (CGT to TGT). In this study, we reported significant differences in genotypic distribution (p = 0.0001) and allele

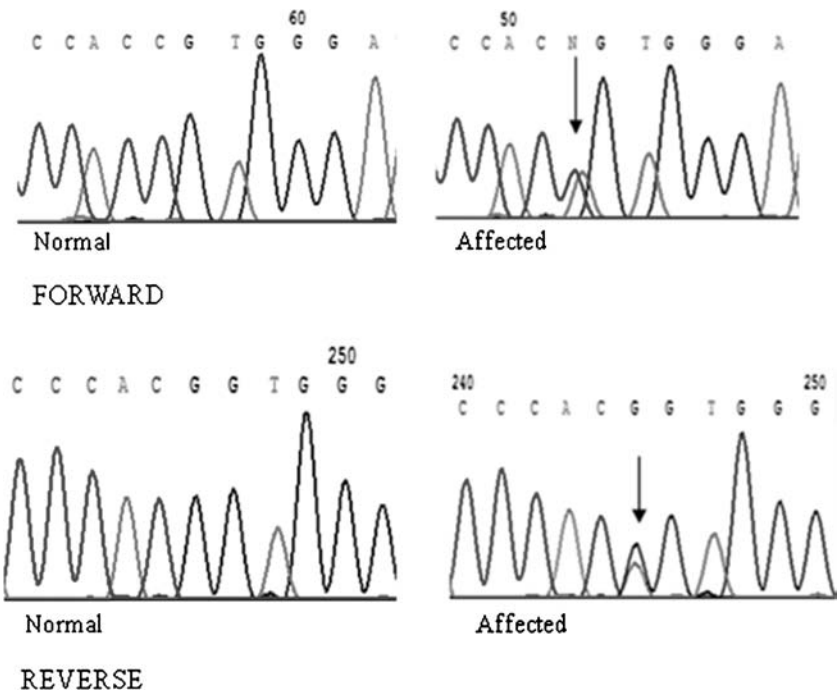

Fig. 2 Chromatogram showing change at codon 7 from C to T in exon of MC4R gene in ethnic Kashmiri population with diabetes

Table 2 Comparison of the distribution of alleles and genotypes of MC4R gene polymorphism at codon 7 in healthy and diabetic subjects

		Controls (n = 220) Number (%age)	Patients (n = 200) Number (%age)	p value	Odds ratio	95 % confidence interval
Alleles	C	440(100.0)	376 (94.0)	<0.0001*	57.32	3.47–946.50
	T	0(0.0)	24(6.0)			
Genotypes	CC	220 (100.0)	180 (90.0)	<0.001*	50.08	3.06–834.42
	CT	0 (0.0)	20 (10.0)			
	TT	0 (0.0)	0 (0.0)			

*Statistically significant (using approximation of Woolf)

frequencies (p = 0.001) of the 7C/T variation between T2DM patients and healthy control subjects. Additionally, we found no association of C/T mutation with anthropometric indices (age, BMI) and metabolic characteristics (TC, TG, FBS, PPBS, RBS) in T2DM patients and healthy control subjects ($p > 0.05$). Although previous studies showed that variation near the MC4R gene is associated with BMI [47], we found a significant difference between metabolic characteristics (TC, TG, LDL, FBS, PPBS and RBS) in controls and diabetic subjects ($p < 0.01$). No significant influence of the MC4R rs17782313 was found on energy metabolism or biochemical variables in obese non-morbid premenopausal women [48]. However, the same variant near MC4R (rs17782313) showed evidence for association with LDL-C and TC in the Chinese Han population [49]. Another study indicated the significant association of rs17782313 polymorphism near the MC4R gene with type 2 diabetes risk, which was independent of BMI [50].

Several missense mutations in different transmembrane domains have been described. Furthermore, the data regarding intracellular transmembrane mutations are limited to the substitution of the asparagines of the codon 62 with a serine, found to produce severe obesity exclusively in the homozygous state in five children from a consanguineous pedigree [14]. Yeo et al. [51] and Vaisse et al. [52] reported the first MC4R mutation in humans in 1998. Later, Gu et al. [53] reported two missense mutations in 140 obese subjects investigated and Hinney et al. [45] reported nine missense mutations and two nonsense mutations in 306 obese children. Recently, Farooqi et al. [14] reported six novel missense mutations while Vaisse et al. [11] reported eight novel mutations. All affected subjects described have been heterozygous but only one homozygous mutation reported by Farooqi et al. [14].

In conclusion, we observed that the frequency of T allele at codon 7 of MC4R was higher in diabetic group than in the control group. People who have T allele at codon 7 may be at greater risk for developing diabetes (Table 2). Therefore, we conclude that MC4R codon 7 mutation is associated with diabetes and is a candidate genetic marker of the disease. This is the first report demonstrating that the 7C/T mutation of MC4R gene is associated with type 2 diabetes patients in Kashmiri population. A carefully designed study is required to examine the functional consequence of C to T mutation at codon 7 in type 2 diabetes patients. The calculated energy for wild-type protein is more (−14,217.289 kj/mol) compared to mutant protein (−14,498.063kj/mol). This change in energy of mutant protein is suggestive of affecting the protein tertiary

Table 3 Association of biochemical parameters and the 7C/T MC4R mutation

	Controls			T2DM			
Parameters	CC	CT	TT	CC	CT	TT	p value
Age (years)	50.32 ± 8.83	–	–	51.01 ± 12.88	53.75 ± 14.64	–	0.37
BMI (kg/m^2)	23.32 ± 1.70	–	–	23.26 ± 1.50	22.94 ± 1.46	–	0.36
TC (mg/dl)	151.55 ± 8.49	–	–	181.71 ± 9.74	182.49 ± 8.03	–	0.73
TG (mg/dl)	127.22 ± 5.38	–	–	154.26 ± 6.05	155.67 ± 5.60	–	0.32
LDL (mg/dl)	87.95 ± 8.80	–	–	104.96 ± 6.05	106.27 ± 6.47	–	0.36
HDL (mg/dl)	33.19 ± 5.98	–	–	32.52 ± 5.41	34.68 ± 4.82	–	0.08
FBS (mg/dl)	92.22 ± 10.71	–	–	190.21 ± 51.22	179.55 ± 29.37	–	0.36
PPBS (mg/dl)	109.65 ± 23.74	–	–	260.84 ± 77.27	242.00 ± 75.02	–	0.30
Random sugar (mg/dl)	110.42 ± 18.76	–	–	271.53 ± 70.52	257.60 ± 69.15	–	0.40

BMI body mass index, *TC* total cholesterol, *TG* triglycerides, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins, *FBS* fasting blood sugar, *PPBS* postprandial blood sugar

structure which may in turn have some impact on protein function. Therefore, further studies are needed to elucidate the actual role of this mutation on protein structure and function. Additionally, the mutation is present in coding sequence of the gene affecting the physico-chemical properties of the protein causing change from polar positively charged arginine to polar and sulfhydryl amino acid cysteine. Further studies are, however, needed to rule out the actual effect of the mutation on protein structure and function. Focused investigation is needed to establish the precise role played by MC4R in the type 2 diabetes development especially in the context of the above observed mutation.

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Author's contributions All authors have read and approved the manuscript. Rubiya Dar formulated and performed all the lab work. Ab Hamid Zargar provided diabetic samples. Tariq Jan analysed the results statistically. Shabhat Rasool helped in the lab work. Khurshid I Andrabi designed the work, edited the manuscript and co-ordinated the group and overall invigilator of the study.

Compliance with ethical standards

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

Research involving human participants and/or animals Humans/animals were involved during the course of research work.

Informed consent The study was performed with informed consent and following all the guidelines for experimental investigations required by the institutional review board or ethics committee of which all authors are affiliated vide letter no. SIMS 131 IEC/2009-2446 dtd: 17-12-2009.

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ORIGINAL ARTICLE

Interleukin-10 as an indicator of chronic course of oral candidiasis in diabetics: an in vitro study

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Abstract The importance of host defense against candidiasis and the role of cell-mediated immunity in host defense have been the subject of many studies. Increased expression of virulence factors in *Candida* isolates from diabetes mellitus (DM) patients with oropharyngeal candidiasis (OPC) has also been reported. To study the difference in T helper type 2 (Th2) and T helper type 1 (Th1) responses and neutrophil respiratory burst response, as a parameter of acute phase and adaptive immune responses, respectively, by using *Candida* isolates from lesions of oropharyngeal candidiasis (OPC) in DM patients compared to oral *Candida* isolates from healthy carriers. Analytical experimental laboratory-based study: (a) yeast identification and speciation was done. (b) Peripheral blood mononuclear cells (PBMCs) and neutrophils, obtained from healthy volunteers, were stimulated with oral *Candida* isolates of DM patients and healthy carriers (controls). Thereafter, cytokine analysis and neutrophil respiratory burst response analysis were done. IL-10 release was uniformly low at all concentrations of *Candida albicans* antigen isolated from healthy controls as compared to when *C. albicans* isolated from DM patients was used as antigenic stimulus. Mean INF-$_\gamma$ concentration was highest when *C. albicans*, isolated from healthy carriers was used as antigenic stimulus for PBMCs. There was no significant difference in neutrophil respiratory burst response among study and control groups. The observations highlight a significant IL-10 dominance, which may be an indicator of the inclination of host immune system toward a chronic progressive disease condition, indicating the pathogenic potential of select *Candida* strains.

Keywords Oral candidiasis · Immunity · Diabetes mellitus · Interleukin 10

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Introduction

Candidiasis is the most common fungal disease found in humans affecting mucosa, skin, nails, and internal organs of the body. *Candida albicans* still remains the most commonly isolated species; however, non-albicans *Candida* are emerging as recent pathogens [1].

Although found as commensal on mucosal surfaces of the body, virtually all patients who have one or more risk factors like patients receiving antibiotic treatment in neutropenia, human immunodeficiency virus (HIV) infection, organ transplantation, and in diabetes mellitus (DM), some form of mucosal candidiasis, most commonly oropharyngeal candidiasis (OPC), appear to manifest clinically.

C. albicans has several known virulence factors contributing to its pathogenicity; in addition, various cell wall antigens like mannan are seen to have immunomodulatory effect on host cells [2]. Hyperglycemia also aids in colonization and growth of a variety of organisms including fungal species; among fungal infections, OPC is seen in these patients in higher frequencies [3].

Hyperglycemia in DM patients is seen to be associated with the selection of strains of *C. albicans* with an enhanced ability to adhere to buccal epithelial cells [4]. *Candida* infection at the

mucosal surfaces can spread into tissues with a Th2 response whereas it is believed that Th1 responses are protective [5].

This study was designed to examine *Candida*-specific Th1/Th2 reactivity and the respiratory burst activity of the naïve immune-reactive cells by using oral *Candida* isolates of DM patients with oral candidiasis and oral *Candida* isolates from healthy carriers (controls). For this study, IL-10 and IFN-$_\gamma$ were taken as markers of Th2 and Th1 immune responses, respectively.

Materials and methods

This laboratory-based study was conducted in the department of microbiology, department of medicine at a tertiary care hospital, Delhi, India. The study was conducted over a period of 12 months including the enrolment, analysis, and compilation of data. The sample size was a randomly selected 30 subjects that included DM patients with clinical evidence of oral candidiasis, admitted in the medicine department, as the study group and 30 age-matched healthy controls. Patients in the study group were from 18 to 60 years of age, diagnosed as diabetic and manifesting oral candidiasis clinically.

Informed consent was obtained from the patients and an ethical clearance was obtained as per institutional guidelines. Every patient was given the right to opt out from study at any stage of the study. The identity of patients in study group was concealed. Patients on corticosteroid, antifungals, or antineoplastic drug therapy and persons diagnosed with malignancies, blood dyscrasias, xerostomia, or chronic renal disease were excluded from the study.

Random blood sugar (RBS) of both patients and control was estimated.

Two sterile cotton-tipped moist oral swabs or scrapings with blunt spatula were used to collect material from the oral lesions in both study and control groups. Three milliliters blood samples were taken from healthy volunteers in ethylenediaminetetraacetic acid (EDTA) vials, maintaining proper aseptic precautions, for separation of peripheral blood mononuclear cells (PBMCs).

Identification of *Candida* species was done as per standard procedure [1, 2, 6].

PBMC isolation from peripheral venous blood was done from healthy volunteers, using HiSep (HiMedia, Mumbai, India). The cells were suspended in 1 ml of Roswell Park Memorial Institute (RPMI) 1640 medium with 10 % fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, 2 mM L-glutamine (HiMedia, Mumbai, India) at a final cell concentration of 1×10^6 cells/ml [7].

Candida antigen was prepared following the protocol of Rimek et al. and stored at −20 °C [8, 9]. Protein concentration of the antigen extract was estimated by absorbance at 280 and at 260 nm.

PBMCs (1×10^6/ml) were distributed in 24-well plates in 1 ml of RPMI 1640 with added antibiotics and 10 % fetal calf serum. PBMCs were stimulated with 0.05, 0.5, and 5 μg/ml of *Candida* antigens. Phorbol myristate-12 acetate (PMA; Sigma-Aldrich, India) at a concentration of 100 ng/ml was used as positive control. Antigen concentrations were selected based on preliminary titration experiments at our laboratory and published literature [8]. Plates were incubated for 48 h at 5 % CO_2 concentration and temperature of 37 °C. Supernatants were transferred in 1.5-ml tubes stored at a temperature of −70 °C for cytokine analysis.

Cytokine analysis was done using IL-10 and IFN-$_\gamma$ ELISA kit (Diaclone, Weldon Biotech, India) for the quantitative determination of IL-10 and IFN-$_\gamma$, respectively, in supernatants. The standard curve was used to accurately determine the concentration of cytokines in the samples tested.

Neutrophil separation was done by dextran sedimentation method [10, 11]. Cells were counted and adjusted to a concentration of 2×10^6/ml in HBSS (HiMedia, Mumbai, India).

Candida isolate suspension was prepared in 5 ml of nutrient broth with 1 % glucose and incubated at 30 °C temperature for 7 to 8 days. Suspension of *Candida* isolates was centrifuged at 1500×*g* for 10 min [10]. Pellet was resuspended in gelatin-HBSS (HiMedia, Mumbai, India) at a concentration of $1–2\times10^7$ cells/ml by counting in Neubauer's chamber.

For nitroblue tetrazolium (NBT) dye reduction, three horizontal wells of an ELISA plate were marked as test, positive control, and negative control. One hundred microliters of suspended neutrophils (2×10^6/ml), 100 μl of NBT, 100 μl of *Candida* cells, and 50 μl of HBSS (HiMedia, Mumbai, India) were added in a test well. Fifty microliters of PMA (Sigma-Aldrich, India) and 50 μl of HBSS (HiMedia, Mumbai, India) were used as positive control and negative control, respectively. The ELISA plate was covered and incubated at 37 °C for 30 min. The amount of formazan produced was measured at 550 nm in an ELISA reader. The results were expressed as absorbance at 550 nm per 2×10^6 cells [10, 11].

Statistical analysis

Standard deviation (SD) and 95 % confidence intervals (CI) were calculated. Quantitative difference in cytokine production by PBMCs to mitogenic and/or antigenic stimuli and quantitative difference in formazan production by NBT dye reduction test, for study and control groups, was identified by the Mann-Whitney *U* post hoc test. Quantitative variables between study and control groups were tested by independent *t* test. In all cases, significance was defined as $p<0.05$.

Results

On examination of oral cavity of patients, white patches on the tongue or mucosa were found to be the predominant lesion (Table 1). Out of a total of 30 samples from diabetic patients, *C. albicans* was isolated from 70 % of the oral cavity lesions. Among healthy volunteers, 7 out of 30 (23.33 %) showed the growth of *C. albicans*, in the rest, no growth was observed.

White patches on the tongue or mucosa were the most common type of lesion present in the cases (15 cases), followed by ulcer on the mucosa or tongue (5 cases), angular cheilosis (4 cases), an erythematous lesion on the tongue (3 cases), white patches and angular cheilosis (1 case), white patches and ulcer on the mucosa (1 case), and angular cheilosis and ulcer on the mucosa (1 case).

The mean IL-10 concentrations were significantly higher than the mean IFN-$_\gamma$ concentrations at antigen concentrations ranging from 0.05 to 5 μg/ml in a diabetic study group. The mean IFN-$_\gamma$ concentrations were significantly higher than the mean IL-10 concentrations at similar antigen concentrations in healthy carriers (control) (Fig. 1).

IL-10 release from antigen-stimulated PBMCs was uniformly high at any concentration when the antigen was derived from *C. albicans* isolated from diabetic study group (Table 1). IFN-$_\gamma$ levels were significantly higher for all antigen concentrations when antigens where derived from healthy carriers (Table 2). Mitogen (PMA at 100 ng/ml) stimulated PBMCs, secreted IL-10 at a concentration of 351.38 pg/ml, and IFN-$_\gamma$ at a concentration of 266 pg/ml.

Neutrophil response on stimulation with *C. albicans* obtained from diabetes mellitus patients showed mean OD of 0.8497 which did not vary significantly with neutrophil response on stimulation with *C. albicans* obtained from healthy carriers (OD of 0.8235). PMA taken as positive control showed mean OD value of 0.8233, indicating the viable state of neutrophils.

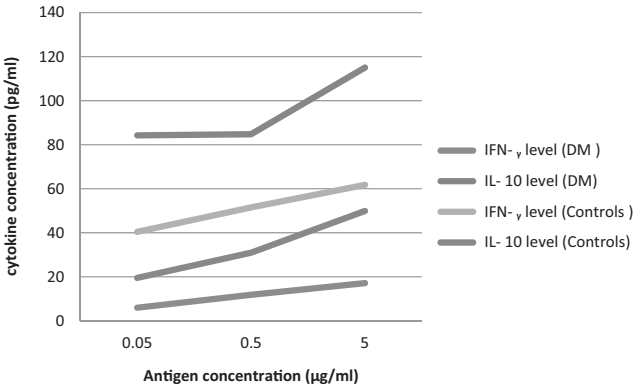

Fig. 1 Interleukin (IL)-10 and interferon (IFN)-$_\gamma$ response of peripheral blood mononuclear cells (PBMCs) to stimulation by oral *Candida* antigens from diabetes mellitus patients presenting with oropharyngeal candidiasis and from healthy controls

Discussion

DM comprises a group of common metabolic disorders that shares the phenotype of hyperglycemia. In 1997, the American Association of Diabetes proposed a classification system for diabetes based on its etiology. Therefore, diabetes is currently classified as type 1 or juvenile diabetes and type 2 or acquired diabetes. According to the World Health Organization (WHO), about 9 % of the world adult population has diabetes [12]. Individuals with DM have a higher frequency and severity of infections. Oral candidiasis due to *C. albicans* has been reported to be increased in patients with DM than in normal control which conforms to the findings of this study [13, 14].

In our study, *C. albicans* carriage among healthy controls was 23.33 %. Other authors have reported similar rates of *C. albicans* carriage [14]. The noninflammatory mechanism of the apparently healthy epithelial cells is a means to protect the colonization of *Candida* in contact with epithelial cells. Probably, the growth inhibitory signals occurring following colonization with *Candida* is a part of a symbiotic relationship where *Candida* sacrifices growth for protection against killing [15].

Table 1 IL-10 response after stimulation of peripheral blood mononuclear cells with *Candida* antigens obtained from the study group and healthy carriers (controls)

Study groups and control	Antigen conc. (μg/ml)	Mean IL-10 cytokine levels (pg/ml)
Candida albicans isolated from healthy carriers	0.05	19.5
	0.5	30.95
	5	49.9
Candida albicans isolated from diabetes mellitus patients	0.05	84.25
	0.5	84.75
	5	115.05
Positive control PMA[a]	100 (ng/ml)	351.38

[a] PMA—phorbol myristate-12 acetate

Table 2 IFN-$_\gamma$ response after stimulation of peripheral blood mononuclear cells with *Candida* antigens obtained from study group and healthy carriers (controls)

Study groups and control	Antigen conc. (μg/ml)	Mean IFN[a]-$_\gamma$ cytokine levels (pg/ml)
Candida albicans isolated from healthy carriers	0.05	40.45
	0.5	51.50
	5	61.75
Candida albicans isolated from diabetes mellitus patients	0.05	6
	0.5	11.83
	5	17.17
Positive control PMA[b]	100 (ng/ml)	266

[a] Interferon gamma

[b] PMA—phorbol myristate-12 acetate

In our study, a pseudomembranous variant (white patch) was the most common clinical presentation of OPC, which is in concordance with previous studies [16]. Also, a mean RBS value of 251.56 mg/dl (SD 95.40, *p* value 0.008) showed a significant association with development of OPC in diabetic patients. This observation is in line with previous studies depicting hyperglycemia as one of the risk factors for development of OPC in diabetic patients [3, 4]. *C. albicans* strains bind to fucose-containing and N-acetylgalactosamine-containing lipids extracted from human buccal cells. The authors concluded that the existence of several adhesin-receptor systems contributes to the virulence of *C. albicans*. The carbohydrate composition of receptors probably plays an important role in the susceptibility to infections in diabetics [4, 17].

The role of *Candida* strain selection under these underlying stressful conditions may lead to an increased expression of virulence attributes, resulting in a more chronic and fulminant course of oral candidiasis, as established by other workers [18, 19].

In our study, we evaluated differences in the acute and adaptive host immune responses to different strains of *Candida* species isolated from diabetic patients and healthy carriers (controls). Neutrophils are capable of ingesting and digesting exogenous antigens, in a process known as phagocytosis, which is an important arm of acute or immediate immune response. During phagocytosis, a metabolic process known as the respiratory burst occurs in activated neutrophils. This process results in the activation of a membrane-bound oxidase that catalyzes the reduction of oxygen to superoxide anion, a reactive oxygen intermediate that is extremely toxic to ingested microorganisms.

In this experiment, the *Candida* strains isolated from both study and control groups stimulated the neutrophils and generated a similar acute immune response. It also highlights as in the previous studies that neutrophils are not the only cells responsible, but adaptive immune responses also have major roles to play as mediators of immune response in *Candida* infections [20].

Oral candidiasis often starts at mucosal level, and CMI is believed to prevent the spread of infection to tissue. The cytokines produced by monocytes and Th1/Th2 subsets of T helper cells determine the progression of an early OPC to a non-healing chronic mucocutaneous candidiasis (CMC) or may even be responsible for its resolution [21].

In our study, Th2 response as represented by mean peak IL-10 (115.05 pg/ml) concentration was highest when *C. albicans* isolated from diabetic patients was used as an antigenic stimulus for healthy PBMCs. Mean peak IL-10 concentration was lowest (49.90 pg/ml) when *C. albicans* isolated from healthy carriers was used as an antigenic stimulus. The increase in IL-10 concentration in study groups, as compared to the controls, probably indicates a possibility of an enhanced expression of virulence factors leading to strain selection in the diabetic patients; moreover, predominance of Th2 response indicates toward the ability of these strains to overcome the protective Th1 responses, as previously documented by other studies or allow their survival [18, 22]. Subsequently, this may predispose to a more fulminant course of oral candidiasis leading to the development of chronicity as a result of an imbalance in the immune system [21].

Mean IFN-γ (61.75 pg/ml) concentration was highest when *C. albicans*, isolated from healthy controls, was used as an antigenic stimulus for PBMCs. This finding can be explained by the fact that in healthy carriers, the overgrowth of *Candida* is kept in check by various innate and adaptive immune response strategies, where Th1 cytokines (IFN-γ) have a predominant role to play and maintain an immunological balance. These commensal strains, when exposed to proper growth conditions, may express the virulence attributes, which may be there in a dormant state in an immunocompetent individual.

The behavior of the *Candida* strains isolated from diabetic patients on the cytokine response highlights the fact that there is a significant IL-10 dominance, which is an adaptation of the host immune system toward a chronic progressive disease

condition indicating the pathogenic potential of these *Candida* strains when encountered by a host. The minimal dose response of IFN-γ only indicates a suppression of Th1 differentiation and its negligible anti-candidicidal role when encountering these pathogens.

It is important understand the adaptive immune response of patients with OPC in diabetes patients to *Candida* antigen, whether influencing the host response toward a favorable outcome or leading to chronicity which may help the clinicians to predict the course of the disease and plan management. Looking at the increased levels of IL-10 in study groups as compared to INFγ, rise in Th2 response can be used as a predictor of chronicity of OPC in these groups of patients, as with the constant stimulation with *Candida* antigen, the immune response tend to become anergic. This can be antigen specific or it may also lead to a dampened immune response to other pathogens, eventually leading to superadded infections. This further strengthens the debate in favor of using immunomodulators as a treatment in mucosal candidiasis in patients showing fungal relapse to conventional antifungals, as documented by previous studies [23, 24]. It is also important to emphasize that in in vivo conditions, many helper T cells do not show either a Th1 or a Th2 profile; individual cells have shown striking heterogeneity in the Th-cell population. One of the best described of these is the Th0 subset, which secretes IL-2, IL-4, IL-5, IFN-γ, and IL-10, as well as IL-3 and GM-CSF [25]. Hence, further studies are needed to understand the observed immunological phenomenon and it is in vivo implications which may determine the outcome of the host to the infections.

Compliance with ethical standards Informed consent was obtained from the patients and an ethical clearance was obtained as per institutional guidelines. Every patient was given the right to opt out from study at any stage of the study. The identity of patients in study group was concealed.

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ORIGINAL ARTICLE

Expression of glucokinase, glucose 6-phosphatase, and stress protein in streptozotocin-induced diabetic rats treated with natural honey

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Abstract Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and associated with oxidative stress. Based on literature, honey components could treat diabetes with unknown mechanism. The present study investigated the hypoglycemic effects and antioxidant activities of honey at the molecular level in STZ-induced diabetic rats. Using an animal model of diabetes, we investigated antidiabetic and antioxidant properties of natural honey. For this fasting blood glucose, malondialdehyde (MDA) level as a marker of lipid peroxidation and total antioxidant capacity were measured in diabetes-induced rats treated with natural honey. The transcript levels of stress proteins including heat shock protein 70 (HSP70), glucokinase (GK), and glucose 6-phosphatase (G6P) were determined using quantitative real-time PCR. Statistical analysis showed that honey significantly decreased MDA levels; in contrast, it increased total antioxidant capacity in diabetic rats ($p<0.05$). Quantitative real-time PCR (QRT-PCR) analysis revealed that expressions of HSP70 and G6P decreased while the expression of GK increased in honey treatment groups in comparison with control group. These findings provide insight into the molecular mechanisms behind the hypoglycemic and antioxidative effects of honey that may be considered for further clinical studies in drug development.

Keywords Antioxidant · Diabetes · Glucokinase · Honey · Oxidative stress

Introduction

Diabetes remains a major public health problem with increasing global prevalence [1]. It is a metabolic disorder characterized by hyperglycemia [2] and a heterogeneous disorder with multiple etiologies [3].

Hyperglycemia can stimulate free radical production which in turn causes cell injury. It is well known that increase in oxidative stress levels, defined as a persistent imbalance between the generation of free radicals [especially reactive oxygen species (ROSs)] and the antioxidant defense system, plays an important role in the development of diabetic complications [4].

Heat shock proteins (HSPs), also called stress proteins, are a highly conserved family of proteins essential for all organisms to maintain cellular homeostasis in multiple stress conditions [5–7]. These proteins are upregulated under stressful conditions such as high temperature, hypoxia, ischemia, endotoxins, heavy metals, and reactive oxygen species and act as chaperones to prevent protein misfolding [7, 8].

Previous studies have demonstrated that antioxidants can reduce markers of oxidative stress in both experimental and clinical models of diabetes [9–11]. Diabetes increase inflammation, oxidation, and glycation; therefore, agents with strong antioxidant properties may have the potential for limiting the progression of diabetes and its related complications [4, 10, 11]. Honey is a natural substance with many medicinal effects such as hypoglycemic and antioxidant effects [12], which

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indicate it as a favorable candidate for diabetes research. Evidence indicates that honey can exert several health-beneficial effects such as gastroprotective [13], hepatoprotective [14], reproductive [15, 16], hypoglycemic [17], antioxidant [17], antihypertensive [18], antibacterial [19], antifungal [20], and antiinflammatory [21] effects. However, the possible beneficial effects of this natural product in controlling the progression of diabetes need more attention.

The aim of the present study was to investigate the role of honey in treatment of diabetes-induced rats by studying the expression levels of key enzymes in glucose metabolism including glucokinase (GK) and glucose 6-phosphatase (G6P).

Material and methods

Study design

In this study, 35 adult male wistar rats aged 6–8 weeks with body mass ranging from 200±20 g were used. The animals were housed in cages, fed a rat chow diet (Pars Dam Co., Tehran, Iran), and given water ad libitum. Diabetes was randomly induced into 28 rats by intraperitoneally injecting streptozotocin (60 mg/kg body mass) 2 weeks prior to the initiation of treatments. After 1 week, animals with a serum glucose concentration exceeding 16 mmol/L were considered to be diabetic. The 28 diabetic rats were randomly divided into four groups (n=7). The first two groups were treated orally with 1 and 2 g of natural honey per kilogram of body weight for 21 days, respectively. The third group was the diabetic control group and received 0.5 mL of distilled water daily. The fourth group of diabetic rats defined as positive control received once daily oral dose of glibenclamide. Seven healthy rats received 0.5 mL of distilled water daily during this period and were defined as the healthy control group. The used doses of natural honey were based on Erejuwa et al. study [17]. Honey sample considered in this study was from jujube plant area that was provided by local beekeepers living in South Khorasan, Iran, during the year 2014 flowering. At the end of the experiment, blood samples were collected and serum levels of glucose and other biochemical factors were measured. For gene expression analysis, the liver of animals were quickly removed and placed in liquid nitrogen. All animal procedures were approved by the ethical committee of Birjand University of Medical Sciences in accordance with the Institutional Animal Ethics Committee.

Sample preparation

At the end of the experimental period (21 days), the rats were anesthetized with ether and blood samples were taken from their hearts. Blood samples were centrifuged at 2500 rpm for 10 min to obtain serum. The serum was kept at −20°C until further testing.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was done according to the procedures of Benzie and Strain (1996) for evaluating total antioxidant power in the serum of the five groups mentioned above. In presence of antioxidant activity and low pH levels in the samples, ferric iron was reduced to its ferrous state and led to the formation of an intensive blue ferrous-tripyridyltriazine complex that could be monitored at a maximum absorption of 593 nm. Two milliliter of FRAP reagent [consisting of 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ) solution in HCl, and 20 mM $FeCl_3$ $6H_2O$ solution in a proportion of 10:1:1 (v/v)] was added to 50 L of sample. After 15 min, the absorbance was measured at 593 nm [22].

Measurement of thiobarbituric acid reactive substances

At the end of the treatment period, blood was collected from the hearts of the rats and used for measuring malondialdehyde (MDA) levels with the thiobarbituric acid reactive substance (TBARS) method [23]. Serum samples (300 μL) were added to 3 mL TBARS reagent (7.5 g trichloroacetic acid, 187 mg TBA, and 6.25 mL chloric acid). Then, the mixture was warmed for 20 min in a boiling water bath. Finally, the absorbance of samples was determined at 532 nm.

Histological studies

For evaluation of pancreatic cell regeneration in response to honey, number of pancreatic islets was calculated. For this, pancreatic tissue was removed and immediately fixed in 10 % formalin. After fixation, paraffin blocks were cut into 5-m-thick sections and stained with hematoxylin and eosin. Then, the stained sections of pancreatic tissue were examined under a light microscope. The total number of islet cells in each section was enumerated and, on graph paper, the total area of each stained section was characterized, and the average number of pancreatic islets per square centimeter was calculated.

RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA from the liver tissue of diabetic and normal rats was isolated using the TRIzol extraction reagent (Bioneer, Korea), according to the manufacturer's recommendations. The integrity of messenger RNA (mRNA) was confirmed by electrophoresis in a denaturing 1 % agarose gel. The RevertAid H Minus First Strand cDNA Synthesis Kit

(Fermentas, USA) was used to reverse-transcribe 1 μg of RNA in a final volume of 2 μL. Specific primers for quantitative real time-PCR of β-actin (reference gene), HSP70, GK, and G6P were listed in Table 1. The reaction mixture of 2 μL consisted of 2× ABI SYBR Green PCR Master Mix, 2 μL cDNA, and 0.2 μL of each primer. Amplifications were performed on the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with 40 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 30 s, and data collection at 80 °C for 20 s. The intensities of the mRNA levels were normalized to those of the β-actin product as ratios of producing arbitrary units of relative abundance. The relative gene expression ($2^{-\Delta\Delta CT}$) between the groups treated with honey and the diabetic control group was assayed using the C_T method [24].

Statistical analysis

Using SPSS version 16 software, the data were statistically computed by one-way ANOVA procedure and subsequently means compared by the Student's *t* test. A p value <0.05 was considered statistically significant. Data are expressed as means±SD. GraphPad Prism software for Windows (version 5) was used for drawing figures.

Results

Effects of honey on oxidative stress status

The effects of honey on biochemical parameters of streptozotocin (STZ)-induced diabetic rats were studied. As it has been shown in Table 2, the level of MDA as lipid peroxidation index in diabetic rats treated with honey significantly decreased when compared with diabetic control rats. The level of reduction was as good as glibenclamide consumption. Moreover, total antioxidant levels in both groups of rats treated with honey remarkably increased in comparison with diabetic control group.

Table 1 Real-time primer sequences

Gene	Sequences
HSPA4 (HSP70)	5′-TGGCATTTTCAGTGTGTCCAG-3′ (forward) and 5′-CACCTGCATCTTCTCTTCTTCCT-3′ (reverse)
GK	5′-TGGTGCTTTTGAGACCCGTT-3′ (forward) and 5′-GAAGCCCCAGAGTGCTTAGG-3′ (reverse)
G6p	5′-CGTCACCTGTGAGACTGGAC-3′ (forward) and 5′-ACGACATTCAAGCACCGGAA-3′ (reverse)
β-actin	5′-GTCCACCCGCGAGTACAAC-3′ (forward) and 5′-GACGACGAGCGCAGCGATA-3′ (reverse)

Effects of honey on blood glucose and beta cell regenation

Table 2 also summarized hypoglycemic effects of natural honey. Honey consumption in diabetic rats decreased dose-dependent fasting blood glucose (FBG) level (25 mmol/L in diabetic rats) to normoglycemic level (11 mmol/L). In line with this alteration level in FBG, the number of pancreatic islets notably increased in diabetic rats treated with honey when compared to diabetic control group. This increase in pancreatic islets is significant in higher dose of honey.

Effects of honey on body weight

The effects of honey on the body weight of the control and honey-treated rats are summarized in Table 3. As shown in the table, the diabetic groups treated with honey exhibited an increase in the body weight ($p<0.05$; initial body weight in comparison with final body weight), with values approximately similar to those observed in the non-diabetic control rats. In fact, honey consumption improved diabetes-induced weight loss.

Effects of honey on glucokinase and glucose 6-phosphatase expressions

Expression levels of GK and glucose 6-phosphatase were assessed after treatment with natural honey. As it has been shown in Fig. 1, the expression level of G6P in diabetic rats notably increased ($p<0.05$) compared with normal groups. Of note, the expression level of this gene significantly ($p<0.05$) decreased when treated with natural honey. Moreover, there was a significant decrease in GK expression of the diabetic rats compared with normal group and oral consumption of honey significantly increased expression level of GK in a dose-dependent manner ($p<0.05$; Fig. 1).

Effect of natural honey on HSP70 expression

As it has been indicated in Fig. 2, significant increase was manifested ($p<0.05$) in mRNA expression of HSP70 levels in diabetic rats when compared to normal tissue of the healthy control group. Honey consumption in two specified doses could decrease transcript level of HSP70 as glibenclamide did.

Discussion

The results of the present study revealed that oral consumption of natural honey could ameliorate oxidative stress and stress protein expression in streptozotocin-induced diabetes. In

Table 2 Effects of natural honey on biochemical parameters of normoglycemic and STZ-induced diabetic rats

Experimental groups[a]	Fasting blood glucose (FBG) (mmol/L)	Malondialdehyde (MDA) (μmol/L)	Total antioxidant (μmol/L)	Number of pancreatic islets (cm^{-2})
Normoglycemic control	7.6±0.4[b, c]	1.27±0.5[c]	780±14[c]	41±3.5[c]
Diabetic control	25±1.2	4.98±0.7	550±21	16±1.8
Diabetic + natural honey (1 g/kg body mass)	12.4±0.7[c]	3.19±0.3	635±18[c]	22±1.9
Diabetic + natural honey (2 g/kg body mass)	11.2±0.4[c]	2.52±0.1[c]	690±16[c]	29±2.6[c]
Diabetic + glibenclamide (0.6 mg/kg body mass)	8.5±0.9[c]	1.9 ±0.4[c]	710±24[c]	38±4.3[c]

[a] For details of experimental conditions, see the text

[b] Data are expressed as mean±SD of seven rats in each group

[c] Significant at $p<0.05$ when compared with the diabetic control group

addition, the present study showed hypoglycemic effect of honey that could be through modifying expression of key metabolic enzyme such as GK and G6P. In diabetes, the liver is susceptible to oxidative stress and damage; therefore, agents with high antioxidant properties may have beneficial effects on hepatic oxidative stress [4, 10, 11]. Several studies have shown the antioxidant properties of different varieties of honey from various countries [25–33]. In addition, it has been previously shown that the antioxidant activity of honey is related to its flavonoids and phenolic compounds [29, 30, 34, 35]. The current study investigated the hypoglycemic and antioxidant effects of natural honey in rats with STZ-induced diabetes. According to the results indicated in Table 2, streptozotocin-induced diabetic rats showed increased levels of MDA and decreased levels of total antioxidant capacity. Treatment with honey improved oxidative stress status, so honey could be a good antioxidant (Table 2). The findings of the current study are in agreement with the results of Erejuwa et al. [36]. According to their report, treating diabetic rats with glibenclamide alone produced no significant reductions in MDA levels or glutathione peroxidase (GPx) and catalase (CAT) activities, but the combination of glibenclamide and honey showed increased CAT activity, reduced MDA levels, and GPx activity. The results of Erejuwa et al. (2011) demonstrated the beneficial effects of honey on oxidative stress parameters in diabetic rats [36].

The average 1:1 ratio fructose to glucose was found in honey. Erejuwa et al. proposed that fructose, as a major constituent in honey, may contribute to its hypoglycemic effects. They also suggested that the fructose in honey might enhance the uptake of glucose via the liver, resulting in improved glycemic control in diabetes mellitus [17]. A hypoglycemic effect of natural honey was reported in another study [37]. Al-Waili et al. showed that administration of natural honey increased insulin and in contrast decreased blood glucose level in diabetic patients. Glucose-lowering effect of natural honey also was seen in present study. Based on our results, honey may decrease blood glucose through activation of glycolysis pathway (increasing the expression level of GK); in contrast, it decreases the expression level of G6P (inhibition of gluconeogenesis) (Fig. 1). The results of the current study showed a significant decrease (fivefold) in G6P expression by honey compared with diabetic control group ($p<0.05$). There was also a significant decrease of GK in diabetic rats when compared with normal tissue, while consumption of honey significantly increased the expression level of GK (fourfold) in a dose-dependent manner ($p<0.05$; Fig. 1), suggesting that honey may be have more specific role in inhibition of gluconeogenesis pathway than induction of glycolysis. Oxidative stress status evaluation in diabetic rats showed high level of stress protein in diabetic rats (eightfold increase of HSP70 in diabetic rats compared with normoglycemic) (Fig. 2). Oral administration of natural honey had an inhibitory effect (nine times decrease) on stress protein expression. Metabolic disorders (hyperglycemia and dyslipidemia) activate heat shock factor (HSF)/HSP pathway, which was associated with oxidative stress, increased generation of inflammatory mediators, vascular inflammation, and cell injury. Knockdown of HSF1

Table 3 Effects of natural honey on body weight of diabetic rats

Experimental[a] group parameters	Non-diabetic control	Diabetic control	Honey (1 gm/kg)	Honey (2 gm/kg)	Glibenclamide (0.6 mg/kg)
Initial body weight (gm)	221±9[b]a	190±10a	193±11a	187±8a	180±10a
Final body weight (gm)	235±8[b]a	170±13b	215±14a	218±9a	228±12a

In each row, figures bearing letters a and b are significantly different at $P\leq0.05$ (one-way ANOVA)

[a] For details of experimental conditions, see the text

[b] Data are expressed as mean±SD of seven rats in each group

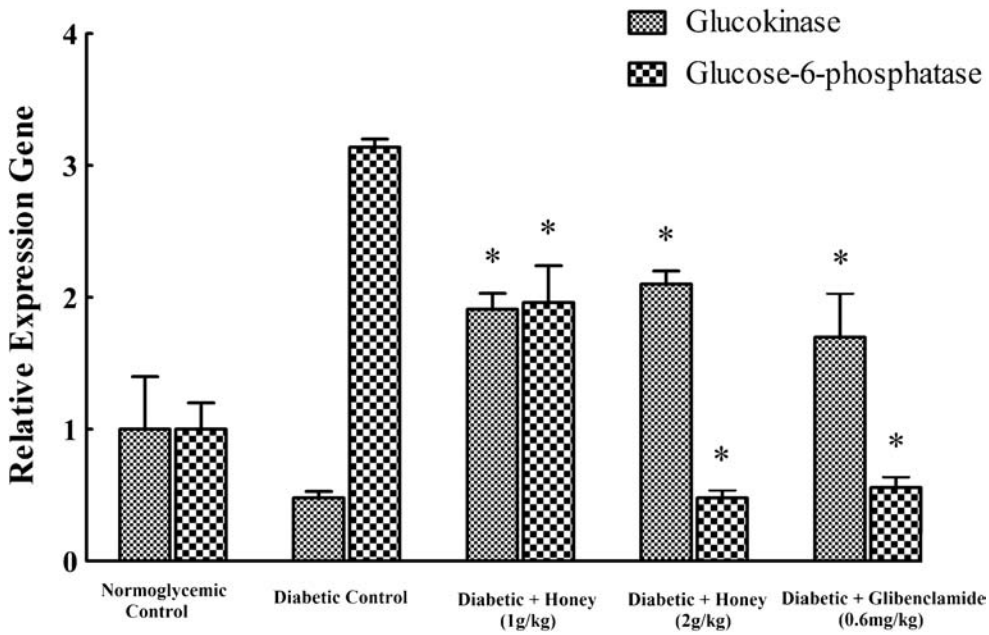

Fig. 1 Effects of natural honey on the expressions of GK and G6P in mRNA levels. Natural honey changed their expression in the liver tissue of diabetic rats in a dose-dependent manner. Data represent relative gene expression (target/β-actin) and mean±SD of three repeats. *$p<0.05$ was considered significant when compared with the diabetic control sample

reduced the activation of key inflammatory mediators in vascular cells [38]. Our findings demonstrate that the expression of HSP70 was significantly increased in the liver tissue of diabetic rats and honey supplementation reduced the expression of HSP70 in the liver tissue of the treated group compared with that of the diabetic control group (see Fig. 2). Hunter-Lavin et al. showed that serum HSP70 levels were higher in non-insulin-treated type-2 diabetes subjects in comparison to insulin-treated subjects. They concluded that HSP70 may therefore be a suitable marker of the severity of the clinical condition and may be useful in the monitoring of type-2 diabetes as well as other diseases associated with oxidative stress [39].

In this study, we showed that hyperglycemia-mediated oxidative stress could induce the expression of HSP70 as a stress protein and honey treatment down-regulate oxidative stress markers and gene-related expressions (Fig. 2).

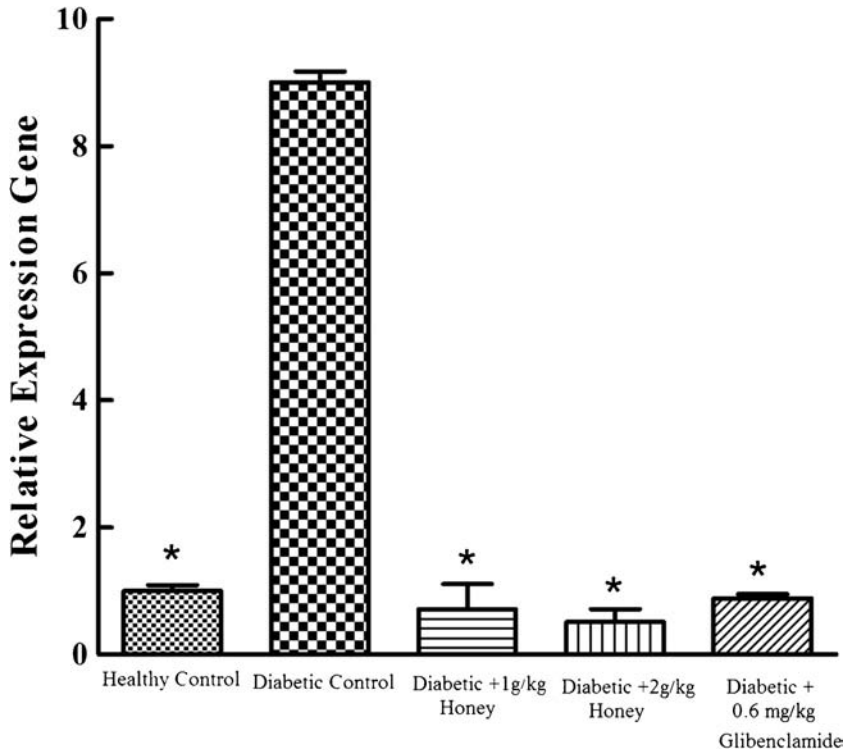

Fig. 2 Effects of natural honey on the expression of HSP70 in mRNA level. Natural honey decreased the HSP70 expression in a dose-dependent manner in the liver tissue of diabetic rats. Data represent relative gene expression (HSP70/β-actin) and mean±SD of three repeats. *$p<0.05$ was considered significant when compared with the diabetic control sample

Although the mechanism by which honey neutralize reactive oxygen species is not understood thoroughly, it seems that the scavenging activity of honey is implemented through its phenolic acid compounds [40]. According to our results, hypoglycemic and antioxidant effects of honey resulted in reduction of malondialdehyde as lipid peroxidation index, G6P enzyme, and stress protein. Correspondingly, it increased total antioxidant capacity and the number of pancreatic islets as well. Interestingly, oral administration of honey played an effective role in improvement of body weight loss in diabetic rats; this may be due to accumulation of adipose tissue (Table 3).

In this regard, it seems that honey can be used as potential therapeutic agent in diabetes mellitus and also for designing of new drugs obtained from natural products; however, further studies on its mechanisms and molecular pathways are required to determine this strategy.

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Compliance with ethical standards

Conflict of interest All contributing authors declare no conflicts of interest.

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SHORT ARTICLE

Helicobacter pylori infection in elderly Chinese patients with type 2 diabetes

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Abstract The relationship between *Helicobacter pylori* infection and diabetes is unclear, especially in elderly people. The aims of this study were to determine the possible relationship between *H. pylori* infection and type 2 diabetes (T2DM) in elderly subjects. In total, 238 T2DM patients (83.66± 3.10 years) and 704 non-diabetic patients (83.29±3.17 years) were included. We measured the glycated hemoglobin level, lipid profile, presence of *H. pylori* using the urea breath test, and fasting serum insulin level. The *H. pylori* infection rate was 45.0 and 39.6 % in T2DM patients and control, respectively. The results were statistically insignificant. In the logistic regression analysis of 238 T2DM patients (including age, sex, diabetes, body mass index, the duration of diabetes, and the levels of serum total cholesterol, triglyceride, hemoglobin A1C, and insulin), no factors selected were associated with *H. pylori* infection. The *H. pylori* infection rate may not be increased in elderly Chinese T2DM patients. However, further studies are needed to confirm our conclusion.

Keywords *Helicobacter pylori* · Elderly · Type 2 diabetes · *H. pylori*

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Introduction

Population aging is a worldwide trend. Elderly people easily suffer from a variety of digestive diseases. Over the past few years, *Helicobacter pylori* has been identified as a risk factor of peptic ulcer disease and gastric malignancies [1], and compelling data have suggested that there is a high prevalence of *H. pylori* infection in old people [2].

Diabetes mellitus is another very common chronic disease that affects older adults' health, diminishes quality of life, and increases health care costs. Diabetic patients are likely to be affected by infection and have a high prevalence of gastrointestinal symptoms, so it is possible that there are some relationships between *H. pylori* and diabetes. However, this relationship is unclear. Some studies have reported high prevalence of *H. pylori* infection in diabetic individuals [3, 4], but some other studies have not reported any association between *H. pylori* infection and diabetes [5, 6]. One study [7] even reported that the prevalence of *H.* pylori infection is lower in diabetic patients.

Thus, it is interesting and meaningful to examine *H. pylori* infection in the context of diabetes, especially in very elderly patients. To our knowledge, few reports have investigated the relationship between *H. pylori* and diabetes in elderly people (aged >75 years). The objective of the present study was to determine the prevalence of *H. pylori* infection in older patients (aged >75 years) with type 2 diabetes (T2DM) and examine the possible association between them.

Patients and methods

This study was approved by our hospital's regional ethics committee, and all patients signed informed consent.

Study population

In total, 238 T2DM patients from the Wangjiangshan Branch of Zhejiang Provincial People's Hospital, Hangzhou between January 2012 and October 2012 were included in the study. Seven hundred and four non-diabetic patients who had undergone a urea breath test (UBT) during the same period were included as a comparison group.

Exclusion criteria for both groups were as follows: those with a peptic ulcer, gastrointestinal tract tumor, prior gastrointestinal surgery; those who had taken antibiotics, bismuth-containing compounds, proton pump inhibitors, or H_2 receptor blockers within 1 month; and those with severe complications such as heart failure and a cerebrovascular accident.

Methods

Fasting blood samples were drawn for glycated hemoglobin (HbA1c), triglyceride (TG), and total cholesterol (TC) levels. HbA1c was measured using the D-10 Hemoglobin Testing System (Bio-Rad Laboratory). The TG and TC levels were measured using an automated clinical chemistry analyzer (Olympus Diagnostica GmbH). *H. pylori* infection was determined by a UBT. All subjects were asked to drink a solution containing ^{13}C-urea. Breath samples (at baseline and 30 min after drinking the solution) were collected. The HCBT-01 breath test automatic instrument (Zhonghe Headway Bio Sci & Tech Co., Ltd.) was used to measure the $^{13}C/^{12}C$ ratio (δ ‰). The $\Delta\delta^{13}CO_2$‰ was calculated, and it was considered positive when $\Delta\delta^{13}CO_2$ was >3.5. Body mass index (BMI) was computed by weight in kilograms divided by height in meters squared.

Statistical analyses

All analyses were performed using SPSS 13.0 (IBM Corp.). Parametric and non-parametric data were compared using a two-sided *t* test and Mann–Whitney *U* analysis, respectively. The chi-square test (χ^2) was used for categorical variables. The significance level was set at $p<0.05$.

Results

Nine hundred forty-two patients (238 T2DM and 704 non-diabetic patients) were included in the study. There was no difference between two groups in terms of age, the sex ratio, BMI, UBT, and the *H. pylori* infection ratio. Table 1 summarizes the parameters examined. In logistic regression analysis (including age, sex, diabetes, and BMI), no factors selected were associated with *H. pylori* infection.

Table 1 Clinical and laboratory parameters of diabetic patients and controls

	Diabetic patients (n=238)	Control (n=704)	P value
Age (years)	83.66±3.10	83.29±3.17	0.053
Sex (F:M)	51:187	131:573	0.341
BMI (kg/m^2)	24.83±3.29	24.30±3.25	0.059
UBT	5.97±9.91	5.05±10.15	0.069
HP (%)	45.0 %	39.6 %	0.149

HP Helicobacter pylori

Of 238 T2DM patients, 107 patients were *H. pylori* positive and 131 were *H. pylori* negative. There was no difference between age, BMI, the duration of diabetes, the level of serum TC, TL, HbA1C, and insulin, except for the sex ratio. Table 2 summarizes the parameters examined. In the logistic regression analysis of 238 T2DM patients (including age, sex, BMI, the duration of diabetes, and the levels of serum TC, TG, HbA1C, and insulin), no factors selected were associated with *H. pylori* infection, except for the sex ratio (odds ratio (OR) 0.352, 95 % confidence interval (CI) 0.169~0.737).

Discussion

Diabetes mellitus has been a public health problem worldwide, and *H. pylori*, which is associated with gastric diseases, is considered as one of the most common human bacterial pathogens. However, the relationship between these two is not clear. Recently, a meta-analysis of >20,000 participants [8] demonstrated a significantly higher prevalence of *H. pylori* infection in those with type 1 diabetes (OR 1.99, 95 % CI 1.52–2.60) and type 2 diabetes (OR 2.15, 95 % CI 1.81–2.55) than in those without diabetes. In our study, although the *H. pylori* infection rate was higher in diabetic patients, there was no statistically significant difference between two groups, which is consistent with previous research

Table 2 Clinical and laboratory parameters of diabetic patients with or without *Helicobacter pylori* (HP) infection

	HP positive (n=107)	HP negative (n=131)	P value
Age (years)	83.68±3.00	83.64±3.19	0.696
Sex (F:M)	36:95	15:92	0.012
BMI (kg/m)	24.54±2.96	25.07±3.53	0.208
TC (mmol/L)	4.56±0.94	4.68±1.02	0.325
TG (mmol/L)	1.44±0.84	1.43±0.83	0.528
HbA1c (%)	6.77±0.85	6.78±0.94	0.749
Duration of diabetes (years)	9.38±7.32	11.15±8.18	0.086
Insulin (μIU/mL)	12.32±17.97	22.77±74.51	0.459

TC total cholesterol, *TG* triglyceride, *HbA1c* hemoglobin A1C

performed in the Hong Kong Chinese population [9]. Age may be a critical factor concerning this discrepancy. Studies conducted in the past have shown that the rate of *H. pylori* infection increases with age, reaching 40–60 % in asymptomatic elderly individuals [10], but a remarkable reduction was noticed in very elderly people (aged >85 years) [11]. Subjects included in our study were older than those in most other studies; thus, the rate of *H. pylori* infection was relatively low in our population, and the difference between groups was not large enough to reach significance. Socioeconomic status may be another important factor. Quite a lot of researches [12] have shown that improvement in the socioeconomic status can minimize *H. pylori* infection. Since most of our subjects in both groups were retired cadres, they had good insurance coverage and experienced favorable medical and sanitation conditions. These factors can reduce *H. pylori* infection and blur the distinction between both groups.

Chronic infections can increase the difficulty of glycemic control by producing proinflammatory cytokines so *H. pylori* eradication can improve HbA1c levels and glucose metabolism, according to some studies [13]. Nonetheless, we compared diabetic patients with or without *H. pylori* infection, and no difference in the HbA1c levels was found. This finding is similar to that from a newly published meta-analysis [14]. Chronic infections also induce disturbances in the lipid metabolism. However, results of the association between *H. pylori* infection and the lipid profiles were controversial, and our study did not show any difference in the levels of TC and TG between diabetic patients with or without *H. pylori* infection. The BMI of patients between these two groups in our study were also similar.

A positive relationship between the duration of the disease and *H. pylori* infection has been noted by some researchers [3, 15]. However, most recent studies have reported a negative relationship in either type 1 diabetes [16] or T2DM [9]. In the present study, we showed a similar diabetes duration between patients with and without *H. pylori* infection.

The mechanism for the contribution of *H. pylori* to diabetes is complicated, and since the information is limited, this remains to be elucidated. The influence of *H. pylori* infection on chronic inflammation, gastric-related hormones, and insulin secretion may be critical factors affecting the progress of diabetes. Firstly, *H. pylori* infection induces the upregulation of several cytokines such as interleukin 6 and tumor necrosis factor α, which results in insulin resistance [17]. Secondly, *H. pylori* affects gut hormones such as leptin and ghrelin homeostasis [18], which are involved in insulin sensitivity and glucose homeostasis. Thirdly, some studies [19, 20] have shown that *H. pylori* infection leads to impaired insulin secretion due to oxidative stress and inflammation. Furthermore, strains of *H. pylori* are also an important factor. A study by Ibrahim [21] reported that cytotoxin-associated gene A positive strains of *H. pylori* are highly correlated with poor glycemic control in T2DM patients.

Although our sample size was relatively large, some limitations should be acknowledged. Our study was limited by its cross-sectional design. We could not avoid selection bias, and longitudinal studies are needed to confirm our results. Furthermore, we did not evaluate patients' gastric symptoms or perform histological evaluations.

In conclusion, the present study lacked a statistically significant difference in the *H. pylori* infection rate in diabetic patients and the controls. Additionally, there was no association between *H. pylori* infection and glycemic control, the lipid profile, BMI, and diabetes duration.

Compliance with ethical standards

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

Ethics approval and consent to participate This study was approved by our hospital's regional ethics committee, and all patients signed informed consent.

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CASE REPORT

Cardiac abnormalities and Wolfram (DIDMOAD) syndrome: a case report

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Abstract Cardiac abnormalities are very rarely reported to be associated with Wolfram (DIDMOAD) syndrome. We report a child who presented with cyanotic heart disease and was operated twice for cardiac lesion. At the age of 6 years, the case developed osmotic symptoms and, during evaluation, diabetes insipidus, optic atrophy and deafness were reported. DIDMOAD syndrome with atrial septal defect, severe pulmonary stenosis and hypoplastic right ventricle is not previously reported. In addition to our case, in a literature survey on all reported cases of DIDMOAD syndrome (more than 700), only 11 cases with cardiac abnormalities have been stated. Common abnormalities found are pulmonary stenosis, followed by tetralogy of Fallot and ventricular septal defects. Impact of DIDMOAD syndrome on cardiovascular development needs to be elucidated.

Keywords Wolfram syndrome · DIDMOAD · Cardiac abnormalities

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Background

Wolfram or DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy and deafness) syndrome is a rare cause of diabetes in children with an estimated prevalence of one case in 770,000 in general population, and the largest quoted data from UK diabetes registry reports an occurrence of one in every 150 patients with young onset of diabetes [1]. It is a neurodegenerative disorder which manifests as diabetes mellitus and optic atrophy as well as diabetes insipidus and deafness in some cases [2]. Additional clinical features include Sensorineural hearing loss, ataxia, dysarthria, neurogenic bladder, dysphagia, dementia, psychiatric disease and endocrine dysfunction, such as diabetes insipidus, hypothyroidism growth retardation and hypogonadism [2]. Cases of upper gastrointestinal bleeds and heart malformations have also been reported in Wolfram syndrome. However, cardiac abnormalities are rarely associated, and there is meagre data on cardiovascular abnormalities in Wolfram syndrome. Till date, little is known about Wolfram syndrome-related diabetes (WSD) and cardiovascular malformations. We surveyed all published series and reports of DIDMOAD syndrome and examined the cardiovascular abnormalities associated with this syndrome. Among all reported cases of DIDMOAD, the most commonly associated malformation was pulmonary stenosis which is reported in eight patients, followed by tetralogy of Fallot and ventricular septal defect reported in two patients (Table 1). Atrial septal defect is not reported till now except in the present case. Medlej et al. in their series of 31 Lebanese Wolfram patients belonging to 17 families found central diabetes insipidus in 87 % of the patients and sensorineural deafness in 64.5% [3]. They also reported valvular heart disease in 16.1 % (5/31) of cases, pulmonary stenosis in 19.3 % (6/31) of cases, and ventricular septal defect in 3.2 % (1/31) of cases [3]. The authors concluded that the percentage of cardiac

Table 1 Literature reports on cardiac abnormalities in Wolfram syndrome

Author	Year	Total number patients	Patients with cardiac lesions	Cardiac lesions
Kinsley et al. [5]	1995	68	3	Tetralogy of Fallot (2) PS (1)
Bekir et al. [4]	2000	1	1	VSD
Medlej et al. [3]	2004	31	6	VSD (1) PS (6)
Ganie et al. [6]	2011	7	1	Not available
Present case	2015	1	1	ASD, hypoplastic right ventricle, PS

PS pulmonary stenosis, *VSD* ventricular septal defects, *ASD* atrial septal defects

abnormalities was significantly higher than the one reported in the general population ($P < 0.001$). Ganie et al. in their series of seven patients of DIDMOAD syndrome found one patient with cardiac lesion, the nature of which is not mentioned [4]. Bekir et al. reported a Turkish family with wolfram syndrome (WFS) affected siblings having ventricular septal defects [5]. Kinsley et al. reported 68 patients of DIDMOAD syndrome and found two with tetralogy of Fallot and one with pulmonary stenosis [6].

Case presentation

A 6-year female born from a nonconsanguineous marriage, by normal delivery and normal mile stones, presented at the age of 3 years with cyanotic spells and easy fatigability to cardiologist. Echocardiography revealed atrial septal defect, severe pulmonary stenosis and hypoplastic right ventricle. Patient was operated twice for cardiac lesions till the age of 6 years and became symptomatically better. She developed osmotic symptoms at the age of 6 years. Blood glucose of 459 mg/dl was reported and urine for ketones was negative. Patient's baseline investigation revealed normal complete blood count except mild anaemia, normal kidney function and liver function tests. Patient had mild hypokalaemia and normal blood pH. Systemic examination revealed body height and weight of 134 cm (<3rd centile) and 34 kg (10 centile). Fundus examination showed bilateral optic atrophy (Fig. 1). Patient's cardiovascular examination showed pan systolic murmur. Patient's neurological examination was normal. Auditory examination showed bilateral Sensorineural deafness. Patient's anti-GAD antibody screen was <0.1 U/ml, (normal anti-GAD antibody range = 0–1.0 U/ml) and post-meal C-peptide was 0.399 nmol/l (normal range 0.366–1.46 nmol/l). Patient was started on insulin but persisted with Polyuria of 6 litres in 24 h. Dehydration tests showed central diabetes insipidus which was confirmed by an absent posterior pituitary bright spot.

Conclusion

We believe that heart malformation features may be present in association with WFS. The pathogenesis of such malformations is not known, but their presence in certain WFS families emphasizes the clinical heterogeneity of the disease and may help to better understand the function of genes and gene products associated with WFS. This is the first study reporting the incidence of cardiac lesions in WFS patients, which is significantly higher than the one reported in the general population. The report illustrates the careful clinical attention warranted in WFS patients at presentation like cardiac lesion and if required should be subjected to electrocardiography.

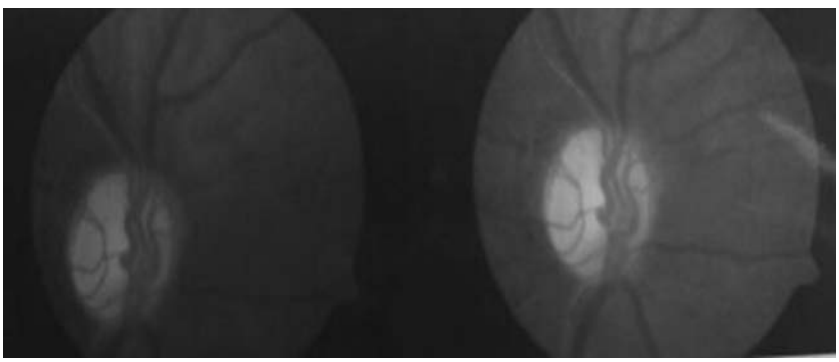

Fig. 1 Bilateral optic atrophy

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Authors' contributions SAM handled the clinical case and conceived the background of this case report, BAS and PAS helped with the literature survey and in the designing of the case report, and IH and IA drafted and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Consent Written informed consent was obtained from the legal guardians of the patient (parents) for publication of this case report and any accompanying images.

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

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- The detailed proposals should include the following:
  - ◊ Title, names of principal and co-investigators, summary, introduction/background, review of literature, aims, methodology, study design, and detailed plan of work and bibliography. Brief biodata of principal investigator and other co-investigators
  - ◊ Importance of work in the context of national priorities. Detailed budget sought along with full justification/ proposed utilization, of funding sought from RSSDI
  - ◊ Whether the project is being partly funded from any other source? If yes, please mention the source and the amount received
  - ◊ Ethical committee clearance of the institution or other bonafide body.

BOOK REVIEW

REVIEW OF 3RD EDITION OF RSSDI TEXT BOOK OF DIABETES MELLITUS : Editor-in-Chief – Hemraj B Chandalia, Executive Editor – Gumpeny Ramachandra Sridhar, Editors – Ashok Kumar Das, Sri Venkata Madhu, Viswanathan Mohan, Paturi Vishnupriya Rao

The third edition of RSSDI text book contains contributions from those who have being practicing / teaching Diabetology for many years, similarly the editors too. Most of the contributors are from within the country with many years of experience behind them. A few non resident Indians have made useful contribution. This edition as pointed out by editor – in – chief has gone on considerable revision from the first two editions. This only shows the importance of making an attempt to have our own text books and keep revising, based on the experience.

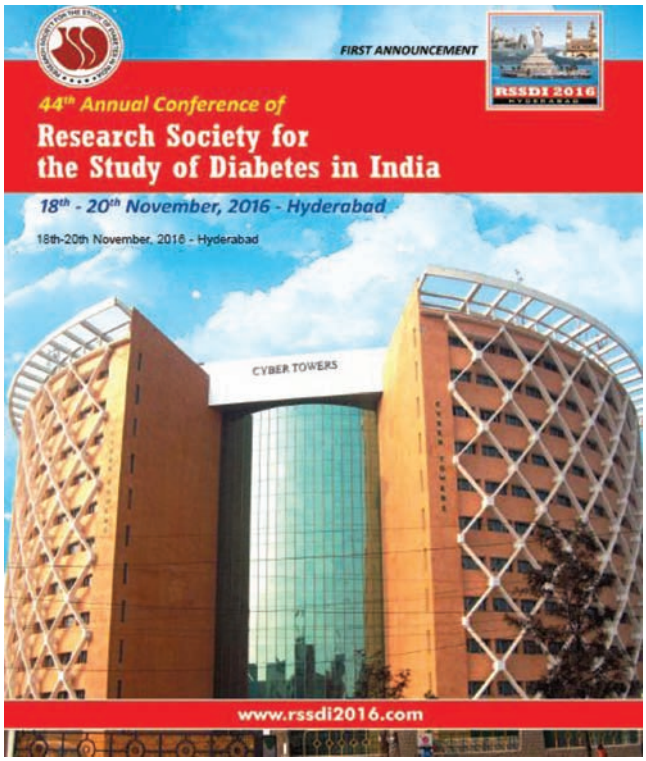
You name any thing in diabetes, this book has it. A few topics which are generally not paid much attention – like complexity of Insulin resistance, the criteria applicable in our country for metabolic syndrome, care of elderly diabetic, musculo-skeletal manifestation in diabetes are well covered. Malnutrition modulated Diabetes Mellitus and late onset of auto immune diabetes (LADA) as seen in our country is dealt with in detail.

The flow chart on management of diabetic keto-acidosis is useful and should be available in all ICU (Intensive Care Unit). The colour pictures of retinopathy and foot lesions are well presented. The usefulness of alternate therapy available in the country is extensively discussed. The Appendix is retained and gives a lot of information applicable to Indian subjects like BMI, waist circumference and laboratory values both in SI units and conventional units. The Index has reached perfection. Some controversial issues are mentioned in individual chapters but I wish an exclusive chapter was dedicated to controversies like classification of LADA and early use of insulin in these patients, need for revising the diagnostic plasma glucose values both in non-pregnant and pregnant diabetic, use of insulin in Type 2 diabetes at the time of detection to overcome the gluco and lipo-toxicity, safety of use of long acting insulin analogs during pregnancy, use of human insulin vis-à-vis insulin analogs, safety of DPP4 inhibitors, SGLT2 inhibitors and other new oral hypoglycemic molecules. Use of quantity and type of fat in the diet, role of low glycemic index diet in the management of diabetes etc.,

This book is a must for anybody who practices and teaches diabetes and students. The availability of this excellent text book has made western text books irrelevant to our country. The Novel feature of this book is mentioning the chapter number on the right edge of each page but single volume covering so many topics is bulky and heavy. I wish it was brought out in two volumes.

Prof. (DR.) C. MUNICHOODAPPA
Bengaluru

Invitation to the RSSDI, 2016 Conference



Welcome to RSSDI-2016,

Dear colleagues,
On behalf of the Organizing Committee of RSSDI 2016 we have great pleasure in welcoming you to the 44th Annual Scientific meeting of the Research Society of Study of Diabetes in India to be held in Hyderabad during 18 -20 November 2016. The RSSDI as you all know has grown to become the largest scientific body of professionals involved in managing diabetes in India and its annual meeting is the major event that all the members of RSSDI and doctors managing Diabetes in India look up to. We are privileged to host this event in Hyderabad. The Scientific program for RSSDI 2016 being crafted by Prof. SV Madhu, Chairman Scientific Committee will be designed to update our knowledge on various aspects of diabetes. The program will not only have Plenary lectures, guest lectures and symposia, but also workshops designed to provide hands-on training in several important practical areas of diabetes management. There would also be ample opportunities for young researchers to present their research work in the form of free papers. The venue for the conference will be the Hyderabad International Convention Centre which has state of art facilities for a conference of this mangnitude. Hyderabad the City of pearls is known for its rich history, food and its multi-lingual culture. It is known for its monuments like the Charminar, Golconda Fort, Falaknuma Palace and for artificially created lakes like the Hussain Sagar, Osman Sagar and the Himayat Sagar. It is also home to the top research institutions like National Institute of Nutrition and Center for Cellular & Molecular Biology and business school like Indian School of Business, IIT, IIIT, and BITS. Hyderabad is well connected by air / train with different parts of the country. We look forward to welcoming you for the RSSDI 2016. The Organizing Committee is working hard to ensure that RSSDI 2016 will be an academically and culturally enriching event for all of you.