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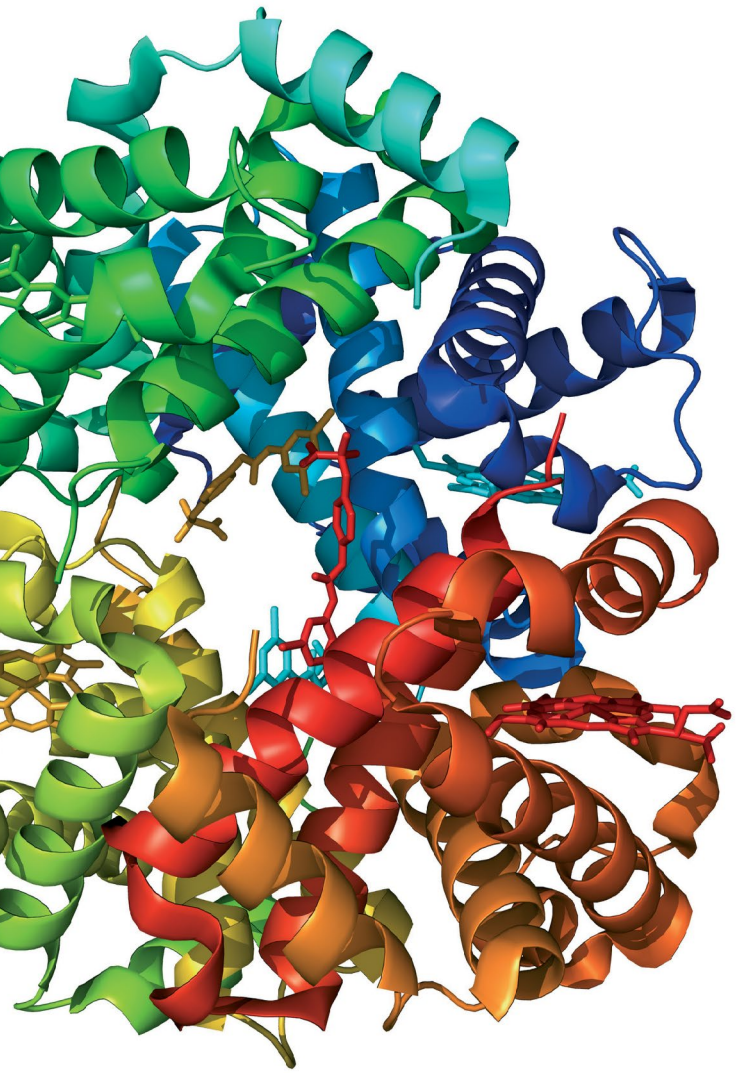

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Editorial Address:
Klinika Diabetologii i Chorób Wewnętrznych
Warszawski Uniwersytet Medyczny
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Za prenumeratę czasopisma „Clinical Diabetology" przysługuje 5 pkt edukacyjnych*

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CLINICAL DIABETOLOGY

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Contents

The Voice of the Editor-in-Chief

Dear Colleagues, Dear Readers,

That is one thing you can be sure of in life: Things change. And so, since May 2019, after the 20th Scientific Congress of Diabetes Poland in Lublin, our Society has been managed by a new Main Board and a new President. And now the time has come for a new Editor-in-Chief of "Clinical Diabetology". Professor Janusz Gumprecht has excellently developed the journal — it appears regularly, contains more and more papers from outside Poland, is included in the Web of Science database and in the Ministry of Science and Higher Education list of scored journals. Step by step, it is getting closer to the PubMed database, which in turn will increase the chance of obtaining the impact factor (IF).

Starting from this issue, the honor and obligation to direct the journal falls on the shoulders of the undersigned. I will of course continue the efforts of previous Editor-in-Chief. I would like "Clinical Diabetology" to develop further, in terms of content enrichment, editorial structure, number of sections, contact with our readers, and current topics. Together with the renewed Editorial Team, we will try to respond quickly to what is happening in diabetology (and a lot is happening at the moment), so that everyone interested in our favorite field can immediately understand the changes in the science of pathophysiology, diagnosis and treatment of diabetes and its complications. Of course, the journal will remain a bimonthly, but we will dynamically comment on the latest research and publish scientific papers and review articles addressing the most current problems of modern diabetology.

The coming time will be special. In 2021, it will be 100 years from the discovery of insulin, and in January 2022, 100 years will pass from its first documented use in humans. It will be an opportunity not only to celebrate the anniversary of one of the most important events in the history of medicine, but above all to broaden social impact and build awareness about diabetes and the need to conduct research on this disease. We are convinced that "Clinical Diabetology" will be the scientific and social medium in this celebration, serving to and strengthening Polish and European diabetology.

However, the most important thing for each and every journal are the readers. We hope that you will very actively participate in developing "Clinical Diabetology", not only by reading published materials, but also by submitting your papers, letters, comments, articles, as well as sending suggestions what we should do to improve the journal, thereby supporting the development of diabetology in our country. On behalf of the Editorial Team, I promise to do our best to accomplish this task.

Editor-in-Chief

Prof. Leszek Czupryniak

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ORIGINAL ARTICLE
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Talaat Abd El Fattah Abd Elaaty[1], Azza Abdelkerim Ismail[1],
Marwa Ahmed Meheissen[2], Nada Ramadan El Essawy[1]
[1]Internal Medicine Department (Unit of Diabetes & Metabolism), Faculty of Medicine, University of Alexandria, Egypt
[2]Medical Microbiology and Immunology Department, Faculty of Medicine, University of Alexandria, Egypt

Bacterial translocation markers in type 2 diabetes mellitus: their association with glycemic control and diabetic kidney disease in Egyptian patients

ABSTRACT

Background. The involvement of bacterial translocation in the pathogenesis of type 2 diabetes mellitus (T2DM) has been highlighted in recent years. The objective of the current study was to evaluate the potential impact of lipopolysaccaride-binding protein (LBP) and DNA translocation on glycemic control and progression to diabetic kidney disease in T2DM patients.

Material and methods. A total of 30 T2DM patients as well as 30 controls were included in a cross-sectional observational study. Plasma LBP levels were determined using an enzyme linked immunoassay. DNA translocation was assessed using polymerase chain reaction targeting *16SrNA* gene.

Results. Plasma levels of LBP were significantly elevated in T2DM patients than in controls ($p = 0.02$). LBP level was significantly and positively correlated with fasting glucose level, glycated hemoglobin, C-reactive protein, albumin-creatinine ratio and negatively correlated with glomerular filtration rate. Receiver operating curve revealed that LBP with a cut off of 15.17 μg/mL succeeded to predict both glycemic control and diabetic kidney disease in T2DM patients. The bacterial *16SrRNA* was detected in almost all blood samples of T2DM patients (28/30) and in about half (16/30) of the control group ($p < 0.001$).

Conclusion. Translocation products could trigger diabetes related complications. Future interventional work should target these products to reverse their effects. (Clin Diabetol 2019; 8, 4: 195–204)

Key words: type 2 diabetes mellitus, diabetic kidney disease, lipopolysaccharide binding protein, DNA translocation, bacterial translocation

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia, insulin resistance and systemic inflammation [1]. Several life-threatening complications are associated with T2DM [1]. Therefore, understanding the possible mechanisms involved in the pathogenesis of T2DM or its complications is of great value to prevent its progression. Recent data suggests a role of the gut microbiota in the induction of systemic inflammation and consequently the regulation of glucose metabolism [2].

Changes in gut bacteria, combined with increased intestinal permeability, stimulate bacterial translocation through the gut barrier; a previously unconsidered source of inflammation. Markers of bacterial translocation include bacterial *16srRNA DNA* and lipopolysaccharide (LPS) [3]. Upon translocation into the bloodstream, LPS induces metabolic endotoxemia followed by low-grade systemic inflammation involving the release of pro-inflammatory cytokines, such

Address for correspondence:
Marwa Ahmed Meheissen
Medical Microbiology & Immunology Department,
Faculty of Medicine, Alexandria University, Kartoum Square,
Azarita, Alexandria, Egypt
Phone: +2 03 4862506, +2 01005667342
Fax: +2 03 486 2506
e-mail: marwa.meheissen@alexmed.edu.eg
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as tumor necrosis factor (TNF), interleukin 1 (IL1), and interleukin 6 (IL6) [4].

LPS binds to LPS-binding protein (LBP), a plasma protein synthesized mainly by the liver, which facilitates the interaction between LPS and cellular targets. Binding of LPS to LBP is the first step in an inflammatory cascade [5].

Since LPS has a short half-life and its measurement in biologic fluids has several limitations, together with the relatively slow rise of LBP, which could serve to monitor the interaction between LPS and innate immune cells, LBP level has been suggested as a good clinical marker of effective metabolic endotoxemia [6, 7]. Several studies have demonstrated that increased circulating levels of LBP were associated with obesity, and T2DM [8–11].

An inflammatory component has been related to the complications of diabetes and diabetic kidney disease. The involvement of inflammatory process draws the attention of researchers to use immunosuppressants to prevent the development of albuminuria and kidney disease [11].

In this context, the influence of DNA translocation or LBP concentrations on glycemic control, and on the progression of diabetic kidney disease in T2DM was evaluated in the present study.

Subjects and methods

A total of 30 T2DM patients, recruited from the Outpatient Clinic of Alexandria Main University Hospital, Egypt were included in this cross-sectional observational study. Subjects were diagnosed as having T2DM according to the report of the Expert Committee for the Diagnosis and Classification of Diabetes Mellitus [12]. Another 30 non-diabetic healthy subjects served as a control group. Patients with type 1 DM, other metabolic diseases, liver or cardiac disease, portal hypertension, infectious diseases, psychiatric problems, and hematological or malignant disease were excluded from the study. Patients on anti-inflammatory drugs as glucocorticoids or those on antibiotic therapy during the last 3 months were also excluded. None of the control subjects were under medication or had evidence of systemic or metabolic disease. An informed written consent was taken from each subject before inclusion in the study. The study was approved by the Ethics Committee of Alexandria Main University Hospital, Egypt.

All subjects were subjected to the following:

History taking and full clinical examination

Demographic data as age and sex were recorded. Body weight and height were measured using standardized methods. Body mass index (BMI) was calculated. Blood pressure was recorded.

Laboratory routine analyses

Sample collection

Venous blood samples (5 mL from each patient) were collected. Sampling was done in the morning after an overnight fast. Fresh serum and plasma samples were used for chemical and hematological tests, while aliquots from frozen whole blood samples and plasma samples were stored at –80°C for PCR and LBP testing respectively.

Chemical and hematological analyses

Routine chemical and hematological tests were performed using automated analyzers. Insulin resistance was calculated on the basis of the homeostasis model assessment of IR (HOMA-IR), using the following formula: [HOMA-IR] = (fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5) [13]. Glomerular filtration rate (GFR) was estimated using CKD-EPI equation [14]. Moderately increased albuminuria was defined as two positive urine samples with urinary albumin-creatinine ratio (ACR) of 30–300 in the past 3 months [14].

Measurement of plasma LBP

Plasma level of LBP was determined by a commercially available double antibody sandwich enzyme linked immunosorbent assay (Assay kit Co., Ltd, USA) according to the manufacturer's instructions.

Detection of bacterial DNA by polymerase chain reaction (PCR)

Detection of bacterial DNA in patients' blood was performed using PCR targeting *16SrRNA* gene present in all bacteria, β-galactosidase gene found in most *E. coli*, and glutamine synthase gene of *Bacteroides fragilis (B. fragilis)*, using specific primers as previously described [15].

DNA extraction from whole blood samples was done using GENEJET whole blood DNA extraction kit (ThermoFisher SCIENTIFIC) according to the manufacturer's instructions.

Each PCR reaction consisted of 12.5 μL master mix (DreamTaq Green PCR master mix (2 ×), 25 picomoles/μL of the primer to be used, 5 μL of extracted DNA and sterile deionized water to a final volume of 25 μL. PCR amplification reaction was carried out using Applied Biosystems 2720, thermal cycler in the following conditions: an initial denaturation step at 95°C for 3 min, followed by 60°C for 45 sec, and 72°C for 10 min. This was followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, and finally an extension step at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gels (Bioline, UK) stained with ethidium bromide.

A 100 bp DNA ladder (Thermo Fisher SCIENTIFIC) was used as a marker.

Statistical analysis

Data were analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range, arithmetic mean, standard deviation and median. Appropriate tests were used to compare quantitative and qualitative variables between the two studied groups. For data correlation Pearson correlation coefficients was used. Receiver operating characteristics (ROC) curve analysis was done to determine the LBP cutoff point, which has the highest sensitivity and specificity. Significance of the obtained results was judged at the 5% level.

Results

Study population characteristics

The mean age of T2DM subjects was 53.3 ± 6.95 years. Twenty-two (73.3%) subjects were females and eight (26.7%) subjects were males. The mean age of the control group was 47.17 ± 11.0 with 23 males (76.7%) and 7 females (23.3%).

Comparison of clinical and laboratory parameters between diabetics and control group

The clinical and laboratory data of the study subjects are reported in Table 1.

Results of bacterial translocation markers

When investigating bacterial translocation markers, it was revealed that the mean LBP plasma level was significantly higher in patients with T2DM compared with control group (19.25 ± 12.69 μg/mL and 13.49 ± 2.25 μg/mL respectively) ($p = 0.02$) as shown in Table 1.

Regarding *16SrRNA DNA*, it was found that most of the T2DM cases (28/30; 93.3%) had circulating DNA compared to about half of the control group (16/30; 53.3%), this difference was statistically significant ($\chi^2 = 12.273^*$, $p \leq 0.001$) (Figure 1).

None of the T2DM cases or the control group was positive for glutamine synthase gene of *Bacteroides fragilis*, or β-galactosidase gene of *E. coli*.

To exclude the effect of obesity, diabetic cases were further subdivided according to BMI into obese ($n = 26$) and non-obese ($n = 4$) group. Comparison of LBP plasma level and the presence of circulating DNA between the two groups demonstrated that the mean LBP level of the obese group was 20.36 ± 13.16 μg/mL compared to 12.07 ± 6.02 μg/mL in the non-obese group, with no statistically significant difference between the two groups ($Z^U = -1.403$, $p = 0.161$). Most of the obese (92.3%) and all non-obese (100%) had positive *16SrRNA DNA* with no statistically significant difference between the two groups. ($\chi^2 = 0.33$, $p = = 1.0$). This finding supports the idea that obesity was not a major cause of the differences.

Correlation of LBP with various clinical and laboratory parameters

Circulating plasma LBP level was significantly and positively correlated with BMI ($r = 0.342$, $p = 0.008$), waist circumference ($r = 0.361$, $p = 0.005$), total cholesterol level ($r = 0.470$, $p < 0.001$), LDL-C ($r = = 0.518$, $p < 0.001$), fasting glucose level ($r = 0.343$, $p = 0.007$), HbA_{1c} ($r = 0.269$, $p < 0.037$), CRP ($r = 0.585$, $p < 0.001$), and ACR ($r = 0.320$, $p < 0.013$). On the other hand, there was a statistically significant negative correlation between LBP level and GFR ($r = -0.289$, $p = 0.025$) (Table 2).

Table 3 shows that the model is highly significant with p value < 0.001, 45% of the variability in LBP level could be explained by this model ($R^2 = 0.451$). CRP was the only variable which is independently and positively associated with plasma LBP levels ($p = 0.001$). The higher the CRP level the higher the LBP; one mg/L increase in CRP will increase the LBP by 0.215 μg/mL holding the other predictors constant (Table 3).

The diagnostic value of LBP for prediction of glycemic control, and diabetic kidney disease

ROC curves were plotted to evaluate the diagnostic value, in terms of area under curve (AUC), of LBP in prediction of glycemic control as well as diabetic kidney disease; the proposed threshold value (cutoff point) of LBP was ≥ 15.17 μg/mL (Figure 2A–D).

Relation of DNA translocation and various clinical and laboratory parameters in diabetics and control subjects

Comparison of different study parameters between subjects with positive and negative circulating *16SrRNA DNA* (whether diabetics or controls) was performed. The mean BMI values in diabetic patients who had circulating DNA (34.68 ± 4.48 kg/m^2), was significantly higher than among controls (29.69 ± 3.27 kg/m^2) ($p \leq 0.001$). Similarly, the waist circumference's mean in diabetic patients who had circulating DNA (116.43 ± ± 5.36 cm) was significantly higher than in the other groups ($p = 0.003$).

Regarding the lipid profile, it was found that the mean values of triglycerides (181.32 ± 73.69 mg/dL), cholesterol (216.04 ± 24.92 mg/dL), LDL-C levels (137.14 ± 19.6 mg/dL), in diabetic patients with circulating DNA, were significantly higher than in the other

Table 1. Comparison between the two studied groups according to clinical and laboratory measures

Clinical and laboratory parameters	T2DM cases (n = 30)	Controls (n = 30)	Test of significance	p value
BMI [kg/m^2]				
Min.–max.	26.29–42.96	24.91–36.15	ZU = –4.459*	**< 0.001***
Mean ± SD	34.79 ± 4.36	29.37 ± 3.03		
Median	35.15	28.05		
Waist circumference [cm]				
Min.–max.	109–127	100–125	t = 3.611*	**0.001***
Mean ± SD	116.0 ± 5.43	110.77 ± 5.79		
Median	115.0	109.5		
Mean blood pressure [mm Hg]				
Min.–max.	80–113.3	86.7–106.7	t = 1.261	0.212
Mean ± SD	101.27 ± 7.71	98.83 ± 7.29		
Median	103.3	101.7		
TG [mg/dL]				
Min.–max.	90–371	98–150	Z^U = –3.466*	**0.001***
Mean ± SD	180.33 ± 71.2	126.03 ± 14.59		
Median	174.0	124.0		
Total cholesterol [mg/dL]				
Min.–max.	176–272	145–210	t = 8.11*	**< 0.001***
Mean ± SD	216.4 ± 24.52	173.63 ± 15.27		
Median	217.0	170.0		
HDL-C [mg/dL]				
Min.–max.	30–67	41–60	Z^U = –3.47*	**0.001***
Mean ± SD	43.3 ± 8.69	49.4 ± 5.59		
Median	42.0	50.0		
LDL-C [mg/dL]				
Min.–max.	104.4–188.8	66–131	t = –3.234*	**0.002***
Mean ± SD	136.74 ± 20.24	99.0 ± 15.75		
Median	133.8	99.5		
Fasting glucose [mg/dL]				
Min.–max.	78–571	80–111	Z^U = –5.836*	**< 0.001***
Mean ± SD	236.87 ± 114.03	93.2 ± 6.98		
Median	215.5	93.0		
Fasting insulin [mLU/mL]				
Min.–max.	5.21–52.2	5.2–15.3	Z^U = –1.738	0.082
Mean ± SD	14.7 ± 8.77	10.49 ± 2.88		
Median	12.0	11.1		
HOMA-IR				
Min.–max.	1.7–23.6	1.1–3.8	Z^U= –5.829*	**< 0.001***
Mean ± SD	8.04 ± 4.58	2.39 ± 0.63		
Median	7.65	2.4		
HbA_{1c} (%)				
Min.–max.	6.2–13.3	4.9–6.0	Z^U = –6.663*	**< 0.001***
Mean ± SD	9.82 ± 2.25	5.33 ± 0.28		
Median	10.0	5.3		
Hb [gm/dL]				
Min.–max.	13–17	13–17	t = –1.267	0.210
Mean ± SD	14.76 ± 1.09	15.1 ± 0.99		
Median	14.7	15.0		

→

Table 1. (cont.). Comparison between the two studied groups according to clinical and laboratory measures

Clinical and laboratory parameters	T2DM cases (n = 30)	Controls (n = 30)	Test of significance	p value
WBCs (cells/cmm)				
Min.–max.	6.3–12.26	4.24–18.9	Z^U = –4.288*	**< 0.001***
Mean ± SD	8.76 ± 1.73	6.79 ± 2.73		
Median	8.35	6.29		
Platelets (cells/cmm)				
Min.–max.	159–400	184–369	t = 0.176	0.861
Mean ± SD	261.5 ± 57.29	259.1 ± 47.66		
Median	252.5	248.0		
ALT [U/L]				
Min.–max.	11–44	11–29	Z^U = –0.423	0.672
Mean ± SD	19.5 ± 7.56	19.1 ± 4.39		
Median	19.5	20.0		
AST (U/L)				
Min.–max.	12–57	11–28	Z^U = –1.312	0.189
Mean ± SD	19.33 ± 10.13	19.07 ± 4.40		
Median	15.5	19.0		
Serum albumin [g/dL]				
Min.–max.	2.9–4.5	3.5–4.9	t = –6.748	**< 0.001***
Mean ± SD	3.47 ± 0.45	4.17 ± 0.34		
Median	3.45	4.1		
Urea [mg/dL]				
Min.–max.	50–200	12–45	Z^U = –6.657*	**< 0.001***
Mean ± SD	111.73 ± 43.25	22.3 ± 9.11		
Median	100.0	19.5		
Creatinine [mg/dL]				
Min.–max.	0.9–3.0	0.4–1.0	Z^U = –6.459*	**< 0.001***
Mean ± SD	1.68 ± 0.52	0.74 ± 0.18		
Median	1.6	0.7		
GFR [mL/min/1.73 m^2]				
Min.–max.	27.41–131.0	92.22–242.01	Z^U = –6.416*	**< 0.001***
Mean ± SD	62.64 ± 22.98	156.68 ± 42.54		
Median	60.7	155.9		
ACR [mcg/mg]				
Min.–max.	22.8–8000.0	10.0–30.0		
Mean ± SD	1506.4 ± 1909.7	21.37 ± 5.48	Z^U = –6.436*	**< 0.001***
Median	835.0	22.5		
ESR [mm/hr]				
Min.–max.	20–132	10–23	Z^U = –6.386*	**< 0.001***
Mean ± SD	60.23 ± 32.35	15.2 ± 3.93		
Median	69.0	14.5		
CRP [mg/L]				
Min.–max.	4–90	3–16	Z^U= –4.557*	**< 0.001***
Mean ± SD	31.07 ± 23.07	10.07 ± 3.76		
Median	21.0	11.0		
LBP [μg/mL]				
Min.–max.	4.14–63.04	11.01–17.9	Z^U = –2.323*	**0.02***
Mean ± SD	19.25 ± 12.69	13.49 ± 2.25		
Median	16.44	12.62		

t — calculated value for Student t-test; Z^U — calculated value for Mann Whitney non-parametric test; * — statistically significant at $p \leq 0.05$; BMI — body mass index; TG — triglycerides; HDL-C — high-density lipoprotein; LDL-C — low-density lipoprotein; HOMA-IR — homeostatic model assessment for insulin resistance; HbA_{1c} — glycosylated hemoglobin; CRP — C-reactive protein; Hb — hemoglobin; ALT — alanine aminotransferase; AST — aspartate aminotransferase; GFR — glomerular filtration rate; ACR — albumin/creatinine ratio; ESR — erythrocyte sedimentation rate; CRP — C-reactive protein; LBP — lipopolysaccharide binding protein

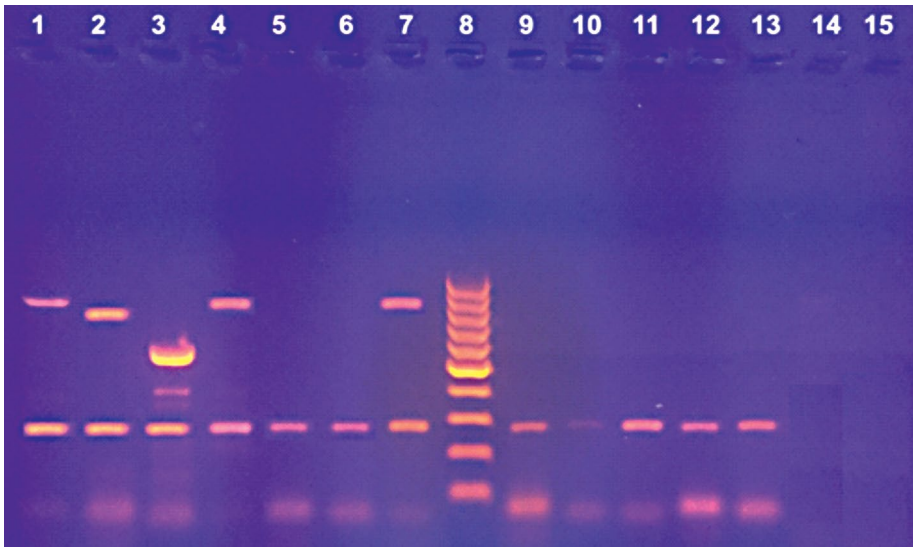

Figure 1. Agarose gel stained with ethidium bromide. A band of 798 bp corresponds to bacterial broad range16SrRNA gene, a band of 762 bp corresponds to *E. coli* β-galactosidase gene (BG), a band of 581 bp corresponds to *Bacteroides fragilis* glutamine synthase (GS) gene, and a band of 268 bp corresponds to internal control β-globin gene. Lane 1: blood sample spiked with *E. coli* ATCC 25922; *16SrRNA* gene positive control (798 bp). Lane 2: blood sample spiked with *E. coli* ATCC 25922; BG gene positive control (762 bp). Lane 3: blood sample spiked with *Bacteroides fragilis* ATCC 25285; glutamine synthase gene positive control (581 bp). Lane 4, 5, 6: Positive Blood sample (positive *16SrRNA* gene in lane 4, negative BG gene in lane 5, negative GS gene in lane 6). Lane 7, 9, 10: Positive blood sample (positive *16SrRNA* gene in lane 7, negative BG gene in lane 9, negative GS gene in lane 10). Lane 11, 12, 13: Negative blood sample for the three genes. Lane 14, 15: Negative controls. Lane 8: molecular size DNA marker (100–1000 bp)

groups ($p = 0.007$; $p \leq 0.001$; $p \leq 0.001$ respectively). Moreover, HDL-C mean (42.32 ± 7.67 mg/dL) was significantly lower in diabetics who had circulating DNA ($p = 0.001$).

In addition, diabetic patients with positive circulating DNA had significantly higher mean levels of fasting blood glucose (234.43 ± 117.76 mg/dL), HOMA-IR score (8.04 ± 4.72), HbA_{1c} (9.69 ± 2.27%) than diabetics or controls with negative DNA translocation ($p \leq 0.001$, $p = 0.008$, $p \leq 0.001$ respectively).

The mean white blood cells count was the highest in diabetic patients with DNA translocation, while the mean serum albumin was the least in the same group. These differences were statistically significant ($p \leq 0.001$). On the other hand, hemoglobin level, platelets count, ALT, and AST did not differ significantly between different groups.

The mean levels of urea (1.68 ± 0.54 mg/dL), creatinine (112.14 ± 44.72 mg/dL), albumin/creatinine ratio (1556.88 ± 1967.5 μg/mg) were significantly higher in diabetic patients with circulating DNA when compared to control subjects with circulating DNA, or subjects who had no circulating DNA whether diabetics or non-diabetics ($p \leq 0.001$). Also, the mean GFR in diabetic patients who had circulating DNA was significantly lower than in subjects who had no DNA whether diabetics or not ($p \leq 0.001$).

Comparing the inflammatory markers between the four groups, revealed that the ESR (59.32 ± 33.32 mm/hr) as well as the CRP (31.93 ± 23.66 mg/L) mean levels in diabetics with circulating DNA were significantly higher than in the subjects with negative DNA translocation ($p \leq 0.001$).

Although, the mean level of LBP was higher in diabetic patients (19.41 ± 13.14 μg/mL) who had circulating DNA compared to controls (14.03 ± 2.19 μg/mL) with circulating DNA, or diabetics and controls who had no DNA (17.09 ± 1.36 μg/mL, 12.87 ± 2.24 μg/mL respectively), these differences were not statistically significant ($p = 0.067$).

Discussion

To our knowledge, this study is the first to examine the effect of endotoxemia and bacterial translocations on glycemic control or progression of diabetic kidney disease in an Egyptian population having T2DM.

The results of the anthropometric and laboratory parameters of the diabetic patients in the present study were similar to data obtained from previous studies [18–20]. Although several studies have investigated the association of LBP and other translocation markers with T2DM [8–11], few studies have focused on the association of LBP or DNA translocation and the progression of diabetes in type 2 diabetic patients.

Tabela 2. Correlations of plasma LBP levels with various parameters

Studied variables	LBP level	
	r	p value
BMI	0.342*	**0.008***
Waist circumference	0.361*	**0.005***
Mean blood pressure	0.117	0.375
Triglycerides	0.182	0.164
Total cholesterol	0.470*	**< 0.001***
HDL-C	–0.229	0.078
LDL-C	0.518*	**< 0.001***
Fasting blood glucose	0.343*	**0.007***
Fasting insulin	–0.166	0.204
HOMA-IR	0.088	0.503
HbA_{1c}	0.269*	**0.037***
Hb	–0.089	0.498
WBCs count	0.056	0.672
Platelets count	–0.237	0.068
ALT	–0.019	0.887
AST	–0.065	0.623
Serum albumin	–0.135	0.304
Urea	0.139	0.289
Creatinine	0.211	0.105
GFR	–0.289*	**0.025***
ACR	0.320*	**0.013***
ESR	0.206	0.114
CRP	0.585*	**< 0.001***

r — Spearman correlation coefficient; *— statistically significant at $p \leq 0.05$. BMI — body mass index; HDL-C — high density lipoprotein; LDL-C — low density lipoprotein; HOMA-IR — homeostatic model assessment of insulin resistance; HbA_{1c} — glycosylated hemoglobin; Hb — hemoglobin; ALT — alanine aminotransferase; AST — aspartate aminotransferase; GFR — glomerular filtration rate; ACR — albumin/creatinine ratio; ESR — erythrocyte sedimentation rate; CRP — C-reactive protein

Tabela 3. Multiple linear regression analysis of factors affecting LBP level

Studied variables	LBP level		
	B	t	p
BMI	0.249	0.817	0.418
Waist circumference	0.032	0.152	0.879
Cholesterol	0.011	0.103	0.918
LDL-C	0.105	0.842	0.404
FBG	–0.002	–0.124	0.902
HbA_{1c}	–0.778	–1.088	0.282
GFR	–0.005	–0.174	0.863
CRP	0.215	3.364	**0.001***
ACR	0.0	–0.336	0.738
F (P)	4.558* (< 0.001*)		
R^2	0.451		

B — regression coefficient; t, p — calculated and p-value of t-test; F (P) — calculated and p-value of ANOVA test; * — statistically significant at $p \leq 0.05$

In the present study, the mean LBP level of the cases was significantly higher than that of the control group ($p = 0.02$). This was in agreement with a previous study, which examined the associations between intestinal permeability and T2DM, LBP was significantly higher in type 2 diabetic patients in comparison with normal individuals [19]. Similarly, Gubern et al., verified higher LBP concentration in T2DM patients and subjects with impaired glucose tolerance compared with non-diabetic subjects [20]. Also, Moreno-Navarrete et al., found that type 2 diabetic patients have higher levels of LBP than controls [9]. On the other hand, Zhou et al., conducted a 5-year nested case-control study on 3510 individuals from the Chinese population. Based on the results of their study, there was no significant difference in LBP levels at baseline between T2DM subjects and controls when matched for age, gender, and BMI. In addition, no association was observed between serum LBP levels and the incidence of T2DM in the 5-year follow-up. They concluded that LBP on its own might not improve diabetes prediction [21].

In the current work, LBP was statistically significantly positively correlated with BMI and waist circumference in diabetic patients. It was also positively correlated with blood sugar level, HbA_{1c}, total cholesterol, LDL-C, ACR and CRP. However, LBP was negatively correlated with GFR. Similarly, Kim et al., assessed LBP as a biomarker of obesity-related insulin resistance in adolescents, the results of their study showed LBP levels were significantly and positively associated with BMI [22]. Furthermore, circulating plasma LBP levels were significantly and positively associated with BMI, systolic blood pressure, aspartate aminotransferase, alanine aminotransferase, total cholesterol, LDL-C, fasting glucose and insulin, and insulin resistance in the study of Moreno-Navarrete et al. [9]. Serum LBP levels were also positively correlated with the parameters of obesity, insulin resistance, and inflammation in diabetic subjects as mentioned in a previous Japanese study [23]. In a prospective observational study involving 49 obese subjects undergoing bariatric surgery and 17 controls, plasma LPS was positively correlated with cardiometabolic risk factors, including triglycerides, systolic blood pressure, and BMI and was negatively correlated with HDL cholesterol [24]. Kim et al., found that plasma LBP levels were significantly and positively correlated with liver enzyme levels, a marker of liver damage and liver involvement in systemic inflammatory disease [22]. This observation was not encountered in our study where the LBP levels were not correlated with liver enzymes.

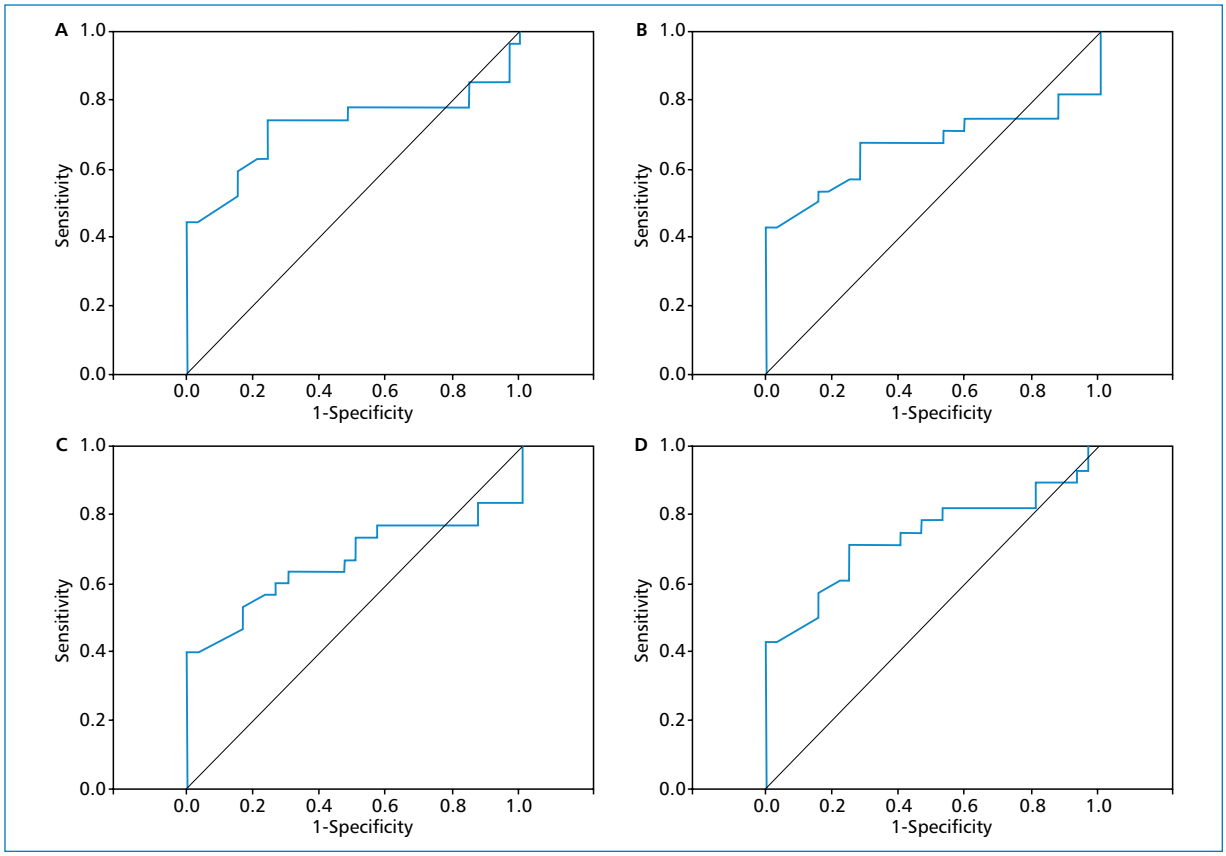

Figure 2A, B. ROC curves for LBP as a predictor of glycemic control in type 2 diabetes mellitus (A. FBG ≥ 126 mg/dL, B. HbA_{1c} ≥ 6.5). A. Area under the curve (AUC): 0.723, proposed threshold value: ≥ 15.17 μg/mL, sensitivity: 74.1%, specificity: 75.8%, positive predictive value (PPV): 71.4%, negative predictive value (NPV): 78.1% (p = 0.003). **B**. AUC: 0.669, threshold value: ≥ 15.17, sensitivity: 67.9%, specificity: 71.9 %, PPV: 67.9%, NPV: 71.9% (p = 0.025). **C**, **D**. ROC curves for LBP as a predictor of diabetic kidney disease (C. ACR ≥ 30 μg/mg, D. GFR ≥ 90 mL/min/1.73 m^2). C. AUC: 0.664, proposed threshold value: ≥ 15.17 μg/mL, sensitivity: 63.3%, specificity: 70.0%, PPV: 67.9%, NPV: 65.6% (p = 0.029). D. AUC: 0.738, threshold value: ≥ 15.17 μg/mL, sensitivity: 71.4%, specificity: 75.0%, PPV: 71.4%, NPV: 75.0% (p = 0.002)

Our results demonstrated that CRP was the only variable which is independently and positively associated with plasma LBP levels (p = 0.001). The higher the CRP level the higher the LBP concentrations. Interestingly, Sun et al., observed a stronger correlation between LBP and inflammatory markers after adjustment for BMI. Moreover, adjusting for hs-CRP and IL-6 almost eliminated the associations of LBP with metabolic syndrome and most of its traits. The explaining mechanism is that LBP triggered an immune response involving formation of interleukins and upregulation of CRP synthesis in the liver [10].

After analyzing ROC curves in the current study, using LBP with a cut off of 15.17 μg/mL succeeded to predict both glycemic control and diabetic kidney disease in T2DM patients. This finding could help to predict complications in T2DM patients in our Egyptian population.

Amar et al., reported previously that bacterial *16S rRNA* gene blood concentration could predict the onset of diabetes, reporting for the first time the clinical importance of bacterial translocation in the development of T2DM [25]. *16SrRNA* is a highly conserved region of bacterial DNA, found in all bacteria; thus by its detection by PCR, all translocated bacteria could be theoretically detected [26]. Ortiz et al., studied DNA translocation in a group of morbidly obese patients candidate for bariatric surgery, they found that inflammatory markers, endotoxin levels, and insulin resistance remained high in patients with bacterial DNA despite weight reduction and were individually affected by the presence or absence of bacterial DNA translocation. They demonstrated increased serum levels of endotoxin in patients with bacterial DNA compared to those without DNA. Moreover, they proved that DNA fragments correspond to commensal gut flora

and coincide with elevated serum endotoxin levels in those patients [27]. Similarly, Sato et al., found that gut bacteria were detected in blood at a significantly higher rate in diabetic patients than in control subjects, and most of these bacteria were Gram-positive anaerobic bacteria [17]. The findings of the previous studies support our results that bacterial translocation to the blood might play important roles in chronic low-grade inflammation in T2DM and could explain the negative PCR results of glutamine synthase gene of *Bacteroides fragilis*, or β-galactosidase gene of *E. coli*.

To further evaluate the role of bacterial translocation on the glycemic control, A French study demonstrated that translocation of commensal bacteria from intestine towards tissue can be reversed with the probiotic strain *Bifidobacterium Lactis*, which proved to improve the epithelial cell gut barrier, thus reducing bacterial translocation and its consequences on inflammation and insulin sensitivity [28].

While some data on translocation markers and their relationship to chronic inflammation is available for chronic kidney disease patients [29], very little is known about this relationship in T2DM patients. The study conducted by Nymark et al., showed that high serum LPS activity contributes to the development of microalbuminuria and diabetic nephropathy in Finnish patients with type 1 diabetes [11]. Disturbance of gut flora and consequently bacterial translocation and increased inflammatory state, lead to progression of diabetic nephropathy, which might be attributed to the gut-kidney axis in which local renin-angiotensin system is possibly involved [30].

Our study has some limitations. Dietary data and treatment data (insulin, or antidiabetis) were limited. Both could affect gut bacteria and LBP levels as well as DNA translocation. Additionally, we only assessed plasma LBP and not LPS levels. Moreover, the PCR method used in the study simply demonstrates the presence of bacterial DNA, does not specify type of bacteria, and does not differentiate between dead or living microorganisms.

Conclusions

In conclusion, our study demonstrated that bacterial translocation markers are present at increased levels in patients with T2DM, and are positively correlated with glycemic control, renal and inflammatory markers. They might then trigger diabetes related complications as diabetic kidney disease.

Future research should focus on interventional protocols to investigate whether manipulation of gut microbiota by dietary interventions or by the administration of probiotics could reduce the rate of bacterial translocation. This might decrease systemic inflammatory response and eventually ameliorate glycemic control, and decrease the risk of progression of diabetic kidney disease.

Conflicts of interest

The authors report no competing interests.

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Nazarii Kobyliak[1], Ludovico Abenavoli[2], Galyna Mykhalchyshyn[1], Tetyana Falalyeyeva[3], Olena Tsyryuk[3], Liudmyla Kononenko[1], Dmytro Kyriienko[1, 4], Iuliia Komisarenko[1]

[1]Department of Endocrinology, Bogomolets National Medical University, Kiev, Ukraine
[2]Department of Health Sciences, University Magna Graecia of Catanzaro, Italy
[3]Taras Shevchenko National University of Kyiv, Kyiv, Ukraine
[4]Kyiv City Clinical Endocrinology Center, Kiev, Ukraine

Probiotics and smectite absorbent gel formulation reduce liver stiffness, transaminase and cytokine levels in NAFLD associated with type 2 diabetes: a randomized clinical study

ABSTRACT

Introduction. In double-blind single center randomized clinical trial (RCT), the efficacy of alive probiotics supplementation with smectite gel *vs.* placebo in type 2 diabetes patient with non-alcoholic fatty liver disease (NAFLD) detected on ultrasonography (US) were studied.

Material and methods. A total of 50 patients met the criteria for inclusion. They were randomly assigned to receive Symbiter Forte combination of probiotic biomass with smectite gel (250 mg) or placebo for 8-weeks. The primary main outcomes were the change in fatty liver index (FLI) and liver stiffness (LS) measured by shear wave elastography (SWE). Secondary outcomes were the changes in transaminases activity, serum lipids and cytokines levels.

Results. All subjects completed the study and received more than 90% of prescribed sachets. In respect to our primary endpoints, FLI and LS insignificant decrease in both interventional and placebo groups. However, when we compare mean changes across groups from baseline, expressed in absolute values, the reduction of both LS (–0.254 ± 0.85 *vs.* 0.262 ± ± 0.77; p = 0.031) were observed. Analysis of secondary outcomes showed that co-administration of probiotic with smectite lead to significant reduction of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, IL-1β, and tumor necrosis factor (TNF-α) after 8 weeks.

Conclusion. In this RCT, we confirmed previously reported animal data, showing that co-administration of probiotic with smectite manifested with reduction of LS, liver transaminases and chronic systemic inflammation. (Clin Diabetol 2019; 8, 4: 205–214)

Key words: diosmectite, nutraceuticals, non-alcoholic fatty liver disease, probiotics, *Lactobacillus, Bifidobacterium, Propionibacterium*

Address for correspondence:
Nazarii Kobyliak, PhD
Assistant of Endocrinology Department,
Bogomolets National Medical University
Kyiv, 01601, Pushkinska 22a str., Ukraine
Phone: +380442356005
e-mail: nazariikobyliak@gmail.com
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Introduction

Non-alcoholic fatty liver disease (NAFLD) is an unique term that combines several components, in particular the simple steatosis with excessive intra hepatic fat accumulation over 5% of organ weight, non-alcoholic steatohepatitis (NASH), constituted by steatosis with development of necroinflammation,

fibrosis and finally cirrhosis and hepatocellular carcinoma [1]. NAFLD is now recognized as the hepatic manifestation of metabolic syndrome and often associated with many pathologies such as type 2 diabetes (T2D), visceral obesity and insulin resistance (IR) [2]. Globally, the incidence of NAFLD is recently growing and has reached in Western Countries 20–30% and 5–18% in Asia [3]. Epidemiology data supports the idea that NAFLD is the most common reason of chronic liver disease, major cause of liver-related morbidity and mortality and urgent problem for global public health [4, 5].

Currently, the treatment of NAFLD is based on lifestyle changes, included enhancement of daily physical activity parallel with calorie restriction [2]. New therapeutic approaches have been under study in pre-clinical and clinical studies. Nowadays, modulation with gut microbiota composition and direct its metabolic effects in a perspective of prevention or treatment of NAFLD [6]. Among the suggested strategies, probiotic supplementation, as the intake of microorganisms capable when administered in acceptable amounts, confers a health benefit on the host [7]. The positive impact of *Lactobacilli* and *Bifidobacteria*, the most studied and commonly used probiotic strains in the treatment and prevention of obesity-associated disorders, have been previously discussed in the literature [8]. Moreover, several potential bacterial candidates, such as *Saccharomyces cerevisiae var*. *boulardii*, *Parabacteroides goldsteinii*, *Enterobacter halii* or *Akkermansia muciniphila*, have been identified and innovative mechanisms of action overriding their beneficial effects for obesity have been elucidated [9, 10]. In this regard, many researchers are trying to describe the role played by the different bacterial strains on the NAFLD management. Our choice of bacteria formulation for the present investigation was based on previous comparative preclinical studies of different probiotic strains intended for treatment and prevention of NAFLD and obesity [11, 12]. Intervention with poly-probiotic mixtures containing both alive and lyophilized strains led to significant reduction of total and visceral adipose tissue weight, steatosis, and necroinflammation, and to enhanced insulin sensitivity in rats with monosodium-glutamate (MSG) induced obesity model [11–13].

Smectite is a natural silicate clay belonging to the dioctahedral smectite class, binds to intestinal mucous, forms multilayer structure with high plastic viscosity and powerful coating properties hence preserving integrity of the mucus, and has the ability to absorb directly bacterial toxins, bacteria, viruses and bile salts [14, 15]. Diosmectite also has a protective effect against intestinal inflammation [16] hence suppressing production of cytokines such as interleukin-8 from secretory epithelial cells [17] and to attenuating the proinflammatory action of tumor necrosis factor-α (TNF-α) [18]. We proposed that all of these pharmacological properties may be beneficial for the treatment of NAFLD.

Based on preclinical data, in rats with MSG-induced obesity supplementation of alive probiotics with smectite gel (Symbiter-Forte formulation) significant reduce chronic systemic inflammatory markers (IL-1β, TNF-α) [19], total NAS (NAFLD activity score) score, with marked decrease of lobular inflammation (0.13 ± 0.09 *vs*. 0.33 ± 0.15) as compared to administration of probiotic alone [20].

Current aims were to provide single center randomized clinical trial (RCT) with double-blind fashion to assess the efficacy of alive probiotics combination with smectite gel (Symbiter-Forte) *vs.* placebo in T2D patient with NAFLD detected on ultrasonography (US).

Material and methods

The recruitment started after the approval of RCT protocol by local commission of bioethics in Kyiv City Clinical Endocrinology Center. All procedures, associated with RCT, were in agreement with the guidelines of the 1975 Declaration of Helsinki. Before study began all participants gave written informed consent and were fully explained with purpose and its methodology.

Study design

This study was registered at *ClinicalTrials.gov* under identifier — NCT03614039 as single-center placebo controlled, double-blind, parallel group study with inclusion of 51 T2D patients. Participants were randomized by the study statistician based on computer-generated list to intervention (Symbiter Forte) or placebo group. The allocation of groups was blind to investigators and patients. Moreover, to maintain blind and parallel study fashion the statistician was not aware of the allocation of participants to intervention. Treatment period continued for 8 weeks. Both placebo and intervention were administered as a sachet formulation with similar organoleptic characteristics (e.g., taste and appearance). The Symbiter Forte was supplied by Scientific and Production Company "O.D. Prolisok" (Ukraine). It contains combination of smectite gel (250 mg), supplemented with biomass of 14 alive probiotic strains: *Bifidobacterium* (1×10^{10} colony forming units — CFU/g), *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), *Acetobacter* (1×10^{6} CFU/g) and short-chain fatty acids producing *Propionibacterium* (3×10^{10} CFU/g) genera. Over a treatment period the participants received 1 sachet (10 g) of probiotic-smectite or placebo per day.

For minimization of the dietary changes effects, 2 weeks before first sachet prescribed, all patients were instructed in one-on-one sessions with a qualified dietitian to follow a therapeutic lifestyle-change diet as classified by the National Cholesterol Education Program (NCEP). In addition, participants were instructed to continue with stable anti-diabetic drugs and received standardized slight physical exercise for 1 hour per day.

Throughout the study, weekly phone follow-up visits were provided for assessment of compliance, adherence to the protocol, as well as the recording of adverse events. The effectiveness of therapy was compared and evaluated separately in the two groups.

Inclusion and exclusion criteria

Current study has similar design to previously reported by our group RCTs were effects of alive probiotic alone or in combination with omega-3 fatty acids in NAFLD were assessed [21, 22]. The main inclusion criteria were: adult T2D patients treated with diet and exercise alone or metformin, SUs and insulin at stable dose at least 4 weeks prior to randomization; body mass index (BMI) $\geq$ 25 kg/m^2; presence of NAFLD detected on US as previously described [21, 22]; aspartate transaminase (AST) and alanine transaminase (ALT) $\leq$ 3 $\times$ upper limit of normal.

The main exclusion criteria were decompensated liver disease including ascites, encephalopathy or variceal bleeding or presence of other chronic diffuse liver diseases such as chronic viral hepatitis associated with HBV, HCV or HEV-infection; drug-induced liver disease; hereditary deficiency of antitrypsin-1, Wilson's disease or idiopathic hemochromatosis. Patients with history over a two-year period or with active alcohol abuse which defined as consumption more than 2 standard drinks (> 20 g/day) for women and 3 standard drinks (> 30 g/d) for men were also excluded. Treatment within 3 months prior to randomization with agents that can impact microbiota composition such as probiotic, prebiotic or antibiotic; vitamin E, omega-3 fatty acids or medications with evidence for effects on NAFLD (pioglitazone, glucagon-like peptide-1 [GLP-1] analogues, dipeptidyl peptidase IV inhibitors, ursodeoxycholic acid) [21, 22]. Presence of uncontrolled cardiovascular or respiratory disease, active malignancy, or chronic infections.

Outcomes assessment

The primary main outcomes were the changes in fatty liver index (FLI) and liver stiffness (LS) measured by Shear Wave Elastography (SWE). The methodology of outcomes assessment was previously described [21, 22].

LS was measured by SWE using a multifrequency convex transducer (2–5 MHz) and Ultima PA ultrasound equipment (Radmir, Ukraine). Firstly, in B-mode we estimated position of liver (the ratio of the edge to the costal arch, the availability of acoustic windows) investigated both lobes of the liver and carried out their antero-posterior size biometrics on inspiration. Even or uneven contour of the liver was assessed, as well as acute or rounded front-bottom corner of the liver. We evaluated the echogenicity (normal, low or high) and echostructure (fine particles — 1–2 mm, medium particles — 3–4 mm, and coarse particles — more than 5 mm). Sound conductivity of the liver parenchyma or opposite US attenuation in the front-rear direction of liver was evaluated by Hamaguchi's B-mode criteria [21, 22].

SWE was carried out by the standard algorithm for 2D SWE. Especially carefully navigated region of interests (ROI) and SV of 2D SWE by B-mode and removed SWE artifacts. We performed 10 valid measurements of LS in every patient, and a median value was calculated, the result being measured in kPa [21, 22].

FLI a validated prediction score for hepatic steatosis severity designed Bedogni et al [23]. FLI was calculated based on laboratory and anthropometric measures, including triglycerides, gamma glutamyl transferase (GGT), BMI, and waist circumference (WC), by using the following formula:

$$FLI = [e^{0.953*\log e\,(\text{triglicerides}) + 0.139*BMI + 0.718*\log e\,(\gamma\text{-GT}) + 0.053*\text{waist circumference} - 15.745}/(1 + e^{0.953*\log e\,(\text{triglicerides}) + 0{,}139*BMI + 0.718*\log e\,(\gamma\text{-GT}) + 0.053*\text{waist circumference} - 15.745})] \times 100$$

Secondary outcomes were the changes in transaminases activity, serum lipids and cytokines (TNF-α, interleukin [IL]-1β, IL-6, IL-8, and interferon [IFN]-γ) levels. All values were determined following a 12-h fasting period, by the hospital clinical laboratory.

Anthropometric data including weight and height were measured to an accuracy of 0.1 kg and 0.5 cm, respectively. BMI was calculated as body weight in kilograms divided by the square of the participant's height in meters (weight/height^2). Waist circumference (WC, narrowest diameter between xiphoid process and iliac crest) was measured as well [21, 22].

Activity of ALT and AST in serum were determined by the standard biochemical methods. Serum concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) concentrations were measured using the standard enzymatic methods with commercially available kits (BioVendor, Czech Republic). Low-density lipoprotein

Table 1. Anthropometric, clinical and laboratory parameters in examined patients (M ± SD or %)

Parameters	Placebo group (n = 24)	Probiotic-smectite group (n = 26)	P
Age (years)	57.38 ± 9.92	53.23 ± 10.09	0.150
Duration of T2D (years)	5.33 ± 2.82	6.46 ± 5.92	0.401
Metformin, % (n)	71.4	70.0	0.905
Sulfonilureas, % (n)	53.6	43.3	0.436
Insulinotherapy, % (n)	25.0	36.7	0.337
BMI [kg/m^2]	32.55 ± 3.62	33.19 ± 4.93	0.601
Weight [kg]	92.3 ± 11.49	94.81 ± 12.04	0.455
Waist circumference [cm]	94.08 ± 4.96	96.57 ± 5.01	0.084
FLI	80.16 ± 10.36	82.11 ± 10.95	0.521
LS [kPa]	7.69 ± 1.33	8.02 ± 1.39	0.401
ALT [IU/L]	35.93 ± 16.32	35.88 ± 16.89	0.991
AST [IU/L]	36.96 ± 17.66	33.31 ± 13.94	0.420
γ-GT [IU/L]	45.33 ± 12.21	47.87 ± 21.84	0.088
TC [mmol/L]	5.92 ± 0.8	6.15 ± 0.83	0.320
TG [mmol/L]	2.50 ± 0,91	2,53 ± 0,99	0.888
HDL-C [mmol/L]	1.4 ± 0.27	1.33 ± 0.23	0.292
LDL-C [mmol/L]	3.41 ± 0.68	3.64 ± 0.77	0.270
TNF-α [pg/mL]	50.52 ± 18.73	51.57 ± 20.35	0.586
IL-1β [pg/mL]	47.69 ± 21.36	44.49 ± 19.88	0.850
IL-6 [pg/mL]	13.89 ± 8.80	13.22 ± 8.40	0.785
IL-8 [pg/mL]	26.05 ± 8.27	29.25 ± 8.04	0.785
γ-INF	168.29 ± 75.51	187.23 ± 75.53	0.380

cholesterol (LDL-C) concentration was calculated using the Friedewald equation [24].

The contents of serum interleukins (TNF-α, IL-1β, IL-6, IL-8, and IFN-γ) were measured by enzyme-linked immunosorbent assay (ELISA) method. The studied cytokines were immobilized in 96-well plates with adsorption surface. They were added with primary and secondary enzyme-labeled antibodies (Sigma). An appropriate substrate was then added and produced a detectable product in the enzymatic reaction. The optical densities of the colored solutions in wells immediately after the enzymatic reaction termination depicted the level of cytokines of the different group.

Statistical analysis

The SPSS statistical package, version 20.0 (SPSS, Inc., Chicago, Illinois) and GraphPad Prism, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical analyses and a *P* value less than 0.05 was considered statistically significant. All data in this study were expressed as mean ± standard deviation (M ± SD) or %. Data distribution was analyzed using the Kolmogorov-Smirnov normality test. The baseline characteristics of participants in the 2 groups were compared using independent sample t-tests and chi-squared (χ^2) test. The changes in outcomes of the participants after the initiation of therapy and end of the trial were compared by paired sample *t*-tests. Analysis of covariance (ANCOVA) was used to identify any differences between the 2 groups after intervention, adjusting for baseline measurements and confounders (BMI and sex).

Results

In this single-center RCT, a totally 51 patients with NAFLD and T2D were randomly allocated to receive probiotic additionally supplemented with smectite (Symbiter Forte group, n = 26) or placebo (n = 25) for 8 weeks, respectively. The groups were homogeneous according to age, sex and diagnostic criteria (Table 1). One patient from placebo group did not complete study due to lost of follow up. In final intention to treat analysis 50 patients were included which received more than 90% of prescribed sachets formulation in double-blind treatment. The compliance rate was comparable between groups — 91.7% in placebo and 92.3% in Symbiter Forte group respectively (p = 0.993). Patients were satisfied with the organoleptic properties of both nutraceuticals formulation. During study period probiotic-smectite and placebo were well tolerated. Across the study patients complained only with several mostly gastrointestinal symptoms.

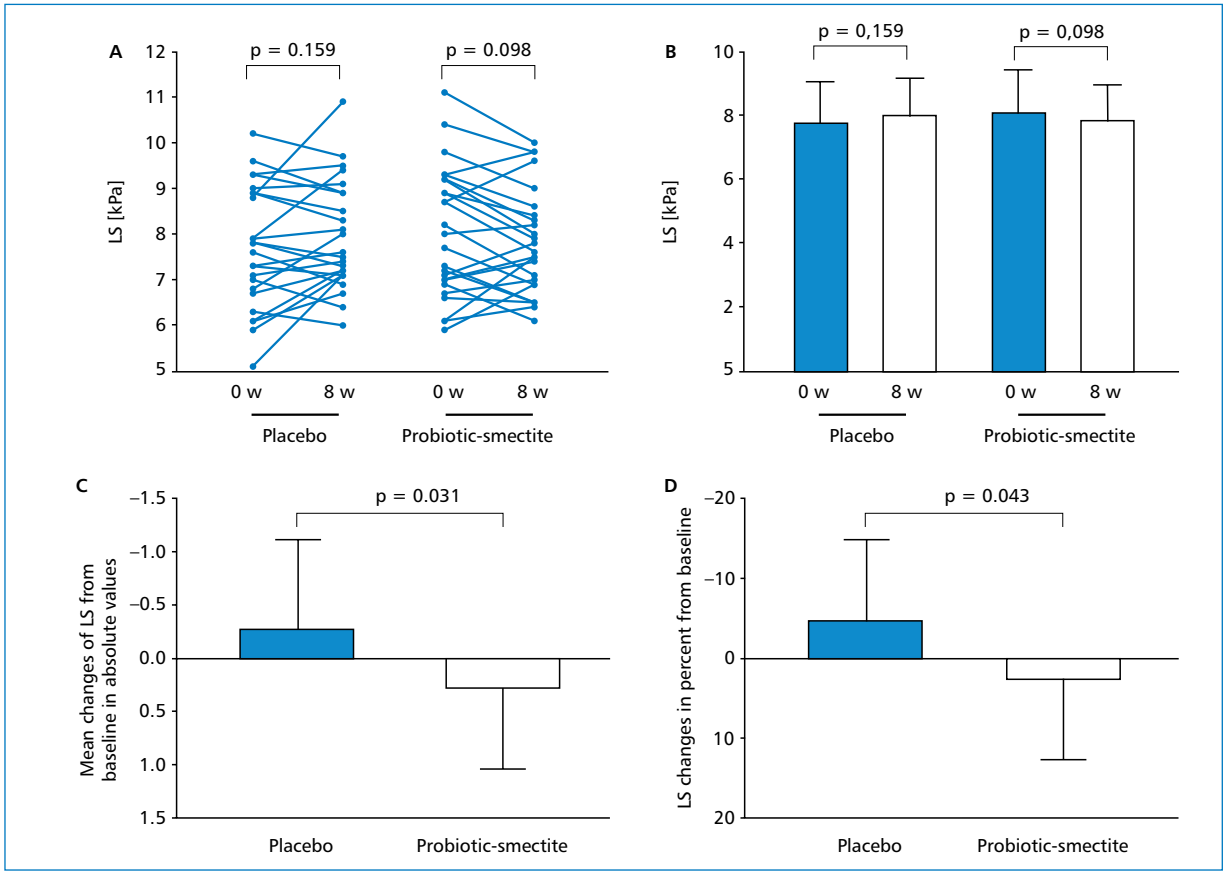

Figure 1. Primary outcomes analysis with accent on LS changes. **A**, **B** — intra-group analysis of changes at baseline and after interventon. Data expressed in mean ± SD (A) and individual values at baseline and after 8 weeks of treatment; **C**, **D** — analysis of inter-group mean changes of absolute values (C) or percentages (D) from baseline to end of treatment throughout the study. ANCOVA was used to identify any differences between the 2 groups after intervention

All adverse events were mild, reversed spontaneously and did not lead to participants withdrawn from the study. Patients received placebo reported adverse events were nausea (n = 2; 8.33%), mild abdominal pain (n = 1; 4.16%) and short-term diarrhea (n = 2; 8.33%). The main complaints in Symbiter Forte group were short-term diarrhea (n = 2; 7.69%) and constipation (n = 1; 3.84%), heartburn (n = 1; 3.84%), mild abdominal pain (n = 1; 3.84%) and dizziness (n = 1; 3.84%). The total prevalence of adverse events was comparable between groups (placebo = 20.8% *vs.* probiotics-smectite = 23.1%, p = 0.848).

There were no significant differences between the groups at baseline in terms of age, sex, diabetes duration, anthropometric and laboratory measurements (Table 1). Participants were treated with oral anti-diabetic agents, insulin or their combination. Recent study suggested that metformin, GLP-1, DPP-4 inhibitors are known to has pleiotropic effects beyond glucose reduction, including improvement of lipid profiles, bile acids and finally gut microbiota [25, 26]. To elude possible interaction between incretin-mimetics and gut microbiota, patients treated with these class of anti-diabetic drugs were excluded from the study. From the other hand metformin nowadays recognized as first line therapy in patients with T2D, so it would be unethical to exclude metformin from participant therapy regimen. In our study to avoid this bias we randomized equal portions of patients, treated with stable doses of metformin at least 4 weeks prior to study start. In general, at baseline proportion of patients on insulin-therapy (p = 0.337), treated with sulfonylureas (p = 0.436) and/or metformin (p = 0.905) were comparable between the groups (Table 1).

Primary outcome changes dynamics from baseline to 8 weeks after intervention are presented in Figures 1 and 2. Both our primary endpoints the LS measured by SWE and FLI insignificant decrease after probiotic-smectite treatment and increase in placebo groups (Figures 1A, B; 2A, B). However, we observed significant differences between mean changes of LS expressed in absolute value (–0.254 ± 0.85 *vs.* 0.262 ± 0.77; p = 0.031) or percentages (–4.427 ± 12.6 *vs.* 2.38 ± 10.25; p = 0.043) from baseline to end of treatment in

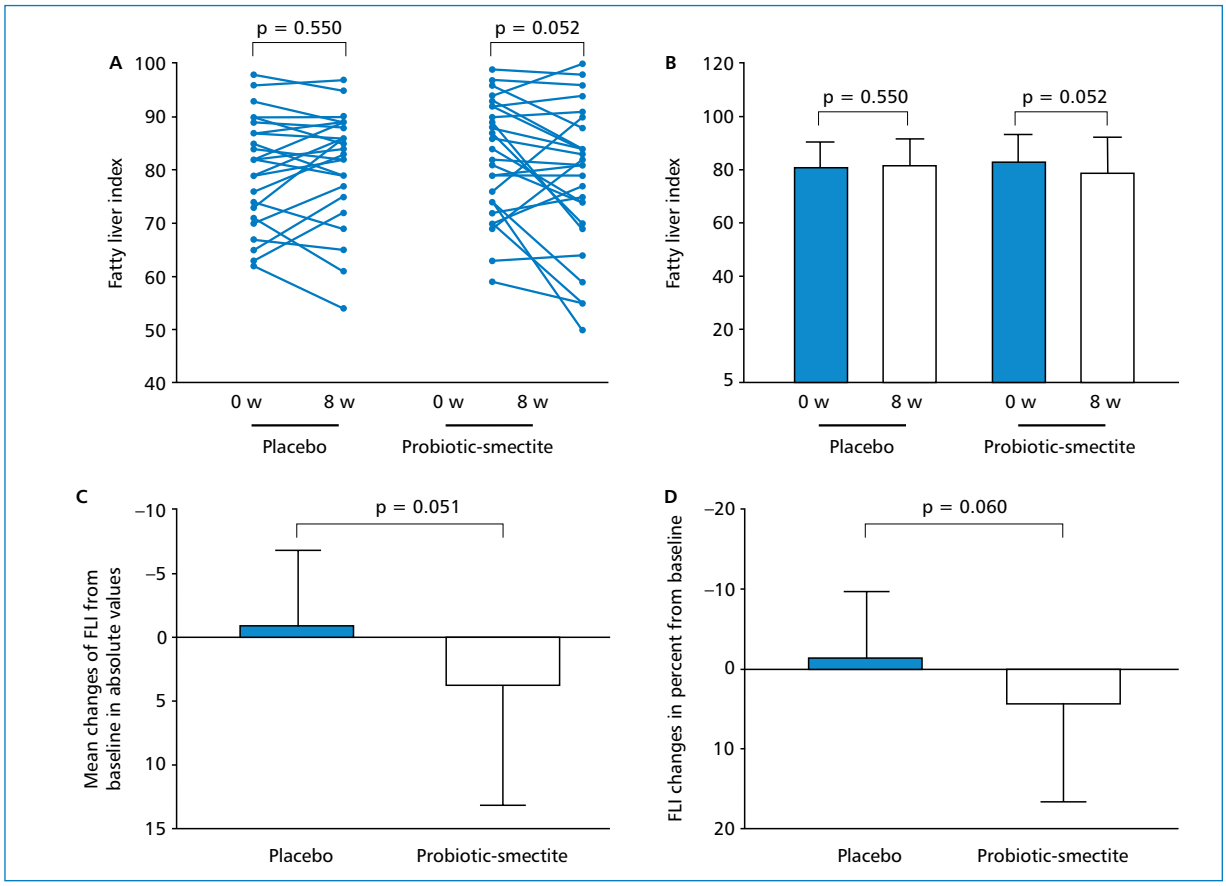

Figure 2. Primary outcomes analysis with accent on FLI changes. **A**, **B** — intra-group analysis of changes at baseline and after interventon. Data expreesed in mean ± SD (A) and individual values at baseline and after 8 weeks of treatment; **C**, **D** — analysis of inter-group mean changes of absolute values (C) or percentages (D) from baseline to end of treatment throughout the study. ANCOVA was used to identify any differences between the 2 groups after intervention

ANCOVA analysis (Figures 1C, D). In respect to another our primary endpoints, FLI mean changes across groups from baseline, expressed in absolute values (–0.750 ± 1.23 *vs.* 3.769 ± 1.84; p = 0.051) or percentages (–1.194 ± 8.43 *vs.* 4.471 ± 12.15; p = 0.06) were insignificant (Figures 2C, D).

Analysis of secondary outcomes showed that co-administration of probiotic with smectite lead to significant reduction of ALT (35.88 ± 16.89 *vs.* 29.25 ± 10.48; p = 0.016), AST (33.31 ± 13.94 *vs.* 30.0 ± 11.67; p = 0.021), TC (6.15 ± 0.83 *vs.* 5.86 ± 0.81; p = 0.010), IL-1β (44.49 ± 19.88 *vs.* 37.75 ± 14.02; p = 0.037) and TNF-α (51.57 ± 20.35 *vs*. 44.81 ± 18.14; p < 0.001) as compared to week 8 (Figures 3–5, Table 2). However, in between groups analysis changes remained significant only ALT expressed in absolute values (p = 0.022, Table 2). In the placebo group, changes were insignificant for all parameters which were included in secondary outcomes analysis.

Discussion

In this single-center RCT, it has been demonstrated that probiotic additionally supplemented with smectite (Symbiter Forte) insignificantly decreased both FLI and LS values measured by SWE in primary outcomes analysis. Analysis of secondary outcomes showed significant reduction of transaminases activity, total cholesterol, IL-1β and TNF-α values in intervention as compared to placebo group after 8 weeks of treatment. However, in between group ANCOVA analysis were mean values from baseline were analyzed, changes remained significant only for LS and ALT activity.

Mofidi et al. [27], similar to the present study, used LS with transient elastography (FibroScan®) and hepatic steatosis (CAP score) measurement, to evaluate the efficacy of synbiotic supplementation in lean NAFLD patients. In the randomized, double-blind, placebo-controlled, clinical trial hepatic steatosis and fibrosis reduction was observed in both groups; how-

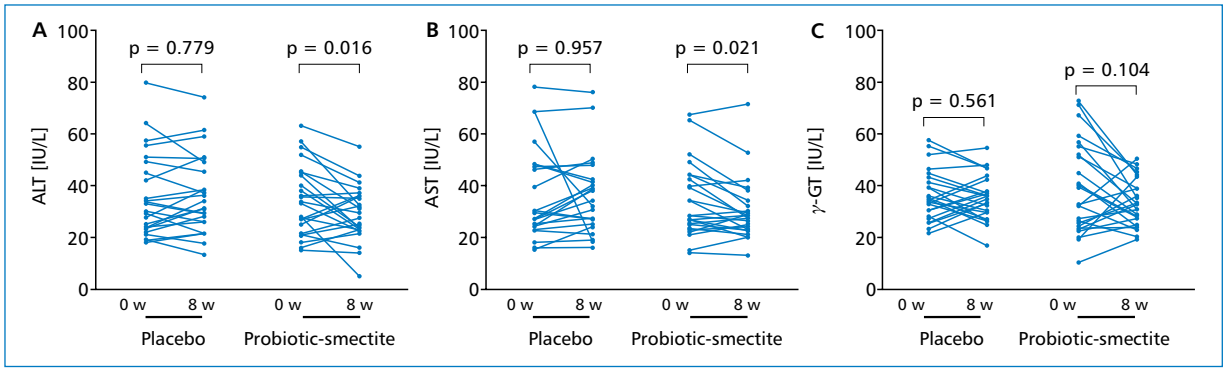

Figure 3. Secondary outcomes analysis with accent on transaminases changes. **A**, **B**, **C** — intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week

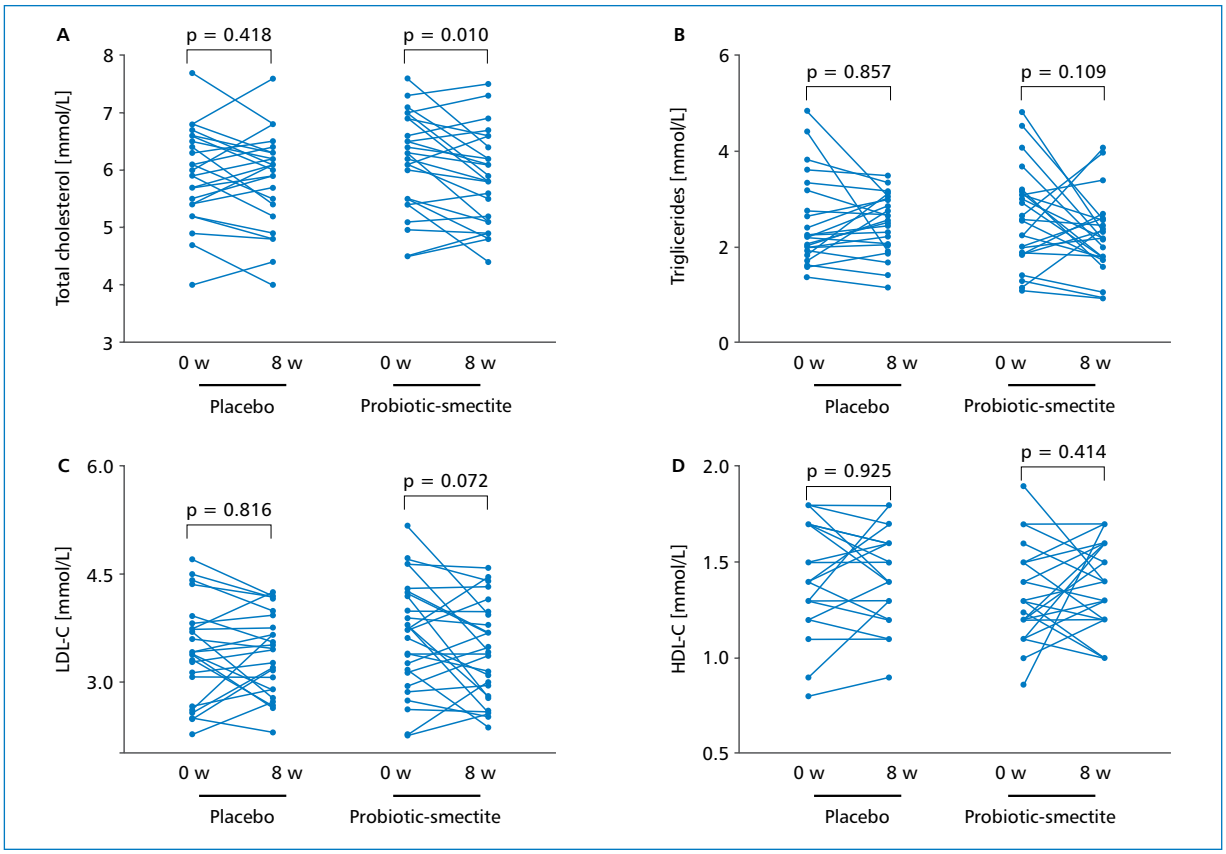

Figure 4. Secondary outcomes analysis with accent on lipid parameters. **A**, **B**, **C**, **D** — intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week

ever, the mean reduction was significantly greater in the synbiotic group rather than in the placebo group ($p < 0.001$) [27].

Recent RCTs with similar design [21, 22] it has showed that administration of alive probiotic, alone or in combination with omega-3 fatty acids, had significant impact on hepatic fat content which characteristic reduction of FLI after intervention. No significant changes were noted LS measured with SWE in both trials [21, 22]. Moreover, co-administration of alive multi-strain probiotic mixture with omega-3 fatty acids once daily for 8 weeks to patients with NAFLD characterized with more pronounced changes in serum lipids and cytokines levels in secondary outcomes analysis as compared to probiotic only or probiotic-smectite, were we observed greatest reduction of transaminases activity.

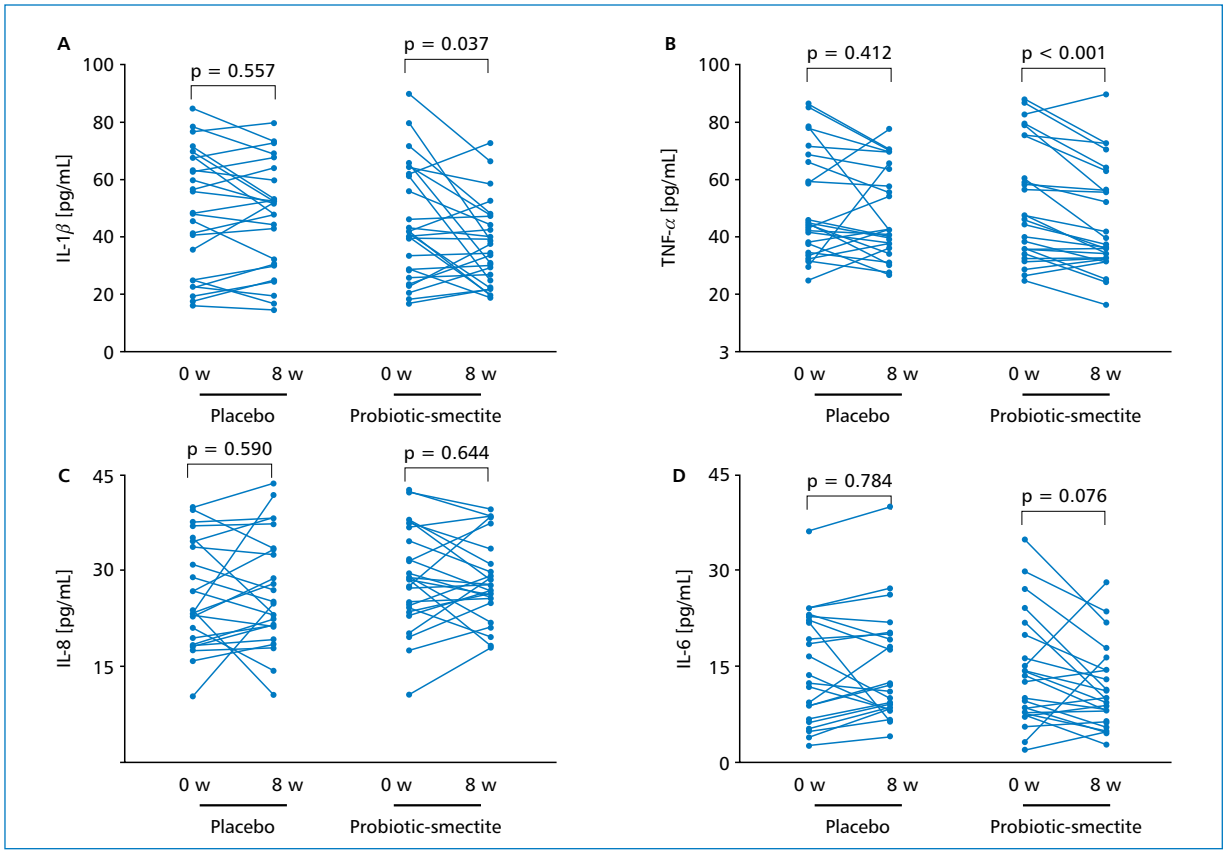

Figure 5. Secondary outcomes analysis with accent on cytokines changes. **A**, **B**, **C**, **D** — intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week

Preparing RCT we have proposed that heartening of probiotic with smectite due to his ability support normal functional activity of intestinal mucosa, immunomodulating and cytoprotective effects, may lead to summation of their single positive effects. Smectite (bentonite) is a natural loamy poly-mineral which are formed by extremely small particles capable for hydration and demonstrate the most physiologically active properties in form of gel [28]. Smectite distributed on the intestinal tract surface and showed cytomucoprotective therapeutic effect by delivering energetic and plastic materials to epitheliocytes, improving the strength of the mucosa barrier and permitting mineral particles to interact with glycoproteins of the mucosa as well as with the microbial biolayer [15, 19]. Another important property of smectite gel is direct absorbing capacity with viruses, toxins, radionuclides, heavy metals and bacterial endotoxins without ''swallowing up'' normal microbiota cells and physiologically important nutrients [17, 20]. Following the mixing of probiotic biomass and smectite gel, the sorbent becomes bound to surface structures of bacterial cells and covers them with a protective layer promoting the increased probiotic biomass survival during its transit through the more aggressive gastrointestinal tract areas [19]. It is worth using nutraceuticals together with probiotics, as then possible to simultaneously improve mucosa cytoprotection and to restore its symbiosis with intestine physiological microflora.

In conclusion, in this RCT we confirmed previously reported animal data, that in NAFLD patients co-administration to, of probiotic with smectite due to his absorbent activity and stabilization mucus layer properties can impact on synergistic enhancement of single effect which manifested with reduction of LS, liver transaminases and chronic systemic inflammation.

The general limitations of our study were the use of the US technique instead of biopsy as the diagnostic criteria for NAFLD, the small sample size, and the absence of a longer term follow up. Therefore, modulation of the gut microbiota with probiotic and different nutraceuticals represents a new branch in NAFLD management, but further studies in larger cohorts are required to determine this beneficial effect.

Conflicts of interest

The authors report that they have no conflicts of interest.

Table 2. Changes in secondary outcomes parameters between baseline and week 8 (M ± SD)

Parameters	Placebo group (n = 24)	Probiotic-smectite group (n = 26)	P
ALT [IU/L]			
Absolute value	–0.37 ± 6.55	6.62 ± 13.07	0.022
Percentage from baseline	–3.79 ± 20.95	10.32 ± 32.1	0.074
AST [IU/L]			
Absolute value	0.15 ± 13.38	3.31 ± 6.88	0.293
Percentage from baseline	–7.59 ± 29.93	6.20 ± 19.22	0.056
γ-GT [IU/L]			
Absolute value	1.23 ± 10.27	6.22 ± 18.8	0.256
Percentage from baseline	–0.76 ± 25.92	–1.36 ± 44.22	0.954
TC [mmol/L]			
Absolute value	0.092 ± 0.54	0.295 ± 0.53	0.195
Percentage from baseline	1.24 ± 9.19	4.39 ± 8.55	0.214
TG [mmol/L]			
Absolute value	0.03 ± 0.79	0.355 ± 1.08	0.236
Percentage from baseline	–4.65 ± 27.35	5.408 ± 41.79	0.323
LDL-C [mmol/L]			
Absolute value	0.02 ± 0.48	0.230 ± 0.62	0.201
Percentage from baseline	–1.04 ± 15.99	4.44 ± 17.04	0.247
HDL-C [mmol/L]			
Absolute value	0.004 ± 0.21	–0.46 ± 0.28	0.435
Percentage from baseline	–1.28 ± 16.57	–6.27 ± 25.75	0.424
TNF-α [pg/mL]			
Absolute value	2.35 ± 13.78	6.75 ± 7.73	0.166
Percentage from baseline	–0.71 ± 33.65	12.17 ± 14.4	0.081
IL-1β [pg/mL]			
Absolute value	1.07 ± 8.87	6.74 ± 15.59	0.125
Percentage from baseline	–1.65 ± 22.13	6.00 ± 33.0	0.345
IL-6 [pg/mL]			
Absolute value	–0.279 ± 4.93	2.28 ± 6.3	0.117
Percentage from baseline	–18.11 ± 43.72	–5.19 ± 94.2	0.542
IL-8 [pg/mL]			
Absolute value	–0.78 ± 7.06	0.51 ± 5.62	0.472
Percentage from baseline	–8.24 ± 38.22	–2.44 ± 24.06	0.521
IFN-α [pg/mL]			
Absolute value	9.84 ± 32.75	21.37 ± 50.33	0.366
Percentage from baseline	3.15 ± 21.33	4.31 ± 32.95	0.885

Authors' contributions

N.K. and L.A. conceived and designed the study. N.K., G.M., L.B., T.F., L.K., D.K. and O.T. wrote the manuscript. All authors enrolled patients and approved the final version of the manuscript.

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Manidip Mandal, Anila Varghese, V.K. Gaviraju, Sangamesh N. Talwar, Suttur S. Malini
Department of studies in Zoology, University of Mysore, India

Impact of hyperglycaemia on molecular markers of oxidative stress and antioxidants in type 2 diabetes mellitus

ABSTRACT

Introduction. The pathogenesis of type 2 diabetes mellitus (T2DM) is strongly linked to oxidative stress mainly caused by chronic hyperglycaemia. The present study investigates the association between hyperglycaemia with oxidative stress markers, antioxidants and lipid profile.

Materials and methods. The case-control study involved two groups, T2DM patients (n = 83) and age and sex matched controls (n = 81). Serum levels of various molecular markers malondialdehyde (MDA), reactive oxygen species (ROS) and nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), vitamin C, total antioxidant capacity (TAC) and lipid parameters total cholesterol, triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured using spectrophotometric assays. Results were analysed to compare and correlate glycaemic levels with these molecular markers.

Results. T2DM patients had a higher body mass index (BMI) and body fat percentage. 2 hour blood glucose, glycated haemoglobin A_{1c} % (HbA_{1c}), total cholesterol, triglycerides and LDL were higher in diabetics, HDL was found to be lower in diabetics than in controls. Mean levels of enzymatic and non-enzymatic antioxidants SOD, CAT, GSH, vitamin C and TAC were significantly lower while oxidative stress markers NO, ROS and MDA were higher in T2DM patients. NO showed a positive correlation ($r = 0.3993$, $p < 0.0001$) whereas TAC showed a negative correlation with glycaemia ($r = –0.4796$, $p < 0.0001$).

Conclusions. Poor glycaemic control in T2DM causes elevated ROS and NO levels with increased lipid peroxidation and lowered antioxidant capacity. MDA and NO being the major risk factors could be used as a parameter along with antioxidants to assess oxidative stress in T2DM patients. (Clin Diabetol 2019; 8, 4: 215–222)

Key words: total antioxidant capacity, nitric oxide, malondialdehyde, type 2 diabetes mellitus, hyperglycaemia

Introduction

Type 2 diabetes mellitus or non-insulin dependent diabetes mellitus (NIDDM) is a metabolic syndrome of multiple etiology characterised by chronic hyperglycaemia resulting from disturbances in glucose and lipid metabolism. Diabetes causes an array of microvascular and macrovascular complications and stroke with an increasing incidences worldwide. The various diabetic complications, β-cell dysfunction and worsening of glycaemic control is linked to increased reactive oxidative stress (ROS) and reactive nitrosative stress (RNS) owing to increased production of free radicals such as the nitric oxide, superoxide radical, hydrogen peroxide and the hydroxide radical and free radical induced lipid peroxidation and a deficiency in the antioxidant defence mechanisms. The lipid peroxidation of tissues which is primarily caused due to increased ROS, is thought to play an important role in the development of atherosclerosis and other microvascular complica-

Address for correspondence:
Suttur S. Malini, PhD
Department of Studies in Zoology, University of Mysore
Manasagangothri, Mysore — 570006, India
Phone: +919686261612
e-mail: ssmalinisri@yahoo.co.in
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tions [1]. During the development of the disease, hyperglycemia causes increase in production of ROS in different tissues by forming advanced glycation end products. Hyperglycaemia-induced mitochondrial superoxide production is the sole underlying mechanism by which it induces cellular damage. The antioxidant defence network maintains the mitochondrial level of ROS within balanced concentrations. However, in hyperglycemia environment, enhanced glucose flux through glycolysis and Krebs cycle causes an overdrive of mitochondrial electron transport chain (ETC) resulting in mitochondrial dysfunction and increased ROS formation [2]. Excessive levels of ROS leads to cellular dysfunction, altered cell cycle, altered cell-signalling, increased inflammation and also is linked to development of insulin resistance, impaired metabolic pathways, diabetes and cardiovascular disorders (CVD) through dysfunction of insulin secretion and metabolism. Antioxidant defence mechanisms involve both enzymatic and non-enzymatic strategies. Common antioxidants include vitamin A, C and E, non-enzymatic antioxidant and cofactor GSH (L-γ-glutamyl-L-cysteinylglycine) and the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). They work in synergy with each other and against different types of free radicals [3]. The impairment of the endogenous antioxidant defence system is produced in many ways during chronic hyperglycaemia.

Lipids with unsaturated double bonds are highly susceptible to damage by free radicals, this process is commonly known as lipid peroxidation and it has deleterious effects in the biological system and it has been strongly linked to diabetes pathogenesis and complications. Malondialdehyde (MDA) is formed as an end product of lipid peroxidation. Elevated MDA levels show adverse physiological consequences which include altering cell membrane structural integrity, inactivating membrane bound enzymes and cell surface receptors. MDA is involved in foam cell formation which leads to atherosclerosis and other cardiovascular diseases. The determination of MDA is an important parameter to evaluate in vivo lipid peroxidation [4]. Nitric oxide (NO) is a gaseous free radical synthesized as a by-product from L-arginine to L-citrulline conversion by the action of nitric oxide synthase (NOS). Endotoxins and cytokines produce an inflammatory response which expresses NOS II, which can generate excess endogenous NO over long periods further aggravating inflammatory and ischemic processes. NO plays a role in angiogenesis, neurotoxicity and is involved in mediating diabetic retinal vascular injury which suggests the possible role of NO in pathogenesis of diabetic retinopathy (DR) [5]. The collective action of all the antioxidants present in plasma and other body fluids can be defined as the total antioxidant capacity (TAC) which provides a composed parameter instead of a sum of all antioxidants. Alterations in TAC levels show a significant role in metabolic syndrome (MetS) and gestational diabetes [6].

Materials and methods

Subjects and study design

The present study was conducted in Department of Studies in Zoology, University of Mysore, Karnataka, India during the period of January–July 2018. The study conformed to the Helsinki declaration and was conducted with the approval of the Institutional ethical and research committee. 83 T2DM patients were recruited from an outpatient department of medicine of a primary health centre. For comparison, 81 age and sex matched healthy controls were randomly selected and recruited. Diagnosis for T2DM was done according to World Health Organisation (WHO) recommendations for the diagnostic criteria for diabetes and intermediate hyperglycaemia (2006). Inclusion criteria for T2DM patients included age group of 25–60, 2 hour blood glucose $\geq$ 11.1 mmol/L and treatment with hypoglycaemic drugs. Exclusion criteria included patients using any kind of vitamin supplements, lipid lowering drugs, antioxidant supplements, anti-inflammatory drugs, pregnant and lactating women, alcoholics, smokers and individuals with tobacco and other drug addiction, past history of chronic illness like tuberculosis, autoimmune disorders, endocrine disorders, patients with type 1 DM and any patient who did not meet the inclusion criteria were excluded from study group. Criteria for controls were based on previous medical and family history of diabetes, free of any metabolic disorders and other major diseases and 2 h blood glucose < 11.1 mmol/L.

Assessment of biochemical parameters

Venous blood samples was drawn to analyse 2 hour blood glucose and the serum was used to analyse HbA_{1c} and lipid parameters. The serum was stored at –20°C for further analysis of molecular markers. All the biochemical estimations were done with the spectrophotometer Varioskan LUX multimode microplate reader instrument (ThermoFisher Scientific, USA). 2 h glucose in blood was estimated by GOD-POD technique. The lipid profile estimation was done using Arkray (Arkray healthcare, Mumbai, India) lipid kit. Serum total cholesterol (TC) was estimated by CHOD-PAP method. Serum HDL was estimated with the HDL precipitation reagent and a HDL standard using the CHOD-PAP kit. Triglycerides (TG) was estimated using GPO-PAP technique. LDL was measured indirectly using the Friedewald formula [7].

Assessment of obesity parameters

The BMI were calculated by dividing weight in kilograms by the square of the height in metres [kg/m^2]. The body fat percentage were measured using OMRON HBF-306 body fat monitor. The Asian Indian criteria of obesity and overweight was used to define the BMI in the subjects (normal BMI: 18.0–22.9 kg/m^2, overweight: 23.0–24.9 kg/m^2, obesity: > 25 kg/m^2) [8].

Molecular markers analysis

The total serum proteins were measured by Lowry method using bovine serum albumin as the standard. SOD activity was measured spectrophotometrically using Fridovich et al. [9] method where photo reduction of riboflavin leading to nitrite formation was measured at 543 nm. Serum Glutathione (GSH) was measured by its reaction with 5-5′ dithiobis (2-nitrobenzoic acid) (DTNB) to give a yellow coloured complex whose absorbance is measured at 412 nm [10]. CAT activity was measured spectrophotmetrically by following the oxidation of H_2O_2 at 240 nm. The decomposition of H_2O^2 can be followed directly by decrease in absorbance at 240 nm. The difference in absorption per unit time is the measure of catalase activity [11]. Vitamin C in serum was determined by dinitrophenyl hydrazine method where ascorbic acid is oxidized to form a coloured complex which is measured at 520 nm. TAC was determined spectrophotometrically through the formation of phosphomolybdenum complex. 100 μL of serum was mixed with 100 μL of 5% trichloroacetic acid (TCA) to precipitate the proteins. The sample was centrifuged and the supernatant was reacted with 1ml of TAC reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) which was incubated at 95°C for 90 minutes. The samples were cooled to room temperature and the absorbance of the aqueous solution was read at 695 nm against a blank [12]. The lipid peroxidation product, MDA, was measured by using thiobarbituric acid reactive substances (TBARS). MDA reacts with thiobarbituric acid at 100°C in an acidic medium to give a pink coloured complex. The colour intensity of the MDA-TBA complex was measured at 535 nm by using a spectrophotometer [13]. Serum NO was measured using the Griess reaction using a modified method of Grisham et al. [14]. Equal volumes of the serum sample and freshly prepared Griess reagent were incubated at 37°C for 10 minutes. A stable decomposition product (NO_2–) formed the coloured complex and its absorbance was measured at 540 nm using a spectrophotometer. ROS was measured fluorometrically using the dichlorofluorescindiacetyl (DCFDA) oxidation method by determining the hydrogen peroxide concentrations present in serum [15]. ROS oxidises DCFDA into a fluorescent compound dichlorofluoroscein (DCF). The fluorescence emitted was determined using a fluorometric reader (Varioskan LUX multimode microplate reader) at an excitation wavelength of 525 nm and an emission wavelength of 488 nm.

Statistical analysis

The subjects were grouped into T2DM (n = 83) and controls (n = 81). The difference in various parameters like age, body mass index, diabetes duration, glycaemic levels, lipid profiles and molecular markers were tested for significance by comparing the two groups using student t-test and the relationship of family history with DM was assessed using chi-square test. The association between hyperglycaemia and diabetes duration with various clinical factors were assessed using linear regression analysis. BMI and Age were entered in the multiple regression analysis model to determine its association with glycaemic status. Pearson's correlation coefficient was used to determine the relationship between glycaemic levels and serum NO and TAC levels. All the data were entered in a Microsoft excel spreadsheet. Statistical analysis was performed using IBM SPSS version 23.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Data are reported as mean ± standard deviation (mean ± SD). The level of significance for all tests was set at $p < 0.05$.

Results

Individuals with T2DM were older compared to non-diabetics ($p < 0.0001$). T2DM patients also had a higher rate of diabetes in family history. The BMI ($p < 0.0001$) and body fat percentage ($p < 0.0001$) were higher in T2DM when compared to the controls. The average DM duration for the whole sample was 9.0 ± 3.1 years, females had a higher DM duration than males (Table 1). The diabetic patients in the study were either undergoing treatment with metformin only (n = 33) or no treatment at all (n = 50). There was no statistically significant difference observed in the levels of various parameters between the treatment and non-treatment groups. The 2-hr blood glucose ($p < 0.0001$), HbA_{1c}% ($p < 0.0001$), total cholesterol ($p < 0.0001$), triglycerides ($p < 0.001$) and LDL ($p < 0.019$) were higher in diabetics when compared to controls while serum HDL ($p < 0.002$) was found to be lower in diabetics than in controls (Table 2). Mean levels of enzymatic and non-enzymatic antioxidants SOD ($p = 0.045$), CAT ($p < 0.0001$), GSH ($p < 0.0001$), vitamin C ($p < 0.001$) and total antioxidant capacity ($p < 0.0001$) were significantly lower in T2DM patients compared

Table 1. Demographic characteristics of the patients

Parameter	Total (n = 164)	Type-2 diabetics (n = 83)	Controls (n = 81)	p
Age (years)	48.1 ± 8.8	50.0 ± 8.3	46.1 ± 8.9	0.0001
Sex ratio (M/F)	99/65	50/33	49/32	0.9092
Family history of T2DM (yes/no)	44/120	32/51	12/69	0.0007
Diabetes duration in years	–		–	0.0001
a. Male		a. 9.6 ± 3.7		
b. Female		b. 8.6 ± 2.5		
BMI [kg/m^2]	27.0 ± 4.1	28.1 ± 4.1	25.9 ± 3.9	0.0001
Body fat (%)	31.3 ± 6.6	33.1 ± 5.8	29.5 ± 6.9	0.0001

Data presented as mean ± SD. T2DM — type 2 diabetes mellitus; BMI — body mass index

Table 2. Biochemical parameters

Parameter	Type 2 diabetics	Controls	95% CI	p
2-hr blood glucose [mmol/L]	14.25 ± 4.46	6.29 ± 1.21	6.94, 8.96	0.0001
HbA_{1c} (%)	10.57 ± 2.80	5.57 ± 0.76	4.36, 5.63	0.0001
Total cholesterol [mg/dL]	159.72 ± 38.69	117.48 ± 32.21	31.32, 53.14	0.0001
Triglycerides [mg/dL]	151.37 ± 48.89	126.0 ± 30.59	10.09, 40.64	0.002
LDL [mg/dL]	58.72 ± 21.40	45.85 ± 30.30	2.26, 23.47	0.019
HDL [mg/dL]	44.44 ± 9.53	48.66 ± 9.31	1.10, 7.33	0.002
Total serum proteins [mg/dL]	6.45 ± 3.80	8.95 ± 3.31	1.37, 3.62	0.0001

Data presented as mean ± SD. HbA_{1c} — glycated haemoglobin; LDL— low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; 95% CI — 95% confidence intervals; CI here indicates difference between two population means lies between lower CI and upper CI

Table 3. Oxidative stress and antioxidant parameters

Parameter	Type 2 diabetics	Controls	95% CI	p
SOD [units/mg protein]	0.0602 ± 0.108	0.1008 ± 0.128	0.001, 0.080	0.045
CAT [nmol H_2O_2 oxidised/min/mL]	0.0284 ± 0.0298	0.0481 ± 0.0350	0.01, 0.03	0.0001
GSH [nmol/mL]	23.316 ± 0.584	24.593 ± 1.120	0.705, 1.849	0.0001
Vitamic C [μmol/L]	31.001 ± 8.693	55.992 ± 18.691	15.957, 34.025	0.0001
TAC [μmol α-tocopherol/L]	119.977 ± 44.103	160.793 ± 60.302	18.985, 62.649	0.0001
Serum NO [μmoles/L]	72.170 ± 26.021	47.022 ± 23.607	17.539, 32.757	0.0001
ROS [mmol DCF formed/min/mg protein]	0.474 ± 0.241	0.388 ± 0.128	0.025, 0.147	0.007
Serum MDA [nmol/mg protein]	0.304 ± 0.178	0.178 ± 0.141	0.076, 0.177	0.0001

Data presented as mean ± SD. SOD — superoxide dismutase; CAT — catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric Oxide; ROS — reactive oxygen species; MDA — malondialdehyde; 95% CI — 95% confidence intervals; CI here indicates difference between two population means lies between lower CI and upper CI

to controls. Oxidative stress markers NO ($p < 0.0001$), ROS ($p = 0.007$) and MDA ($p < 0.0001$) were higher in T2DM patients when compared to controls (Table 3).

Linear regression analysis showed a positive association between hyperglycaemia and oxidative stress markers, BMI, total cholesterol, triglycerides and LDL and negative association with antioxidant levels and HDL (Table 4). There was no correlation observed between diabetes duration with different molecular markers and lipid profile, a positive association between diabetes duration and glycaemic levels, BMI and fat % whereas a negative association with TAC was observed (Table 5). Multiple regression analysis was used to test if age and BMI significantly affects the glycaemic status in T2DM patients. The results of the regression indicated the two predictors explained 7.3% of the variance [$R^2 = 0.073$, $F(2,161) = 6.34$, $p = 0.002$]. It was found that BMI significantly affected glucose

Table 4. Association of hyperglycaemia with different parameters

Parameter	*R^2	Slope	95% CI	F	p
Age	0.0194	0.2395 ± 0.1335	–0.0220, 0.5011	3.221	0.0746
BMI	0.0328	0.1461 ± 0.0623	0.0239, 0.2682	5.497	0.0203
Fat %	0.0316	0.2285 ± 0.0993	0.0337, 0.4231	5.290	0.0227
Cholesterol	0.1629	3.233 ± 0.5758	2.105, 4.362	31.53	0.0001
HDL	0.0952	–0.666 ± 0.1731	–1.006, –0.3276	14.84	0.0002
LDL	0.0642	1.482 ± 0.5601	0.3699, 2.595	7.003	0.0094
TG	0.0564	2.072 ± 0.8391	0.4058, 3.739	6.099	0.0152
CAT	0.0675	–0.0017 ± 0.0004	–0.0026, –0.0007	11.74	0.0008
SOD	0.0347	–0.0048 ± 0.0021	–0.0090, –0.0006	5.143	0.0248
GSH	0.1552	–0.1022 ± 0.0324	–0.1673, –0.0370	9.918	0.0027
Vitamin C	0.1298	–0.3521 ± 0.1479	–0.6517, –0.0525	5.667	0.0224
TAC	0.1558	–5.009 ± 1.138	–7.268 to –2.750	19.37	0.0001
ROS	0.0497	0.0090 ± 0.0033	0.002512, 0.01551	7.384	0.0074
NO	0.1304	1.945 ± 0.3945	1.172 to 2.718	24.30	0.0001
MDA	0.0500	0.0074 ± 0.0026	0.0023, 0.0125	8.055	0.0052

*Linear regression analysis — goodness of fit. 95% CI — 95% confidence intervals; BMI — body mass index; TG: — triglycerides; LDL — low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; SOD — superoxide dismutase; CAT — catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric oxide; ROS — reactive oxygen species; MDA — malondialdehyde

Table 5. Association of diabetes duration with different parameters

Parameter	*R^2	Slope	95% CI	F	p
Glucose	0.5537	1.092 ± 0.1089	0.8745, 1.309	100.5	0.0001
BMI	0.0472	0.2940 ± 0.1467	0.0015, 0.5864	4.015	0.0484
Fat %	0.059	0.4664 ± 0.2070	0.05392, 0.8789	5.078	0.0269
Cholesterol	0.0185	1.730 ± 1.399	–1.059, 4.519	1.529	0.2199
HDL	0.0055	–0.2809 ± 0.4835	–1.248, 0.6861	0.3375	0.5635
LDL	0.0243	1.390 ± 1.393	–1.425, 4.205	0.9960	0.3243
TG	0.0158	2.718 ± 3.391	–4.135, 9.570	0.6425	0.4276
CAT	0.0057	–0.0007 ± 0.0010	–0.0029, 0.0014	0.4694	0.4952
SOD	0.0386	–0.0091 ± 0.0057	–0.0206, 0.0024	2.495	0.1193
GSH	0.0398	–0.0866 ± 0.0699	–0.2284, 0.0551	1.535	0.2231
Vitamin C	0.0382	–0.0712 ± 0.0842	–0.2483, 0.1057	0.7161	0.4085
TAC	0.2906	–8.923 ± 2.323	–13.64, –4.208	14.75	0.0005
ROS	0.0139	0.0059 ± 0.0064	–0.0069, 0.0187	0.8504	0.3601
NO	0.0048	0.5972 ± 0.9477	–1.292, 2.486	0.3971	0.5304
MDA	0.0095	0.0058 ± 0.0069	–0.0080, 0.0198	0.7128	0.4012

*Linear regression analysis — goodness of fit. 95% CI — 95% confidence intervals; BMI — body mass index; TG — triglycerides LDL — low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; SOD — Superoxide dismutase; CAT — Catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric oxide; ROS — reactive oxygen species; MDA — malondialdehyde

levels (β = 0.207, p = 0.007) as well as age (β = 0.184, p = 0.017). Glycaemic levels showed a significant negative correlation with total antioxidant status (r = –0.4796, p < 0.0001) (Figure 1A), whereas glycaemic levels showed a significant positive correlation with oxidative stress marker NO (r = 0.3993, p < 0.0001) (Figure 1B).

Discussion

Oxidative stress has focus interest in various clinical research in recent times. There is a growing evidence connecting the action of oxidative stress to the pathogenesis and complications in diabetes mellitus and many other diseases. Oxidative stress plays a role in pathogenesis of insulin resistance and β-cell dysfunc-

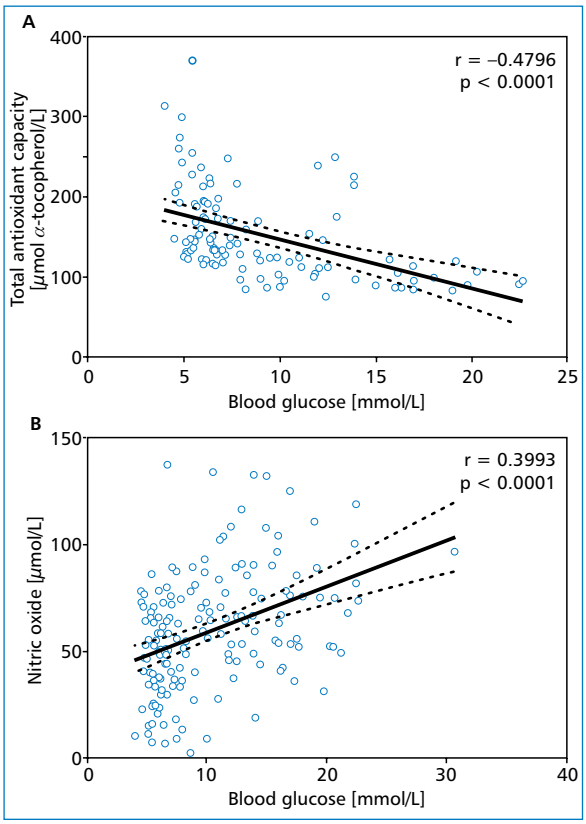

Figure 1A. Correlation between total antioxidant capacity (TAC) and blood glucose; **B.** Correlation between serum nitric oxide (NO) and blood glucose

tion, caused by dysregulation of cell homeostasis and metabolism [4]. Hyperglycaemia is the principal metabolic alteration which is associated with diabetes mellitus, and increased glycaemic levels in bodily fluids has been implicated to increase oxidants, cause cellular damage, vascular dysfunction and pathogenesis of vascular disease.

This study reveals a detailed information on the impact of hyperglycaemia on oxidative stress status in T2DM patients. Present study shows a two-fold higher blood glucose as well as HbA_{1c} levels in diabetic compared to normal subjects, which is an indication of worsening glycaemic control. Hyperglycemia increases the levels of free radical generation which activates several pathways involved in pathogenesis of complications of diabetes: polyol pathway, increased AGE formation and activation of protein kinase C (PKC) isoforms [16]. This ultimately leads to irreversible damage of biomolecules like proteins, lipids and nucleic acids and loss of its functional ability [17]. Dyslipidaemia is a major risk factor for CVD in diabetes mellitus. It is characterised by high total cholesterol, serum triglyceride concentrations, high LDL cholesterol and lowered HDL cholesterol concentrations. Dyslipidaemia is attributed to free fatty acid flux secondary to insulin resistance [18]. Data from table 2 shows a considerable increase in total cholesterol, serum triglycerides and LDL levels and a significant decrease in HDL levels in this study indicating a dyslipidaemia condition in the diabetic patients although the LDL and triglyceride parameters in diabetics do not exceed the American Diabetes Association 2011 guidelines for lipid goals [19]. Dyslipidaemia in diabetes have the ability to generate oxidative stress which possibly leads to the development of macroangiopathy causing endothelial dysfunction and atherosclerosis.

The study also showed a significant drop in both enzymatic and non-enzymatic antioxidants. There was a drop in serum CAT activity which was in agreement with the findings of Pasaoglu et al. [20]. Several studies on serum and erythrocyte SOD levels have shown increased, decreased as well as unchanged enzyme levels. In this study there was a decrease in SOD activity. This finding is in accordance with Kesavulu et al. We observe a decrease in CAT activity and consequently SOD activity. A possible explanation for the fall in SOD activity could be linked to glycation of the SOD enzyme in serum due to hyperglycaemic condition. Decrease in SOD activity consequently leads to decrease in CAT activity as both the enzymes function in unison to neutralise superoxide ion to water and oxygen molecule [21]. Another possible explanation for the fall in CAT activity could be due to downregulation of serum CAT due to catalase gene mutations caused by elevated hydrogen peroxide levels [22]. The study also observed decrease in serum GSH levels which was consistent with that of Gallou et al. [23]. GSH a key intracellular antioxidant is involved in redox regulation of protein thiols and hydrogen peroxide in mitochondria [21]. Several studies have shown that reduced/oxidized glutathione ratio is lower in diabetics [24]. In hyperglycaemic conditions, glucose is preferentially used in polyol pathway which consumes NADPH that is necessary for GSH regulation by the GR enzyme. Thus, hyperglycaemia indirectly causes GSH depletion. Vitamin C is a powerful dietary antioxidant, it donates electrons thus helping in scavenging free radicals. Vitamin C reduces the overall hyperglycaemic state by reducing blood glucose, reducing glycosylation of proteins and by decreasing the production of sorbitol. Several clinical studies have shown that vitamin C levels in diabetics are lowered and that supplementing with vitamin drugs helps to slightly alleviate the diabetic complications [25]. In this study, the level of vitamin C, is significantly decreased in diabetics. A possible explanation for the low vitamin C levels in diabetics could be linked to increased ascorbic acid

oxidation or impaired regeneration from its oxidized state [21]. There has been limited studies describing the total antioxidant status in T2DM. This study concluded that diabetics had a lower TAC level than controls. The correlation study showed that TAC was negatively correlated with glycaemic levels indicating worsening plasma antioxidant capacity with increased hyperglycaemia in T2DM. Opara et al. observed a decrease in TAC levels in diabetic patients whereas Korkmaz et al. observed increased TAC levels in early diabetic stages and Savu et al. observed an increase in TAC levels synergistically with other antioxidants as well as MDA [25–27]. As the results stand controversial and the mechanisms are poorly understood, further investigation is needed to validate the status of TAC for monitoring antioxidant levels in diabetes.

T2DM is characterised by prolonged and increased intracellular and extracellular ROS generation. In this study ROS levels were studied by determining the peroxide concentrations in serum. The ROS levels in diabetics were slightly higher than in controls although there was no significant difference between them. Free radicals attack membrane phospholipids causing lipid peroxidation and high levels of these oxidised products have been correlated with development of vascular complications. The high levels of MDA in serum can be linked to failure of antioxidant system to curb the deleterious action of free radicals and hence it serves as a reliable stress marker to assess free radical induced tissue damage [7]. The increase in lipid peroxidation reflected by the increase in serum MDA levels in diabetics in the present study are in accordance with previous studies that hyperglycaemia increases lipid peroxidation from overproduction of free radicals in diabetics [20]. The study showed significantly higher levels of serum NO in T2DM patients than in controls. Additionally, NO levels were positively correlated with increasing glucose levels which shows worsening glycaemic control accelerates NO production in serum. These findings are in agreement with Maejima et al. and Ozden et al. [28, 29]. Ozden et al. explains increased NO synthesis is a result of compensatory mechanism due to oxidative stress causing NO inactivation by inhibiting NO mediated endothelial function. Contrary to this study, Ghosh et al. reported decreased serum NO levels in T2DM patients [30]. Elevated serum NO levels have been attributed to pathogenesis of diabetes and diabetic retinopathy, hence assessing NO levels could be an important tool to measure the severity of diabetes in patients.

The regression analysis shows hyperglycaemia to be strongly associated with obesity and CVD-risk parameters showing a positive association with BMI, body fat, total cholesterol and LDL. It also showed a strong positive association with MDA and NO levels and a negative association with CAT, GSH, vitamin C and TAC levels. In contrast, diabetes duration and oxidative stress parameters and lipid profile showed no correlation. This finding is consistent with the results of Aouacheri et al. [31]. The positive association between diabetes duration with glycaemic levels and negative association with TAC in the analysis indicates prolonged hyperglycaemic conditions greatly reduces the antioxidant capacity of cells, leading to oxidative stress. This study shows that poor glycaemic control in T2DM elevates oxidative and nitrosative factors, decreases antioxidants and alters lipid profile all of which are strongly linked to diabetic and vascular complications.

Conclusions

This study shows the importance of monitoring levels of antioxidants in parallel with NO and MDA along with the usual glycaemic and lipid markers in T2DM which could be useful to assess the degree of oxidative stress present and provide important identification cues for patients to undergo antioxidant treatment since administration of antioxidants is found to be effective only in selective group of patients with depleted natural antioxidants and elevated oxidative stress levels. Some of the limitations of this study are the small patient population to assess the different parameters and the need to perform assays on protein carbonyls to further understand the extent of damage caused by oxidative stress in T2DM. More clinical studies are needed on larger patient populations to understand the underlying physiological effects of ROS, RNS and different antioxidants in T2DM and the potential of using these parameters to determine oxidative stress in the patients.

Conflict of interest

The authors declare no conflict of interest.

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Hanna Kwiendacz[1], Magdalena Domek[1], Katarzyna Nabrdalik[1], Anna Maj-Podsiadło[2], Magdalena Marcisz[3], Karolina Drożdż[3], Janusz Gumprecht[1]

[1]Department of Internal Medicine, Diabetology and Nephrology, Medical University of Silesia, Zabrze, Poland
[2]Department of Internal Medicine and Diabetology, Prof. Stanisław Szyszko Independent Public Clinical Hospital No 1, Medical University of Silesia, Katowice, Poland
[3]Student's Scientific Association by the Department of Internal Medicine, Diabetology and Nephrology, Medical University of Silesia, Zabrze, Poland

An unusual use of personal insulin pump by a patient with type 1 diabetes on a ketogenic diet — a case report

ABSTRACT

In this case report we present a 28-year-old woman with type 1 diabetes mellitus on a ketogenic diet for 5 months, using a personal insulin pump in an unusual way. The patient was admitted to the Department of Internal Medicine and Diabetology due to vomiting and diarrhea that had lasted for several days. On a daily basis, she used personal insulin pump for only several hours a day (a 5-hour basal rate of 0.6 units/hour of fast-acting insulin) in order to avoid dawn phenomenon, without any prandial insulin, and she used continuous glucose monitoring for 24 hours a day for glycemia control. Additionally she was taking 30 units of long acting insulin analog before sleep. The patient was unwilling to change her treatment method and she was discharged from the hospital against medical advice. Due to the increase in popularity of ketogenic diet, there is a need for large studies assessing its safety and efficacy. Moreover, our case draws attention to the fact that patients can use modern technologies, which are developed to improve the glycemic control, in unconventional ways. (Clin Diabetol 2019; 8, 4: 223–226)

Key words: diabetes mellitus type 1, ketogenic diet, personal insulin pump, insulin, continuous glucose monitoring

Introduction

Over the last decades, there has been a significant technological progress in the treatment of type 1 diabetes, mainly regarding new insulin preparations, continuous glucose monitoring and personal insulin pumps. However, many patients still do not achieve glycemic targets [1] and have difficulty controlling postprandial hyperglycemia, which significantly influences HbA_{1c} values [2]. Only 30% of adults with type 1 diabetes reach the HbA_{1c} target of < 7% [3]. Treatment of type 1 diabetes with intensive functional insulin therapy, including continuous subcutaneous insulin infusion (personal insulin pumps), is a recognized and effective method of treatment [4]. Clinical recommendations for dietary management of patients with diabetes indicate the need to individualize the daily amount of carbohydrates consumed in the range of 25–60% of the total daily energy requirement [5]. According to the 2019 Guidelines of Diabetes Poland, there is insufficient scientific data to determine one optimal carbohydrate dietary intake for patients with diabetes. However, it is believed that carbohydrates should account for approximately 45% of the daily energy requirement and a reduced amount of carbohydrates, i.e. 25–45% can be consumed temporarily by patients who are not

Address for correspondence:
lek. Hanna Kwiendacz
Katedra i Klinika Chorób Wewnętrznych, Diabetologii i Nefrologii, Śląski Uniwersytet Medyczny, Wydział Lekarski z Oddziałem Lekarsko-Dentystycznym w Zabrzu
ul. 3 Maja 13–15, 41–800 Zabrze
Phone/fax: +48 32 370 44 15
e-mail: hanna.kwiendacz@gmail.com
Translation: lek. Małgorzata Kamińska
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physically active. Fats should constitute 25–40% of the daily energy intake [6]. Due to the fact that both the type and amount of carbohydrates consumed affect postprandial glycemia, patients as well as researchers are very interested in low-carbohydrates diets, including a diet with a very low amount of carbohydrates, i.e. ketogenic diet, also in patients with type 1 diabetes [7–9]. The ketogenic diet has already been used in the treatment of type 1 diabetes before the invention of insulin [10], and therefore, the history has come full circle. There is no single definition of a diet with a very low carbohydrate content; however, the most common daily amount of carbohydrates in such a diet is less than 50 g or less than 10% of the total daily energy intake, with increased amount of fats and proteins consumed [3, 11]. When a ketogenic diet is used, ketosis occurs as a result of an increased production of so-called ketone bodies: acetoacetate, betahydroxybutyrate and acetone. Ketonemia reaches the maximum value of 7–8 mmol/L, at blood pH within a normal range. When using a balanced diet, the average blood ketone concentration is < 0.3 mmol/L, whereas in diabetic ketoacidosis blood ketone concentration can exceed 20 mmol/L with coexisting decrease in blood pH [12, 13]. Nowadays, patients are looking for alternative therapeutic methods, especially for various types of diets, that would improve their health. Popular websites often publish information about alleged benefits of these methods which have not been confirmed in scientific studies [14]. In recent months there has been a debate in the scientific community, caused by the publication of the paper by Lennerz et al. [15], regarding the relevance of promoting a diet with a very low carbohydrate content in patients with type 1 diabetes [9, 16]. These authors, using an online questionnaire filled out by patients, evaluated glycemic control and adverse events, such as the occurrence of diabetic ketoacidosis, hypoglycemia, and hospitalization due to decompensation of diabetes, in children and adults with type 1 diabetes who were on a ketogenic diet [15]. The study participants were recruited from a Facebook group established in April 2014 and associating, at the time of the study, 1,900 people with type 1 diabetes on a very low carbohydrate diet (up to 30 grams/day). Finally, 493 people completed the study questionnaire, of which 316 were qualified for further observation. The results obtained indicated a good glycemic control (mean HbA_{1c} was 5.67 ± 0.66%), a low rate of hypoglycemia (in 2 patients) or ketoacidosis (in 4 patients). One can get the impression that the results of the cited work encourage the use of a ketogenic diet; nevertheless, both the authors themselves and experts commenting on the study underline the need to verify these results in well-designed, large clinical trials.

Data on the prevalence of diabetic ketoacidosis (DKA) in adults with type 1 diabetes, unlike data regarding children, are limited. Based on a recent systematic review of the literature, the incidence of DKA was estimated at 50–100 cases per 1,000 adult patients with type 1 diabetes [17]. The data on the incidence of DKA at the level of 2% of patients presented by Lennerz et al. [15] are lower than the estimated annual risk of DKA episode in patients not using the ketogenic diet [17]; however, due to the fact that these data come from a small survey, it is not possible to conclude on that basis that the risk of developing ketoacidosis in patients on a ketogenic diet is low and that in this aspect the diet can be considered safe. Moreover, generally acknowledged contraindications to the use of the ketogenic diet, which include disorders of lipid metabolism, porphyria and pyruvate carboxylase deficiency, should be taken into account [18].

In this report we present a case of a patient with type 1 diabetes on a ketogenic diet, using a personal insulin pump in an unusual way.

Case presentation

A 28-year-old female, diagnosed with type 1 diabetes at the age of 13, who has been treated for 5 years with personal insulin pump (Medtronic Minimed G640) with compatible continuous glucose monitoring system (CGMS) was admitted to the Department of Internal Medicine and Diabetology on July 2017 due to vomiting and diarrhea that had lasted for several days. The patient explained that she used personal insulin pump for only several hours a day, providing a 5-hour infusion of fast-acting insulin analog (insulin aspart, Novo Nordisk, 0.6 units/hour) in order to avoid dawn phenomenon between 2 a.m. and 7 a.m. Therefore, she connected the insulin pump tubing before going to sleep and disconnected it after waking up. Additionally she administered a single daily dose of basal insulin (30 units of insulin glargine U300, Sanofi Aventis) before going to sleep. She did not administer insulin boluses before meals.

In addition, for 5 months the patient has been using a ketogenic diet to avoid the need for prandial insulin. This behavior was probably caused by the lack of acceptance of the disease (the patient refused a psychologist consult). She declared the following daily intake of nutrients: 10 g carbohydrates, 15 g proteins, 120 g fats. On the day of admission to the Diabetology Department, the patient was in a good general condition. A physical examination did not reveal any abnormal findings, except for dryness of the oral mucosa. The BMI was 20.5 kg/m^2 (body weight 62 kg, height 174 cm). The only abnormal results of the laboratory tests were

Table 1. Blood glucose levels during hospitalization [mg/dL]

Date\Time	3:00	8:00	12:00	16:00	21:00	24:00
14.07.2017	–	–	–	147	113	87
15.07.2017	72	79	106	75	82	107
16.07.2017	107	116	100	110	104	85
17.07.2017	71	100	144	85	114	–
18.07.2017	–	80	–	–	–	–

the increased concentration of betahydroxybutyrate in the venous blood (6 mmol/L), with normal blood pH, and ketonuria (++++). Blood glucose at admission was 147 mg/dL. During the hospitalization glycemic values were within normal range, which is presented in Table 1. The results of other laboratory tests (complete blood count, serum aminotransferases [AST and ALT] and lipid concentration, venous blood gasometry) did not show significant deviations from the reference values.

After intravenous rehydration (the patient did not consent to the administration of glucose either orally or intravenously) and treatment with proton pump inhibitor administered intravenously, dyspeptic symptoms resolved and the patient was discharged on the fourth day of hospitalization at her own request, against medical advice. During the hospitalization, the patient did not agree to diet modification or administration of prandial insulin.

According to the patient, after she had been diagnosed with diabetes her mean HbA_{1c} values oscillated around 7%. The introduction of the ketogenic diet resulted in a reduction in HbA_{1c} value from 6.4% to 5.4%, and the blood glucose self-monitoring values remained in the range of 60–90 mg/dL. Moreover, she did not observe body weight reduction while being on the diet. The patient monitored her blood ketone concentration on a daily basis using an Optium Xido Abbott Diabetes glucose meter, thus assessing the state of ketosis. While on a ketogenic diet, the patient had never had symptoms like the one observed just before hospitalization. She claimed that the way she use a personal insulin pump in combination with basal insulin injected with a pen and a ketogenic diet has been accepted by her diabetologist; however, she did not have any medical documentation confirming this information. It is also worth noting that the amount of fat consumed by the patient in proportion to the other nutrients (about 90% of the total daily caloric supply) is not in accordance with any recommendations of diabetic or dietary associations. Average daily blood glucose values of 60–90 mg/dL, which in the long-term perspective may have a negative effect on neurocyte function, also raise concern [19].

It should be emphasized that due to the lack of randomized, prospective trials assessing the safety of the ketogenic diet, its impact on individual metabolic pathways, the body composition by percent of mass or long-term systemic effects is not known. However, it has been proven that excessive protein supply may adversely affect kidney function in people with reduced renal filtration, and patients with type 1 diabetes are at particularly high risk of this complication [20, 21]. In addition, high-fat products contribute to the progression of atherosclerotic lesions and increased visceral fat, which increases insulin resistance [22].

Taking into account lack of the patient's consent to psychological consultation, strict carbohydrate restriction and BMI values at the lower limit of the normal range, anorexia-related eating disorders should be considered. It is also important that the patient, although using insulin reservoirs and infusion sets for insulin administration for only a few hours daily, is subject to the same reimbursement principles as the patients who use them 24 hours a day, which is not negligible, given the reimbursement-related costs incurred by the payer.

Summary

The presented clinical case shows that the patients can use the recommended forms of therapy in their own way, not necessarily consistent with the current principles of the treatment of type 1 diabetes [4, 5]. According to our knowledge, this is the first report describing the unconventional use of a personal insulin pump in the treatment of type 1 diabetes, where the pump therapy, used only to prevent the dawn phenomenon in the early morning hours, is combined with the single daily injections of a long-acting insulin analog, while eliminating prandial insulin boluses through the use of a ketogenic diet. Modern technologies developed to improve blood glucose monitoring and insulin therapy certainly facilitate obtaining the desired glycemic control by patients using different diets; however, it should be noted that the presented diet does not meet the principles of healthy nutrition in type 1 diabetes, so it should not be accepted by a health care provider. The case of the described patient

points out the need to carry out large trials assessing short- and long-term safety, but also the effectiveness of the ketogenic diet, which patients use more and more often. It also indicates that modern tools, in this case an insulin pump, can be used by patients in an unconventional way.

Conflict of interests

The authors declare no conflict of interest.

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VIA MEDICA
CASE REPORT
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Sarvenaz Kashefi[1], Ramesh Omranipour[2, 3], Habibollah Mahmoodzadeh[3], Hamid Ahmadi[2], Abbas Mirshafiey[1]

[1]Department of Immunology, School of Public Health, Tehran University of Medical Science, Tehran, Iran
[2]Breast Cancer Research Center (BDRC), Tehran University of Medical Science, Tehran, Iran
[3]Department of Surgical Oncology, Tehran University of Medical Science, Tehran, Iran

Clinical improvement of diabetes mellitus type 1 by β-D-mannuronic acid (M2000) in a breast cancer patient — as a case report

ABSTRACT

A 56 years old female with breast cancer (BC) and poor controlled diabetes mellitus type 1 (DM1) which has registered in a clinical trial for assessment of therapeutic efficacy of β-D-mannuronic acid (M2000) on pre-surgical BC patients is described in this case report. After receiving M2000, the patient was followed for 9 weeks. During this period, cancer mass details, fasting blood glucose (FBG) levels, 2-hour post-prandial blood glucose (2HPP), blood uric acid (BUA) level and urine analysis (UA) were continuously controlled. After 9 weeks of treatment with M2000, her FBG, BUA and UA decreased significantly. This finding was exactly in accordance with our published experimental data about the anti-diabetic effect of M2000 in an animal model. Therefore, it might be concluded that M2000 is probably able to improve DM1 by reducing FBG level, BUA level, glycosuria, ketonuria and proteinuria. (Clin Diabetol 2019; 8, 4: 227–229)

Key words: β-D-mannuronic acid, M2000, breast cancer, diabetes mellitus, NSAID

Address for correspondence:
Dr. Habibollah Mahmoodzadeh,
Prof. Abbas Mirshafiey
Tehran University of Medical Science, Tehran, Iran
Phone: +982161192541, fax: +982188485429
Phone, fax: +982188954913, e-mail:
e-mail: hmahmoodzadeh@tums.ac.ir;
mirshafiey@tums.ac.ir
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Introduction

BC patients with DM1 have poor prognosis with unknown mechanism [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the drugs prescribed for BC and DM1 [1]. However, their excessive use can be associated with adverse effects in the gastrointestinal tract, which limits their use in certain cases [2].

Alginates are linear copolymers found in brown seaweed and consist of β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) which are bound together by 1,4 glycoside bonds. M2000 (Patent PCT/EP2017/067919) is a low molecular weight small molecule ($C_6H_{10}O_7$) which is produced from sodium alginate, based on the protocol of Mirshafiey, et al. The purity of the M2000 has been confirmed using Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) and Fourier Transform Infrared Spectroscopy (FT-IR) [3]. Some data showed M2000 is a NSAID with a prominent immunosuppressive feature which can be used safely [4]. The molecular mechanism of therapeutic efficacy of this novel drug is based on its inhibitory effects on matrix metalloproteinase 2, 9 (MMP2, 9) activity, decrease in immune cells infiltration in inflammatory foci, blocking the toll-like receptor 2, 4 (TLR2, 4) downstream signaling transduction pathway, reduction of the level of inflammatory cytokine such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, a decrease in antibody production and induction of apoptosis using fibrosarcoma cell line [5, 6]. In this study, another effect of M2000 in the treatment of DM1 was presented for the first time as a case report in an ongoing phase I and II clinical trials on Iranian BC patients. In this report, a 56-year-old patient diagnosed with BC and poor controlled DM1 is introduced.

Table 1. Laboratory data changes during treatment with M2000

Parameter	A day before treatment	3rd week after treatment	6th week after treatment	9th week after treatment
FBG [mg/dL]	189	122	101	91
2HPP [mg/dL]	260	246	223	196
Uric acid [mg/dL]	8.2	7.6	6.3	5.8
Proteinuria [mg/dl]	321	255	208	117
Glycosuria [mmol/l]	1.9	1.5	1.2	1
Ketonuria [mmol/l]	2.3	1.4	1	0.7

FBG — fasting blood glucose; 2HPP — 2 hour post-prandial

Case report

A 56 years old female with the body mass index of 30.1 kg/m^2 (height: 164 cm, weight: 81 kg) patient with BC that refused surgery and chemotherapy selected. She accepted treatment with M2000 for BC. She also had uncontrolled DM1 diagnosed 8 years ago, receiving 68 units of Lantus and Novorapid insulin daily (24 units in the morning, 22 units in the afternoon and 22 units at night). Mean daily glycaemia was 206 ± 15 mg/dL. Patient had no comorbid diseases nor diabetic complications. Treatment with M2000 was started at a dose of 1000 mg per day for 9 weeks to evaluate its effect on breast tumor growth. Laboratory data changes are shown in Table 1. The patient did not report side effects during the treatment.

Discussion

The dysregulated metabolism in poorly controlled diabetes causes a long-term pro-inflammatory condition characterized by increased levels of IL-6, TNF-α, C-reactive protein (CRP) and other markers of chronic inflammation [2]. Recent evidence suggested that, persistent inflammation may contribute to genetic instability and chronic forms of inflammation can increase the risk of cancer [1]. This finding is also confirmed by credible evidence regarding the reducing effect of NSAIDs on the risk of certain types of cancer [1, 6].

The mechanism of inflammation in diabetic patients is unclear. Chronic oxidative stress is associated with chronic inflammation. Oxidants affect almost all stages of the inflammatory response process, including the release of inflammatory cytokines, sensing by innate immune receptors of TLRs, nucleotide-binding oligomerization domain-like receptors and activating the triggering signaling of the adaptive cellular response to such signals. Reactive oxygen species may damage lipids, proteins and DNA and then begin carcinogenesis [2]. In addition, chronic inflammation is associated with the increased levels of TNF-α, which strongly activates nuclear factor kappa B (NF-κB) and enhances the downstream signaling transduction, ultimately leading to progression

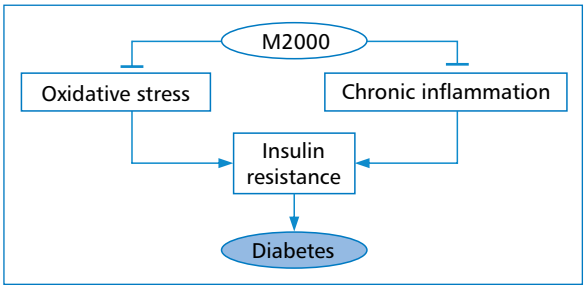

Figure 1. Schematic hypothesis: the anti-diabetic effect of M2000

of many tumors [7]. NF-κB protein complex contributes to the proliferation and survival of malignant cells, increases angiogenesis, metastasis, damages adaptive immunity and mediates the response to hormones and chemotherapy drugs. Therefore, long-term exposure to chronic inflammation and oxidative stress results in conversion of susceptible cells to malignant cells [8].

The main objective of this study was to improve BC by reducing tumor growth using M2000. M2000 was introduced as a novel NSAID with immunosuppressive effects due to its impact on the expression and activity of cyclooxygenase 1, 2 (COX1, 2) genes. In terms of the molecular mechanism, this new drug exerts its therapeutic effect through inhibiting the activity of MMP2 and MMP9, reducing the infiltration of immune cells in inflammatory foci, decreasing the level of inflammatory cytokines IL6 and IL17, reducing antibody production and inducing apoptosis using fibro sarcoma cell line. The results of a study showed that, this new NSAID can affect the M2000-treated mice through decreasing the expression levels of blood glucose, Scavenger receptor-A (SR-A), Lipoxygenase-1 (LOX-1), CD36 and CD68 compared to untreated diabetic rats (Figure 1) [9, 10].

Collectively, after 9 weeks of follow-up, there was a significant improvement in the level of FBG, 2HPP and UA in the patient with DM1. Therefore, it might be concluded that M2000 is a new NSAID that can improve DM1 in patients with BC.

Conflicts of interest

The authors report that they have no conflicts of interest.

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