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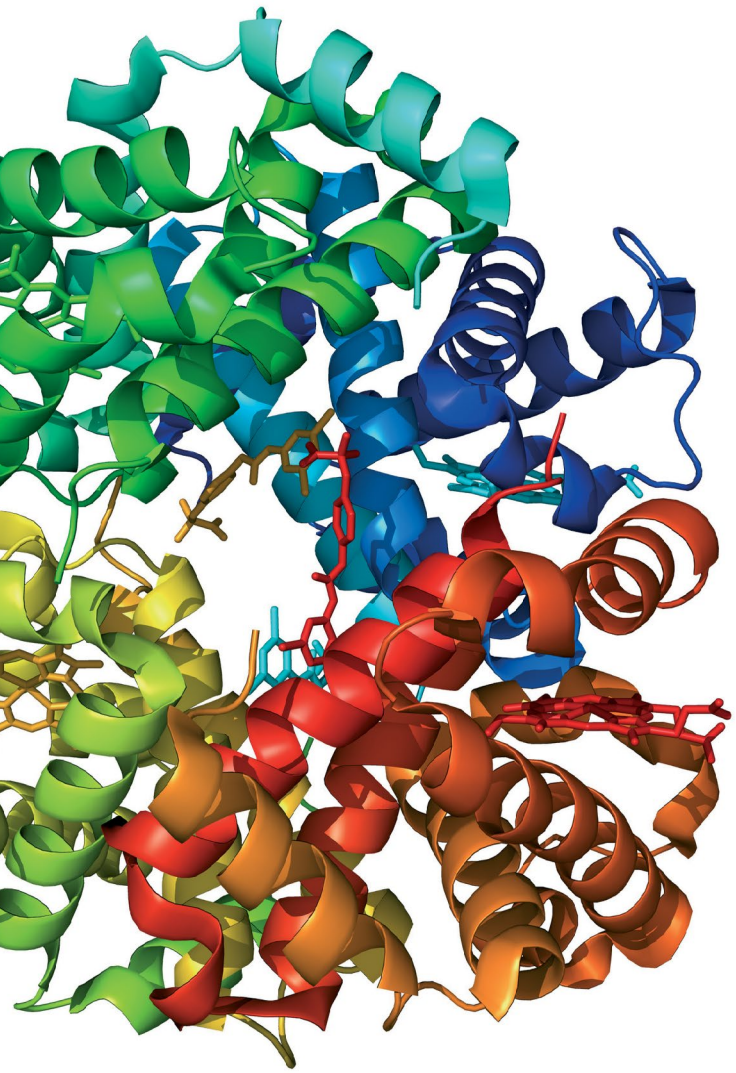

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Contents

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Changes in hematological parameters during first days of diabetic ketoacidosis treatment in children with type 1 diabetes mellitus

ABSTRACT

Background. Diabetic ketoacidosis (DKA) is a life-threatening complication of newly diagnosed type 1 diabetes (T1DM) and is associated with severe dehydration.

The aim of the study was to evaluate the changes in hematological parameters (RBC, Hct, Hb, MCV, PLT, WBC) and their correlations with acidosis level and dehydration during ketoacidosis treatment.

Methods. The study group consisted of 262 children with newly diagnosed type 1 diabetes. Clinical data were collected from hospital discharge charts. Data considering hematological parameters were collected from two timepoints: first at admission and second up to 6 days since admission.

Results. Ketoacidosis was present in 76 patients (29.01%). The DKA group had significantly higher values of baseline RBC ($p = 0.0026$), Hct ($p = 0.0019$), Hb ($p = 0.0235$), PLT ($p = 0.0427$) and WBC count ($p < 0.0001$) *vs.* patients without DKA. Interestingly, baseline MCV level was similar between the groups ($p = 0.9869$). During the first days of diabetes treatment, all hematological parameters such as RBC, Hct, Hb, PLT and WBC significantly decreased in both groups (all p values < 0.0001), while MCV significantly increased after treatment ($p < 0.0001$). However, the latter was evident only in no-DKA group. Changes in all hematological parameters correlated positively with pH (all $R > 0.3$ and all p values < 0.05) in DKA group but not in no-DKA group. However, weak, positive correlations at the margin of statistical significance with pH were observed for changes in PLT ($p = 0.0609$) and WBC ($p = 0.0811$) in no-DKA group.

Conclusion. Monitoring dynamics of hematological parameters at T1DM diagnosis may be useful in estimating patients' hydration status. (Clin Diabetol 2020; 9; 3: 149–160)

Key words: type 1 diabetes mellitus, diabetic ketoacidosis, dehydration, blood cell count, fluid therapy

Introduction

Diabetes mellitus refers to a group of metabolic disorders which are characterized by high glucose concentration resulting from lack of or deficiency in insulin secretion, action or both. In children and adolescents with diabetes, type 1 diabetes (T1DM) is the one most often diagnosed with its underlying cause being autoimmune pancreatic β-cell destruction leading to insulin deficiency [1]. The prevalence of diabetes is dramatically rising, with the projected number of

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patients estimated to increase by 54% from 2017 to 2045 to a staggering 693 million [2]. At the same time the incidence of childhood T1DM increases 3% per year worldwide [2]. Insulinopenia in patients with type 1 diabetes quickly leads to diabetic ketoacidosis (DKA). In this condition insulin deficiency is accompanied by a surge of counterregulatory hormones (glucagon, cortisol, catecholamines and growth hormone), which leads to hyperglycemia, increased lipolysis and ketogenesis. Dehydration sets in soon after, due to glycosuria further aggravated by concomitant vomiting [3]. Eventually, DKA leads to cerebral edema, coma or even death [4–6]. Mild to moderate dehydration in DKA may be managed predominantly by oral rehydration therapy (ORT), which may be bolstered by intravenous (IV) fluid administration in more severe cases, aiming to replenish fluid deficiency over 24 to 48 hours [6]. Cerebral edema is the most severe complication of excessive or too rapid fluid administration. This complication occurs in approximately 0.5% to 0.9% of children with newly onset T1DM and concomitant DKA with mortality rate of cerebral edema reaching 24% [6]. Therefore it is crucial to accurately estimate the degree of dehydration before initiating fluid therapy in DKA. This is not an easy estimate to make though, as dehydration is not directly correlated with the severity of DKA assessed by blood gas values [7].

One of the method used to estimate volume depletion is the Clinical Dehydration Scale (CDS) which takes into consideration: general appearance, sunken eyes, moisture of mucous membranes and tears production. This is a simple and noninvasive indicator but the accuracy of this scale is limited due to the present subjectivity of the performers [8]. Body weight changes during water depletion are also indicative for the degree of dehydration, but given the severity of DKA it is unfeasible in guiding therapy [7]. Moreover, the restitution of initial weight during DKA treatment is also linked to renutrition and insulin administration. Therefore, alternative means are developed to non-invasively, and rapidly evaluate fluid deficiency or overload. One such tool was recently described by Colucci et al. [9]. Their sensor uses nuclear magnetic resonance (NMR) to identify fluid overload in patients with end-stage renal disease (ESRD) at their bedside. Clearly, similar devices may be soon implemented to asses hydration status in children with DKA.

Laboratory measurements are helpful in evaluating hydration in an invasive manner. Plasma osmolality is the most direct test for dehydration, with 300 mOsm/kg being the commonly accepted threshold for dehydration [10]. However, among patients with DKA, equation for plasma osmolality may underestimate patient's osmolality leading to phenomenon known as osmolal gap (difference between calculated and directly measured osmolality) [11].

Serum sodium concentration alone is also used to estimate extracellular fluid (ECF) volume. However, apart from initial measurement, sodium concentration loses its usefulness during treatment. As plasma glucose concentration decreases due to insulin action, serum sodium concentration increases [12]. Therefore, in DKA one has to rely on corrected sodium concentration or bolster it with blood urea nitrogen measurements as described by Ugale et al. [7].

An alternative approach to estimate the extent of dehydration is the use of blood cell count parameters. Hematocrit (Hct) and hemoglobin (Hb) concentration are two such examples [13, 14]. However hemoglobin measurement is believed to be more verifiable indication of hydration status than hematocrit [15]. Dehydration is also reflected by decreased mean corpuscular volume (MCV) of erythrocytes, elevated platelet (PLT) and white blood cell (WBC) counts but all these attributes are influenced by a variety of other clinical factors [16].

Given this data it seems reasonable that hematological parameters such as increased hematocrit, hemoglobin concentration or platelet count might be useful indicators of deficit in extracellular fluid volume in children with DKA, despite their limitations [6, 12]. The benefit of this approach is the serial character of blood cell count parameters, routinely measured several times during DKA treatment. This in turn allows to estimate the baseline values for each patient, i.e. those preceding the onset of DKA, and guide fluid replenishment therapy using this data.

Therefore we aimed in this study to evaluate the changes in hematological parameters (RBC [red blood cells], Hct, Hb, MCV, PLT, WBC) and their correlations with acidosis level and dehydration during ketoacidosis treatment.

Methods

Data collection

All parents gave their informed consent to use their children's medical documentation for clinical studies. Clinical data from hospital discharge charts were obtained from patients hospitalized in the Department of Pediatrics, Oncology, Hematology and Diabetology of the Medical University of Lodz (currently Department of Pediatrics, Diabetology, Endocrinology and Nephrology) between 2009–2015. In our analysis we included patients with age under 18 and newly diagnosed T1DM. DKA and its severity at onset were assessed by using the International Society for Pediatric and Adolescent

Diabetes [6] consensus guidelines (mild: venous pH < 7.3 or serum bicarbonate concentration < 15 mmol/L; moderate: pH < 7.2 or serum bicarbonate concentration < 10 mmol/L; severe: pH < 7.1 or serum bicarbonate concentration < 5 mmol/L). We collected clinical data at T1DM diagnosis about: sex, date of birth, date of diagnosis, diabetes parameters (glucose concentration, HbA_{1c}, C-peptide, daily dose of insulin per kilogram of body weight (DDI), insulin therapy mode, presence of ICA and anti-GAD antibodies), ketosis parameters (pH, HCO_3^-, BE), renal profile (urea, creatinine, urine specific gravity), sodium (Na^+) and potassium (K^+) concentrations, CRP level and hematological parameters (RBC, Hct, Hb, MCV, PLT, WBC). Data considering hematological parameters were collected from two timepoints: first at admission and second up to 6 days since admission. Second measurement was considered as baseline value for each parameter before the DKA onset. Thus, changes of hematological parameters, calculated as a difference between second measurement minus first measurement, may reflect changes in water depletion. We could not provide ketone bodies concentration due to the fact that during diabetes onset they were not routinely examined. Effective osmolality [mOsm/kg] was calculated from following formula [17]: 2 [Na^+] + glucose/18. As acidosis increases the serum potassium concentration independently of intracellular potassium changes, for every 0.1 unit change of extracellular pH, there is an average 0.6 mEq/L inverse change of the serum potassium concentration [18]. Thus, following corrected [K^+] [mEq/L] formula was used for pH < 7.35 : serum potassium + ((pH – 7.35) *0.6 *10). eGFR [mL/min/1.73 m^2] was calculated using the Schwartz formula [19]: 0.413* (height/serum creatinine). Estimated dehydration [%] was calculated from multivariate analysis model of measured dehydration provided by Ugale et al. [7]. Formula was as follow: –22.60 + (0.16 *0.357 *urea at admission) + (0.18 *sodium concentration at admission).

Data analysis

Continuous data were presented as median with interquartile range and categorical data were presented as number with respective percentage. For comparison of continuous variables, we used Mann-Whitney U test and categorical data between two groups were compared with Chi-square, Yates correction and Fisher exact test, respectively. Wilcoxon signed-rank test was used to compare changes in hematological parameters after treatment of diabetic ketoacidosis. Spearman rang correlation coefficients were utilized for analysis of correlation. We used multivariate linear regression models for analysis of factors affecting changes in hematological parameters. In order to select variables entering multivariable linear model building for each hematological parameter, from Table 1. We firstly selected variables with p < 0.15 for correlation in DKA group with each hematological parameter. Afterwards, we checked collinearity of selected variables and excluded one of the variables (with lower R for correlation with outcome) from highly correlated pair (R > 0.4). The final model for each hematological parameter changes was adjusted to age and sex and contained variables with p < 0.15 in multivariate model. A p-value at the level of < 0.05 was considered as statistically significant for remaining analysis. All statistical analysis was performed with STATISTICA 13.1 software (TIBCO Palo Alto, CA, USA).

Results

Study group characteristics

Among 400 young patients (age < 18) diagnosed with new onset of T1DM between October 2009 and October 2015, we excluded 138 (34.5%) patients without two results of blood tests meeting predefined criteria. Characteristics of the remaining study group were shown in Table 1. DKA was present in 76 patients (29.01%).

The DKA group was characterized by significantly higher values of baseline RBC (p = 0.0026), Hct (p = 0.0019), Hb (p = 0.0235), PLT (p = 0.0427) and WBC count (p < 0.0001) *vs.* patients without DKA. Interestingly, baseline MCV level was similar between the groups (p = 0.9869). Children with DKA had also higher glucose concentration, HbA_{1c} level at diagnosis, effective osmolality, and lower eGFR than those without DKA (p < 0.0001, 0.0126, < 0.0001, < 0.0001, < 0.0001 respectively). At the discharged, daily dose of insulin (DDI) was significantly higher in DKA group (Me: 0.79 U/kg (25–75%: 0.62–0.97 U/kg) *vs.* Me: 0.58 U/kg (25–75%: 0.42–0.78 U/kg) p < 0.0001).

During the first days of diabetes treatment, we observed significant decrease of all hematological parameters (all p values < 0.0001) except for MCV which significantly increased after treatment (p < 0.0001). For changes in RBC, Hct, Hb concentration and platelets count there was no significant correlation with time between the blood tests measurements and the magnitude of parameters' change (all absolute R < 0.1 and p values > 0.3). However, the change of MCV correlated positively with time between the blood tests measurements (R = 0.14, p = 0.0303) while WBC change showed a negative correlation (R = –0.21, p = 0.0017). There was no significant difference in time between two hematological parameters measurements between DKA and no-DKA patients (Me: 3.0

Table 1. Study group characteristics at T1DM diagnosis

Characteristic	DKA (N = 76) N (%)	No-DKA (N = 186) N (%)	P value
Sex			
Males	41 (53.95%)	108 (58.06%)	0.5414
Females	35 (46.05%)	78 (41.94%)	
Type of therapy			
MDI	71 (93.42%)	175 (95.11%)	0.8055
CSII	5 (6.58%)	9 (4.89%)	
The presence of antibodies:			
ICA	36 (69.23%)	84 (70.00%)	0.9196
GAD	40 (75.47%)	90 (74.38%)	0.9705
Severity of DKA			
Mild	30 (39.47%)	NA	**NA**
Moderate	33 (43.42%)	NA	
Severe	13 (17.11%)	NA	
	Me (25–75%)	**Me (25–75%)**	
Age at onset (years)	9.65(4.37–12.54)	9.24 (6.03–13.50)	0.3033
Glucose concentration [mg/dL]	517.50 (390.64–685.00)	402.33 (302.87–550.00)	**< 0.0001**
HbA_{1c} (%)	12.30 (11.10–13.85)	11.70 (10.00–13.30)	**0.0126**
C-peptide [ng/mL]	0.33 (0.21–0.52)	0.37 (0.21–0.64)	0.3787
pH	7.24 (7.17–7.30)	7.38 (7.36–7.41)	**< 0.0001**
HCO_3^- [mmol/L]	9.15 (6.55–12.30)	21.60 (19.00–23.05)	**< 0.0001**
BE [mEq/L]	–16.85 (–21.40 to –12.70)	–3.00 (–5.50 to –1.60)	**< 0.0001**
Effective osmolality [mOsm/kg]	300.71 (291.17–309.67)	292.62 (288.02–300.62)	**< 0.0001**
Na [mEq/L]	135.00 (132.00–138.00)	135.00 (132.90–137.00)	0.6123
K [mEq/L]	4.36 (4.05–4.90)	4.40 (3.98–4.72)	0.2933
Corrected K [mEq/L]*	3.88 (3.02–4.29)	4.37 (3.98–4.70)	**< 0.0001**
Urea [mg/dL]	27.00 (21.40–36.00)	27.91(22.10–34.40)	0.9497
Creatinine [mg/dL]	0.72 (0.55–0.95)	0.60 (0.49–0.78)	**0.0017**
eGFR [mL/min/1.73 m^2]	77.47 (63.83–96.05)	103.25 (88.69–119.55)	**< 0.0001**
Urine specific gravity [kg/L]	1.0225 (1.0150–1.0300)	1.0250 (1.0150–1.0300)	0.6097
Estimated dehydration (%)	3.24 (2.54–4.03)	3.27 (2.77–3.85)	0.8857
CRP [mg/dL]	0.85 (0.20–2.05)	0.30 (0.10–1.47)	0.0769
Before therapy			
RBC [10^{12}/L]	5.15 (4.78–5.42)	4.90 (4.60–5.23)	**0.0026**
Hct (%)	42.00 (39.00–45.50)	40.00 (37.20–42.80)	**0.0019**
Hb [g/dL]	14.30 (13.40–15.50)	13.90 (13.20–14.80)	**0.0235**
MCV [fL]	82.00 (79.00–85.00)	82.00 (78.00–86.00)	0.9869
PLT [$10^3/\mu$L]	315.00 (261.00–354.00)	289.50 (240.00–335.00)	**0.0427**
WBC [$10^3/\mu$L]	13.29 (8.50–18.00)	8.79 (7.30–10.90)	**< 0.0001**
After therapy			
RBC [10^{12}/L]	4.57 (4.32–4.90)	4.65 (4.38–5.00)	0.1401
Hct (%)	37.70 (35.65–39.90)	38.20 (36.40–41.10)	0.0524
Hb [g/dL]	12.80 (12.20–13.70)	13.20 (12.50–14.10)	**0.0143**
MCV [fL]	82.00 (79.00–85.00)	83.00 (79.00–87.00)	0.4655
PLT [$10^3/\mu$L]	237.00 (188.00–289.00)	255.00 (207.00–300.00)	0.0888
WBC [$10^3/\mu$L]	6.70 (5.40–8.20)	6.40 (5.30–8.30)	0.6946

*Corrected by 0.6 [mEq/L] for every 0.1 unit reduction of pH; MDI — multiple daily insulin injections; CSII — continuous subcutaneous insulin infusion; DDI — daily dose of insulin; Hct — hematocrit; Hb — hemoglobin; MCV — means corpuscular volume; PLT — platelets; Me — median; 25–75% — interquartile range; RBC — red blood cells; WBC — white blood cells

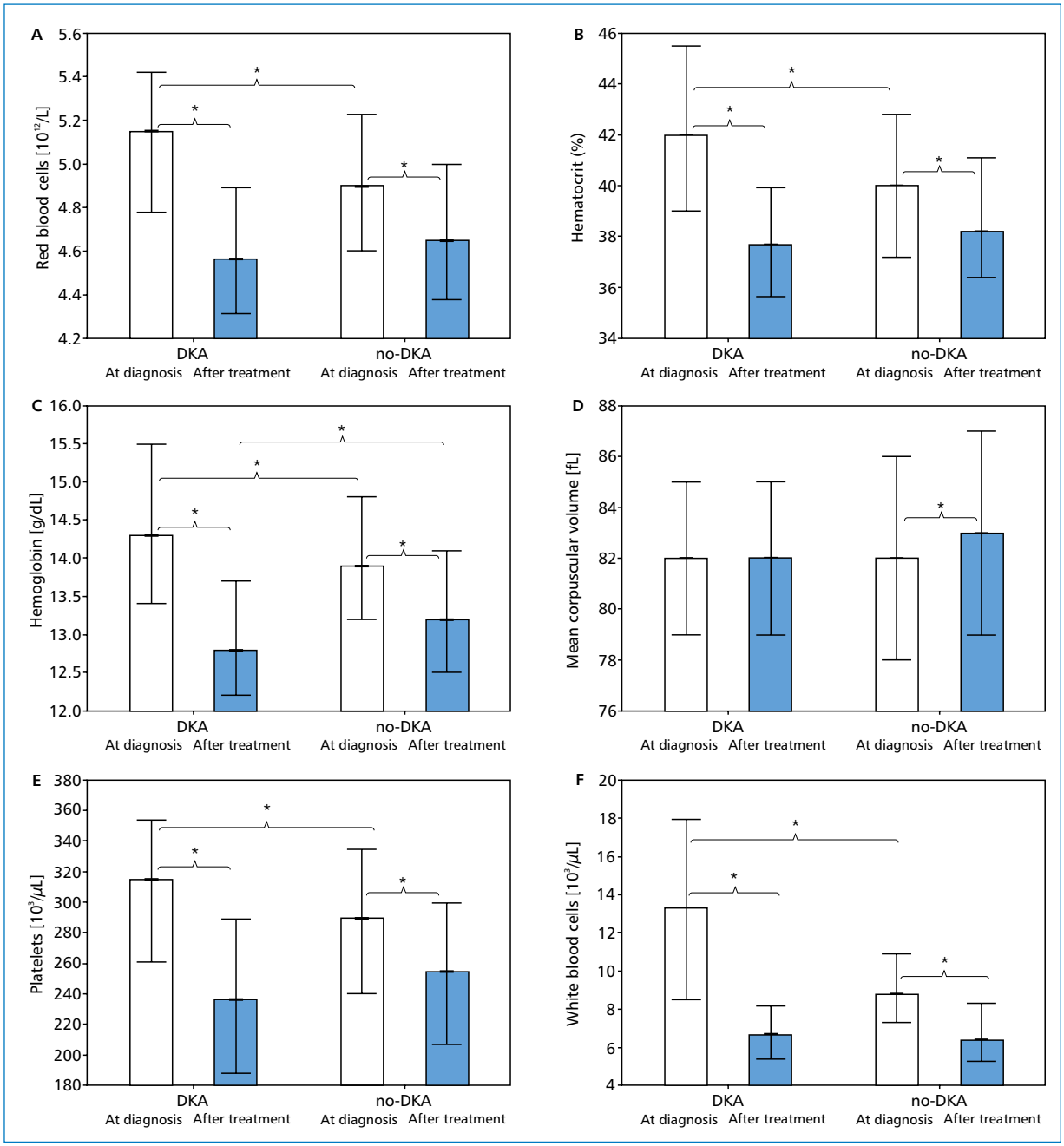

Figure 1. Changes of hematological parameters during first days of newly diagnosed T1DM treatment in children with DKA and without DKA. Red blood cells — **A**; hematocrit — **B**; hemoglobin concentration — **C**; mean corpuscular volume — **D**; platelets — **E**; white blood cells — **F**

days [25–75%: 2.0–4.0 days] *vs.* Me: 3.0 days [25–75%: 2.0–4.0 days] p = 0.2638).

In general linear models analysis with interaction between group allocation and timepoint there was a significant difference in hematological parameters dynamics change between the groups (all p values < 0.01). Parameters such as RBC, Hct, Hb, PLT and WBC were characterized by greater drop from their baseline values in DKA group *vs.* non-DKA group (all p values < 0.0001) (Figure 1 A–C, E–F). MCV level increased significantly but only in the no-DKA group (p < 0.0001) (Figure 1D).

Correlations between changes in hematological parameters and clinical factors

In order to establish the relationship between patients clinical status at admission with hematological parameters in DKA and no-DKA patients we correlated

Table 2. Correlations between blood cell parameters at admission of newly diagnosed type 1 diabetes treatment and clinical parameters

	pH		Effective osmolality [mOsm/kg]		Estimated dehydration (based on Na+ and urea) (%)		eGFR [mL/min/ /1.73 m^2]		C-peptide [ng/mL]	
DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
RBC [10^{12}/L]	**–0.39**	**0.0006**	**0.31**	**0.0076**	**0.34**	**0.0030**	**–0.36**	**0.0221**	**0.28**	**0.0246**
Hct (%)	**–0.36**	**0.0014**	**0.35**	**0.0022**	**0.32**	**0.0070**	**–0.43**	**0.0046**	**0.36**	**0.0041**
Hb [g/dL]	**–0.42**	**0.0002**	**0.50**	**< 0.0001**	**0.43**	**0.0002**	**–0.51**	**0.0006**	**0.43**	**0.0005**
MCV [fL]	–0.06	0.6327	0.13	0.2734	–0.02	0.8392	–0.07	0.6677	0.25	0.0517
PLT [10^3/μL]	0.03	0.8247	0.13	0.2866	0.05	0.6780	–0.15	0.3338	0.15	0.2484
WBC [10^3/μL]	**–0.53**	**< 0.0001**	**0.28**	**0.0155**	0.23	0.0560	**–0.44**	**0.0042**	0.01	0.9119
No-DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
RBC [10^{12}/L]	0.01	0.9397	0.02	0.8300	0.09	0.2378	**–0.23**	**0.0289**	**0.18**	**0.0255**
Hct (%)	–0.09	0.2309	< 0.01	0.9996	0.01	0.8922	–0.05	0.6385	**0.25**	**0.0023**
Hb [g/dL]	–0.10	0.2089	0.12	0.1256	**0.20**	**0.0115**	–0.12	0.2620	**0.21**	**0.0093**
MCV [fL]	–0.11	0.1343	–0.05	0.5197	**–0.16**	**0.0337**	**0.22**	**0.0301**	0.12	0.1415
PLT [10^3/μL]	–0.06	0.4582	–0.08	0.3002	0.11	0.1682	–0.11	0.3090	**–0.19**	**0.0204**
WBC [10^3/μL]	**–0.19**	**0.0122**	0.02	0.8032	0.05	0.4991	**–0.21**	**0.0452**	**–0.25**	**0.0019**

Table 3. Correlations between magnitude of blood cell parameters changes during first days of newly diagnosed type 1 diabetes treatment and clinical parameters

	pH		Effective osmolality [mOsm/kg]		Estimated dehydration (based on Na+ and urea) (%)		eGFR [mL/min/ /1.73 m^2]		C-peptide [ng/mL]	
DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
Delta RBC [10^{12}/L]	**0.45**	**0.0001**	–0.21	0.0847	–0.19	0.1295	0.20	0.2113	**–0.34**	**0.0084**
Delta Hct (%)	**0.45**	**0.0001**	**–0.26**	**0.0284**	–0.23	0.0648	0.28	0.0737	**–0.35**	**0.0067**
Delta Hb [g/dL]	**0.45**	**0.0001**	–0.21	0.0847	–0.19	0.1295	0.20	0.2113	**–0.34**	**0.0084**
Delta MCV [fL]	**0.30**	**0.0111**	**–0.32**	**0.0075**	**–0.28**	**0.0220**	**0.49**	**0.0012**	–0.11	0.4053
Delta PLT [10^3/μL]	**0.30**	**0.0136**	**–0.31**	**0.0113**	–0.21	0.0840	**0.37**	**0.0183**	–0.25	0.0535
Delta WBC [10^3/μL]	**0.59**	**< 0001**	**–0.25**	**0.0354**	–0.13	0.2804	**0.47**	**0.0022**	–0.08	0.5421
No-DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
Delta RBC [10^{12}/L]	0.02	0.8355	–0.04	0.6014	–0.03	0.7535	0.16	0.1355	0.07	0.4414
Delta Hct (%)	0.05	0.5712	0.04	0.5888	0.04	0.6666	0.08	0.4344	0.10	0.2502
Delta Hb [g/dL]	0.03	0.7131	–0.05	0.5147	–0.03	0.6878	0.14	0.1831	0.08	0.3522
Delta MCV [fL]	–0.06	0.4575	0.16	0.0536	**0.19**	**0.0206**	–0.06	0.5489	0.01	0.8633
Delta PLT [10^3/μL]	0.15	0.0609	< 0.01	0.9955	–0.02	0.7760	**0.27**	**0.0101**	0.15	0.8633
Delta WBC [10^3/μL]	0.14	0.0811	–0.13	0.0981	–0.16	0.0555	0.18	0.0841	–0.05	0.5769

them with pH, effective osmolality, estimated dehydration, eGFR and C-peptide (Tables 2 and 3) and with age, HCO_3^-, BE, time between the blood tests results, urine specific gravity and DDI (Tables S1 and S2).

Firstly, we correlated clinical data with hematological parameters at admission (Tables 2 and S1). RBC, Hct and Hb in DKA group correlated significantly, negatively with pH and eGFR and positively with effective osmolality, estimated dehydration and C-peptide. Among those, only correlation between C-peptide with RBC, Hct and Hb were significant also in no-DKA group. WBC correlated negatively and significant with pH and eGFR in both groups. Relationships between BE and hematological parameters at admission (Table S1)

Table S1. Correlations between blood cell parameters at admission during first days of newly diagnosed type 1 diabetes treatment and clinical parameters

	Age at onset (years)		HCO_3^- [mmol/L]		BE [mEq/L]		Time between the blood tests measurements (days)		Urine specific gravity [kg/L]		DDI [units/kg]	
DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
RBC [10^{12}/L]	**0.51**	**< 0.0001**	**–0.23**	**0.0442**	**–0.30**	**0.0087**	–0.06	0.6161	0.30	0.1000	0.01	0.9219
Hct (%)	**0.67**	**< 0.0001**	–0.10	0.3921	**–0.27**	**0.0189**	–0.05	0.6730	0.17	0.3577	0.03	0.8532
Hb [g/dL]	**0.71**	**< 0.0001**	–0.19	0.1031	**-0.34**	**0.0030**	–0.07	0.5315	0.26	0.1478	0.11	0.4664
MCV [fL]	**0.37**	**0.0011**	0.12	0.2981	–0.06	0.6233	0.07	0.5467	–0.17	0.3525	0.11	0.4429
PLT [$10^3/\mu$L]	–0.11	0.3322	–0.06	0.6354	< 0.01	0.9694	–0.02	0.8444	0.07	0.7220	0.08	0.5990
WBC [$10^3/\mu$L]	0.11	0.3480	**–0.50**	**< 0.0001**	**–0.55**	**< 0.0001**	0.05	0.6673	0.21	0.2409	–0.03	0.8142
No-DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
RBC [10^{12}/L]	**0.28**	**0.0002**	0.13	0.0975	0.11	0.1612	–0.06	0.4323	0.10	0.3839	0.10	0.3214
Hct (%)	**0.50**	**< 0.0001**	**0.16**	**0.0407**	0.08	0.2736	< 0.01	0.9537	0.14	0.2031	0.12	0.1990
Hb [g/dL]	**0.49**	**< 0.0001**	**0.16**	**0.0343**	0.10	0.1894	–0.04	0.5556	0.15	0.1719	0.08	0.4310
MCV [fL]	**0.28**	**0.0002**	0.02	0.8190	–0.03	0.6974	0.02	0.8150	0.03	0.7765	0.12	0.2055
PLT [$10^3/\mu$L]	**–0.50**	**< 0.0001**	**–0.16**	**0.0305**	**–0.22**	**0.0033**	0.02	0.7451	0.07	0.5156	–0.14	0.1495
WBC [$10^3/\mu$L]	**–0.43**	**< 0.0001**	**–0.38**	**< 0.0001**	**–0.41**	**< 0.0001**	0.02	0.7703	–0.01	0.9314	0.02	0.8612

Table S2. Correlations between magnitude of blood cell parameters changes during first days of newly diagnosed type 1 diabetes treatment and clinical parameters

	Age at onset (years)		HCO_3^- [mmol/L]		BE [mEq/L]		Time between the blood tests measurements (days)		Urine specific gravity [kg/L]		DDI [units/kg]	
DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
Delta RBC [10^{12}/L]	**–0.38**	**0.0013**	**0.32**	**0.0073**	**0.42**	**0.0003**	–0.09	0.4690	0.01	0.9470	–0.04	0.7593
Delta Hct (%)	**–0.42**	**0.0003**	**0.30**	**0.0108**	**0.41**	**0.0004**	–0.05	0.6943	< 0.01	0.9979	–0.06	0.6635
Delta Hb [g/dL]	**–0.38**	**0.0013**	**0.32**	**0.0073**	**0.42**	**0.0003**	–0.09	0.4690	0.01	0.9470	–0.04	0.7593
Delta MCV [fL]	–0.12	0.3348	**0.25**	**0.0352**	**0.27**	**0.0224**	0.13	0.2845	–0.17	0.3760	–0.01	0.9359
Delta PLT [$10^3/\mu$L]	–0.22	0.0666	0.23	0.0571	**0.26**	**0.0331**	0.05	0.6758	–0.23	0.2324	–0.01	0.9279
Delta WBC [$10^3/\mu$L]	**–0.24**	**0.0441**	**0.50**	**< 0.0001**	**0.59**	**< 0.0001**	–0.20	0.1002	–0.17	0.3754	0.02	0.9039
No-DKA	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**	**R**	**P value**
Delta RBC [10^{12}/L]	0.10	0.2111	0.12	0.1256	0.13	*0.0994*	–0.07	0.3980	–0.08	0.4853	–0.15	0.1333
Delta Hct (%)	0.06	0.4843	0.10	0.2002	0.14	*0.0798*	–0.01	0.8681	–0.18	0.1304	–0.12	0.2487
Delta Hb [g/dL]	0.11	0.1754	0.12	0.1241	0.14	*0.0800*	–0.04	0.6448	–0.07	0.5784	–0.16	0.1035
Delta MCV [fL]	–0.06	0.4785	–0.08	0.3147	–0.07	0.3583	**0.16**	**0.0443**	–0.04	0.7657	–0.03	0.7290
Delta PLT [$10^3/\mu$L]	0.08	0.3248	0.07	0.3547	0.14	0.0862	–0.10	0.2181	–0.19	0.1038	0.05	0.6280
Delta WBC [$10^3/\mu$L]	0.06	0.4573	**0.16**	**0.0421**	**0.19**	**0.0178**	**–0.18**	**0.0226**	–0.10	0.3919	0.07	0.5052

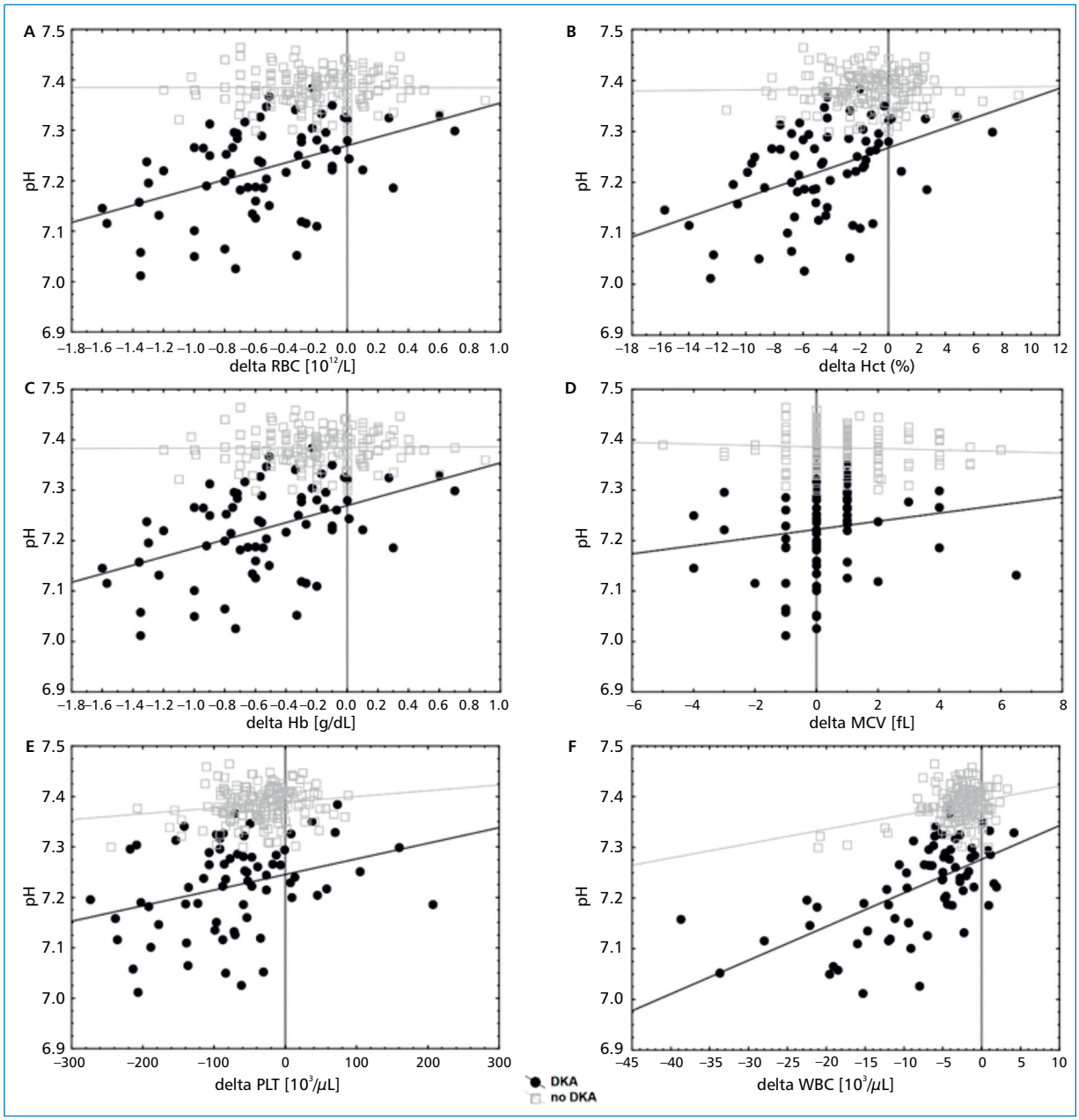

Figure 2. Correlations between pH and changes in morphological parameters during first days of newly diagnosed T1DM treatment in children with DKA and without DKA. RBC (red blood cells) — **A**; Hct (hematocrit) — **B**; Hb (hemoglobin) — **C**; MCV (mean corpuscular volume) — **D**; PLT (platelets) — **E**; WBC (white blood cells) — **F**

showed similar dynamics as correlations with pH in the DKA group. For HCO_3^- only correlations with RBC and WBC were significant. Interestingly, in no-DKA group significant and negative correlations between PLT and both BE and HCO_3^-, were observed. Additionally, also only in no-DKA group significant and positive correlations between HCO_3^- with Hct and Hb were observed.

After that, we correlated patients' clinical status with changes in hematological parameters since admission (Tables 3 and S2). In the DKA group changes in all hematological parameters correlated positively with pH (all $R > 0.3$ and all p values < 0.05) (Figure 2). Relationships in the DKA group between changes in hematological parameters and both HCO_3^- (Table S2) and base excess (Table S2), showed similar dynamics to pH. In comparison, in the no-DKA group which was 2.5-times more numerous than DKA group, no correlations with pH were noted for changes in RBC, Hct, Hb and MCV. However, for change in PLT and WBC weak, positive correlations with pH at the margin of

statistical significance were noted. For correlations with HCO_3^- and base excess, no significant associations with hematological parameters changes were found in the no-DKA group, except for WBC and both HCO_3^- and BE (Table S2) and weak positive correlations at the margin of statistical significance of BE and changes in RBC, Hct, Hb and PLT.

Effective plasma osmolality significantly, negatively correlated with all hematological changes but only in DKA group (delta RBC and delta Hb were at the margin of statistical significance). Dehydration estimated by equation provided by Ugale et al. [7] correlated negatively (with borderline significance) with all hematological changes but also only in DKA group. Strangely, the change of MCV correlated significantly but negatively with estimated dehydration in DKA group but significantly and positively in no-DKA group. eGFR correlated strongly positively with the changes of MCV and WBC in DKA group and with the change of PLT both in DKA and no-DKA group. C-peptide correlated significantly and negatively with changes in RBC, Hct and Hb only in DKA group and at the borderline significance with the PLT change.

When correlation between each change in hematological parameters were performed, all but MCV, changes significantly, positively correlated with each other (all $R > 0.4$, all p values < 0.0001). The change of MCV correlated only with the change of Hct ($R = 0.21$, $p = 0.0017$).

Multivariate models explaining changes in hematological parameters during DKA treatment

In order to evaluate which clinical variables can explain changes of hematological parameters in DKA group, we performed multivariate analyses (Tables S3). The change of RBC and Hb were both independently positively associated with pH and negatively with C-peptide. Similar associations were found in the multivariate model for change in Hct which additionally showed significant negative association with creatinine concentration. For the change of RBC, Hct and Hb built models explained more than 30% of parameter variation (adjusted R^2).

The change of MCV was significantly and negatively associated with glucose concentration. Our model explained only 7% of parameter variance suggesting that other variable (not included in our database) are associated with MCV changes during DKA treatment.

PLT changes was associated positively with pH and negatively with glucose concentration. The model explained 17.5% of parameter variation.

For the change of WBC we were able to create model comprising 3 variables: BE (positive association), creatinine level (negative association) and potassium level (negative association). Model explained almost 42% of changes in WBC variation.

Discussion

In this study we found that hematological parameters such as RBC, Hct, Hb, PLT and WBC decreased after fluid therapy among children with newly diagnosed T1DM. At the diagnosis, abovementioned hematological parameters were higher in DKA group compared to no-DKA group. Also, these parameters had greater magnitude of changes from their baseline values in DKA in comparison to no-DKA group. Interestingly, MCV increased after fluid therapy but only in no-DKA group.

MCV changes may be associated with changing plasma osmolality during fluid therapy. Dehydration may influence MCV but the direction of change depends on plasma osmolality. During hyperosmolar dehydration the plasma volume is decreasing (mainly water), which leads to uneven ion concentration between inside of red blood cells and plasma itself. In order to rebalance the concentration, water is moving from the inside of RBC to the extracellular space. As the consequence, RBC shrinks and thus MCV is reduced.

Effective plasma osmolality was higher in DKA group compared to no-DKA group. Hence, odd is the fact that in our study the DKA group had the same MCV values at T1DM diagnosis as no-DKA group. Moreover, in no-DKA group MCV increased significantly after fluid therapy, but this dynamics were not present in DKA group. We believe that the increased MCV may suggests over hydration in patients from no-DKA group. To support this hypothesis, we observed positive correlation of the delta MCV with time between the blood tests measurements suggesting that longer time of fluid therapy is associated with bigger change of MCV. On the other hand, the lack of increase in MCV in DKA group after fluids therapy may suggest improper restoring of plasma osmolality after treatment.

Decreased MCV may also result from iron or copper deficiency and different types of hemoglobinopathies [20]. MCV may increase due to the anemia, vitamin B12 or folate deficiency, alcohol abuse and many other disorders [21]. However, due to short period of time between blood test measurements, we can assume that those factors did not affect MCV in our studies.

Other hematological parameters (RBC, Hct, Hb, PLT and WBC) had the same dynamics of change in both DKA and no-DKA group, but greater magnitude of changes were observed in DKA group. It could suggest that children with DKA were more dehydrated and it was reflected by higher blood condensation at diabetes diagnosis.

Table S3. Multivariate linear models for changes in RBC, Hct, Hb, MCV, PLT and WBC

Delta RBC	**R^2**	**R^2 adj.**	**P value for the model**	**Delta MCV**	**R^2**	**R^2 adj.**	**P value for the model**
	38.91%	34.47%	**< 0.0001**		11.18%	7.15%	**0.0484**
Variables	**Beta**	**Beta***	**P value**	**Variables**	**Beta**	**Beta***	**P value**
Intercept	**–16.7650**		**0.0004**	Intercept	**1.5844**		**0.0053**
Sex — male	–0.0093	–0.02	0.8607	Sex — male	–0.0886	–0.05	0.6466
Age (years)	**–0.0248**	**–0.25**	**0.0345**	Age (years)	–0.0190	–0.06	0.6428
pH	**2.2952**	**0.42**	**0.0004**	Glucose concentration [mg/dL]	**–0.0020**	**–0.30**	**0.0145**
C-peptide [ng/mL]	–0.3138	–0.21	0.0720				
Delta Hct	**R^2**	**R^2 adj.**	**P value for the model**	**Delta PLT**	**R^2**	**R^2 adj.**	**P value for the model**
	44.44%	39.20%	**< 0.0001**		22.35%	17.50%	**0.0025**
Variables	**Beta**	**Beta***	**P value**	**Variables**	**Beta**	**Beta***	**P value**
Intercept	**–127.4183**		**0.0027**	Intercept	–1549.9744		0.0883
Sex — male	0.0433	0.01	0.9252	Sex — male	8.5233	0.09	0.4130
Age (years)	–0.1601	–0.18	0.1607	Age (years)	–3.0931	–0.16	0.1659
pH	**17.6857**	**0.36**	**0.0023**	pH	216.9799	0.21	0.0817
Creatinine [mg/dL]	–2.9730	–0.23	0.0897	Glucose concentration [mg/dL]	**–0.1094**	**–0.29**	**0.0162**
C-peptide [ng/mL]	–2.7970	–0.20	0.0661				
Delta Hb	**R^2**	**R^2 adj.**	**P value for the model**	**Delta WBC**	**R^2**	**R^2 adj.**	**P value for the model**
	38.91%	34.47%	**< 0.0001**		46.26%	41.92%	**< 0.0001**
Variables	**Beta**	**Beta***	**P value**	**Variables**	**Beta**	**Beta***	**P value**
Intercept	**–16.7650**		**0.0004**	Intercept	**17.1585**		**0.0021**
Sex — male	–0.0093	–0.02	0.8607	Sex — male	–0.7409	–0.10	0.3040
Age (years)	**–0.0248**	**–0.25**	**0.0345**	Age (years)	–0.0899	–0.06	0.6173
pH	**2.2952**	**0.42**	**0.0004**	BE [mEq/L]	**0.7697**	**0.51**	**< 0.0001**
C-peptide [ng/mL]	–0.3138	–0.21	0.0720	Creatinine [mg/dL]	–4.7149	–0.21	0.0931
				K [mEq/L]	–1.6080	0.15	0.1326

Based on correlations between changes in each hematological parameters (RBC, Hct, Hb, MCV, PLT and WBC) with pH, we assumed that severity of DKA is associated with dehydration expressed as greater change of each parameter from the baseline level. Our findings are not consistent with those provided by Ugale et al. [7]. This difference might arose from fact that they measured dehydration as a change of body mass. Measuring change of body mass as a dehydration status may be affected by e.g. body fat percentage. Thus, when patient has higher percentage of body fat and loses e.g. 10% of total body water, the change in total body weight will be smaller in comparison to the lean patient with the same percentage of lost total body water. Additionally, renutrition and insulin administration may affect body mass. These might be the reasons of the different results between our study and Ugale's one. Also, estimated dehydration provided in Ugale's study did not differ significantly between DKA and no-DKA group in our study. This result is rather strange and we believe that it may indicate that this equation is not accurate in estimating dehydration in newly diagnosed T1DM patients as the difference in hydration status between those two group was shown by lower eGFR and higher effective plasma osmolality in DKA group.

Furthermore, our study showed that only RBC, Hct and Hb at admission correlated significantly and negatively with eGFR and pH and positively with estimated dehydration, effective osmolality and C-peptide. This could further support hypothesis that higher RBC, Hct and Hb at diagnosis and their further drop after fluid administration is strongly associated with patients

hydration status and could help clinicians to estimate patients hydration status at T1DM diagnosis. However, those correlations were not evident in no-DKA group what could emerge from lesser dehydration in this group of patients (eGFR and plasma osmolality were mostly within normal limits in this group).

Interestingly, even though most of above-mentioned correlations were not present in no-DKA group, positive correlation between C-peptide and RBC, Hct, Hb was significant in both groups. We presume that higher C-peptide concentration at admission (preserved residual beta-cell function) was associated with longer development of full symptomatic type 1 diabetes that forced patients and their parents to seek medical counselling. Thus, with longer time, those children may develop greater dehydration as shown by lower RBC, Hct and Hb without much changes in plasma osmolality and eGFR due to compensatory mechanisms.

Also we observed increased level of PLT count at T1DM diagnosis and higher levels of this parameter in DKA group. Venous thrombosis compilations are rare but well-known consequences of DKA [22]. Increased PLT count may partially explain this phenomenon in addition to already known increased platelet aggregation, elevated levels of procoagulants and decreased activity of anticoagulants in patients with DKA [23]. All of this, could suggest consideration of antithrombotic treatment among patients with DKA and high PLT count.

Despite above-mentioned parameter, also WBC shared the same dynamics of change (significant drop after treatment and higher levels in DKA group at admission). It is known that WBC reflects systemic inflammation level but taking into consideration that most of our patient had CRP levels with normal limits, it suggest that here higher WBC could be also a marker of blood condensation. Interestingly, WBC values most strongly correlated with pH level in DKA group and this association was also present in no-DKA group and this phenomenon was also observed by others [24].

The main limitation of our study was lack of directly measured dehydration. Thereby, we were not able to associate blood morphology parameters and their changes with true patients' hydration status. Additionally, the amount of administered fluids was not registered and we draw our conclusion on effect of fluid therapy using time between the blood samples collection as a surrogate of administered fluid volume. Also, absence of ketone bodies concentration can be consider as a study limitation. Due to retrospective character of our study we were not able to obtain measurements of this parameter. However taking into consideration that all patients were newly diagnosed with T1DM and improved with insulin therapy we may assume that only diabetic ketoacidosis was an underlying cause of acidosis among those patients. Finally, we used hematological parameter after treatment as baseline values as we were not able to obtain their values shortly before development T1DM. Another solution would be to verify hematological parameters 6 months after hospitalization, but we believe that other factors (e.g. diet) might influenced them in such a long period of time and disturbed the results.

Conclusions

Hematological parameters measured at T1DM diagnosis may be useful in estimating patients' hydration status. Monitoring of their dynamics during fluid therapy may inform about the treatment effectiveness in restoring total body water.

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Statement of competing interests

The authors have no conflicts of interest to disclose.

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Magnesium, zinc and iron serum levels as potential parameters significant for effective glycemic control in children with type 1 diabetes

ABSTRACT

Background. Various trace elements contribute to the development of diabetes and its complications through their roles in glucose metabolism and the oxidative stress response. The aim of this study was to ascertain the difference in serum magnesium, zinc and iron concentrations between healthy children and children with type 1 diabetes mellitus (T1DM). This study also aimed to determine whether serum concentrations of magnesium, zinc, and iron in children with T1DM correlated with the duration of the disease and the quality of glycemic control in this group.

Material and methods. A total of 99 children with T1DM and 40 healthy children were included in this study. Magnesium, zinc and iron serum levels were assessed using the photometric method.

Results. Significantly lower levels of magnesium and zinc ($P < 0.001$) were observed, between the T1DM group and the healthy control group but no statistically significant differences were found in iron levels ($P = 0.13$) between the two groups. While there were no statistically significant differences in serum concentrations with respect to the duration of disease, it was, however, discovered that children with poorer glycemic control had significantly lower serum zinc concentrations ($P < 0.001$) while magnesium and iron levels remained similar ($P = 0.07$ and 0.21 respectively).

Conclusion. This study found that while there was no significant difference in iron serum levels in children with T1DM compared to healthy controls, children with T1DM did have more significantly decreased magnesium and zinc serum levels than the control group. Serum zinc levels in this study also directly correlated to poorer glycemic control. Further studies are required to explore whether magnesium and zinc supplementation, or nutritional intake, could potentially be used to achieve better glycemic control in children with T1DM.

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Key words: iron, magnesium, zinc, diabetes mellitus, type 1, child, supplements

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Introduction

Despite continuous efforts and rapid technological as well as pharmaceutical advances, diabetes mel-

litus continues to be one of the most common health problems in the general population. Children with type 1 diabetes mellitus (T1DM) are especially interesting patient group, because any findings concerning the mechanisms of diabetes and the development of diabetic complications in these children can be used to improve their future quality of life. In view of that, recent studies have explored the effect of various elements in the formation of oxygen-free radicals as well as the effect of the ensuing oxidative stress on the development of diabetic complications [1, 2].

Magnesium is involved in the synthesis of glutathione, a major antioxidant, through ensuring the proper functioning of the enzyme gamma glutamyl transpeptidase (GGT) [3]. It also plays a vital role in glucose metabolism, and its deficiency has been most commonly associated with diabetes mellitus [4] through its effect on the development of dyslipidaemia, increased insulin resistance and carbohydrate intolerance [5]. Hypomagnesemia also increases the possibility of developing diabetic complications, particularly atherosclerosis [6], diabetic retinopathy [7] and end-stage renal disease (ESRD) [8]. Another important element in glucose metabolism is zinc (Zn), which plays a role in secretion of insulin from pancreatic cells [9] where they form a Zn-insulin complex [2]. It further increases hepatic binding of insulin, as well as having a role in the modulation of insulin actions. As an antioxidant, zinc stabilizes membranes and reduces hydroxyl radicals (OH) through the induction of metallothionein synthesis [10]. Both of these functions are diminished in cases of zinc deficiency, which has impacts on glycaemic control. In children with T1DM, it has been observed that Zn deficiency directly correlates with poorer glycemic control and increased HbA_{1c} [11].

In regards to serum iron levels, iron deficiency has been correlated with an increased HbA_{1c} [12] in older patients with T1DM, although the same correlation was not noted in most children with diabetes [13]. Children with newly discovered T1DM, however, were found to have lower serum levels of iron than children who have had T1DM for a number of years [14]. Therefore, both increased and decreased serum levels of iron may have possible negative outcomes in patients with T1DM. On one hand, iron deficiency causes microcytic anemia, which is common in patients with T1DM and is associated with the progression of diabetes, as well as the development of its co-morbidities such as micro- and macroangiopathies [15]. On the other hand, through the Fenton reaction, increased serum levels of iron can generate reactive oxygen species, damaging tissues or cells [16].

Since various elements are closely linked to the progression of diabetes and its co-morbidities, the aim of this study was to ascertain the difference in serum magnesium, zinc and iron concentrations between healthy children and children with T1DM. This study also aimed to determine whether serum concentrations of magnesium, zinc, and iron in children with T1DM correlated with the duration of disease and the quality of glycemic control in this group.

By fulfilling these aims, this study could help increase understanding about the mechanisms of magnesium, zinc and iron involvement in T1DM, as well the possibilities of supplementing those elements to ensure better glycemic control in children with T1DM.

Material and methods

Subjects

In this case-control study, a total of 139 children under 18 years of age were included, among which 99 children had previously diagnosed T1DM, while 40 represented a healthy control group. The control group was comprised of children who had undergone a standard systematic examination in a paediatric practice, and were not suffering from diabetes mellitus, acute infections, malignant, autoimmune or chronic diseases. For the purpose of this study the following tests were performed to rule out underlying diseases in the control group: DM was excluded in the control group based on blood glucose measurements (fasting plasma glucose < 5.6 mmol/l) and the absence of DM symptoms [17]. Acute infections were excluded by complete physical examination and normal laboratory values of white blood cells (WBC) and C-reactive protein (CRP). The T1DM group consisted of children that satisfied the criteria defined by the International Society for Paediatric and Adolescent Diabetes (ISPAD) Clinical Practice Consensus Guidelines 2018 Compendium [18]. Blood glucose levels, levels of islet cell autoantibodies (ICA), glutamic acid decarboxylase 65 (GAD-65) autoantibodies, and insulinoma-associated protein-2 (IA-2), as well as the presence of usual symptoms accompanying T1DM were used to determine the diagnosis of T1DM. Children with T1DM who were suffering from associated autoimmune diseases (such as celiac disease or Hashimoto thyroiditis) were not included in this study. In children with T1DM, serum levels of magnesium, zinc and iron were compared and correlated with the duration of disease and the quality of disease control. Regarding T1DM duration, children were grouped as either duration of T1DM under or over 1 year [17]. Furthermore, in accordance with the criteria defined by ISPAD [18], in children with long lasting disease

(> 1 year), glycemic control was defined as either good ($HbA_{1c} \leq 7\%$) or poor glycemic control ($HbA_{1c} > 7\%$).

Methods

Blood samples were collected in two tubes. Clot activator tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) were used for glucose, as well as for serum zinc, iron and magnesium levels. The coefficients of variation (CV %) for these elements were as follows: for Zn within-run (1.7), between-run (2.1) and reference values (10.7–18.4 μmol/l); for Mg within-run (1.02), between-run (1.15) and reference values (0.74–0.97 mmol/l); for Fe within-run (1.02), between-run (2.09) and reference values (6–31 μmol/l). Samples were centrifuged for 10 minutes at 1500 g. K2EDTA tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) were used for WBC count and haemoglobin A_{1c} (HbA_{1c}) measurement. Blood samples were collected in the fasting state according to the recommendations of the Clinical Laboratory Standard Institute (CLSI) between 7:00 and 9:00 a.m. [19]. All tests were performed immediately. CRP was measured by immunoturbidimetric assay (Roche Diagnostics GmbH, Mannheim, Germany), glucose was measured by the enzymatic method (Beckman Coulter Inc., Brea, USA), and zinc, magnesium as well as iron serum levels were measured with the photometric method using Beckman Coulter AU680 analyser (Beckman Coulter Inc., Brea, USA). WBC count was determined using a Sys-mex SF-3000 automated haematology analyser (Sysmex Corporation, Kobe, Japan) while HbA_{1c} was determined by the immunoturbidimetric method on a Dimension EXL with an LM analyser (Siemens Healthcare Diagnostics Inc., USA).

Statistical analysis

Data were reported using descriptive statistical methods. The Mann–Whitney U test was used to compare the medians between the two groups. All P values are two-sided. The level of significance is set at alpha = 0.05. Statistical analysis was performed by the statistical program MedCalc Statistical Software version 19.0.5 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2019).

Ethical statement

Informed consent was obtained from parents or legal guardians of all children enrolled in the study. The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Osijek University Hospital and the Faculty of Medicine, University of Osijek (12 March, 2018, IRB number: 2158-61-07-18-14).

Table 1. Distribution of respondents by gender in studied groups (N = 139)

Gender	N (%)		Total	P-value†
	Control group	T1DM* group		
Boys	18 (45.0)	47 (47.5)	65 (46.8)	0.85
Girls	22 (55.0)	52 (52.5)	74 (53.2)	
Total	40 (100)	99 (100)	139 (100)	

*T1DM group — children with type 1 diabetes mellitus; †Fisher exact test

Table 2. Demographic and clinical characteristics of study groups (N = 136)

Factor	Median (interquartile range)		P-value†
	Control group	T1DM* group	
Age (years)	13 (13.0–17.0)	12 (8.0–14.0)	0.09
BMI [kg/m^2]	16.9 (15.1–18.1)	17.1 (14.9–21.3)	0.11
HbA_{1c} (%)	5.1 (4.7–5.5)	7.9 (7.2–8.6)	**0.03**

BMI — body mass index; HbA_{1c} — haemoglobin A_{1c}; *T1DM group — children with type 1 diabetes mellitus; †Mann–Whitney U test

Results

This case control study included 139 children, of which 99 (71.2%) were diagnosed with T1DM and 40 (29.5%) who were used as a healthy control group after ruling out T1DM, acute infections or other underlying diseases. There were 74 girls (53.2%) and 65 boys (46.8%) with no significant difference between the groups (Table 1).

The mean age of children in the two groups was 14 years (Table 2). Table 2 further describes the characteristics of the study groups (age, BMI and HbA_{1c}).

In children with T1DM, the median (interquartile range) of disease duration was 4.1 (3.9–4.3) years. Out of the total number (N = 99) of children with T1DM, 10 (9.9%) children were treated with an insulin pump, while the other 89 (90.1%) children used intensive insulin therapy. In comparing the two groups, it was shown that the T1DM group had significantly lower levels of both magnesium ($P < 0.001$), and zinc ($P < 0.001$) than the control group. However, there was no significant difference in measured serum iron levels between the two groups (Table 3).

With respect to the duration of disease within the T1DM group, there were no significant differences in serum magnesium, zinc and iron levels (Table 4).

Significantly lower zinc serum levels were measured in children with poorer glycemic control ($P < 0.001$). However, no statistically significant difference was observed in serum iron and magnesium levels (Table 5).

Table 3. Differences in serum magnesium, zinc and iron levels in studied groups (N = 139)

Elements	Median (interquartile range)		HL diff.	95% CI	P-value
	Control	T1DM			
Magnesium [mg]	0.87 (0.83–0.90)	0.80 (0.75–0,84)	–0.08	–0.1 to –0.05	< 0.001*
Zinc [mmol/L]	12.9 (11.8–13.8)	10.7 (9.3–12.1)	–2.1	–2.7 to –1.4	< 0.001*
Iron [μmol/L]	15.9 (10.6–20.5)	14.4 (9.7–18.03)	–1.7	–4.1 to 0.6	0.13

HL diff. — Hodges–Lehmann median difference; *significant at P ≤ 0.05

Table 4. Comparison of serum magnesium, zinc and iron levels in children with T1DM (N = 99) in regard to the duration of T1DM

Elements	Median (interquartile range)		HL diff.	95% CI	P-value
	Under 1 year (n = 7)	Over 1 year (n = 92)			
Magnesium [mg]	0.75 (0.72–0.79)	0.70 (0.75–0.84)	0.04	–0.02 to 0.09	0.12
Zinc [mmol/L]	10.7 (10.2–11.1)	10.7 (9.2–12.1)	–0.3	–1.6 to 1.2	0.73
Iron [μmol L]	15.4 (12.4–17.8)	13.9 (9.7–18.1)	–1.5	–5.5 to 2.6	0.45

HL diff. — Hodges–Lehmann median difference

Table 5. Comparison of serum magnesium, zinc and iron levels in children with T1DM (> 1 year) according to the quality of glycemic control in (N = 92)

Elements	Median (interquartile range)		HL diff.	95% CI	P-value
	HbA_{1c} ≤ 7% (n = 48)	HbA_{1c} > 7% (n = 44)			
Magnesium [mg]	0.82 (0.76–0.85)	0.79 (0.75–0.82)	–0.02	–0.04 to 0.01	0.21
Zinc [mmol/L]	11.2 (10.2–13.1)	9.9 (8.8–11.2)	–1.4	–2.2 to –0.6	< 0.001*
Iron [μmol/L]	15.2 (10.9–18.8)	11.5 (9.6–16.5)	–2.3	–4.7 to 0.10	0.07

HL diff. — Hodges–Lehmann median difference; *significant at P ≤ 0.05

Discussion

In recent times, T1DM in paediatric patients has become one of the most researched endocrinological disorders in the world. Among the many things studied, the correlation between the imbalance of trace elements in the human body and their effects on the severity of T1DM through the effects of oxidative stress is particularly interesting [2]. It has been suggested that achieving the balance of various trace elements in the body could improve glycemic control in T1DM patients by using relatively simple means of pharmaceutical and nutritional correction. Magnesium and zinc are the obvious potential candidates due to their important roles in glucose metabolism and antioxidant response.

The results of this study demonstrate significantly lower serum levels of magnesium and zinc in children with T1DM when compared to healthy controls. These results are in accordance with other similar studies [2, 20, 21]. Decreased magnesium serum levels in children with T1DM were reported in the study by Lin et al. [11] but, without the similar decrease of serum zinc levels when compared with healthy controls. Similar serum zinc levels between T1DM and healthy group were also reported in other studies [22, 23].

Zn plays a major role in the stabilisation of insulin hexamers and the pancreatic storage of the hormone, and is an efficient antioxidant. Zn may also have an indirect insulin-like effect, with genetic studies identifying the islet-restricted Zn transporter ZnT8 as a likely player in the control of insulin secretion. When the serum Zn concentration falls, there is a concomitant reduction in insulin secretion and peripheral insulin sensitivity [24]. Ahmed and Helal [20] in their study comprised of 25 children with T1DM and 13 apparently healthy controls, reported decreased magnesium and zinc serum levels in T1DM children compared to the healthy controls. These results could be explained by the fact that magnesium and zinc levels may decrease due to urinary loss by osmotic action, glucosuria and hyperglycemia [20].

With respect to the duration of the disease within the T1DM group, there were no significant differences

found in this study in serum magnesium, zinc and iron levels. This finding was similar to the study by Estakhri et al. in which zinc serum levels did not correlate to the duration of disease [23]. However, Ahmed and Helal [20] in their study report a negative correlation of serum magnesium and zinc levels with the duration of T1DM. Possible reasons for these contradictory results can be due to the difference in duration of disease between patient populations, quality of glycemic control, genetic, dietary characteristics, and environmental factors [23].

Significantly, in this study, lower zinc serum levels were observed in children with poorer glycemic control. Similarly, decreased levels of serum zinc in children with T1DM and poorer glycemic control were also described in the studies of Alghobashy et al. from 2018 [1] and Lin et al. from 2014 [11]. The negative correlation of serum zinc level with the degree of disease control was also observed by Ahmed and Helal [20], however, the study by Estakhri et al. [23] showed that the level of serum zinc was not influenced by the quality of the glycemic control. Although, this discrepancy may be explained by the fact that the study of Estakhri et al. [23] included a significantly smaller number of children with T1DM (N = 30) whose disease duration was significantly shorter (2.5 years). Lower levels of zinc increase the production of hydroxyl radicals, facilitating oxidative stress in children with T1DM, through the decrease of iron binding to the cell membrane as well as through the inhibition of metallothionens [11]. The negative correlation of zinc levels with HbA_{1c} levels in children with poorer glycaemic control is one observable negative effect of this oxidative injury [2]. Decreased zinc serum levels are also associated with impaired utilisation of glucose and decreased insulin sensitivity [25].

The aforementioned negative correlation of serum Zn levels with respect to HbA_{1c} level can be explained by increased urinary micronutrient loss due to increased osmotic diuresis caused by hyperglycemia [1]. The absence of a correlation between disease duration and serum HbA_{1c} level is most likely the result of good glycemic control and relatively short average disease duration in our study group of children with T1DM (4.1 years). In contrast, the study results of Al Ghobashy et al. [1] indicate a positive correlation of disease duration with HbA_{1c} levels in children with T1DM. However, the children with T1DM in this study had a mean age of illness of 4.8 years, with 75% of them defined as having poorly controlled disease.

Zinc supplementations can, through the increase of serum zinc levels, help to decrease diabetes-induced oxidative stress [2]. The fact that zinc levels in this study directly correlated to poorer glycemic control, represented by higher HbA_{1c}, seems to further suggest that zinc correction could be beneficial to children with T1DM.

Indeed, several studies, such as Jayarwardena et al. [25] and the meta-analysis by Capdor et al. [26] indicated that the use of zinc supplementation showed a tendency to decrease HbA_{1c} levels in patients with T1DM. Magnesium supplements were administered to children with T1DM during 3 months in the study conducted by Shahbah et al. [27] and were shown to significantly decrease HbA_{1c} levels. Introducing more magnesium and zinc rich food in the diet of children with T1DM probably represents an easy and beneficial method to balance the element's serum levels and improve glycemic control.

While iron supplementation can certainly alleviate the iron deficiency anemia associated with diabetes [14] as well as its possible negative effects on non-verbal intelligence as observed by Mojs et al. [28], there is currently not enough clinical evidence on their use in children with T1DM.

Limitations of this study include its cross-sectional design, as well as the fact that data were collected from a single centre and therefore may not be extrapolate to other population groups.

In conclusion, magnesium and zinc supplementation or nutritional intake could potentially be included in the standard treatment of children with T1DM to achieve better glycemic control, however, further multi-centre, longituidal studies are required. Furthermore, the usage of iron supplementation in diabetic children is currently controversial and also requires further research.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Molecular genetic analysis of leucine tRNA in relevance to type 2 diabetes mellitus

ABSTRACT

Background. Several point mutations in the mitochondrial DNA cause maternally inherited metabolic disorders. The most common type of mutation A3243G in the gene of transfer RNA leucine ($tRNA^{Leu(UUR)}$) is thought to be responsible for the prevalence of type 2 diabetes mellitus. This study was designed to analyze the $tRNA^{Leu(UUR)}$ gene of mtDNA of the diabetic individuals with familial history of diabetes to identify the point mutations A3243G.

Material and methods. Saliva samples were preferred as a source of DNA to minimize the risk of infection. DNA was successfully extracted from their saliva. Samples of high-quality DNA was amplified with PCR and sequenced in Macrogen Inc. Korea.

Results. The m.3243A>G mutation in mitochondrial *tRNA*$^{Leu(UUR)}$ gene was not observed.

Conclusion. The result shows that the m.3243A>G mutation in mitochondrial *tRNA*$^{Leu(UUR)}$ gene is not frequent cause of type 2 and some other factors may be possible i.e. genetic, behavioral or environmental. It is recommended that the sample size for diabetic individuals need to be increased for a future study and screened for the mitochondrial as well as other mutations of nuclear origin. (Clin Diabetol 2020; 9; 3: 167–173)

Key words: diabetes mellitus, leucine tRNA, mitochondrial DNA, point mutation

Introduction

Diabetes mellitus (DM) is a common disease affecting many individuals worldwide. DM encompasses a range of metabolic failures, characterized by hyperglycemia developed from impairment of beta cells of pancreas and mutations in genomic DNA. Genetic impairment is involved in both peripheral insulin sensitivity and glucose linked insulin secretions. Mitochondria has important role in glucose insulin secretion, mutations in mitochondrial DNA (mtDNA) can cause insulin secretion impairment. In mitochondria, oxidative phosphorylation variation in the ration of intracellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) may trigger the exocytosis of insulin [1].

Mitochondrial DNA follows a maternal pattern of inheritance [2]. More than 220 mutations have been related with syndromes in the 22 genes of human tRNA and 40 in human $tRNA^{Leu(UUR)}$ gene. Epidemiological studies indicate prevalence of approximately 1 in 5,000 mtDNA mutations in adults, making mtDNA the most common carriers of genetic disorders [3]. Mutations in mitochondrial tRNA genes are linked with multiple human diseases, including heart failure, neuromuscular disorders, diabetes, visual and hearing loss. Particularly, mutation at position A3243G of $tRNA^{Leu(UUR)}$ gene causing MELAS Syndrome (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) is

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responsible for about 2% of cases of type 2 diabetes [4]. Point mutation A3243G in the $tRNA^{Leu(UUR)}$ gene is one of the most common mtDNA defect, firstly diagnosed in children with MELAS Syndrome. Some families also express diabetes and deafness known as MIDD (maternally inherited diabetes and deafness) [5, 6]. In general population, MIDD is responsible for 0.5 to 1.5% of all diabetes cases. MIDD pathophysiological mechanism are complex and may involve impairment of glucose toxicity, insulin secretion and resistance. In mitochondrial diabetes, insulin impairment response to glucose challenge is in an early stage and indicates major factor in the pathogenesis of hyperglycemia. However, insulin response to glucagon and arginine rather than glucose is not clear [7, 8].

The most notorious mutation is A3243G in $tRNA^{Leu(UUR)}$ gene that causes impairment in the arrangement of functional proteins of Electron Transport Chain (ETC). This type of mutation is non-adoptive and brings impairment in the production of insulin [9]. A3243G point mutation becomes pathogenic when its concentration reaches 10–30%.Most commonly, considered as the frequent cause of insulin independent diabetes mellitus [10]. Mutation in $tRNA^{Leu(UUR)}$ gene at position A3243G cause defects in function of tRNA to properly assemble proteins in respiratory chain complexes due to which oxidative phosphorylation will not properly produce enough ATP to activate ATP-sensitive potassium channels in the β-cells to secrete insulin in response to high blood sugar level which will eventually cause type 2 DM. The Adenine to Guanine at nucleotide position A3243G in the tRNA gene play a major role in the methylation amino-acylation codon recognition and tertiary union of the molecules assembly. The mutation responsible for this maternally inherited diabetes mellitus is varying in different ethnic groups [11, 12].

The population of tribal areas of Pakistan is less explored and little literature is available on this specific ethnic group. Therefore, this study population has been assessed for mitochondrial DNA mutation which is more frequently found in population affected with type 2 diabetes mellitus. The aim of this study was to determine mitochondrial $tRNA^{Leu(UUR)}$ gene mutation in MIDD in the population of tribal areas of Pakistan as this might help to determine the molecular mechanism of the type 2 DM. Such type of finding will pave the way for better treatment, genetic counselling and prenatal diagnosis of maternally inherited diabetes.

Materials and methods

The approval of this study was obtained from the Hazara University Institutional Review Board and written informed consent was obtained from all the study individuals. A detailed medical history of the study individuals was recorded and detailed pedigree (Figure 1, Table 1) was drawn at the time of visit to the affected families. Saliva samples (5 ml) were obtained from fifteen diabetic individuals and DNA was extracted successfully. Only five (N = 5) samples had optimum quantity of DNA required for sequencing. The collected amount of saliva 5 ml was important to get high amount of buccal epithelial cells for high concentration of the genomic DNA. The samples were stored at –20 °C soon after collection to recede the risk of contamination.

DNA extraction and PCR amplification

Genomic DNA was extracted from buccal epithelial cells using a modified protocol [13]. The extracted DNA was quantified using NanoDrop measurement at ratio of absorbance at 260/280 nm as shown in Table 2. After quantification whole genomic mitochondrial DNA was amplified using standard kit method (Qiagen Repli-G mitochondrial DNA kit). The fragments encompassing np 3243 of mitochondrial DNA were amplified with PCR using AmpliTaq DNA polymerase. The sets of reverse and forward primer were used. The nucleotide sequence of forward primer was F-5'-CAAATTCCTCC-CTGTACGAAAGG-3' and the reverse primer was R-5'--AATGAGGAGTAGGAGGTTGGCC-3'. PCR was carried out in a total volume of 25 μl master mix containing 50 ng of extracted DNA, 2.0 μl each dNTP, 11.5 μl ddH2O, 2.5 μl MgCl2, and 0.5 μl of *Taq* polymerase. The DNA was initially denatured at 94°C for 3 min and subjected to 35 PCR cycles of 94°C for 45 sec, 59°C for 1 min, and 72°C for 3 min. The PCR products after amplification were electrophoresed on 1% agarose gel and stained with ethidium bromide (Figure 2).

Gene clean

TIANgel Midi Purification Kit, Cat# DP209-02.PCR was used according to manufacturer's instructions for elution of the PCR amplificated DNA.

Sanger sequencing of the targeted mtDNA region

DNA sequencing were carried out using the Big-Dye terminator cycle sequencing method and reaction products were analyzed on an ABI-Prism 377 automated sequencer at Macrogen Inc. Korea (www.macrogen.com).

The resulted sequencing data was analyzed using NCBI online tool BLAST. The revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA was used as reference for analysis of the DNA samples.

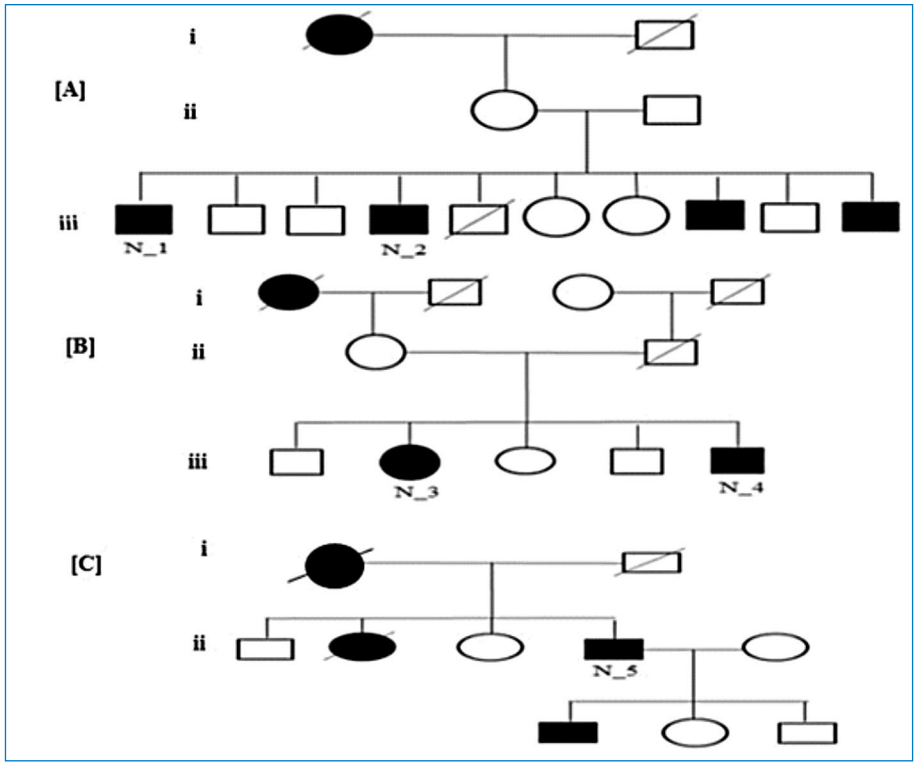

Figure 1. Family pedigree of all the three families (A, B and C) including three generations. The circles represent the female members while the squares representing the male members. Black square and circles represent the diseased one and white show the normal non diseased one. N_1 to N_5 are the type 2 diabetes individuals inlcuded in the final analysis

Table 1. Characteristics and clinical history of all the study subjects (N = 5)

Sample number	Gender	Age (years)	Weight [kg]	Complications	Medications
N_1	Male	60	60	Renal disease	Insulin
N_2	Male	55	75	Blindness, joint problems, high blood pressure	Oral hypoglycemic agent
N_3	Female	50	65	Joint pain, infertility, skin and renal disease	Insulin
N_4	Male	43	80	High blood pressure, hearing loss, renal and heart disease	Oral hypoglycemic agent
N_5	Male	75	55	High blood pressure, renal disease	Insulin

Table 2. DNA concentrations of samples (N = 5)

Sample number	DNA concentration [ng/μl]
N_1	1.81
N_2	1.96
N_3	1.78
N_4	1.90
N_5	1.84

Results

Individual's clinical and social history was recorded with the help of questionnaires. Characteristics of the individuals and most common clinical complications related to type 2 DM are presented in Table 1. The $tRNA^{Leu(UUR)}$ gene from the proband N_1, N_2, N_4 and N_5 from the diabetic families were sequenced. Sample 3 (N_3) was wasted and not included in the

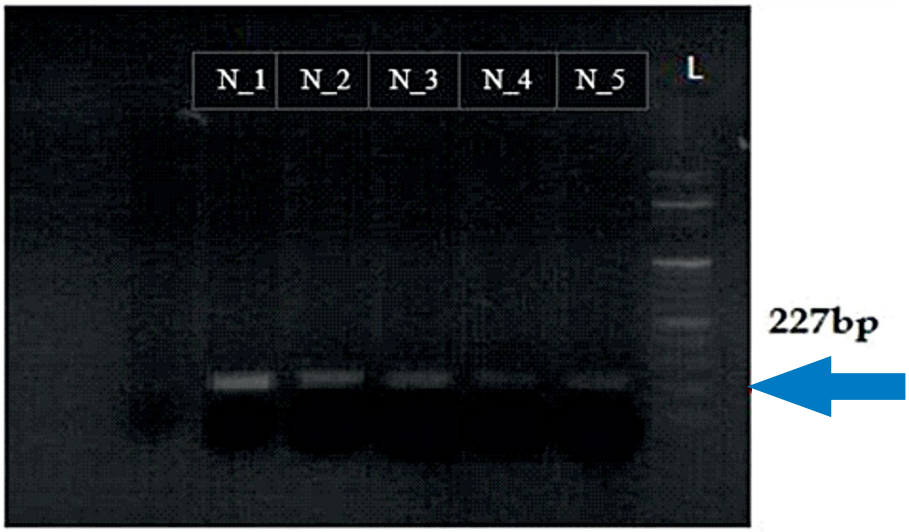

Figure 2. PCR amplified tRNA$^{Leu(UUR)}$ gene products from sample N_1 to N_5 of 227 bp DNA fragments, L = 100 bp DNA marker

final analysis. Sequences were aligned using online tool using NCBI. The obtained sequence data samples were aligned with rCRS with accession No NC-012920.1 (Figure 3). After careful analysis of sequenced data, point mutation tRNA$^{Leu(UUR)}$ gene at position 3243 A-G was not observed.

Discussions

In Pakistan, presently 6.9 million individuals suffer from diabetes. It is assumed that by 2030, Pakistan will be ranked fifth for residing most number of DM individuals [14]. The inheritance of diabetes is different in different ethnic groups i.e. some ethnic groups in a particular area are more affected than others. Also, social, behavioral and environmental factors contribute to the onset of disease [15, 16]. Pakistani tribal areas are still not explored with respect to mtDNA mutation associated with MIDD. In this research study, we assessed and analyzed mtDNA tRNA$^{Leu(UUR)}$ gene mutation in families suffering from diabetes, living in tribal areas of Pakistan. During recording of the questionnaires, most of the individuals had experienced emotional stress before the onset of type 2 DM. Emotional stress is a risk factor for type 2 DM that should also be considered [17]. The outcomes of the current study for mutation in mitochondrial tRNA$^{Leu(UUR)}$ gene in subjects with type 2 DM in population from tribal areas of Pakistan identified none of the individual as a carrier of this mutation.

Diabetes mellitus is a group of metabolic disorders such as destruction of beta cells of pancreas and genomic DNA mutations in genes linked to type 2 DM [18]. Gene mutations are involved in peripheral insulin sensitivity and glucose induced insulin secretions. mtDNA has 10 times more spontaneous mutations as compared with nuclear genome and is responsible for more than 80% of MELAS cases due to lack of protective histone and DNA repair system [19]. Therefore, mitochondrial DNA mutation may lead to impaired insulin secretion due to its role in glucose induced insulin secretion in pancreatic beta cells and pancreatic islets cells easily effected by disturbance of oxidative phosphorylation. Diabetes mellitus associated with mtDNA is transmitted maternally while the most common point mutation associated with DM was mitochondrial DNA tRNA gene (i.e. A3243G). Pakistani population is not explored in respect to disease associated with mitochondria particularly type 2 DM in relation with mt tRNA-Leu gene [20]. It is still not explored that which type of mtDNA mutation is responsible for type 2 DM.

In mtDNA, point mutations can occur due to deletion, insertion and substitution of nucleotide so comprehensive analysis and screening of entire mtDNA is required. However, the ratio of mutated mtDNA varies between tissues in relation to wild type mtDNA, being high ratio in post mitotic tissues (pancreas, brain and skeletal muscles) while low in rapidly dividing tissues (blood leukocytes). According to a study from Hart et al., the defect in the mitochondrial tRNA$^{Leu(UUR)}$ gene is associated with type 2 DM [11]. Martikainen et al. reported that 1% of DM emergence associated with A3243G mutation in mtDNA tRNA$^{Leu(UUR)}$ gene [21]. The response of impaired insulin to glucose in patients with tRNA$^{Leu(UUR)}$ gene mutation is an early and critical abnormality in the development of type 2 DM [7]. However, it should not be excluded that other mutations such as tRNAGlu 14709 T → C, ND-13316 G → A,

Figure 3. Sequence alignment of sample N_1 to N_5 obtained from Macrogen (www.macrogen.com) with revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA (Accession NO-012920.1)

ND-13394 T → C, ND-13426 A → G, ND-412026 A → G, tRNALeu 3256 C → T, tRNALys8296 A → G, tRNALys8344 A → G, tRNALys8363 G → A, tRNASer 12258 C → A in genes have active role in etiology of disease [22].

The pathogenic mutation in the mitochondrial genome is very common due to the lack of efficient mitochondrial DNA mutation repair machinery. The mitochondrial genome has overlapping coding regions and mutation in any region can cause severe phenotypic effects. Since 1988, more than 270-point mutations have been described, affecting every mtDNA gene. Remarkably, more than half of these mutations are located in tRNA genes, even though tRNA comprise only about 10% of the total coding capacity of the genome. Among the point mutations the most common are an A→G transition at position 3243 in the $tRNA^{Leu(UUR)}$ gene. It was long believed that A→G transition at position 3243 in the $tRNA^{Leu(UUR)}$ gene is the common cause of type 2 DM [23, 24].

The leucine tRNA gene in the mitochondrial genome appears to be a frequent spot for point mutations, as several different mutations have been described so far. The most common mutation occurs at base pair (bp) 3243, and it accounts for approxi-

mately 80% of cases of the MELAS syndrome. For this reason, mtDNA testing were preferred while MODY has a high inheritance rate and represents one end of a continuum of monogenic forms of diabetes that includes neonatal diabetes [25, 26]. The frequency of the A3243G mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene vary in the members of different ethnicities. In a study conducted for the confirmation of A3243G mutation in $tRNA^{Leu(UUR)}$ gene being the frequent cause of type 2 DM has concluded that this mutation is not the frequent cause of type 2 DM [27].

In this study, all the selected families had type 2 DM familial vertical history. The clinical description included phenotypic features including sensorineural hearing loss, diabetes mellitus, cardiovascular disease, renal disease, blindness, arthritis, hypertension, and infertility. Other potential diabetic phenotypes include noninsulin-dependent diabetes mellitus (NIDDM), insulin-dependent diabetes mellitus (IDDM), malnutrition related diabetes mellitus (MRDM) and other diabetes associated syndromes such as Wolfram syndrome-diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) and Maturity-onset diabetes of the young (MODY) [28]. NIDDM is a heterogeneous disorder with different pattern of inheritance that appears after forty year of age and is characterized by defect in beta cell function and insulin resistance [29]. IDDM is a polygenic disease characterized by defects in secretion and action of insulin. Several molecular alteration such as decrease in receptor tyrosine kinase activity, insulin receptor number, and IRS-1 phosphorylation contribute to insulin resistance [30]. MRDM also known as tropical pancreatic diabetes mellitus is rare diabetes linked with long term malnutrition, characterized by hyperglycaemia, insulin resistance, insulinopenia, and dysfunctional of the beta cells of pancreas [31]. DIDMOAD is a mitochondrial DNA disorder, can cause DM as the start of symptoms [32]. MODY is a monogenic disorders in seven different genes mutations lead to alter secretion of insulin [33]. Some diabetic patient, diagnosed as type 2 DM do not indicate evidence of circulating autoantibodies and overweight, are medicated using oral hypoglycemic drugs. This type of diabetes is classified as latent autoimmune diabetes of adults (LADA) [34].

The outcomes of the current study for mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene in subjects with type 2 DM in population from tribal areas of Pakistan identified none of the individual as a carrier of A3243G mutation in $tRNA^{Leu(UUR)}$ gene. The mitochondrial DNA is highly vulnerable to pathogenic mutation almost at any site, therefore in the etiology of this disease other gene variation should not be excluded. The clinical spectrum of mtDNA mutations are extremely broad, identical clinical signs and symptoms can be caused by nuclear genes and mtDNA mutations. If the suspect are obese or overweight with fasting hyperglycaemia, glycosuria in the presence of normoglycaemia and having strong family history of DM recommends mtDNA tests. Also, the large sample size and comprehensive sequencing of the entire mtDNA molecule is needed in Pakistani population.

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Conflict of interest

The authors declare that they do not have any competing interests.

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Correlation of red cell distribution width with inflammatory markers and its prognostic value in patients with diabetes and coronary artery disease

ABSTRACT

Background. Recent studies have shown red blood cell distribution width (RDW) as a marker for severity and prognosis in coronary artery disease patients. Other studies have also correlated RDW with diabetes mellitus and inflammation. However, such correlation and prognosis in patients with concomitant coronary artery disease and diabetes after percutaneous intervention remains unclear.

Material and methods. Our study group comprised of 730 subjects including 700 patients (cases) and 30 normal subjects (control group). Patients who presented with coronary artery disease were divided into diabetic and non-diabetic groups. All patients had RDW measured at admission and percutaneous intervention was done. Follow-up for adverse events was carried out between 6 to 12 months.

Results. RDW was elevated in patients as compared to control group ($p < 0.05$). RDW correlated well with inflammatory markers including erythrocyte sedimentation rate, C-reactive protein, HbA_{1c}, white blood cells and troponin. RDW was higher with more severe atherosclerosis based on SYNTAX and Gensini scores ($p < 0.05$). Prognosis was found to be worse in patients with high RDW as well as in diabetics.

Conclusions. RDW has positive correlation with other inflammatory marker. It may be used as a marker in determining the severity and prognosis in diabetic patients with coronary artery disease. (Clin Diabetol 2020; 9; 3: 174–178)

Key words: red cell distribution width, coronary artery disease, diabetes

Introduction

Guidelines for acute ST elevation myocardial infarction (STEMI) patients recommend primary PCI as the preferred reperfusion strategy [1]. Inflammation plays a critical role in the initiation and propagation of the atherosclerotic process [2, 3]. Numerous inflammatory markers and indices have been studied recently in relation to atherosclerosis. One such marker is red cell distribution width (RDW). Use of RDW has been established in the investigation of the etiology of anemia [4]. Moreover, recent studies are showing an increasing evidence linking elevated RDW with adverse outcomes, in patients with coronary artery disease, heart failure (HF) and with metabolic syndrome. Diabetes mellitus (DM) is an independent risk factor for heart failure (HF) [4–6].

HbA_{1c} is associated with type 2 diabetes patients and is a marker of long term glucose homeostasis as well as adequacy of glycemic control in diabetic patients [7]. HbA_{1c} level increase with age, chronic subclinical

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inflammation and possibly oxidative stress and also in conditions that can adversely affect red blood cell survival. Correlation of RDW and diabetes in coronary artery disease patients has not been studies well.

Therefore, the aim of this exploratory study was to investigate the correlation and prognostic value of RDW in diabetes and coronary artery disease patients.

Patients and methods

It is a retrospective clinical study which included patients admitted to our hospital with coronary artery disease and diabetes. For the purpose of this analysis patients were divided into low RDW, high RDW, diabetic and non-diabetic groups. ST elevation myocardial infarction (STEMI) was defined as typical chest pain lasting for at least 30 min with new ST-segment elevation at the J point in more than two contiguous precordial or inferior leads (> 0.2 mV in V1 through V3 and > 0.1 mV in the other leads) [8]. Diabetes was defined according to definition previous stated elsewhere [9].

Hypertension was defined as repeated SBP measurements at least 140 mm Hg, repeated DBP measurements at least 90 mm Hg or chronic treatment with antihypertensive medications. Diabetes was recorded when it was reported by the patient and appeared in their medical records or if the patient was receiving regular treatment with oral hypoglycemic agents or insulin. Dyslipidemia was defined as a low-density lipoprotein-cholesterol level above the target according to National Cholesterol Educational Program-3 recommendations, as a high-density lipoprotein-cholesterol level below 40 mg/dL, or chronic treatment with lipid-lowering drugs prior to hospitalization [10].

All patients with an ACS received aspirin (325 mg loading dose) on admission and 100 mg daily as well as clopidogrel (300 mg loading dose for patients < 75 years of age and 75 mg for patients > 75 years of age). PCI was done for all STEMI patients.

Blood samples were taken on admission from all patients at the time of hospitalization and prior to the administration of medication. All hematological measurements were performed using Cobas B221 and 6000, Roche-Switzerland. Our hospital ethics committee approved this study and informed consent was obtained from all study participants according to the declaration of Helsinki.

Statistical analysis

Data was analyzed with Statistical Package for the Social Sciences (SPSS 20) for Windows (SPSS Inc., Chicago, Illinois, USA). Continuous data are presented as the mean along with standard deviation. The Kolmogorov-Smirnov test was used to evaluate data normality.

Independent-samples t-test was used to compare two groups showing normal distribution. Categorical variables were summarized as percentages and compared with the chi-square test. Pearson's and Spearman correlation analysis tests were used to determine the correlation between variables. P value less than 0.05 was considered to indicate a significant difference.

Results

730 subjects were enrolled in this study which included 30 normal and disease free subjects and 700 coronary artery disease patients. Mean RDW in control group was compared to RDW in patients which showed significant difference [(12.4 *vs.* 14.5) ($p < 0.05$)]. All patients underwent percutaneous intervention at about 8 ± 5 hours after onset of chest pain. The patients were divided into groups according to RDW and diabetic status. The mean age of the patients was 64 ± 7.92 years. Patients were assessed at baseline according to RDW levels (low and high). There was significant difference between low and high RDW groups with regard to hemoglobin, HbA_{1c}, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cells (WBC) and troponin levels. Other demographic and clinical baseline characteristics between the two groups did not show any significant difference (Table 1).

Angiographic characteristics and scores of the study groups are presented in Table 2. SYNTAX Score ranged from 5 to 47 and with a mean of 23.75 ± 10.12 while Gensini Score ranged from 8 to 10 and with a mean of 48.31 ± 25.47. SYNTAX and Gensini score were divided into low and high based on mean values. RDW levels in relation to angiographic scoring were assessed in diabetics ($HbA_{1c} \geq 7$) and non-diabetics ($HbA_{1c} < 7$). RDW levels were significantly higher with high SYNTAX score, Gensini scores and multi-vessel disease and as well as in diabetics ($p < 0.05$) (Table 2).

The Pearson and Spearman correlation coefficients were used to assess the correlations between RDW and various clinical and laboratory variables. Significant positive correlation was found between RDW and age, ejection fraction, ESR, HbA_{1c}, WBC, CRP and troponin while there was significant negative correlation with hemoglobin ($p < 0.05$) (Table 3).

Prognosis was assessed on the basis of five indices (angina, myocardial infarction, heart failure, stroke and death) during a period of 6 months to one year after percutaneous intervention. Comparison was made between low and high RDW groups as well as diabetic and non-diabetics. It showed significant increase in myocardial infarction, heart failure and death in both high RDW and diabetic groups. Moreover, frequency of angina was also more in diabetic group ($p < 0.05$) (Table 4).

Table 1. Baseline characteristics of study groups according to RDW

Variable	RDW < 16 (n = 300)	RDW ≥ 16 (n = 400)
Age (years)	63 ± 7.10	65 ± 8.75
Ejection fraction (%)	50.62 ± 8.65	48.78 ± 8.10
Hypertension	99 (33%)	140 (35%)
Smoking	69 (23%)	108 (27%)
Renal failure	30 (10%)	48 (12%)
Hyperlipidemia	63 (21%)	96 (24%)
HbA_{1c}	5.5 ± 0.5	7.5 ± 1.0*
Erythrocyte sedimentation rate [mm/h]	18 ± 5	39 ± 10*
C-reactive protein [mg/L]	0.7 ± 0.4	1.9 ± 0.6*
Troponin (high sensitivity) [ng/l]	545 ± 370	2360 ± 574*
Hemoglobin [gm/dl]	15 ± 1.0	11 ± 1.5*
White blood cells [$\times 10^9$/L]	9.50 ± 2.32	14.48 ± 2.25*
Aspirin	300 (100%)	400 (100%)
Clopidogrel	291 (97%)	396 (99%)
Beta blocker	285 (95%)	372 (93%)
Renin antagonists	264 (88%)	364 (91%)
Lipid lowering agents	240 (80%)	336 (84%)

*$p \leq 0.05$

Table 2. Relationship between RDW and angiographic variables in diabetic ($HbA_{1c} \geq 7$) and non-diabetic groups ($HbA_{1c} < 7$)

Variable	RDW	
	$HbA_{1c} < 7$	$HbA_{1c} \geq 7$
SYNTAX score		
Low (< 23)	12.85 ± 1.10	13.45 ± 1.7
Moderate-high (23–32)	14.24 ± 1.15	17.85 ± 1.14*
Gensini score		
Low (< 47)	12.55 ± 1.18	12.55 ± 1.18
High (≥ 47)	14.37 ± 1.21	18.12 ± 1.11*
No. of vessels involved		
Single vessel disease	11.97 ± 1.25	14.10 ± 1.36
Multi-vessel disease	13.78 ± 1.75	18.82 ± 1.61*

*$p \leq 0.05$

Table 3. Correlation of RDW with clinical and laboratory variables

Variable	Correlation coefficient (P)
Age	0.487 (0.030)
Systolic blood pressure	0.037 (0.061)
Diastolic blood pressure	0.002 (0.346)
Ejection fraction	0.030 (0.048)
ESR	0.561 (0.001)
Serum creatinine	0.258 (0.979)
BMI	0.003 (0.0686)
Total cholesterol	0.014 (0.725)
Hemoglobin	–0.486 (0.025)
HbA_{1c}	0.529 (0.001)
WBC	0.580 (0.001)
CRP	0.349 (0.029)
Troponin	0.412 (0.017)

ESR — erythrocyte sedimentation rate; BMI — body mass indeks; WBC — white blood cells; CRP — C-reactive protein

Table 4. Prognosis in relation to RDW and diabetes after percutaneous intervention

	RDW < 16 (n = 300)	RDW ≥ 16 (n = 400)	Non-diabetics (n = 380)	Diabetics (n = 320)
Angina	18 (6%)	28 (7%)	20 (5.26%)	26 (8.12%)†
Myocardial infarction	4 (1.33%)	12 (3%)*	5 (1.32%)	11 (3.44%)†
Heart failure	3 (1%)	14 (3.50%)*	4 (1.05%)	13 (4.06%)†
Stroke	0 (0%)	2 (0.50%)	1 (0.26%)	1 (0.31%)
Death	3 (1%)	10 (2.50%)*	4 (1.05%)	9 (2.81%)†

*$p \leq 0.05$ comparison between RDW < 16 and RDW ≥ 16 groups; †$p \leq 0.05$ comparison between non-diabetic and diabetic groups

Discussion

This study showed good correlation of RDW with other inflammatory markers. The underlying pathophysiologic mechanisms for the association between a high RDW and atherosclerosis is unknown, but it is believed that inflammation might play a role with cytokines induced changes in the red cell membrane, leading to an increased RDW [11]. Erythrocyte malformation and hence increased RDW may be caused by oxidative stress, inflammation, and increase in cholesterol levels in erythrocyte membrane. Inflammation or oxidative stress may cause increased RDW by impairing iron metabolism, inhibiting the production of or response to erythropoietin, and shortening red blood cell survival [12, 13].

Lippi et al. [14] showed an increase in RDW levels during acute coronary syndrome and its usefulness for the risk stratification. Previous studies have also assessed the relationship between an increased RDW and poor reperfusion results after STEMI, increased risk and morbidity in hospital as well as mortality [15]. A possible explanation for this result is the intense inflammatory response in the setting of STEMI.

In our study, the RDW levels have been shown to be higher in patients with diabetes. A number of risk factors such as age, hypertension, hyperlipidemia, high CRP levels may play a role [12].

Fatemi et al. showed high RDW levels in patients with two or more vessel disease and with early phase of atherosclerosis [16, 17].

Osadnik et al. [18] demonstrated prognostic value of RDW in stable angina pectoris patients undergoing PCI by showing higher mortality in these patients with a high RDW level.

RDW is a marker of inflammation and has prognostic value in diabetic patients [19, 20]. The association between DM and RDW has been initially examined by Subharshree [21] showed correlation between BNP and RDW in diabetic patients with HF. Malandrino et al. [22] demonstrated the relationship between RDW and microvascular and macrovascular complications of diabetes mellitus (DM).

RDW may reflect an underlying inflammatory process and inflammation is considered a vital component in the diabetic disease process as well [4, 23], which led to Sherif et al. [24] suggesting that RDW could be used as a marker of inflammation in type 2 DM.

Elevated glucose levels may affect erythrocytes in multiple ways such as changes in erythrocyte membranes which include increased rigidity, changes in osmotic fragility due to changes in Na+/K+-ATPase activity and tubulin acetylation and increased aggregation. Other changes include defective oxygen binding of hemoglobin and alterations in cell as well [25–27]. These changes can result in increase in shear stress on the endothelial wall and increased blood viscosity [28].

Conclusion

RDW has positive correlation with other inflammatory markers. It is an inexpensive and easily accessible marker which can be used in determining the severity and prognosis in diabetic patients with coronary artery disease after percutaneous intervention. More pronounced inflammatory process may be responsible for increased severity and hence poorer prognosis in diabetic patients.

Conflict of interest

The authors declare to have no conflict of interest.

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Evaluation of *Helicobacter pylori* IgG levels in type 2 diabetes mellitus patients

ABSTRACT

Background. Diabetes mellitus (DM) is a common and debilitating chronic disease with increasing prevalence in the world and in Iran. *Helicobacter pylori* (*H. pylori*) is a gram-negative bacillus that causes gastritis and peptic ulcer disease and stomach cancer. It is more common in developing countries. Several studies have shown the possible association between *H. pylori* infection and DM. We performed this study to evaluate *H. pylori* infection in type 2 diabetes mellitus (T2DM) patients in comparison to non-diabetic individuals.

Methods. In a case-control study 99 T2DM patients (aged 31 to 96 years) who referred to Urmia Imam Khomaini hospital and 96 non-diabetic controls were included. Venous blood samples were received from all participants and fasting blood glucose (FBG), HbA_{1c} and serum *H. pylori* IgG levels were measured. For all individuals demographic data, including age, sex and body mass index (BMI) were recorded. *H. pylori* IgG levels greater than 10 U/ml was considered as *H. pylori* infection. *H. pylori* IgG serum levels of all of T2DM patients and control group were compared with each other. Data were analyzed using SPSS version 17. We used independent T-test, Chi-square and Fisher exact test for statistical analysis. The level of significance was considered as p-value < 0.05.

Results. Means age of T2DM patients and control group were 59.77 ± 13.25 and 63.43 ± 13.16 years respectively and there was not significant difference between two groups (p = 0.05). Frequency of positive *H. pylori* serology in T2DM patients was 69.7% and in non-diabetic group was 66.7% and there was not significant difference between two groups in this regard (p = 0.65). Mean ± SE serum *H. pylori* IgG levels in T2DM and non-diabetic subjects was 45.78 ± 4.82 and 44.35 ± 4.83 U/ml respectively (p = 0.83). Mean HbA_{1c} level was significantly higher in T2DM patients compared to control group (8.40 ± 2.02 and 5.29 ± 0.45 respectively, p < 0.001).

Conclusions. According to the results of this study frequency of *H. pylori* infection and also serum *H. pylori* IgG levels in diabetic patients does not differ from non-diabetics subjects. (Clin Diabetol 2020; 9; 3: 179–183)

Key words: diabetes mellitus type 2, *Helicobacter pylori*, *Helicobacter pylori* serology, IgG

Introduction

Diabetes is a common and debilitating chronic disease with increasing prevalence both in the world and in Iran [1–3]. *Helicobacter pylori* (*H. pylori*) is a gram-negative bacillus that causes gastritis and peptic ulcer disease and stomach cancer [4]. It is more common in developing countries [5]. Some studies have shown that *H. pylori* infection has been associated with non-gastrointestinal diseases such as ischemic heart disease, neurologic diseases, and autoimmune thyroid disorders [5–10]. Several studies have shown the association between *H. pylori* infection and diabetes mellitus [5, 11, 12]. The issue of whether *H. pylori* infection causes diabetes or those with diabetes are more likely to develop *H. pylori* infection is still not fully understood [12]. One of the proposed mechanisms of developing diabetes in patients with *H. pylori* infection can be increased insulin resistance [5]. Other

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Table 1. Demographic characteristics and laboratory test results of type 2 diabetes mellitus (T2DM) patients and control group

Parameter	T2DM patients (n = 99)	Control group (n = 96)	p value
Age (years)	59.77 ± 13.25	63.43 ± 13.16	0.05
Female (%)	53 (53.5%)	49 (51%)	0.72
Weight [kg]	73.56 ± 14.44	66.54 ± 14.34	0.001
Height [cm]	163.99 ± 9.81	161.02 ± 10.46	0.04
BMI [kg/m^2]	27.45 ± 5.28	25.61 ± 4.47	0.009
HbA_{1c} (%)	8.40 ± 2.02	5.29 ± 0.45	< 0.001
FBG [mg/dL]	201.69 ± 78.71	90.39 ± 24.25	0.001

Data are expressed as mean ± SD. FBG — fasting blood glucose; BMI — body mass index

mechanisms include chronic inflammation, reduced insulin secretion, and increased synthesis of some of the diabetogenic hormones such as leptin that leads to insulin resistance [5]. Furthermore decreased ghrelin level in patients with *H. pylori* infection leads to reduced energy consumption and weight gain [5].

In some studies, there is even a significant correlation between microvascular complications of diabetes and helicobacter infection such as microalbuminuria and neuropathy [13, 14]. In contrast, in some studies, there was no association between *H. pylori* infection and diabetes mellitus found [15].

Given the controversy and limited studies in this field in Iran, we designed this study to investigate the association of *H. pylori* infection with type 2 diabetes.

Materials and methods

In this case-control study 99 type 2 diabetes mellitus (T2DM) patients over the age of thirty (aged 31–96 years) who referred to Endocrinology department of Urmia Imam Khomaini hospital and 96 non-diabetic controls were included. Subjects who had autoimmune, infectious or rheumatologic diseases and those taking proton pomp inhibitors within one month before the study as well as patients treated with immunosuppressive drugs were excluded from study.

The subjects in our study had no predominant symptoms of upper gastrointestinal tract involvement and they had not been treated with anti-helicobacter drugs for a month before study initiation. The control group was selected from non-diabetic healthy individuals who referred to the ophthalmology department of Imam Khomaini hospital and did not present any exclusion criteria.

The study began after approval of Urmia University of medical sciences ethic committee and after obtaining written consent from all individuals. Venous blood samples were received from participants in the fasting state for fasting blood glucose (FBG), HbA_{1c} and serum *H. pylori* IgG levels. FBG and HbA_{1c} were measured by enzymatic method (Pars Azmun kite, Tehran, Iran) and turbidimetric immunoassay (Aptec kite, Belgium) respectively and serum *H. pylori* IgG level was measured by ELISA method (Pishtazteb kite, Tehran, Iran). For all subjects demographic data, including age, sex weight, height and body mass index (BMI) were recorded. *H. pylori* IgG levels greater than 10 U/ml was considered as *H. pylori* infection. *H. pylori* IgG serum levels of all of T2DM patients and control group were compared with each other. Data were analyzed by independent T-test, Chi-square and Fisher exact tests using SPSS version 17. The level of significance was considered as p-value < 0.05.

Results

Demographic characteristics and laboratory test results of two study groups are demonstrated in Table 1.

As shown in Table 1, there is no statistically significant difference between the two groups in terms of age (p = 0.05). Also, the percentage of female population in the two groups was not statistically significant.

Both FBG and HbA_{1c} mean values were significantly higher in T2DM patients than in control group (Table 1). Frequency of positive *H. pylori* serology in T2DM patients was 69.7% (n = 69) and in non-diabetic group was 66.7% (n = 64) and there was not significant difference between these two groups in this regard (p = 0.65). Mean ± SE (standard error) serum *H. pylori* IgG levels in T2DM and non-diabetic subjects were 45.78 ± 4.82 and 44.35 ± 4.83 U/ml respectively and the difference between these two groups was not statistically significant (p = 0.83).

FBG, HbA_{1c} and BMI mean values did not differ significantly between diabetic patients with and without *H. pylori* infection (Table 2). Also as shown in Table 3 the difference between FBG, HbA_{1c} and BMI mean values was not statistically significant between control subjects with *H. pylori* infection and without it.

Table 2. Glycemic parameters and BMI in diabetic patients with and without *H. pylori* infection

	DM *H. pylori*+	DM *H. pylori*–	p-value
FBG [mg/dL]	191.66 ± 64.33	224.7 ± 102.18	0.05
HbA_{1c} (%)	8.33 ± 1.95	8.55 ± 2.22	0.63
BMI [kg/m^2]	27.47 ± 5.22	27.40 ± 5.49	0.96

DM — diabetes mellitus; FBG — fasting blood glucose; BMI — body mass index

Table 3. Glycemic parameters and BMI in control group with and without *H. pylori* infection

	Control *H. pylori*+	Control *H. pylori*–	p-value
FBG [mg/dL]	92.73 ± 27.86	85.71 ± 13.8	0.1
HbA_{1c} (%)	5.34 ± 0.48	5.19 ± 0.37	0.13
BMI [kg/m^2]	25.83 ± 4.3	25.16 ± 4.85	0.49

FBG — fasting blood glucose; BMI — body mass index

Moreover in the total population of our study, the mean value of BMI was 26.68 ± 4.85 kg/m^2 in *Helicobacter* infected subjects and 26.25 ± 5.25 kg/m^2 in non-infected individuals, and there was no statistically significant difference between these two groups ($p = 0.57$).

Discussion

Type 2 diabetes mellitus is a common systemic disease with serious complications [1, 2, 16]. In recent years, several studies have examined the association between T2DM and *H. pylori* infection and some of them have confirmed this association but others have not shown any relationship between these two. In the present study, the percentage of T2DM patients with *H. pylori* infection was 69.7% and in the control group it was 66.7% and the difference between these two groups was not statistically significant. Similar to our study in a cross-sectional study conducted by Jafarzadeh et al. on 100 T2DM patients and 100 healthy controls in 2011 in Rafsanjan city of Iran, there was no significant difference between the prevalence of positive *Helicobacter* antibodies (IgG) levels among patients with type 2 diabetes and healthy subjects (76% *vs*. 75% respectively). But in their study healthy individuals compared with diabetic patients, had significantly higher levels of anti-*Helicobacter* IgG antibodies (131.63 ± 11.68 *vs.* 54.43 ± 4.50 U/ml; $p < 0.0001$) [17]. Our results are consistent with study by Jafarzadeh et al. regarding the similar rate of *H. pylori* seropositivity in the two study groups and lack of association between T2DM and *H. pylori* infection [17]. However, in our study, the mean serum level of anti-*Helicobacter* IgG antibiodies in diabetic patients was higher than in the control group (45.78 ± 4.82 and 44.35 ± 4.83 respectively), but this difference was not statistically significant ($p = 0.83$).

In a study by Bener and colleagues on 210 patients with type 2 diabetes and the same number of non-diabetic patients, a higher percentage of diabetic patients than non-diabetic ones had positive *H. pylori* antibody (IgG) levels (76.7% *vs.* 64.8% respectively, $p = 0.01$) which is against our study results [18].

In the research conducted by Devrajani et al. on 74 diabetes cases and 74 non-diabetic controls in Pakistan, the percentage of positive *Helicobacter pylori* stool antigen was 73% and 51.4%, respectively and the difference between these two groups in this regard was statistically significant ($p = 0.0001$). They suggested that patients with diabetes are susceptible to *H. pylori* infection, therefore they recommended screening for *H. pylori* infection in diabetic patients [19].

Furthermore Bajaj et al. in the study on 80 diabetic patients and 80 controls, showed that the prevalence of *H. pylori* infection in diabetic patients is considerably higher than in the control group (77.5% *vs*. 58.3% respectively, $p = 0.02$). Also, in their study the average HbA_{1c} levels in the diabetic group with *H. pylori* was significantly higher than those without *Helicobacter* infection [20].

Therefore, they suggested that *H. pylori* infection is associated with higher HbA_{1c} levels and inappropriate blood glucose control, but our study results are not consistent with the results of the study by Bajaj et al.

In a study by Zojaji et al. on 85 T2DM patients in Iran, the mean serum HbA_{1c} level 3 months after treatment of *H. pylori* infection was significantly reduced compared to its pre-treatment level, however, fasting blood glucose did not change significantly after treatment compared to the pretreatment state. They

concluded that *H. pylori* eradication has beneficial effects on the glycemic control in diabetic patients [21]. However, Wada and colleagues, in a retrospective study in Japan, showed that treatment of *Helicobacter pylori* infection in T2DM patients does not improve HbA_{1c} levels [22].

Also Vafaeimanesh et al. in the study on T2DM patients who were treated with oral medications showed that *H. pylori* infection therapy has no beneficial effects on blood glucose profiles, in addition they had lower rates of successful response to anti *H. pylori* regimen compared with non-diabetic subjects [23].

The study of Quatrini et al. in Italy, also supports the higher percentage of *H. pylori* infection in diabetic patients population (insulin dependent and non-insulin dependent) compared with non-diabetic subjects (69% and 46% respectively, $p = 0.007$) [24]. Although the different outcomes of their study comparing to our study may be due to the fact that they used respiratory urease test for the detection of *H. pylori* infection, while we have used ELISA method (anti *H. pylori* IgG levels).

Our study shows that there is no significant relationship between *H. pylori* infection and type 2 diabetes.

H. pylori infection diagnostic tests are classified into two categories of invasive and non-invasive tools and various previous studies have used either one method alone or a combination of several methods [25, 26].

Although urea breath test is more accurate and commonly used as a standard diagnostic test [25, 26], we did not use it because of its high cost and unavailability in our center.

Among the different methods for *H. pylori* infection detection, we used serology (ELISA) becauseof its convenience and availability and its low cost in this study. Although serologic results also usually not influenced by antibiotic or PPI (proton pump inhibitors) treatment [26], we did not include subjects that were taking PPI or anti-helicobacter medications within one month before the study.

One of the limitations of our study is the use of only one method (ELISA) for the diagnosis of *H. pylori* infection. As mentioned earlier given the high cost of respiratory urease test and its unavailability as well as the financial limitations we could not use neither this test nor any additional method such as *H. pylori* fecal antigen test to confirm the diagnosis of *H. pylori* infection.

Another limitation of our study was the lack of assessment of the upper gastrointestinal symptoms in diabetic patients and not considering it as the inclusion criteria, although most of participants were asymptomatic and did not report any significant upper gastrointestinal symptoms.

Factors such as race, geographic region, economic state, different dietary and drug regimens used to control blood glucose, as well as different diagnostic methods used to detect *H. pylori* infection, can influence the results of different studies.

More prospective studies with long-term follow-up of *H. pylori* infected patients in terms of the risk of type 2 diabetes development are needed. These studies should use more accurate methods or a combination of several diagnostic tools for *H. pylori* infection what can be helpful in revealing the relationship between *H. pylori* infection and diabetes. As well as future research on the role of oral hypoglycemic drugs may be useful in the development of H. pylori infection in diabetic type 2 patients.

Conclusion

Based on the results of this study, it seems that there is no relationship between *H. pylori* infection and type 2 diabetes.

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Conflict of interest

The authors declare to have no conflict of interest.

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REVIEW ARTICLE
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Diabetic kidney disease — state-of-the-art knowledge in 2020

ABSTRACT

Diabetic kidney disease is one of the most common complications of diabetes. For many years, it has also been the most common cause of end stage renal disease. The diagnosis of DKD is based on determining the urinary albumin-to-creatinine ratio and calculating the estimated glomerular filtration rate. Recently, the disease phenotype has changed and instead of the classical diabetic kidney disease presentation characterized by albuminuria followed by progressive renal failure, patients nowadays more often present only with reduced eGFR but normal urinary albumin excretion. The nephroprotective properties of new antidiabetic drugs, such as sodium-glucose co-transporter-2 inhibitors and glucagon-like peptide-1 analogues, are the novelty of recent years. Moreover, there are ongoing outcome trials with renal safety as the primary endpoint, and their results may extend the knowledge about using antidiabetic drugs for renal risk reduction not only in patients with diabetes but also in those without carbohydrate metabolism disorders. (Clin Diabetol 2020; 9; 3: 184–188)

Key words: diabetes mellitus, diabetic kidney disease, microangiopathy

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Introduction

Over the last several decades, diabetic kidney disease (DKD) has become the major cause of end stage renal disease (ESRD) in the developed countries [1, 2]. This microangiopathic complication of diabetes develops in about 30% of patients with type 1 diabetes mellitus (T1DM) and about 40% of patients with type 2 diabetes mellitus (T2DM) [1, 3]. An increased DKD incidence reflects a dramatic rise in the number of diabetic patients, and therefore diabetes has been dubbed the non-infectious epidemics of the 21st century [4]. It has been estimated that in 2017 the number of adults with diabetes worldwide was 451 million (including more than 2.2 million in Poland), and this number is predicted to increase to 693 million by 2045 [5]. The natural history of DKD was believed to begin with hyperfiltration followed by microalbuminuria, overt albuminuria, and reduction in the glomerular filtration rate, ultimately leading to ESRD [6], but in the recent years, it has been questioned as this complication has become increasingly heterogeneous. It is currently believed that the phenotype of DKD evolves, with an increasing number of patients with T2DM who present with a reduced estimated glomerular filtration rate (eGFR) without concomitant albuminuria [7]. In addition, the development of DKD in both T1DM [8] and T2DM [9] is associated with an increased cardiovascular event rate and mortality. Thus, a reduction in the risk of DKD's occurance and/or its progression is vital for reducing the risk of both cardiovascular mortality and the development of ESRD requiring renal replacement therapy. The conventional multifactorial approach to the management of DKD includes lifestyle modifications, optimal control of diabetes, blood pressure and lipid profile, and the use of renin-angiotensin-aldosterone (RAA) system inhibitors. Recently, however, large cardiovascular outcome trials (CVOT) of new antidiabetic drugs, such as sodium-glucose co-transporter-2 (SGLT2) inhibitors and

glucagon-like peptide-1 (GLP-1) analogues, have shed a new light on the benefits of these drugs for the reduction of not only cardiovascular but also renal risk [10].

The present article discusses the most important aspects of DKD diagnosis and management based on the evidence reported in recent years.

Diagnosis of DKD

As DKD remains asymptomatic for a long time, an extremely important aspect of managing diabetic patients is screening for DKD, which should be repeated annually since the diagnosis of T2DM and starting from 5 years after the diagnosis of T1DM [11]. The diagnosis of DKD is based on the clinical evaluation including the presence of albuminuria and a reduced eGFR [11]. Currently, the preferred approach to the evaluation for albuminuria is a measurement of the urinary albumin-to-creatinine ratio (UACR) [11, 12] in a spot urine sample, preferably collected in the morning hours. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula should be used for eGFR calculations [12–14]. Large cross-sectional studies showed that many patients with T2DM and a reduced eGFR had normal urinary albumin excretion. In these studies, the proportion of subjects with normoalbuminuria despite a reduced eGFR (< 60 mL/min/1.73 m^2) ranged from 16% to 33% [15–18]. These observations were also confirmed in patients with T1DM [19], with the proportion of patients with normoalbuminuria and a reduced eGFR up to as much as 50–60% in some studies [20–22]. The proposed explanations include improved blood glucose and blood pressure control, and a widespread use of RAA system inhibitors [23]. Thus, although the rate of ESRD in diabetes has not changed, the DKD phenotype evolved from a classical one, associated with albuminuria, to the one with normal urinary albumin excretion observed in some patients [24]. Also of note, the rate of nondiabetic chronic kidney disease (CKD) has increase secondary to an increasing mean life expectancy, unhealthy lifestyle, and more frequent acute kidney injury events of both ischaemic and toxic aetiology [25–27]. Due to these factors, CKD in diabetic patients is partially related to the presence of non-diabetic kidney disease. Thus, CKD in diabetic patients may be "pure" DKD (resulting from diabetes per se), non-diabetic kidney disease, or a combination of diabetic and non-diabetic kidney disease, and these types may be truly distinguished only based on renal biopsy findings [28]. In the largest study to date that evaluated renal biopsy samples in patients with T2DM, including 620 patients with the mean diabetes duration of 10 years, DKD was identified in 37% of renal biopsy samples, non-diabetic kidney disease in 36%, and concomitant diabetic and non-diabetic kidney disease in 27% [29]. However, renal biopsy, although considered a diagnostic gold standard, is not a routine diagnostic method in the evaluation of DKD and thus for the statistical and registry purposes, patients are generally categorized as having DKD if they have been diagnosed with both diabetes and CKD.

Management of DKD

The major issue in diabetes is to define the principles of a long-term, safe, well-tolerated and effective therapy in patients with a varying degree of vascular complications. Antidiabetic drugs, in particular new molecules, should not only affect blood glucose control but also contribute to a reduction in cardiovascular and renal morbidity and mortality. For this reason, the U.S. Food and Drug Administration's (FDA) scientific advisory committee published a document in December 2008 that mandated the need for cardiovascular risk assessment (performing CVOT) for all the novel antidiabetic drugs. These studies must be powered to show no excess cardiovascular and renal risk (i.e., prove cardiovascular and renal safety) of antidiabetic drugs compared to placebo. As CKD, and in particular DKD, is associated with an increased cardiovascular risk and mortality in both T1DM [8] and T2DM [9], the goal of the therapy includes both reducing progression of DKD and preventing the development of cardiovascular disease [31]. To achieve these goals, a multifactorial approach to the management of DKD should include lifestyle modifications and optimal control of blood glucose, blood pressure (including use of RAA system inhibitors) and lipid profile [32–34]. Until recently, RAA system inhibitors were the only drugs with proven nephroprotective properties [35, 36]. However, their use continued to be associated with a high residual risk of DKD progression and the development of ESRD [37]. This stagnation in the area of nephroprotection in DKD has ended with the results of CVOT of SGLT2 inhibitors and GLP-1 analogues [38–44]. Of SGLT2 inhibitors, cardiovascular benefits were proven for dapagliflozin, empagliflozin, and canagliflozin in the following studies respectively: the Dapagliflozin Effect on Cardiovascular Events–Thrombolysis in Myocardial Infarction 58 (DECLARE-TIMI 58) study, the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME), and the Canagliflozin Cardiovascular Assessment Study (CANVAS) [38–40]. These studies evaluated predefined endpoints in T2DM patients with coronary artery disease or at high cardiovascular risk. In addition to a significant reduction of the combined primary endpoint of cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke,

a significant reduction in hospitalization due to heart failure and a significant nephroprotective effects was shown, with a relative risk reduction of renal disease progression by about 40%. To further substantiate these very promising results in regard to nephroprotection, subsequent studies with these drugs were designed with the primary endpoint of renal disease progression. These include: the Dapagliflozin And Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD) study [45], The Study of Heart and Kidney Protection With Empagliflozin (EMPA-KIDNEY) [46], and the Canagliflozin and Renal Outcomes in Type 2 Diabetes and Nephropathy (CREDENCE) study [47]. One of these studies, the CREDENCE trial with canagliflozin, was terminated earlier than planned due to a proven independent nephroprotective effect of the drug, and the results were published in April 2019. Canagliflozin treatment was shown to result in a significant reduction of the primary combined endpoint that included ESRD (defined as the need for dialysis therapy or kidney transplantation, or persistent eGFR reduction to < 15 mL/min/1.73 m^2), doubling of serum creatinine, and renal or cardiovascular death. Several months later, in September 2019, FDA approved canagliflozin for the prevention of ESRD, doubling of serum creatinine, cardiovascular mortality, and hospitalizations due to heart failure in patients with T2DM, DKD, and albuminuria > 300 mg/d [48]. The newest SGLT2 inhibitor available in Poland is ertugliflozin, and its CVOT, the Cardiovascular Outcomes Following Ertugliflozin Treatment in Type 2 Diabetes Mellitus Participants With Vascular Disease (VERTIS CV) study, is currently underway. Regarding GLP-1 analogues, CVOT which first showed nephroprotective properties of these drugs based on the assessment of secondary endpoints included: the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) study [41], the Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes (SUSTAIN-6) study [42], and the Dulaglutide and Cardiovascular Outcomes in Type 2 Diabetes (REWIND): A Double-Blind, Randomised Placebo-Controlled Trial [44]. These studies revealed that GLP-1 analogues contribute to a reduced risk of renal function worsening, and their nephroprotective effect is mostly related to a reduction in albuminuria. To date, no studies of GLP-1 analogues have been published that would evaluate a primary endpoint of renal safety (reduced CKD progression, reduced mortality due to renal causes). The only ongoing trial with a GLP-1 analogue that evaluates a primary endpoint of renal safety is A Research Study to See How Semaglutide Works Compared to Placebo in People With Type 2 Diabetes and Chronic Kidney Disease (FLOW), and its results are expected in August 2024 [49].

Summary

The above evidence of a beneficial effect of SGLT2 inhibitors and GLP-1 analogues on the reduction of cardiovascular and renal risk led to a modification of the therapeutic approach and guidelines on the management of patients with diabetes. The 2018 consensus of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) [50] first highlighted the need for combining the previously recommended multifactorial approach based on the results of STENO and STENO 2 (Intensified Multifactorial Intervention in Patients With Type 2 Diabetes and Microalbuminuria) studies [32, 51] with treatment individualization based on the presence of cardiovascular disease, CKD, an elevated risk of hypoglycemia, the need for weight reduction, and economic restraints. It should be noted that cardiovascular and renal safety are intrinsically and bidirectionally related and cannot be considered separately. In an update of the 2018 ADA/EASD consensus that was published in December 2019, it was suggested that adding an SGLT2 inhibitor or a GLP-1 analogue to metformin should be considered regardless of baseline HbA_{1c} value in T2DM patients at high cardiovascular risk to reduce the risk of major adverse cardiovascular events (MACE), hospitalizations due to heart failure, cardiovascular mortality, and CKD progression, and these recommendations have been echoed in the 2020 Diabetes Poland guidelines [52, 53]. According to them, GLP-1 analogues with proven cardio- and nephroprotective properties should be preferentially used in patients with an atherosclerotic cardiovascular disease, and SGLT2 inhibitors should be preferred in patients with a heart failure and reduced ejection fraction (< 45%) or CKD (eGFR 30–60 mL/min/1.73 m^2 or UACR > 30 mg/g). The 2020 Diabetes Poland guidelines do not recommend using SGLT2 inhibitors in patients with eGFR below 60 mL/min/1.73 m^2 yet. The ongoing trials should clarify in the near future whether these drugs may be indicated for nephroprotection also in primary prevention, and also in subjects without diabetes.

Conflict of interests

The authors declare to have no conflict of interests.

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SGLT-2 inhibitors as adjunctive to insulin therapy in type 1 diabetes

ABSTRACT

The absolute insulin deficiency that occurs in type 1 diabetes mellitus (T1DM) is associated with the need for intensive functional insulin therapy as the only appropriate treatment model. In the recent years, introduction of new classes of glucose-lowering drugs has led to an increasing interest in adjunct therapies for T1DM. These therapies are designed to support exogenous insulin therapy in achieving the therapeutic goal while reducing the risk of hypoglycaemia and exerting a beneficial effect on body weight. One potential therapeutic option are sodium-glucose co-transporter 2 (SGLT-2) inhibitors. In the present paper, we reviewed the current clinical research on SGLT-2 inhibitors as add-on therapy to insulin in patients with T1DM. This therapy modification contributes to an improvement in metabolic control without increasing the risk of severe hypoglycaemia and with a beneficial effect on body weight, translating to improved compliance, quality of life, and patient satisfaction with treatment. However, due to possible adverse effects including euglycaemic diabetic ketoacidosis, the decision to use SGLT-2 inhibitors in patients with T1DM should be made with caution, and patients require proper education regarding the prevention and treatment of acidosis. (Clin Diabetol 2020; 9; 3: 189–192)

Key words: type 1 diabetes, adjunct therapy, SGLT-2 inhibitors

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Introduction

Due to the pathophysiological mechanism of absolute insulin deficiency that mediates the development of type 1 diabetes mellitus (T1DM), the affected patients require insulin substitution therapy along with its all inconveniences. Despite advances in insulin therapy over the last hundred years and introduction of glucose monitoring systems, many patients still do not attain optimal blood glucose and metabolic control and thus are at risk of more rapid development of chronic disease complications. In addition, even with adequate metabolic control, the risk of cardiovascular mortality in patients with T1DM is increased nearly 3-fold [1].

According to the Diabetes Poland guidelines, the recommended treatment model for T1DM is intensive functional insulin therapy using multiple subcutaneous insulin injections or continuous subcutaneous insulin infusion by a personal insulin pump [2]. However, this therapy continues to be associated with a risk of hypoglycaemia, which often makes the optimal blood glucose control more challenging, increases treatment costs and reduces compliance, which ultimately reduces the quality of life. In addition, overweight or obesity and metabolic syndrome coexist in an increasing number of patients with T1DM. In young patients with T1DM, insulin sensitivity is reduced compared to their healthy peers with similar body weight, physical activity level, and body fat content. Exogenous insulin therapy promotes further increase in body weight, which increases insulin requirement, thus creating a pathophysiological vicious circle, and may increase atherogenesis, accelerating the development of late diabetes complications including cardiovascular disease. All these factors result in an increasing interest in adjunct therapies to support exogenous insulin therapy in achieving the therapeutic goal while reducing the risk of hypoglycaemia and exerting a beneficial effect on body weight [3, 4].

Effects of sodium-glucose co-transporter 2 inhibitors in type 1 diabetes mellitus

One potential therapeutic option are sodium-glucose co-transporter 2 (SGLT-2) inhibitors. Inhibition of SGLT-2 leads to a number of beneficial effects including urinary caloric loss (leading to a reduced insulin requirement), body weight reduction, increased insulin sensitivity, blood pressure lowering, and reduced progression of albuminuria and diabetic nephropathy [5], all delaying the development of chronic complications of diabetes. SGLT-2 inhibitors are also effective in reducing the cardiovascular risk in patients with type 2 diabetes mellitus. It was also shown that adding a SGLT-2 inhibitor reduces the risk of hypoglycaemia. Of note, SGLT-2 inhibitors act independently of insulin. Interestingly, SGLT-2 inhibitors are also believed to exert a protective effect on beta cell function, extending their insulin-secreting function [6].

Possible adverse effects of adjunct SGLT-2 inhibitor therapy in T1DM should be taken into account, of which clinically most important are urogenital infections and in particular euglycaemic diabetic ketoacidosis (EDKA). These effects may not only interfere with the therapeutic process but also call for a careful patient selection for such therapy. EDKA is clearly a controversial issue. It is promoted by a reduced carbohydrate availability coupled with a reduced insulin dose. SGLT-2 inhibition increases glucosuria which leads to a reduced plasma insulin level, while the amount of exogenous insulin is reduced and at the same time glucagone level is increased. A lower insulin to glucagone ratio stimulates ketogenesis and lipolysis (with circulating free fatty acid levels increased by 40% during a meal), which leads to increased lipid oxidation (on average by 20%) at the expense of carbohydrate oxidation [7]. Factors triggering EDKA include infections, reduced food and fluid intake, reduced insulin dose, and alcohol intake. Pathophysiologically, EDKA is similar to diabetic ketoacidosis (DKA), except for glucosuria induced by SGLT-2 inhibitors which 'artificially' lowers blood glucose level.

Overview of clinical studies using SGLT-2 inhibitors in in type 1 diabetes mellitus

Currently, more and more reliable data indicate the efficacy of SGLT-2 inhibitors as adjunct therapy in T1DM.

The first SGLT-2 inhibitor approved for T1DM was dapagliflozin, the efficacy and safety of which was assessed in the multicentre, randomized, double-blind, placebo-controlled DEPICT-1 and DEPICT-2 (Efficacy and Safety of Dapagliflozin in Patients with Inadequately Controlled Type 1 Diabetes) studies. The first of these studies was performed in Europe and North America, and the other included patients from North and Latin America, Europe, and Japan. The DEPICT studies assessed the efficacy and safety of a 24-week dapagliflozin treatment in adult patients (18–75 years of age) with chronic inadequate diabetes control (haemoglobin A_{1c} [HbA_{1c}] levels 7.5–10.5%) who received dapagliflozin 5 mg (n = 259), dapagliflozin 10 mg (n = 259), or placebo (n = 260) daily. In the study protocol, the patients were advised to reduce the daily insulin dose by not more than 20% after taking the first dapagliflozin dose. The DEPICT-1 trial showed a significant reduction of HbA_{1c} level (primary endpoint) by 0.42% in the 5 mg group and 0.45% in the 10 mg group ($P < 0.0001$ for both doses). A reduction was also noted in the daily insulin dose (by 8.8% and 13.2%, respectively; $P < 0.0001$ for both doses) and body weight (by 2.96% and 3.72%, respectively; $P < 0.0001$ for both doses). The proportion of patients with HbA_{1c} level reduction by $\geq 0.5\%$ without severe hypoglycaemia was significantly higher in both dapagliflozin groups compared to placebo. In addition, in the patient subgroup that used continuous glucose monitoring (CGM), addition of dapagliflozin was shown to result in a significant improvement of the mean daily glucose levels, with an increase of the time in blood glucose level target range by 9.1% in the 5 mg group and 10.1% in the 10 mg group ($P << 0.0001$ for both doses). Severe hypoglycaemia occurred in 8%, 6%, and 7% of patients, respectively, in the 5 mg, 10 mg, and placebo groups. Urogenital infections were noted more frequently in the active treatment groups, while the rates of other adverse effects were similar in all study groups. The incidence of DKA was also similar in all study groups. EDKA was reported in only 2 patients in the 10 mg dapagliflozin group. The positive effects of adjunct dapagliflozin therapy were maintained at the end of extended follow-up period (overall 52 weeks), with a significant reduction in HbA_{1c} level and body weight. During this period, overall 9 cases of EDKA were reported, including one in the placebo group [8]. The DEPICT-2 trial was performed in a study population of a similar size and showed consistent results regarding the efficacy of dapagliflozin in the treatment of T1DM. CGM data showed a similar reduction in blood glucose levels in the active treatment groups, and similar reductions were noted in HbA_{1c} levels, body weight, and daily insulin dose [9]. In March 2019, based on the DEPICT-1 and DEPICT-2 study results, the European Medicines Agency (EMA) approved dapagliflozin at the dose of 5 mg daily as an adjunct therapy in patients with T1DM and body mass index (BMI) ≥ 27 kg/m^2 in whom insulin therapy only is not sufficient for optimal metabolic control [10], but this therapy was not approved by the U.S. Food and Drug Administration (FDA).

Another somewhat unusual drug of this class is sotagliflozin. It has not been approved for the treatment of type 2 diabetes mellitus and is active against both SGLT-2 and SGLT-1. Sotagliflozin has been approved by EMA was for the treatment of T1DM based on the results of the inTandem studies [11] but again, no approval was granted by FDA. The inTandem study program included 3 multicentre, randomized, double-blind, placebo-controlled trials. The inTandem-2 and inTandem-1 studies evaluated sotagliflozin 200 mg and 400 mg compared to placebo. The primary endpoint was HbA_{1c} level change at 24 weeks of therapy. The first of these studies was conducted in Europe and the other one in North America. Both included similar numbers of patients (sotagliflozin 400 mg: n = 263 in the inTandem-2 study, n = 262 in the inTandem-1 study; sotagliflozin 200 mg: n = 261 and n = 263, respectively; placebo: n = 258 and n = 268, respectively). In both studies, the protocol called for the greatest reduction in postprandial insulin dosing (by 30%), which was likely related to the additional incretin effect of this drug. Both trials showed significant reductions in HbA_{1c} level ($P < 0.001$ for both doses), body weight (by 2–3.5 kg, $P < 0.001$ for both doses), and daily insulin dose (by 6.2–9.7%, $P < 0.001$ for both doses). In the inTandem-2 study, the proportion of patients with achieved HbA1c level < 7% was 27.2%, 27.8%, and 15.5%, respectively, in the sotagliflozin 200 mg, sotagliflozin 400 mg, and placebo groups. In the inTandem-1 study, these proportions were 30%, 35.5%, and 20.9%, respectively. In patients using CGM systems, adjunct therapy with sotagliflozin was shown to increase the time in blood glucose level target range by 5.4% and 11.7%, respectively, for the 200 mg and 400 mg doses ($P = 0.026$ for the 200 mg dose; $P < 0.001$ for the 400 mg dose). These positive effects on HbA_{1c} levels, body weight, and daily insulin requirement were maintained at 52 weeks of follow-up in both sotagliflozin groups, and the reported satisfaction with treatment increased significantly. The rates of severe hypoglycaemia were lower, while diarrhoea and fungal genital infections were more common in the sotagliflozin groups. The rate of DKA at 52 weeks in the inTandem-2 study was 2.3%, 3.4%, and 0%, respectively, in the sotagliflozin 200 mg, sotagliflozin 400 mg, and placebo groups. In the inTandem1 study, these rates were 3.4%, 4.2%, and 0.4%, respectively. Of 36 cases of DKA reported in these two studies combined, 13 cases occurred with blood glucose levels < 250 mg/dL. The inTandem3 study showed that addition of sotagliflozin 400 mg contributed to an improved metabolic control, with a significantly higher proportion of patients with HbA_{1c} level < 7.0% at 24 weeks of follow-up (28.6% *vs.* 15.2%, $P < 0.001$). In addition, the active treatment was associated with positive effects regarding the reduction of HbA_{1c} level (−0.46%), body weight (−2.98 kg), systolic blood pressure (−3.5 mm Hg), and daily insulin dose (−2.8 units daily) ($P \leq 0.002$ for all comparisons). The rate of severe hypoglycaemia was similar in both groups (3.0% *vs.* 2.4% in the placebo group). The rate of ketoacidosis was higher in the sotagliflozin group (3.0% *vs*. 0.6% in the placebo group), while the rates of other adverse effects were similar in both groups [12].

Long-term safety and efficacy in the treatment of T1DM has also been documented for empagliflozin. The EASE-2 and EASE-3 (Empagliflozin as Adjunctive to inSulin thErapy) studies evaluated the effect of add-on empagliflozin therapy on HbA_{1c} levels in adult patients with chronic inadequate T1DM control (HbA_{1c} level 7.5–10%). The EASE-2 trial studied empagliflozin 10 mg (n = 243) and 25 mg (n = 244) *vs*. placebo (n = 243), and the EASE-3 trial studied empagliflozin 2.5 mg (n = 241), 10 mg (n = 248) and 25 mg (n = 245) *vs*. placebo (n = 241).

Study participants were advised to reduce the daily insulin dose by 10% at the trial initiation. At 26 weeks, a significant reduction of HbA_{1c} level was noted for all empagliflozin doses compared to placebo ($P < 0.0001$). Both trials showed a reduction of body weight and blood glucose level variation, and an increase in the CGM time in range. Systolic blood pressure and daily insulin requirement were also reduced. In addition, the EASE-2 study showed that the positive effects of empagliflozin were maintained during a longer follow-up of 52 weeks. Severe hypoglycaemia occurred in 1.2%, 4.1%, 2.7%, and 3.1% of patients receiving empagliflozin 2.5 mg, 10 mg, 25 mg, or placebo, respectively. The rate of genital infections was insignificantly higher in the active treatment groups. DKA was reported in 0.8%, 4.3%, 3.3%, and 1.2% of patients in the empagliflozin 2.5 mg, 10 mg, 25 mg, and placebo groups, respectively. These studies indicated that the 2.5 mg dose was both effective at improving metabolic control in T1DM and safe, as it was not associated with an increased risk of severe hypoglyceamia and EDKA [13]. Of note, this dose is not sufficiently effective in the treatment of type 2 diabetes mellitus.

Similar results of phase 3 clinical trials are currently not available for canagliflozin and ertugliflozin. Smaller phase 2 clinical trials with canagliflozin showed that both 100 mg and 300 mg doses were effective in reducing HbA_{1c} level, body weight, and daily insulin requirement [14].

Summary

In view of the studies reviewed above, SGLT-2 inhibitors seem an effective and relatively safe thera-

peutic option as an adjunct therapy in T1DM. Most benefits from such therapy may be expected in patients with chronically uncontrolled diabetes, abnormal body weight (overweight or obesity), and those using relatively large insulin doses. As noted above, the risk of ketoacidosis including EDKA is a limitation that necessitates careful patient selection for adjunct SGLT-2 inhibitor therapy in T1DM. Poor candidates for such therapy include patients with risky behaviours including excessive alcohol intake, use of psychoactive substances/ /illicit drugs, and use of a low-calorie low-carbohydrate diet. Inadequate access to a physician is also a contraindication for adding a SGLT-2 inhibitor in patients with T1DM. According to the American Diabetes Association expert consensus published in February 2019, patients with HbA_{1c} levels > 10% are also not candidates for SGLT-2 inhibitor therapy due to a high rate of ketoacidosis in this group (> 15% during a 5-year follow-up) even without SGLT-2 inhibitor therapy [15].

The patients must be educated not to reduce the insulin dose by more than 10–20% when initiating SGLT-2 inhibitor therapy. The smallest effective dose of any drug of this class should be used. In addition, the drug should be immediately withdrawn in acute conditions associated with a possible DKA trigger such as fasting, infection or other acute illness. The drug should also be withdrawn 72 hours before elective surgery. Patients treated with SGLT-2 inhibitors require monitoring of not only blood glucose levels but also the presence of ketone bodies in serum and urine. Of note, EDKA is not associated with the warning of symptomatic hyperglycaemia, and thus patients should be alert to such symptoms as nausea, vomiting, lack of appetite, fatigue, and dyspnoea, occurring even with blood glucose levels < 250 mg/dL. The management of EDKA associated with SGLT-2 inhibitor use is summarized by the STICH mnemonic (STopping SGLT-2 inhibitor therapy, Injecting insulin, consuming Carbohydrates, Hydrating, monitoring ketones) [16].

In summary, adding a SGLT-2 inhibitor as an adjunct to insulin therapy in T1DM has been shown to be an effective therapeutic approach. Such therapy modification contributes to a better disease control as evidenced by lower HbA_{1c} levels and body weight and improved CGM parameters without an increased risk of severe hypoglycaemia. It also seems that these benefits translate to improved compliance, quality of life, and patient satisfaction with treatment. However, not all patients with T1DM are candidates for such therapy due to possible adverse effects including the most dangerous complication of (euglycaemic) DKA. Patients in whom SGLT-2 inhibitor therapy is considered must be well educated, with an emphasis on the identification of DKA symptoms and triggers, and the management of this condition should it occur.

Conflict of interests

The authors declare no conflicts of interests.

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Protective effect of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor antagonists (ARBs) on microalbuminuria in diabetic patients

ABSTRACT

This review assesses the protective effect of ACEIs and ARBs on microalbuminuria in diabetic patients and identifying the preferred type based on their beneficial effects in addition to their blood pressure-reducing effect in diabetic patients with microalbuminuria and adverse drug reaction profile. In this review, articles published between 2001 and 2019 are included and MEDLINE search was used with key words such as diabetes, microalbuminuria, angiotensin II receptor antagonists, and ACEIs. ARBs reduced the risks of end stage renal disease (ESRD) and two-fold rise in the serum creatinine level; ACEIs did not reduce the risks of ESRD in an analysis of studies including both type 1 and type 2 diabetic patients. However, a meta-analytical review or study needs to be conducted to evaluate the comparative effects of ARBs and ACEIs in either type 1 or type 2 diabetic patients. Early treatment with ACEIs or ARBs decreased the risk of microalbuminuria in patients with type 2 diabetes. Telmisartan is found to be beneficial in microalbuminuria or diabetic nephropathy. Long-term therapy with higher dose of irbesartan resulted in consistent protective effects on the renal functions even after its withdrawal. ACEIs or ARBs are consideredas the 1st line therapy in both type 1 and 2 diabetic patients with microalbuminuria. ARBs are definitely preferred for patients who cannot tolerate ACE inhibitors. ARBs may be preferred over ACEIs due to their predominant renal protective effects in addition to their beneficial effect of improving blood pressure in type 2 diabetes mellitus. However, the comparative effects of ARBs and ACEIs in either type 1 or type 2 diabetic patients with microalbuminuria needs to be further evaluated in a randomized controlled study. (Clin Diabetol 2020; 9, 3: 193–200)

Key words: angiotensin, enzyme inhibitors, receptor blockers, microalbuminuria, diabetes, renal, protective effects

Introduction

Albuminuria is developed in 1/3rd of the diabetic patients. There is higher risk of all-cause mortality and end-stage renal disease (ESRD) in diabetic patients with albuminuria. Cardiovascular disease and mortality can be predicted on the basis of microalbuminuria as it is an indicator of endothelial dysfunction. Microalbuminuria is also reported in cases with hypertension, dyslipidaemia, renal malfunction, obesity, and smoking; all these are contributors to the development of atherosclerosis. Hyperactivity of renin–angiotensin system leads to the development of cardiovascular events in diabetic patients with microalbuminuria [1–3].

It has been reported in studies that angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor antagonists (ARBs) lead to a reduction in the cardiovascular mortality rate and an improvement in glomerular filtration rate in hypertensive diabetic

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patients with microalbuminuria, macroalbuminuria, or normoalbuminuria [1, 4, 5].

This review is carried out to assess the protective effect of ACEIs and ARBs on microalbuminuria in diabetic patients and to identify the preferred ACEIs and ARBs based on their beneficial effects in addition to their blood pressure-reducing effect in diabetic patients with microalbuminuria and adverse drug reaction profile. The safety aspects of ACEIs and ARBs are also discussed.

Methodology

This review includes articles published between 2001 and 2019. A MEDLINE search was used for it. The following words were used while searching articles: diabetes, microalbuminuria, angiotensin II Receptor antagonists, angiotensin-converting enzyme inhibitors. Articles on clinical trials, meta-analysis reports, guidelines and past reviews are included. Thus, human clinical studies in relation to the effect of ACEIs and ARBs including ramipril, perindopril, olmesartan, losartan, irbesartan, telmisartan etc. especially on microalbuminuria in diabetic patients are considered. Non-renal parameter-related clinical studies and preclinical studies were excluded for this review. Possible mechanisms in the protective effect of ACEIs and ARBs in diabetic patients with microalbuminuria are also discussed in this review.

Microalbuminuria in diabetes

A review article by Satchell et al. [6] indicates the following: microalbuminuria is a predominant risk factor for CV (cardiovascular) disease and advancement in renal impairment, especially in diabetic population, though it seems to be valid in non-diabetic individuals as well; it is well-known that the diabetic population has the tendency to develop renal complications. It is essential to know the pathological and physiological mechanisms behind the development of microalbuminuria to strategize therapies needed to prevent and treat microalbuminuria [6].

Damage to the endothelial glycocalyx is an essential feature. Various mediators are involved in the damage process, such as reactive oxygen species (ROS), vascular endothelial growth factor (VEGF) and proinflammatory cytokines. Impairment in the pathway of the endothelium cell and podocyte leads to endothelial damage and aggravates the impairment. Impairment in glomerular podocyte and its loss is involved during a damaging process of advancement of microalbuminuria to overt nephropathy (macroalbuminuria) [6].

It is reported that damage to the endothelial glycocalyx is predominantly involved in microalbuminuria and generalised endothelial dysfunction, and in microvascular and macrovascular complications. Therefore, treatments targeting the endothelial glycocalyx could be beneficial in microalbuminuria in the diabetic population. A decrease in microalbuminuria could help improve endothelial dysfunction and may be beneficial in reducing the risk of cardiovascular diseases [6].

The American Diabetes Association suggests the evaluation of albuminuria in type 2 diabetes population initially at the time of diagnosis of diabetes and every year afterwards [7].

Another review by Basiet al. [8] indicates that ACE inhibitor or ARB therapy is recommended for patients with microalbuminuria or obvious proteinuria. Maximization of the dosages of ACE inhibitor or ARB therapy is useful during the management of albuminuria with regular monitoring. ACE inhibitor or ARB therapy is useful to reduce hypertension. But, if the blood pressure is not controlled, add-on therapy with other antihypertensives could be beneficial to maintain the blood pressure. Other therapies, such as statins, renin inhibitors, and glycosaminoglycans, have also been found to be effective in reducing urinary albumin excretion [8].

Clinical efficacy of ACEIs and ARBs on microalbuminuria in diabetic patients

As per a systematic review and meta-analysis by Wang et al. [1] ACEIs and ARBs result in similar improvement in microalbuminuria in patients with diabetes and albuminuria. It was noted that ARBs led to a significant reduction of the risk of end-stage renal disease (ESRD) in patients with diabetes and albuminuria. Most importantly, ACEIs did not show this reno-protective effect of reducing the risk of ESRD. This was a meta-analysis of twenty-six randomized controlled clinical studies including a big population of nearly ten thousand patients. ACEIs and ARBs also showed reduction in the risk of two-fold increase in the serum creatinine level. The improvement in microalbuminuria was similar with ACEIs and ARBs. It can be concluded from this meta-analysis that ARBs might be the preferred therapy over ACEIs due to their beneficial effect on renal functions. However, the researchers incorporated both type 1 and type 2 diabetic patients with albuminuria in this meta-analysis. Therefore, there could be the risk of bias. Thus, a meta-analytical study needs to be conducted to evaluate the comparative effects of ARBs and ACEIs especially in either type 1 or type 2 diabetic patients [1].

Persson et al. [4] evaluated the impact of ACEIs or ARBs on the prevention of microalbuminuria in patients with type 2 diabetes and normoalbuminuria. They concluded that the risk of microalbuminuria for patients with type 2 diabetes and normoalbuminuria is significantly lower with ACEIs or ARBs. The conclusion was

reached based on the meta-analysis of six randomized controlled clinical trials on nearly seventeen thousand patients with type 2 diabetes and normoalbuminuria. Unlike the meta-analysis report by Wang et al. [1], all-cause mortality was reduced to a certain extent with both ACEIs and ARBs by Persson et al. Studies on the following ACEIs or ARBs were considered for this review: enalapril, ramipril, trandolapril, candesartan, perindopril and olmesartan [4].

Uzuet al. [3] reported in their study that both ARBs and aliskiren, a direct renin inhibitor (DRI), reduced albuminuria significantly in hypertensive patients with type 2 diabetes. Moreover, albuminuria decreased significantly with ARBs in patients with high-normal albuminuria and DRI did not reduce albuminuria in similar patients. ARBs showed similar effectiveness to that of DRI in reducing urinary excretion of albumin, angiotensinogen, urinary albumin-to-creatinine ratio, and systolic and diastolic blood pressure. Thus, it indicates that DRI is not superior to ARBs. Patients were treated with the following ARBs in this study: valsartan, telmisartan, olmesartan, candesartan, losartan, irbesartan, azilsartan [3].

A choice of ACEIs and ARBs in diabetic patients with microalbuminuria

The following are the results from different studies for individual ACEIs and ARBs to conclude a choice of ACEIs and ARBs in diabetic patients with microalbuminuria.

Clinical efficacy of ramipril

As per the DIABHYCAR study (2004), no beneficial effect was found on ESRD or two-fold rise in serum creatinine with the ACE inhibitor ramipril. The same study indicates no beneficial effect with low dose (1.25 mg) ramipril once daily on cardiovascular and renal outcomes of patients with type 2 diabetes and albuminuria, although a slight reduction in blood pressure and urinary albumin was observed [9].

Clinical efficacy of perindopril

In a placebo-controlled clinical trial conducted by Yao et al. on Asian patients, perindopril reduced urinary albumin excretion rate (AER) significantly in patients with initial stage of diabetic nephropathy with normal blood pressure and microalbuminuria during the one-and-half-year treatment [10].

Jerums et al. [11] reported the following results in a placebo-controlled i.e. six-year follow-up study with perindopril, maintenance of albumin excretion rate (AER), reduction in GFR in Australian type 2 diabetic patients with normal blood pressure and microalbuminuria. The blood pressure remained normal in 83% of patients with perindopril. A significantly smaller number of patients developed macroalbuminuria [11].

Another double-blind study conducted by Kopf et al. concluded that urinary albumin excretion rate was maintained by perindopril in patients with insulin-dependent diabetes mellitus and mild-to-moderate hypertension and stable microalbuminuria, and perindopril therapy was found to be safe as well [12].

Clinical efficacy of olmesartan

In a study conducted by Raff et al. [13] it was found that olmesartan (OLM) prolonged the onset of the development of microalbuminuria in type 2 diabetic patients. Moreover, OLM prolonged the development of ECG signs of cardiac structural adaptation and left ventricular remodelling in type 2 diabetic patients [13].

Clinical efficacy of losartan

The RENAAL study seems to be a landmark work to show the following benefits with losartan therapy in patients with type 2 diabetes and nephropathy: reduction in the incidence of ESRD and two-fold rise in the serum creatinine level, 35% showed reduction in the level of proteinuria and good tolerance were documented with this drug. However, this study did not report the effect of the therapy on microalbuminuria. Moreover, there was no impact of losartan on the rate of death [14, 15].

A review of a clinical study by Ruilope et al. [16] indicates that losartan appears to be an essential therapy in type 2 diabetic patients with nephropathy in addition to dietary therapy for proteinuria and diabetes. In addition to blood pressure lowering effect, a renal protective benefit of losartan helps delay the initiation of dialysis or kidney transplantation [16].

According to a study conducted by Woo et al., losartan leads to an effective reduction in the urinary excretion of transforming growth factor (TGF)-beta and albumin in type 2 DM patients with microalbuminuria during a six-month therapy in addition to its property of effective reduction in arterial blood pressure [17].

A long-term randomized clinical study conducted by Weil et al. [18] indicated that losartan therapy reduced the mesangial fractional volume in type 2 diabetic American-Indian patients with microalbuminuria. Thus, losartan therapy helped preserve the kidney structure in these patients [18].

A randomized controlled-study by Agha et al. [19] concluded that losartan demonstrated a significant reduction in proteinuria in patients with normotensive type 2 diabetes mellitus (T2DM) patients with early nephropathy. This study indicates that the effect

of losartan seems to be beyond its blood pressure reducing effect. This was a ten-month study on 361 patients. Eighty percent of the patients reported significant reduction in albuminuria by more than 30%. The anti-albuminuric effect of losartan was a reversible impact [19].

A similar beneficial effect of losartan on the urinary excretion of albumin was demonstrated in a multicentric randomized, double-blind, placebo-controlled clinical study conducted by Zandbergen et al. [20]. A 25–30% relative reduction was observed in this study with two different dosages through a ten-week therapy. This reemphasizes the effect of losartan on proteinuria possibly not associated with blood pressure reducing effect. Additionally, the safety of losartan was established with normotensive patients [20].

Clinical efficacy of irbesartan

In a double-blind placebo-controlled randomized study, Andersen et al. [21] reported a persistent reduction of microalbuminuria after withdrawal of a two-year high-dose (300 mg, once daily) irbesartan treatment on hypertensive type 2 diabetic patients with persistent microalbuminuria. This indicates a long-term renal protective effect of high-dose irbesartan treatment. This study also concluded that placebo and irbesartan 150-mg groups demonstrated increase in urinary albumin excretion. Most importantly, it was decreased persistently and significantly by 47% in the high-dose irbesartan group [21].

Clinical efficacy of telmisartan

Furat et al. [2] reported in a controlled study that telmisartan was beneficial for decreasing systemic inflammation and levels of urinary albumin excretion in patients who had type 2 diabetes mellitus and had undergone coronary artery bypass surgery. Microalbuminuria levels between the groups differed significantly in the pre-operative period, first hour postoperatively and fifth day post-operatively. C-reactive protein levels between the groups differed significantly on the fifth day post-operatively [2].

A review by Schmieder et al. [22] demonstrated that the effect of telmisartan on kidney function benefits in patients with microalbuminuria or overt diabetic nephropathy. They reported that telmisartan offers benefits at all the stages of the renal abnormalities in patients with type 2 diabetes. Telmisartan delays the progression to overt nephropathy in patients with microalbuminuria. The effectiveness of telmisartan is similar to angiotensin-converting enzyme inhibitors, but with a greater tolerance than angiotensin-converting enzyme inhibitors. The effect of telmisartan on protein excretion in diabetic nephropathy appears to be better than that of losartan and equivalent to that of valsartan. In the ONTARGET study, telmisartan offered a comparable cardiovascular protection to ramipril in diabetic patients [22].

Clinical efficacy of ACEIs plus ARBs

In a study conducted by Joshi et al. [23] it was found that the fixed dose combination of losartan and ramipril demonstrated a good to excellent efficacy in approximately 98% patients and accomplished a target blood pressure in nearly 79% patients with a 12 week--therapy. The combination offered a reduction in the urinary albumin excretion in most of the patients with microalbuminuria and proteinuria. This was an open, non-comparative, multicentric clinical study conducted on Indian patients. All the patients were treated with combination of losartan + ramipril in two fixed doses. Nearly 21% patients obtained normoalbuminuria with this therapy [23].

However, a study conducted by Tütüncü et al. concluded that ACE inhibitors and angiotensin II receptor blockers lead to comparable efficacy in treating diabetic microalbuminuria, and the combination of the two drugs did not add any further benefit. In this prospective, randomized clinical trial, the efficacy of treatment with enalapril or losartan, or both enalapril and losartan, was compared in patients with microalbuminuria [24].

Moreover, a review by Mercier et al. [5] indicated that dual RAAS inhibition with ACE inhibitors, ARBs or ACE inhibitors, and direct renin inhibitors failed to improve cardiovascular or renal outcomes and predisposed patients to adverse events. Thus, more studies need to be conducted to assess the efficacy and safety of the ACEI plus ARB therapy [5].

Based on the reno-protective effects demonstrated above, ARBs (losartan, telmisartan high-dose irbesartan) may be preferred for diabetic patients with albuminuria.

Guidelines on the role of ACEIs and ARBs on microalbuminuria in diabetic patients

American Diabetes Association (ADA) Guidelines

ADA recommends the following: "An ACE inhibitor or ARB, at the maximum tolerated dose is indicated for blood pressure treatment as first-line treatment for hypertension in patients with diabetes and urinary albumin-to-creatinine ratio (UACR) ≥ 300 mg/g creatinine or 30–299 mg/g creatinine. If one class is not tolerated, then it should be replaced with the other" [25] (Figure 1).

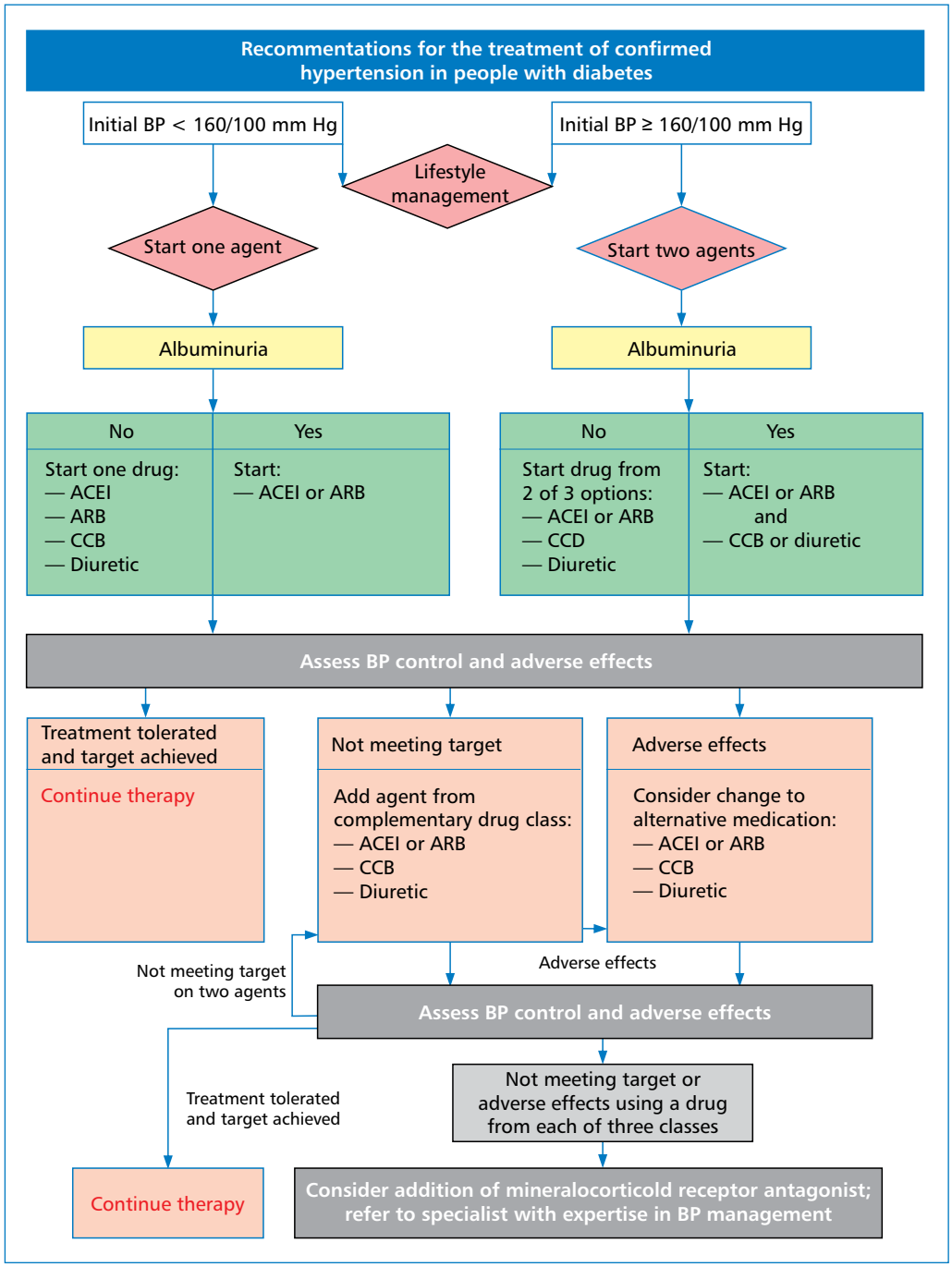

Figure 1. Recommendations by ADA for the treatment of confirmed hypertension in people with diabetes

NICE guideline recommendations

Type 1 diabetes:

— "ACE inhibitors should be initiated with the usual precautions and titrate to the maximum dose in all adults with diagnosed nephropathy (including those with microalbuminuria alone) and type 1 diabetes".

— "If ACE inhibitors are not tolerated, then it should be replaced with angiotensin II receptor antagonists. The combination therapy is not recommended".

Type 2 diabetes:

— "Initiate ACE inhibitors with the usual precautions and titrate to the maximum dose in all individuals with confirmed raised albumin excretion rate (> 2.5 mg/mmol for men, > 3.5 mg/mmol for women)".

— "Replace an angiotensin II-receptor antagonist for an ACE inhibitor for a person with an abnormal albumin:creatinine ratio if an ACE inhibitor is poorly tolerated" [26].

Guidelines from National Institute for Health and Care Excellence (NICE) 2014 and Kidney Disease Outcomes Quality Initiative (KDOQI) 2012

— "Use an ACE inhibitor or an ARB in patients with diabetes and albuminuria, even for subjects with microalbuminuria and normal blood pressure" [27, 28].

Results and discussions

It is cited in the ADA guidelines that to reduce the risk of aggravation of kidney disease, treatment needs to be initiated with ACEIs or ARBs on patients with albuminuria (urine albumin-to-creatinine ratio ≥ 30 mg/g) [25].

A systematic review and meta-analysis by Wang et al. [1] showed that ARBs significantly reduced the risks of ESRD by approximately 26% and doubling of the serum creatinine level by nearly 25% [1].

As per a meta-analysis of 28 studies by Vejakama et al., ACEI/ARB lead to persistent protective effect on renal functions over other antihypertensive drugs, predominantly CCBs, and placebo in patients with type 2 diabetes. This reno-protective benefit seems to be beyond antihypertensive effect as there was no difference in reduction in the blood pressure between ACEI/ARB and active comparators. In this study ACEI/ /ARB demonstrated significant reduction in the risk of two-fold rise in serum creatinine, macroalbuminuria, and albuminuria compared to other antihypertensive drugs [29].

An inhibition of the renin–angiotensin II system (RAS) seems to be very important in preventing or delaying albuminuria in diabetic patients. It is reported that the mechanism behind a decrease in albuminuria in diabetic nephropathy with ARBs, e.g. olmesartan, obstructs the podocyte apoptosis in the kidney [30]. An animal study showed a rise in p27 (Kip1) mRNA and protein expression in diabetic glomeruli and podocytes. An ARB therapy reduces p27 (Kip1) expression; this therapy helps reduce renal hypertrophy [31]. A preclinical study illustrated a reduction in albuminuria and advancement in glomerulosclerosis type 2 diabetes with valsartan by decreasing podocyte injury, oxidative stress and inflammation in renal tissues [32].

A pilot study by Esmatjes et al. demonstrated reduction in TGF-β_1 plasma level and urinary albumin excretion with losartan on hypertensive type 2 diabetes mellitus patients with microalbuminuria. The researchers suggested TGF-β_1 as an indicator of beneficial reno-protective effect with inhibition of the renin–angiotensin system. Such a mechanism of decrease in the synthesis of TGF-β for a beneficial reno-protective effect with the use of losartan was further substantiated by Houlihan et al. through a study on hypertensive type 2 diabetic patients with elevated albumin excretion [33, 34].

ACEIs are also beneficial in blocking increased expression of TGF-β type II receptor in diabetic nephropathy [35]. ACEIs also appeared to be responsible for an alteration in matrix degradation pathways and that may reduce matrix accumulation in diabetic nephropathy [36].

Based on an animal study, Bonnet et al. suggested prevention of renal gene and protein expression of nephrin as a possible mechanism for the beneficial effect of ARBs in reducing proteinuria in hypertensive diabetics. The researchers used irbesartan in this study [37].

A preclinical study by Ertürküner et al. [38] indicates that perindopril helps prevent impairment in renal corpuscle (Mesangial matrix and podocyte) diabetic rats [38].

Cordonnier et al. projected another mechanism of inhibition for interstitial cell growth ARBs. Researchers found out that perindopril reduces excessive interstitial cell growth in patients with diabetic glomerulopathy suffering from hypertension [39].

Low-dose irbesartan therapy seems to be inadequate to protect the kidney due to inadequate inhibition of the RAAS in patients with type 2 diabetes and microalbuminuria. This inadequate inhibition of the RAAS was demonstrated by dose-dependent rise in plasma renin levels. Researchers also suggested evaluation of a 300 mg dose of irbesartan. Implementation of high-dose irbesartan treatment offers a consistent decrease in microalbuminuria may be due to reversal of renal structural and/or biochemical anomalies that contribute to long-term renal protective effects [21].

A systematic review and meta-analysis of twelve randomized controlled trials done by Caldeira et al. leads to the conclusion that treatment with ARBs should be implemented in patients who cannot tolerate ACE inhibitors. The researchers noticed that ARBs lead to minimal incidences of cough and angioedema, which are common side effects of ACE inhibitors [40].

Conclusion

ACEIs or ARBs are considered to be the 1st line of therapy for both type 1 and 2 diabetic patients with microalbuminuria. ARBs are preferred for patients who cannot tolerate ACE inhibitors. ARBs (e.g. losartan or telmisartan or higher dose of irbesartan) may be preferred over ACEIs due to their predominant renal protective effect in addition to their blood pressure improving beneficial effects of improving blood pressure in type 2 diabetic patients. However, the comparative

effects of ARBs and ACEIs in either type 1 or type 2 diabetic patients with microalbuminuria need to be further evaluated.

Conflict of interest

The authors declare to have no conflict of interest.

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Angiopoietin-2 and vascular complications of type 2 diabetes

ABSTRACT

Cardiovascular diseases are the leading cause of death among patients with diabetes mellitus type 2 (T2DM), and microvascular complications of diabetes are associated with high morbidity and diminished quality of life. Angiopoietin-2 (Ang-2) is essential for endothelial physiology and plays an important role in vascular-related diseases. Its concentration in blood is elevated in patients with T2DM in comparison to those with normal glucose tolerance and in subjects with diabetic macrovascular complications compared to those without them. As for microvascular complications, it was found that serum Ang-2 concentration was significantly higher among T2DM patients with diabetic retinopathy compared to diabetic patients free of this complication. Moreover, in an animal model, Ang-2 mRNA expression was elevated in endothelial cells isolated from diabetic mice's kidneys when compared to non-diabetic controls, which suggests its role in the development and progression of diabetic nephropathy. Targeting molecular Ang-2 pathway may become a therapeutic aim, especially that anti-angiogenic therapies are considered to be effective treatment methods in this field. (Clin Diabetol 2020; 9; 3: 201–204)

Key words: diabetes mellitus, biomarker, vascular complications, angiopoietin-2, angiopoietin

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Introduction

451 million people globally have diabetes, with the vast majority of them suffering from T2DM, and its prevalence is projected to reach 693 million people by the year 2045 [1]. Cardiovascular diseases (CVD) are the leading cause of death among patients with T2DM and diabetes reduces life expectancy curve by as much as ten years [2, 3]. The microvascular complications of diabetes are responsible for increased morbidity and diminished quality of life. Diabetic retinopathy became a leading cause of vision loss, diabetic nephropathy is a primary cause of end-stage renal disease, and the diabetic foot is the most often non-traumatic amputations of lower extremities [4, 5].

Endothelial dysfunction is an important factor involved in the pathogenesis of diabetes-related macro- and microangiopathies [6]. Angiogenesis and vascular remodeling are modulated by vascular growth factors, among others, angiopoietins with two major ones well characterized, which are angiopoietin-1 (Ang-1) and Ang-2 [7, 8].

Ang-2 is exclusively expressed in the endothelium cells, and both Ang-1 and Ang-2 bind to the receptor Tie-2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2) with the same affinity [9, 10]. Ang-2 possesses the opposite physiological properties to Ang-1. Ang-1 signaling through Tie-2 is responsible for endothelial wall stabilization and protects the endothelium from excessive activation by growth factors and cytokines [11], whereas the rapid release of Ang-2 from epithelium can prevent Ang-1 from binding to the receptor and in this manner disrupt the protective Ang-1/Tie-2 signaling [8, 11, 12].

Expression of Ang-2 is influenced by inflammatory factors like thrombin [13] and conditions like hypoxia [13]. There is some evidence for the association of elevated concentration of circulating Ang-2 with the incidence

of CVD [14] and cardiovascular mortality in the general population [15]. Dysfunction in angiogenesis has also been proposed as a common factor predisposing to vascular complications in diabetes [16].

Moreover, the selective increase in plasma Ang-2 concentration may lead to neovascularization and endothelial abnormalities, which are linked to the pathophysiology of microvascular and atherosclerotic complications in T2DM [17]. In the study by Lim et al. it was proven for the first time that plasma Ang-2 concentration is elevated among patients with diabetes in comparison to the ones without glucose metabolism disorders [18].

The aim of this review article is to present scientific data related to Ang-2 and its association with micro- and macro- vascular complications in T2DM.

Ang-2 and macroangiopathy

Rasul et al. reported that subjects with diabetic macrovascular complications, in particular with the CVD, had higher serum Ang-2 concentration than those without these complications [19]. This was in line with previous in vitro study, proving that hyperglycemia may lead to an increase in Ang-2 concentration, causing increased myocardial apoptosis, increased infarction size, and impaired myocardial angiogenesis [20].

Li et al. [21] found that serum Ang-2 concentration is associated with angiopathy in T2DM, where it was significantly higher in patients with diabetic macro- and microvascular complications compared to patients without angiopathy. In this study, which included 32 patients with macroangiopathy (cerebrovascular disease, heart disease, and peripheral arterial disease), and 52 patients with microangiopathy (diabetic nephropathy, diabetic retinopathy, and diabetic peripheral neuropathy) serum Ang-2 concentration was positively correlated with HbA_{1c} and HOMA-IR. It may implicate that Ang-2 is closely associated with insulin resistance, glucose metabolism disorders, and vascular complications in patients with T2DM. In the same study, it was also found that Ang-2 concentration was the highest among patients with macroangiopathy and the lowest in the group of T2DM patients without any vascular complications. The in-between values of Ang-2 concentration were found in the group of T2DM patients with microangiopathy. Besides, these authors suggest that hypothetically Ang-2 has exerted pro-angiogenic activity, and high Ang-2 blood concentrations may cause local inflammation, and in the consequence the vessels' permeability may be increased [21].

Further, inflammation stimulates Ang-2 release and its binding to the Tie-2 receptor, therefore, promoting proinflammatory and prothrombotic pathways. The same authors performed a subsequent study involving a higher number of patients (240 participants *vs.* 120 patients in the previous one) with diabetes and confirmed that serum Ang-2 concentration is positively correlated with the number of micro and macrovascular complications of T2DM [22].

Ang-2 and microangiopathy

Retinopathy

Diabetic retinopathy (DR) and diabetic macular edema (DME) have been, for years, thought to be the leading cause of blindness in the populations from the majority of developed countries [23]. Hyperglycemia leads to Ang-2 transcription, and this may lead to migration and apoptosis of retinal pericytes through Tie-2 activation. While searching for the probable explanation of this fact, one can use an animal model in which tested animals can be knocked out of specific genes. In order to explain this phenomenon, the Ang-2-deficient mice model was created, and retinal pericytes' migration, and apoptosis were observed. This proves that the inhibition of Ang-2/Tie-2 could be a potential therapeutic intervention [24, 25].

One of the features of DR, which may cause DME and vision impairments is vascular leakage [26]. It has been previously reported that serum Ang-2 concentration is significantly higher among T2DM patients with DR, both non-proliferative and proliferative ones, in comparison to patients with diabetes but without DR [26]. On the basis of animal studies with streptozocin-induced DR model, it was established that an increase in Ang-2 concentration was associated with vascular leakage, which can be blocked by intravitreal administration of antibodies neutralizing Ang-2 [27].

A study by Campochiaro et al. performed in patients with diabetes and DME, has shown that the administration of a vascular endothelial-protein tyrosine phosphatase, which promotes Tie-2 receptor activation (AKB-9778) for four weeks, caused DME reduction and vision improvement [28].

Nephropathy

Angiopoietins are important for glomerular capillaries in physiological conditions where they are responsible for blood flow regulation and permeability of the vascular wall. Imbalance of different growth factors, among others angiopoietins, promotes endothelial dysfunction and has been linked to the early pathological changes in glomerular function in diabetes, namely changes in blood flow and vascular wall's permeability [12].

Based on the 8-week observation of rats with streptozocin-induced diabetes, the authors concluded

that diabetes was associated with a disproportionate increase in Ang-2 comparing to Ang-1, where Ang-1 expression was reduced in the diabetic kidney after eight weeks of the experiment [29]. Similarly, only the Ang-2 mRNA level was elevated in whole glomeruli or glomerular endothelial cells isolated from diabetic mice when compared to non-diabetic counterparts, and no changes in relation to Ang-1 were observed [30].

Additionally, it was proven that high blood glucose concentration leads to the downregulation of Ang-1 mRNA in high-glucose-treated podocytes in comparison to normal-glucose treated cells [31]. This information supports the hypothesis that Ang-2/Ang-1 may take part in diabetic glomerular disease onset and progression [32].

Also, studies performed in humans report the negative role of Ang-2 on glomerulus where Ang-2 mRNA expression was increased in glomeruli isolated from patients with diabetes comparing to specimens obtained from non-diabetic live donors and no difference was observed in Ang-1 expression [31]. Moreover, urinary Ang-2 concentration was increased in patients with T2DM and associated with albuminuria [33]. Besides, there is preliminary evidence that Ang-2 might be an independent predictor of adverse outcomes related to kidneys' function in chronic kidney disease (CKD) in patients from general as well as diabetic population [34].

Especially relevant may be the information that elevated serum Ang-2 concentration is linked to systemic inflammation in patients with CKD and may predict mortality [35]. Additionally, plasma Ang-2 concentration has also been associated with arterial stiffness, which is known to be a risk predictor of cardiovascular mortality in T2DM [36].

Most recently, it has been proven that high serum Ang-2 concentration is independently associated with the increased risk of composite outcomes of either major adverse cardiac events (MACEs) or all-cause mortality in patients with diabetic nephropathy. The authors of this work suggest that serum Ang-2 could be a potential predictive factor for MACEs in patients with diabetic nephropathy at high risk of macrovascular complications [37].

Conclusion

Patients with diabetes are at risk of microvascular and macrovascular complications [38]. Ang-2 is essential for endothelial physiology and plays an important role in vascular-related diseases as it regulates endothelial permeability and angiogenesis. A selective increase in circulating Ang-2 concentration may favor abnormal neovascularization and endothelial disruption, which are linked to both microvascular and atherosclerotic vascular complications in T2DM [17, 39]. Ang-2 plays a role in vascular diseases, and perhaps targeting Ang/Tie signaling pathway may become a therapeutic approach because anti-angiogenic therapies are considered to be effective treatment methods in this field, especially in relation to microvascular complications of diabetes.

Conflict of interest

All the authors declare no conflict of interest in the field covered by this paper.

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