ORIGINAL ARTICLE

Analysis of Apoptotic, Clinical, and Laboratory Parameters in Type 1 Diabetes and Early Diabetic Nephropathy: Clustering and Potential Groups Evaluation for Additional Therapeutic Interventions

Ⓘ Ievgeniia Burlaka

Bogomolets National Medical University, Department of Pediatrics, Kyiv, Ukraine

What is already known on this topic?

Diabetic nephropathy (DN) is marked by pathological changes occurring in the renal glomeruli that lead to the development of albuminuria, hypertension, and progressive decline in renal function. Traditionally, different factors, such as the duration of diabetes, puberty, age at onset, family history of diabetic complications, family history of insulin resistance, type 1 and 2 diabetes, genetic factors, race/ethnicity, modifiable, glycemic (metabolic) control, smoking, hyperlipidemia, intrauterine exposure, obesity, pregnancy, social status discussed in terms of risk factors and predictors of type 1 diabetes (T1D) complications, including DN. However, complex studies dedicated to evaluating the possibility of using clinical markers in combination with markets of apoptosis and hypoxia for T1D and DN stratification in children have not yet been performed.

What this study adds?

We believe that our results showing that T1D pediatric patients with increased platelets (PLT) count, hyperfiltration and reduced anti-apoptotic defense may be a cornerstone group for therapeutic interventions, i.e. antioxidants along with optimal glycemic control. DN group found to have somewhat increased PLT count, high frequency of diabetic ketoacidosis episodes/year, high microalbuminuria, prominent increase in HIF level, prominent disturbances in apoptosis controlling factors BcL-xL and caspase-3 requires additional therapeutic interventions, i.e. antioxidants, anti-apoptotic effectors along with optimal glycemic control, management of hypertension and albuminuria.

Abstract

Objective: Type 1 diabetes (T1D) is one of the most prevalent chronic illnesses diagnosed in childhood. Diabetic nephropathy (DN) is one of the commonest complication of T1D. Therefore the development of specific treatment that arrests progression of DN based on an individual approach would be beneficial. Analysis of criteria of apoptosis, and clinical, and laboratory characteristics in T1D and early DN in the framework of clustering may be helpful in the identification of potential groups for additional therapeutic interventions.

Methods: A survey of 104 children (62 males, 42 females) with T1D and DN aged 2 to 17 years in the Endocrinology unit of Clinical Pediatric Hospital No 6 (Kyiv, Ukraine) was performed. Clinical data (age, gender, disease duration, blood pressure), conventional laboratory markers including complete blood count, serum cholesterol, hemoglobin A1c (Hb1Ac), glomerular filtration rate (GFR), and microalbuminurea (MAU), and markers of apoptosis (BcL-xL, caspase-3) and transcriptional factor HIF-1alfa were analyzed.

Results: A cluster group in T1D children was characterized by somewhat higher number of platelets (PLT) - $344.9 \pm 7.88 \cdot 10^9$/L, increased GFR up to hyperfiltration level 124.5 ± 8.86 mL/min/1.73 m² and decreased anti-apoptotic defense - BcL-xL 144.9 ± 2.35 a.u. was identified. Children with DN may be divided into three groups based on age, body mass index, systolic blood pressure, PLT count, erthyrocyte sedimentation rate, albumin/globulin ratio, serum cholesterol, Hb1Ac, number of diabetic ketoacidosis (DKA) episodes, GFR, MAU, HIF-1alfa, Bcl-xL, caspase-3 levels. Among children with early DN a cluster characterized by the following parameters was found: PLT count - $311. \pm 12.05 \cdot 10^9$/L, frequency of DKA episodes - 4.82 ± 0.26 episodes/year, MAU - 112.0 ± 10.12 mm/24 h, HIF - 200.5 ± 3.49 a.u., BcL-xL - 128.8 ± 3.1 a.u., and caspase-3 - 159.6 ± 5.5 a.u.

Address for Correspondence: Ievgeniia Burlaka MD, Bogomolets National Medical University, Department of Pediatrics, Kyiv, Ukraine
Phone: + 38-097-432-49-44 **E-mail:** evgbur1982@gmail.com **ORCID:** orcid.org/0000-0001-6043-7325

Conclusion: Thus, we hypothesize that T1D pediatric patients with increased PLT count, hyperfiltration and reduced anti-apoptotic defense may represent a group for additional therapeutic interventions, such as antioxidants along with stndard therapies to achieve optimal glycemic control. Within the DN group there was a sub-group with somewhat increased PLT count, high frequency of DKA episodes/year, high MAU, prominent increase in HIF level, prominent disturbances in apoptosis controlling factors BcL-xL and caspase-3 tht may require additional therapeutic interventions, again including antioxidants, but may additionally benefit from anti-apoptotic effectors along with optimal glycemic control, and management of hypertension and albuminuria.

Keywords: Early diabetic nephropathy, T1D, hypoxia, HIF-1 alfa, apoptosis, predictors

Introduction

An estimated 1.1 million people under 20 years of age are affected by type 1 diabetes (T1D) worldwide (1,2). T1D represents 5-10% of the global diabetes burden and is not a disease of childhood alone, with almost half diagnosed in adulthood (3,4). Overall annual increase in T1D is estimated at 3% (2-5%) (5). Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus, affecting 25 to 40% of patients with T1D. It is the single most common cause of end stage renal disease (ESRD) in adults in the Western world (6,7). DN is marked by pathological changes occurring in the renal glomeruli that lead to the development of albuminuria, hypertension, and progressive decline in renal function (6,7,8).

Traditionally, different factors, such as the duration of diabetes, puberty, age at onset, family history of diabetic complications, family history of insulin resistance, type 1 and 2 diabetes, genetic factors, race/ethnicity, glycemic (metabolic) control, smoking, hyperlipidemia, intrauterine exposure, obesity, pregnancy, and social status have ben identified in terms of risk factors and predictors of T1D complications, including DN (9,10).

An alternative approach, attmpting to identify subgroups of patients with T1D would be to make an evaluation of patients based on different clinical, anamnestic, and pathogenic characteristics. This approach could use a global space-time clustering for cases of T1D. This appraoch has been used to identify possible sex-related differences in response to an infectious agent in patients with T1D (11).

In this study, in addition to the main clinical data, we analyzed pediatric patients with T1D and early DN markers of apoptosis, including proteins belonging to the Bcl-2 family. It is been shown previously that the Bcl-2 family of proteins plays a central role in monitoring the genetic programs of the organism. We also measured hypoxia-inducible factor 1 alfa (HIF-1alfa) in all subjects. The rationale for this was as follows. Hypoxia is present in animal models and may be found as early as three days after the induction of diabetes, predominantly in the medullary region (12). However, comprehensive studies to evaluate the possibility of using clinical markers in combination with markets of apoptosis

and hypoxia for T1D and DN stratification in children have not done yet.

The aim of the present study was to evaluate cluster groups of children with T1D and DN, based on levels of transcription factor and marker of intracellular hypoxia including HIF-1alfa and anti-apoptotic factor BcL-xL and the proapoptotic factor, caspase-3, together with basic clinical and laboratory parameters in order to attempt to identify potential subgroups of patients with T1D and DN that may be amenable for additional therapeutic interventions.

Methods

Patients

The study included data from 2013 to 2020 with a total of.104 children (62 males and 42 females) with T1DM and early stage of DN followed-up in the Endocrinology unit of Clinical Pediatric Hospital No 6 (Kyiv, Ukraine). The study was approved by the Ethics Committee of the Bogomolets National Medical University (approval No 142). All informed consents were signed by children (≥12 years old) themselves and/or by their parents and kept in medical records. Medical records and data, including anamnesis was analyzed in all patients. All diabetic patients were seen every 3 months and all were on multiple flexible dosing intervals of insulin treatment. Chronological age, diabetes duration, weight, height, body mass index (BMI), blood pressure, hemoglobin A1c (Hb1Ac), serum cholesterol, complete blood count, urinalysis, and urine albumin excretion was measured and recorded at each visit to hospital.

Patients with T1D and without signs of DN with urinary albumin excretion within physiological range prior the study inclusion and at each follow-up visit were designated group T1D (n = 57). Disease duration in this group was ≥1 year.

The DN group (n = 48) were children with DN observed during 1 year after the first documented episode of albuminuria. Diagnosis of the DN was based on the measurement of abnormal levels of urinary albumin in diabetic patients after the exclusion of other causes of albuminuria. Two out of three samples falling within the microalbuminuria (MAU) (30 to 300 mg of albumin/24 h)

or macroalbuminuria (more than 300 mg of albumin/24 h) range confirm the presence of DN. Urinary MAU/albumin excretion measured in 24-hour urine collection samples using basic conventional technique established in Clinical Pediatric Hospital No 6. Causes of albuminuria were excluded in all patients in the DN group.

Excludion criteria included severe chronic and acute diseases, such as chronic inflammatory diseases, autoimmune diseases, transplantation, viral hepatitis B or C, liver cirrhosis, or other severe liver diseases, acute and chronic gastrointestinal diseases, previous acute kidney injury, chronic kidney disease (CKD), major surgery within 12 months before study, AIDS, heart disease, and cancer.

Glomerular filtration rate (GFR) was used to assess kidney function. The Schwartz formula for children and adolescents 1 to 17 years old was used (13). The demographic and clinical characteristics of the patients included in the study is shown in Table 1.

Immunoblotting for Detection of HIF-1alfa, BcL-xL, Caspase-3

Plasma samples were used to measure markers of apoptosis and intracellular hypoxia response. Proteins suspended in Laemmli sample buffer were resolved in polyacrylamide gels by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked in 5% non-fat

milk in TBS-T (136 mM NaCl, 10 mM Tris, 0.05% Tween 20) and immunoblotted using antibodies specific for Bcl-xL and HIF-1α, and caspase-3 (Cell Signaling Technology, Danvers, MA USA) for 1 hour at room temperature. An actin mouse monclonal antibody was used as a loading control. After three washes with TBS-T, the membranes were incubated with secondary anti-rabbit or anti-mouse antibodies labeled with horseradish peroxidase for 1 hour at room temperature. Membranes were washed three times with TBS-T. Protein bands were visualized by Enhanced chemiluminescence substrate. Quantification of the protein content was done by densitometric analysis.

Statistical Analysis

The data are expressed as means ± standard error of the mean. ANOVA followed by post-hoc Kruskal-Wallis test for multiple comparisons was used to test significance of differences. Data was analyzed using GraphPad Prism 9.0 Software for Windows (San Diego, CA, USA). Two-step clustering was done using Statistica 10.0 software. An intelligent clustering method in which the optimal clustering number is automatically determined was performed. This identifies clusters by two processes: first, preclustering, followed by hierarchical clustering. Hierarchical algorithms were used to estimate the optimal clustering number based on the silhouette width, the calculation of the distance using the log-likelihood and clustering in accordance with Schwarz's Bayesian criterion. P values < 0.05 were considered statistically significant.

Results

Clinical Characteristics of Patients

Identification and Characteristics of Three Clusters by Remodeling the Cluster Analysis Based on Fourteen Variables in Children with T1D

The clustered results, based on nine variables - disease course, age, BMI, systolic blood pressure (SBP), platelet (PLT) count, erythrocyte sedimentation rate (ESR), albumin/globulin ratio, serum cholesterol, Hb1Ac, number of diabetic ketoacidosis (DKA) episodes, GFR, HIF-1alfa, Bcl-xL, caspase-3 are shown as three subgroups in T1D patients (Figure 1).

These cluster groups were designated cluster I, cluster II, and cluster III. Disease duration and mean age values did not show any difference between the clusters (Figure 2A, 2B). The mean BMI was also similar in clusters I-III (Figure 2C). Mean SBP values did not show statistical differences between the clusters (Figure 2D).

Table 1. Clinical characteristics of patients

Parameter, mean ± SEM	T1D (n = 57)	DN group (T1D with diabetic nephropathy) (n = 47)
Age, years	12.74 ± 0.77	13.25 ± 0.56
Boys/girls	29/28	33/14
Boys, age, years	11.73 ± 0.82	12.82 ± 0.76
Girls, age, years	13.85 ± 1.33	14.2 ± 0.66
Duration of T1D	4.9 ± 0.5	6.0 ± 051
BMI, kg/m^2	18.75 ± 0.63	19.72 ± 0.55
Boys, BMI, kg/m^2	18.14 ± 0.63	19.65 ± 0.72
Girls, BMI, kg/m^2	19.42 ± 1.13	20.05 ± 0.9
Systolic blood pressure, mmHg	106.5 ± 1.44	126.4 ± 1.34***
Diastolic blood pressure, mmHg	71.02 ± 0.88	71.94 ± 1.11
PLT, 10^7/L	267.6 ± 8.14	262,9 ± 8,83
ESR, mm/h	4.8 ± 0.13	10.45 ± 0.53**
Albumin/globulin ratio	1.26 ± 0.04	1.00 ± 0.03**
Serum cholesterol, mMol/L	4.58 ± 0.15	5.83 ± 0.14**
GFR, mL/min/1.73 m^2	135.5 ± 24.21	85.87 ± 2.19***
Hb1Ac, %	9.41 ± 0.3	10.22 ± 9.55

**p < 0.01, ***p < 0.001.
SEM: standard error of the mean, T1D: type 1 diabetes, DN: diabetic nephropathy, BMI: body mass index, PLT: platelets, ESR: erythrocyte sedimentation rate, GFR: glomerular filtration rate, Hb1Ac: hemoglobin A1c

PLT count, ESR, albumin/globulin ratio, serum cholesterol level were selected as basic laboratory markers. The mean PLT count in cluster I was $344.9 \pm 7.88 \cdot 10^9$/L, in cluster II - $257.4 \pm 3.02 \cdot 10^9$/L (p < 0.01) and cluster III - $205.1 \pm 12.52 \cdot 10^9$/L (p < 0.001 - cluster I vs. cluster II value and p < 0.0001 - cluster I vs. cluster III) (Figure 3A). Similar mean ESR values were found in the three clusters (7.54 ± 1.53 mm/h, 6.06 ± 0.83 mm/h and 8.22 ± 2.5 mm/h, respectively; p > 0.05) (Figure 3B). The albumin/globulin ratio did not

show difference between cluster I, II and III (1.2 ± 0.05, 1.26 ± 0.05 and 1.32 ± 0.16, respectively; p > 0.05) (Figure 3C). Furthermore, the mean serum cholesterol level was also similar between the clusters (4.85 ± 0.18 mMol/L in cluster I, 4.49 ± 0.17 mMol/L in cluster II and 4.77 ± 0.62 mMol/L in cluster III; p > 0.05) (Figure 3D).

Hb1Ac, number of DKA episodes, and GFR were selected as markers of T1D compensation and kidney function. The mean Hb1Ac value did not differ between the clusters (Figure 4A). However, the average number of DKA episodes/year in cluster II was somewhat higher than in cluster I (2.12 ± 0.26 episodes/year vs. 1.91 ± 0.42 episodes/year) but this was not significant. In cluster III the mean number of DKA episodes per year was 2.19 ± 0.31 (p > 0.05 - cluster I vs. cluster II and III) (Figure 4B). Finally, GFR as a direct indicator of kidney function was investigated in all subjects. The mean GFR value was similar in cluster II and III (98.13 ± 2.99 and 91.9 ± 5.82 mL/min/1.73 m^2, respectively; p > 0.05). Cluster I GFR value was 124.5 ± 8.86 mL/min/1.73 m^2 which was significantly higher compared to cluster II and cluster III (p < 0.05) (Figure 4C).

The expression of proapoptotic factor, caspase-3, anti-apoptotic factor BcL-xL, and the marker of intracellular hypoxia, HIF-1alfa were also analyzed. HIF-1alfa was selected as a marker of chronic hypoxia but was similar in cluster I, II and III (165.4 ± 3.83 a.u., 165.0 ± 1.6 a.u. and 158.2 ± 3.19 a.u., respectively; p > 0.05) (Figure 5A). The Bcl-xL level in cluster I was 144.9 ± 2.35 a.u. which is significantly lower compared to the value in cluster III at 160.0 ± 2.4 a.u. (p < 0.001). In addition this value in cluster II was 140.6 ± 1.57 and was found to be significantly lower
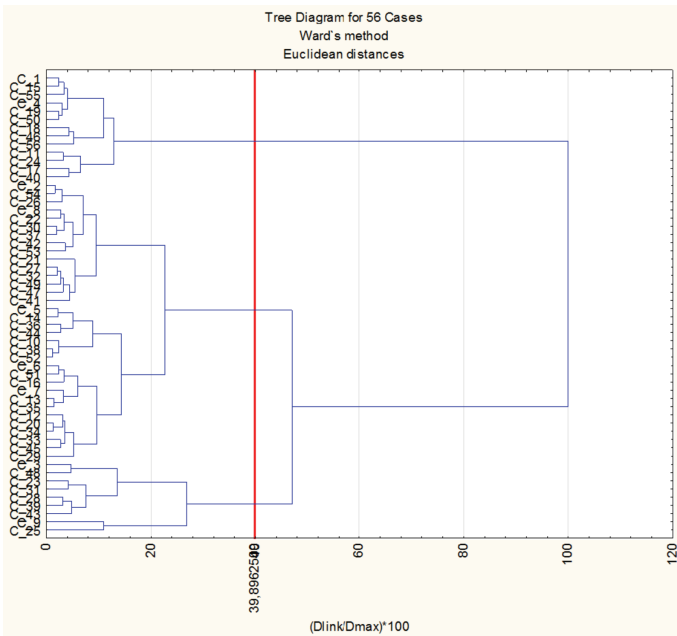

Figure 1. Identification of three clusters by remodeling the cluster analysis based on fourteen variables in children with type 1 diabetes
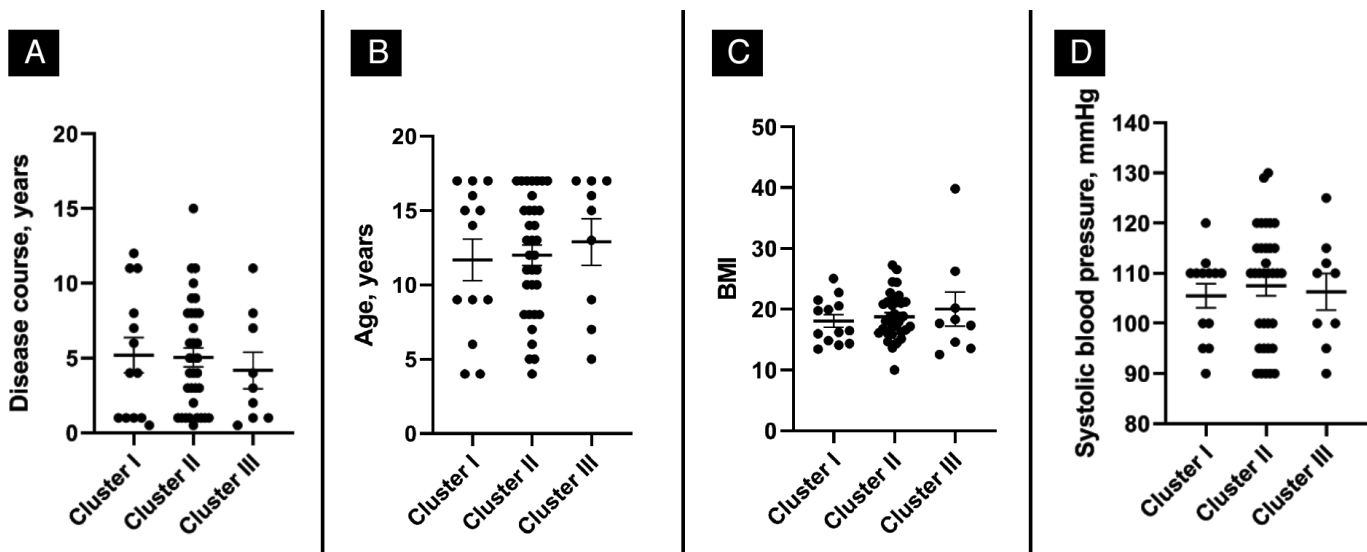

Figure 2. Disease course (A), age (B), BMI (C), SBP (D) in cluster groups of children with T1D. Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

BMI: body mass index, SBP: systolic blood pressure, SEM: standard error of the mean, T1D: type 1 diabetes

compared to cluster III (p < 0.05) (Figure 5B). The level of caspase-3 in cluster I, cluster II and cluster III was similar at 137.7 ± 3.28 a.u., 137.6 ± 2.13 a.u. and 136.0 ± 2.99 a.u., respectively (p > 0.05) (Figure 5C).

Identification and Characteristics of the Three Clusters by Remodeling the Cluster Analysis in Children with Early DN

The clustered groups were designated based on fourteen variables, including age, BMI, SBP, PLT, ESR, albumin/globulin ratio, serum cholesterol, Hb1Ac, number of DKA episodes, GFR, MAU, HIF-1alfa, Bcl-xL, caspase-3 in children with early DN (Figure 6).

No difference was documented in mean age (Figure 7A), mean BMI, and SBP values between clusters in the DN group (Figure 7B, 7C) when compared clusters I-III.

The mean PLT in cluster I was $311. \pm 12.05 \cdot 10^9$/L, which is higher when compared to cluster II ($260.4 \pm 11.12 \cdot 10^9$/L; p < 0.01) and cluster III ($273.4 \pm 8.05 \cdot 10^9$/L; p < 0.05) (Figure 8A). The ESR level was similar in the three clusters at 10.18 ± 1.55 mm/h, 9.36 ± 1.22 mm/h and 8.91 ± 0.92 mm/h, respectively (p > 0.05) (Figure 8B). The albumin/globulin ratio was also not different between the three clusters at 1.55 ± 0.04, 1.12 ± 0.05 and 1.19 ± 0.05, for clusters I, II and III respectively (p > 0.05) (Figure 8C).
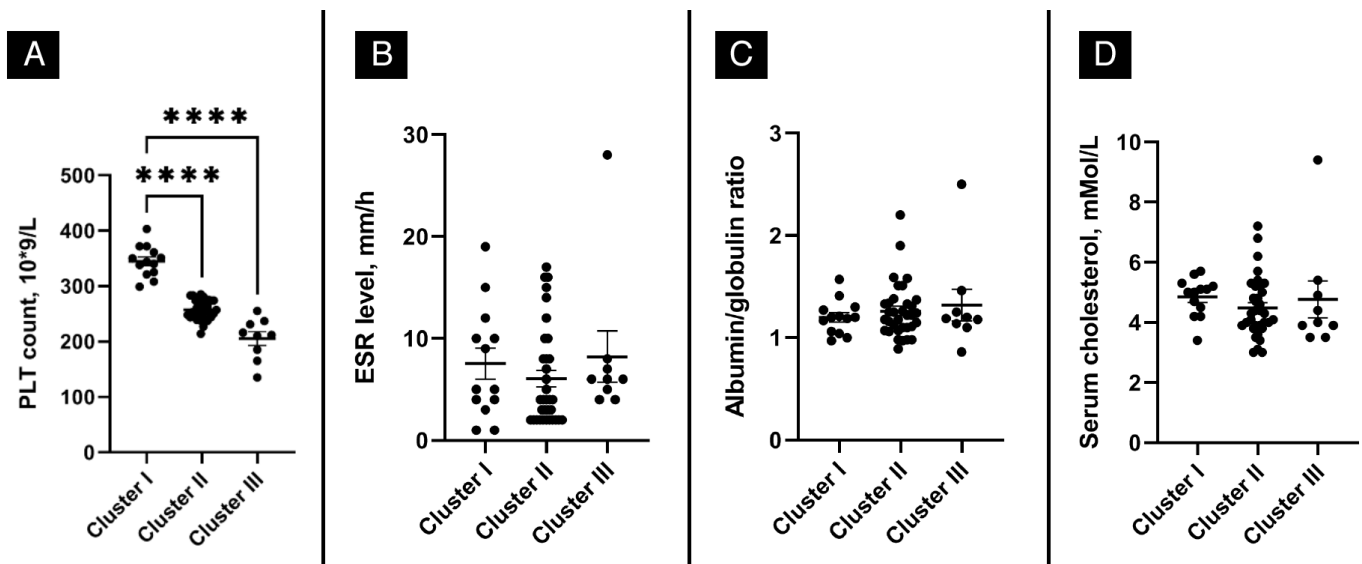

Figure 3. PLT count (A), ESR (B), albumin/globulin ratio (C) and serum cholesterol (D) levels in cluster groups of children with T1D (****p < 0.0001). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

SEM: standard error of the mean, T1D: type 1 diabetes, PLT: platelets, ESR: erythrocyte sedimentation rate
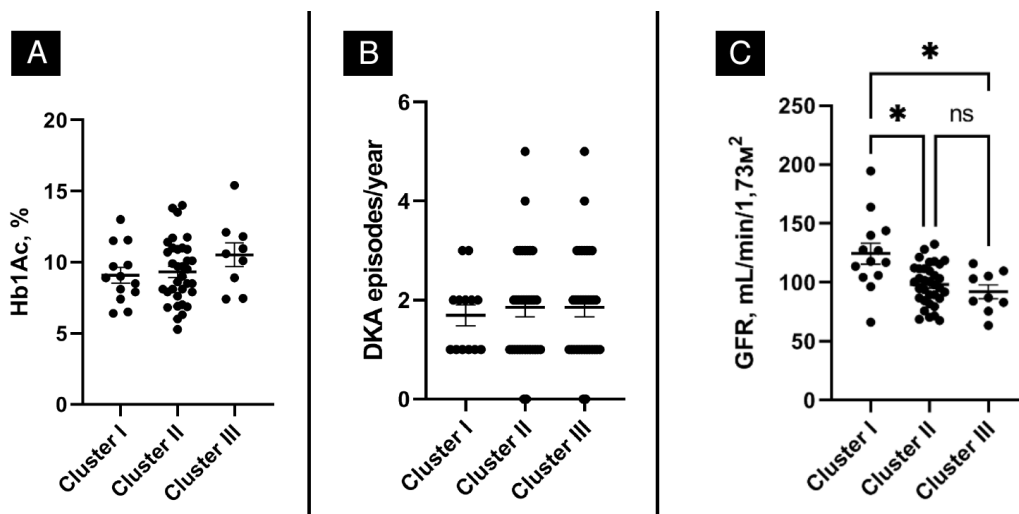

Figure 4. Hb1Ac (A), number of DKA episodes (B), GFR (C) levels in cluster groups of children with T1D. Ns: not significantly different (*p < 0.05). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

Hb1Ac: hemoglobin A1c, DKA: diabetic ketoacidosis, GFR: glomerular filtration rate, SEM: standard error of the mean, T1D: type 1 diabetes
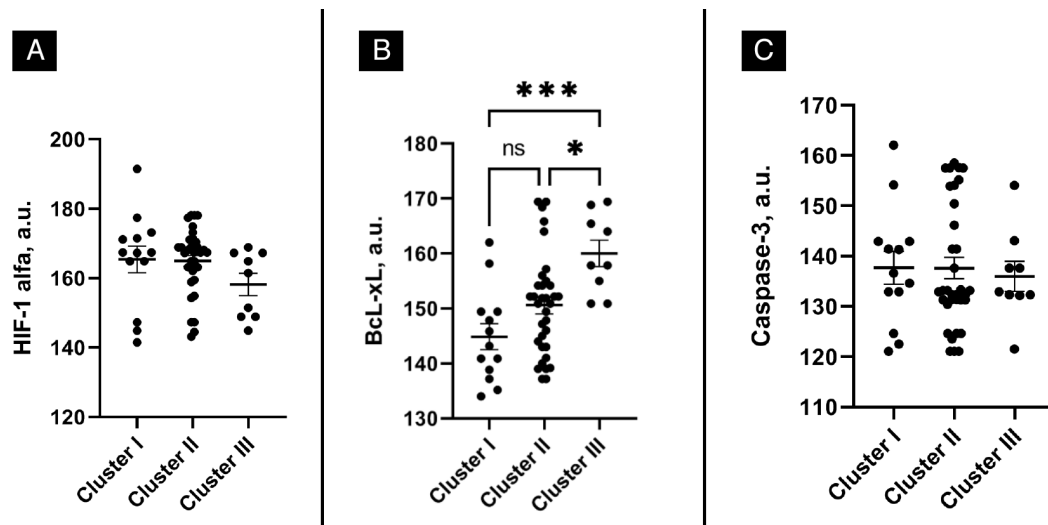
Figure 5. HIF1-alfa (A), BcL-xL (B), caspase-3 (C) levels in cluster groups of children with T1D. Ns: not significantly different (*p < 0.05, *p < 0.001). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test
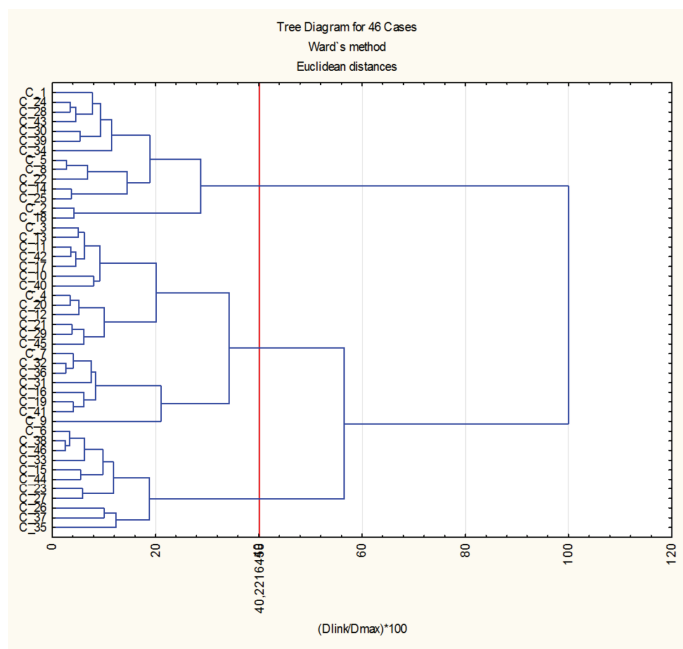
SEM: standard error of the mean, T1D: type 1 diabetes

Figure 6. Identification of three clusters by remodeling the cluster analysis based on fourteen variables in children with early DN

DN: diabetic nephropathy

Furthermore, the serum cholesterol level was also similar at 5.86 ± 0.23 mMol/L in cluster I, 5.66 ± 0.31 mMol/L in cluster II and 5.84 ± 0.19 mMol/L in cluster III (p > 0.05) (Figure 8D).

The mean Hb1Ac was not different between the three clustering groups; 10.81 ± 0.73 % in cluster I, 9.92 ± 0.7 % in cluster II, and 9.83 ± 0.42 % in cluster III (p > 0.05) (Figure 9A). The average number of DKA episodes/year in cluster I

was 4.81 ± 0.26 episodes/year, in cluster II it was 3.21 ± 0.42 episodes/year and in cluster III it was 4.29 ± 0.31 episodes/year (p > 0.05) (Figure 9B).

GFR as a direct indicator of kidney function was evaluated in all children with T1D. The mean GFR value did not show any difference between cluster I, II and III at 87.57 ± 3.8, 85.05 ± 3.58 and 83.62 ± 3.71 mL/min/1.73 m², respectively (p > 0.05) (Figure 9C). MAU excretion as a direct indicator of kidney damage was analyzed in all children with DN. The mean MAU value in cluster I was 112.0 ± 10.12 mg/24 h and was significantly higher compared to cluster II 38.25 ± 6.32 mm/h (p < 0.001) and cluster III at 35.64 ± 2.82 mm/h (p < 0.001) (Figure 9D).

The expression of HIF-1alfa, BcL-xL and caspase-3 was analyzed in clusters of children with DN. The values of HIF-1 alfa were similar in cluster II and cluster III at 182.5 ± 5.11 a.u. and 185.2 ± 3.28 a.u., respectively (p > 0.05). However, HIF-1 alfa in cluster I was significantly higher than in clusters II and II at 200.5 ± 3.49 a.u. (p < 0.05) (Figure 10A). The mean value of Bcl-xL in cluster I was 128.8 ± 3.1 a.u. which was significantly lower compared to the cluster II value of 146.3 ± 3.27 a.u. (p < 0.05) but did not differ from the cluster III value of 137.2 ± 2.67 a.u. (p > 0.05) (Figure 10B). Caspase-3 results were similar between cluster I, cluster II and cluster III at 159.6 ± 5.5 a.u., 137.7 ± 3.64 a.u. and 146.3 ± 2.67 a.u., respectively (p > 0.05) (Figure 10C).

Discussion

Recent trends have indicated that the incidence of diabetes is increasing rapidly worldwide, with a dramatic increase in

prevalence in the Middle Eastern countries, among both adults and children (14,15). DN is the leading cause of end-stage renal disease worldwide. Chronic hyperglycemia and high blood pressure are the main risk factors for the development of DN. In general, screening for MAU should be performed annually, starting five years after diagnosis in T1D.

The pathogenesis of DN development and progression is complex and multifactorial with the involvement of many pathways and mediators (16). Conventionally, the developmental mechanism of DN is the result of abnormal homeostasis, which includes hemodynamic abnormalities, metabolic disorders, and hormone synthesis, such as Ang-

II. The renin-angiotensin-aldosterone system, advanced glycation end product (AGE) formation, activation of transforming growth factor-β1, connective tissue growth factor, protein kinase C, mitogen-activated protein kinase, and reactive oxygen species are important pathways to the development and progression of DN (17). This is why the exact pathogenic mechanism and molecular incidence of DN are still not fully understood and the contribution of each pathway in inducing DN is not clear and thus the early identification of risk groups is challenging.

As with many other CKD, the diagnosis of DN is based on changes in urinary albumin excretion rate and GFR.
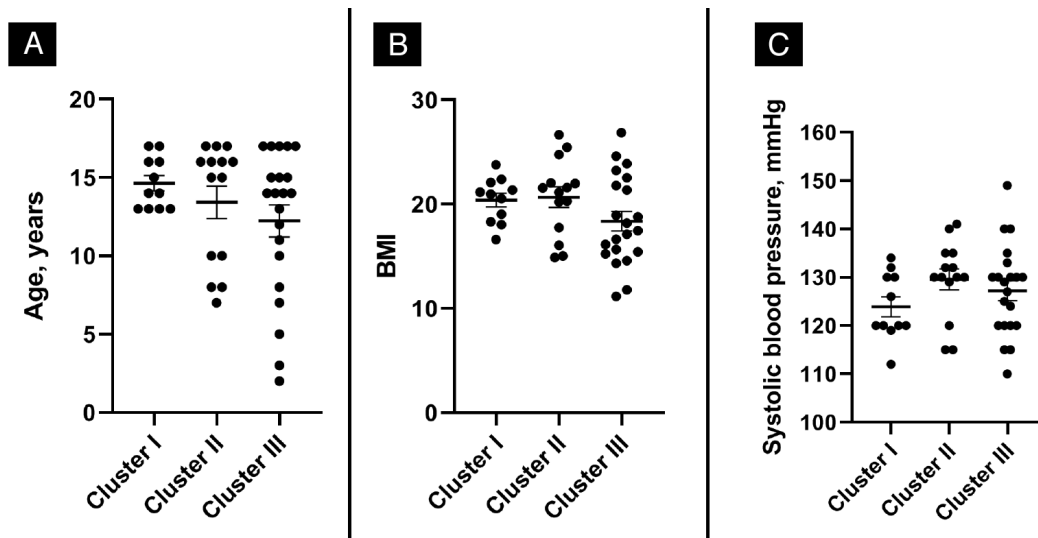

Figure 7. Age (A), BMI (B), SBP (C) in cluster groups of children with early DN. Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

BMI: body mass index, SBP: systolic blood pressure, DN: diabetic nephropathy, SEM: standard error of the mean
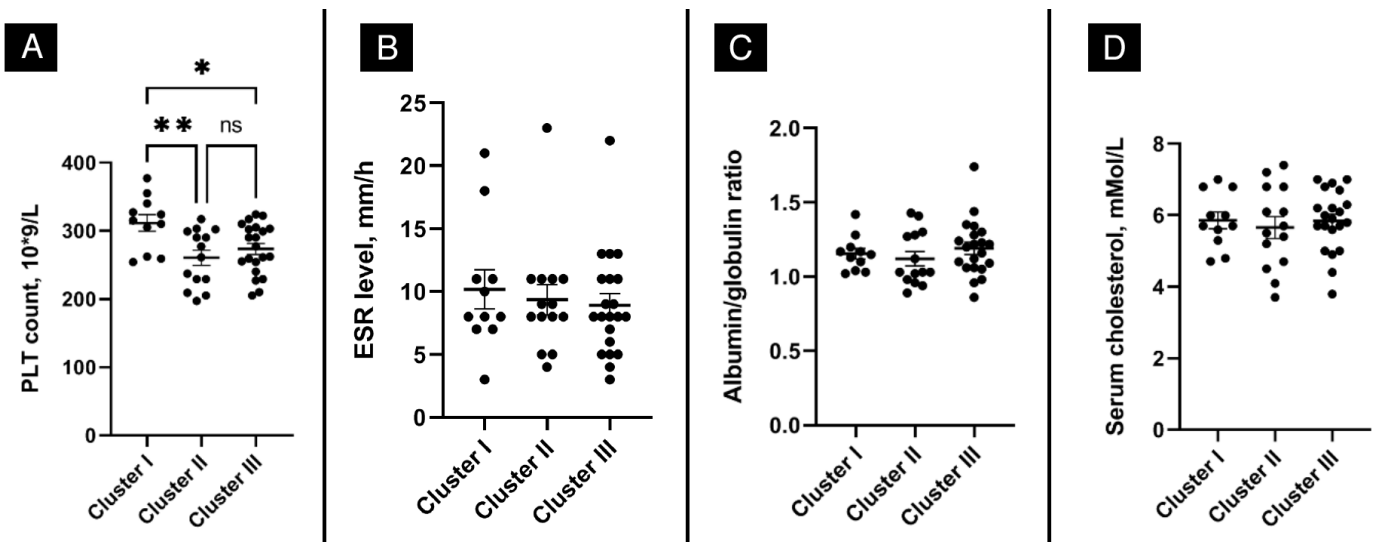

Figure 8. PLT count (A), ESR (B), albumin/globulin ratio (C) and serum cholesterol (D) levels in cluster groups of children with early DN ($*p < 0.05$, $*p < 0.01$). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

PLT: platelets, ESR: erythrocyte sedimentation rate, DN: diabetic nephropathy, SEM: standard error of the mean

Structural changes may be observed in kidney biopsies as early as the first few years after the onset of diabetes, but the disease has a long "silent period" in its development (18). Thus, our current understanding of the trajectory of DN in children and adolescents suggests that advanced CKD and kidney failure take decades to develop after the onset/diagnosis of diabetes, which means that the data on the prevalence and time course of these outcomes in childhood-onset diabetes is largely derived from adult studies (19,20). This presents a dilemma for any rigorous study of diabetic kidney disease (DKD) in children and adolescents because understanding any aspect of DN, for example biomarkers, risk factors for progression, and assessment of response to interventions, has had to rely on intermediate outcomes, such as albuminuria, and hyperfiltration. This requires identification of risk hroups of patients based on conventional clinical tests results and novel pathogenic biomarkers.

The lack of reliable surrogate markers for DN progression during childhood and adolescence makes identification of
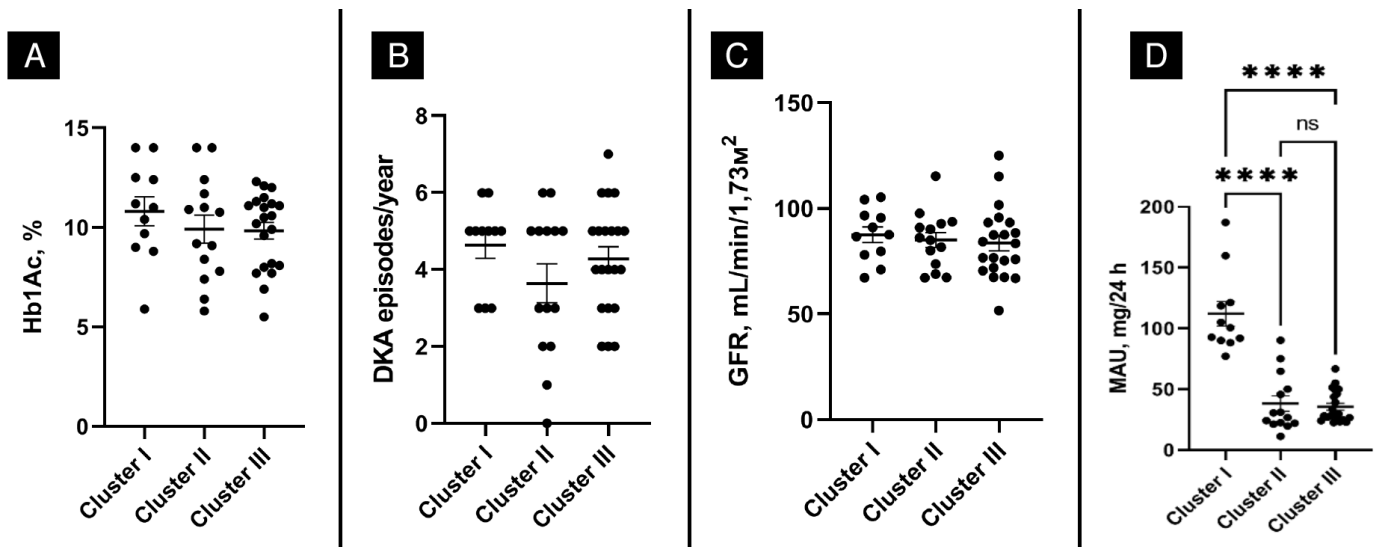

Figure 9. Hb1Ac (A), number of DKA episodes (B), GFR (C), MAU (D) levels in cluster groups of children with early DN. Ns: not significantly different, (****$p < 0.0001$). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

Hb1Ac: hemoglobin A1c, DKA: diabetic ketoacidosis, GFR: glomerular filtration rate, MAU: microalbuminurea, SEM: standard error of the mean, DN: diabetic nephropathy
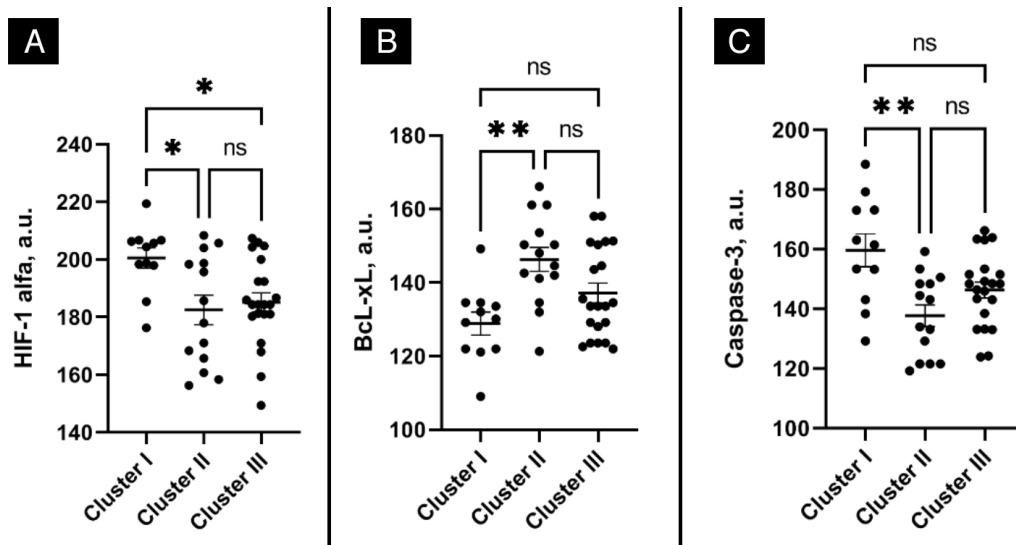

Figure 10. HIF1-alfa (A), BcL-xL (B), caspase-3 (C) levels in cluster groups of children with early DN. Ns: not significantly different, (*$p < 0.05$, *$p < 0.01$). Histograms represent means ± SEM. Statistical analysis performed using the post-hoc Kruskal-Wallis test

DN: diabetic nephropathy

novel markers of early disease in youth even more critical than it is in adults. Most published studies report cross-sectional associations between various urinary/serum protein biomarkers and intermediate outcomes, such as albuminuria, with a smaller number of studies examining these associations using longitudinal data.

Rare studies are notable in bypassing the reliance on these flawed surrogate markers and examining the association between putative biomarkers, such as plasma AGEs or plasma bradykinin with early kidney structural changes in youth with T1D. In adults, serum tumor necrosis factor receptor 1 (TNFR1) and TNFR2 have been found to be associated with the early structural changes of DN as well as with DN progression, highlighting the contribution of inflammatory pathways to the disease process (21). Other potential biomarkers for adult DN are urinary neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, N-acetyl-β-D-glucosaminidase, and liver fatty acid-binding protein (LFABP) (22,23). None of these putative markers or others is currently a part of routine clinical care in adult or pediatric DKD.

Therefore, the focus of the present study was stratification of children with T1D and early DN using conventional laboratory markers in combination with markers of apoptosis and chronic hypoxia.

We chose members of the Bcl-2 family BcL-xL and caspase-3 as markers of apoptosis. The Bcl-2 family has long been identified for its role in apoptosis regulation. In an *in vivo* model, Wada et al. (24) showed that puromycin-induced podocyte apoptosis was p53 dependent and associated with changes in Bcl-2-related proteins and apoptosis inducing factor AIF translocation. The protective effects of dexamethasone on PA-induced apoptosis were associated

with decreasing p53, increasing Bcl-xL, and inhibition of AIF translocation (25).

We measured the level of HIF-1alfa in all patients with T1D and early DN which is an important transcriptional factor regulating many cellular functions. HIF-1 is a heterodimer composed of the rate limiting factor HIF1α and the constitutively expressed HIF-1β (26). DN is associated with chronic, low-grade inflammation under the persistent influence of MAU and glucose (25). Hypoxia can induce apoptosis by causing hyperpermeability of the inner mitochondrial membrane, which leads to the release of cytochrome C and apoptosis induction (27).

Our results show that all examined children with T1D may be divided into three groups based on fourteen variables, - disease course, age, BMI, SBP, PLT count, ESR, albumin/globulin ratio, serum cholesterol, Hb1Ac, number of DKA episodes, GFR, HIF-1alfa, Bcl-xL, caspase-3 levels. Cluster I can be defined as a risk group characterized by somewhat higher PLT, increased GFR up to hyperfiltration level and decreased anti-apoptotic defense. Cluster II and cluster III did not show these characteristics.

Children with DN may be divided into three groups based on age, BMI, SBP, PLT count, ESR, albumin/globulin ratio, serum cholesterol, Hb1Ac, number of DKA episodes, GFR, MAU, HIF-1alfa, Bcl-xL, caspase-3 levels. Cluster I was found as the most different and its characteristics are PLT count, frequency of DKA episodes, mean MAU in 254 hour urine collection, and levels of HIF, BcL-xL and caspase-3.

We speculate that cluster I in the T1D group, characterized by somewhat of an increase in PLT, hyperfiltration and reduced anti-apoptotic defense should be considered as a potential risk group for further complications, including DN and cardiovascular events. Previously, we have shown that
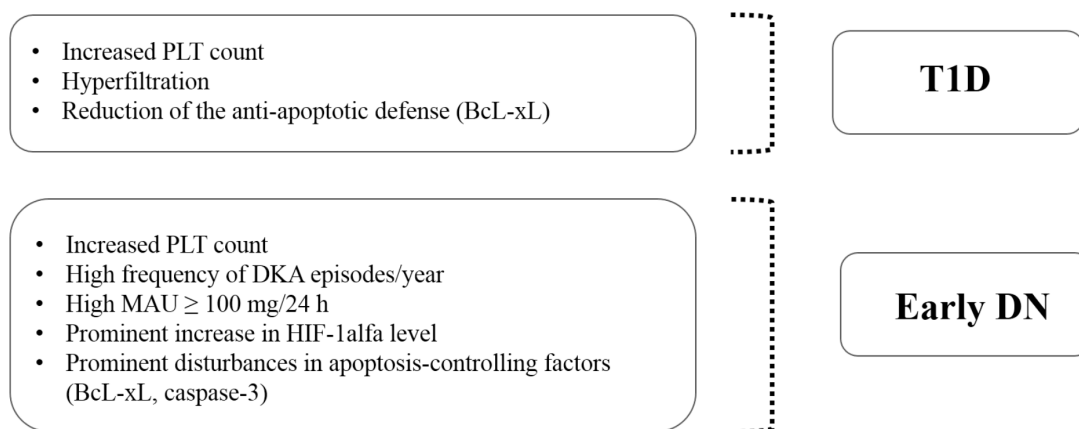

Figure 11. Summarized scheme of the parameters of the clustered risk groups of children with T1D and early DN

DN: diabetic nephropathy, T1D: type 1 diabetes, PLT: platelets, DKA: diabetic ketoacidosis

children with T1D have increased GFR (28). This finding is in line with other research showing that a 25-50 % elevation in the GFR is seen early in the course in up to one-half of patients with T1D, an abnormality that is exaggerated after ingestion of a protein load. Glomerular hypertrophy and increased kidney size typically accompany the rise in GFR (29,30). PLT may be a factor possibly contributing to future cardiovascular events as well. It has been shown that enhanced PLT reactivity is considered a main determinant of the increased atherothrombotic risk of diabetic patients. Thrombopoietin, a humoral growth factor able to stimulate megakaryocyte proliferation and differentiation, also modulates the response of mature PLT by enhancing both activation and binding to leukocytes in response to different agonists (31). Cluster I in the DN group was found to have a somewhat increased PLT count, high frequency of DKA episodes/year, high MAU, prominent increase in HIF level, and prominent disturbances in apoptosis controlling factors BcL-xL and caspase-3. We speculate that in addition to the pathogenic effects from modestly incresed PLT and DKA-related cardiovascular and circulatory disorders due to poor metabolic control and glycemic variability, albuminuria causes additional stimulating effect on apoptosis. Albuminuria is a potent apoptotic agent. Albumin uptake in primary rat renal epithelial cells is accompanied by a time- and dose-dependent mitochondrial accumulation of the apoptotic factor Bax, down-regulation of the antiapoptotic factor Bcl-xL and mitochondrial membrane depolarization (32). A summarized scheme of the parameters of the risk groups of children with T1D and early DN is given in Figure 11.

Study Limitations

This study has certain limitations that must be acknowledged. Our pilot study was cross-sectional, at a single center with modest patient numbers. The strength is that enrolled patients were studied for the full range of clinical, laboratory, and anamnestic markers in parallel with markers of hypoxia and apoptosis measurement.

Conclusion

Thus, we hypothesize that T1D pediatric patients with increased PLT, hyperfiltration and reduced anti-apoptotic defense may constitute a group requiring therapeutic interventions, such as antioxidants along with conventional treatment and optimal glycemic control. Within the DN group, there was a sub-group with somewhat increased PLT count, high frequency of DKA episodes/year, high MAU, prominent increase in HIF-1 alfa level, prominent disbalance in level of apoptosis controlling factors BcL-xL and caspase-3

which may also require additional therapeutic interventions, once again including antioxidants, but may also warrant anti-apoptotic effectors along with optimal glycemic control, management of hypertension and albuminuria.

Ethics

Ethics Committee Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Bogomolets Medical University (protocol code: 142, date: 22.02.2022).

Informed Consent: Informed consent was obtained from all subjects involved in the study.

Peer-review: Externally peer-reviewed.

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