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Association of Xanthine Oxidase Activity, Oxidative Stress and Early Tubular Kidney Injury in Children with Type 1 Diabetes

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Abstract

Background: Xanthine oxidase (XO) is an important source of reactive oxygen species (ROS) and may be involved in oxidative stress-related renal injury. However, the relationship between XO activity, oxidative stress and early renal tubular injury in children with type 1 diabetes (T1D) and early diabetic nephropathy (DN) remains insufficiently studied. Methods: This cross-sectional study included 150 participants aged 10–18 years: 50 children with T1D without DN, 50 with with T1D and DN and 50 healthy controls. Plasma XOR and XDH activities were determined spectrophotometrically, and XO activity was calculated as the difference between XOR and XDH activities. Superoxide production was assessed spectrophotometrically by monitoring cytochrome c reduction. The Urinary Kidney Injury Molecule (KIM-1) was assessed as a marker of tubular injury. Associations between oxidative stress markers, KIM-1 and DN were examined using correlation and multivariable regression analyses. Results: Plasma XO activity increased progressively across the study groups, from 10.25 (9.49–11.12) μU/mL in controls to 27.95 (26.31–29.29) μU/mL in children with T1D and 54.79 (52.62–57.73) μU/mL in those with DN ($p < 0.0001$). Principal component analysis showed clustering of oxidative stress and tubular injury biomarkers distinct from conventional renal function measures. Multivariable regression identified XO and superoxide production as independently associated with urinary KIM-1 levels in the DN group. T1D duration, BMI, KIM-1, XO and superoxide production were independently associated with DN. The combined model demonstrated excellent discrimination (AUC = 0.933). Conclusions: Increased XO activity is associated with oxidative stress and urinary KIM-1 in children with T1D, with the highest levels observed in DN group. The combined associations of XO, superoxide, KIM- and clinical factors with DN suggest a potential link between oxidative stress and early tubular injury in pediatric T1D. These biomarkers may be associated with early involvement and require further investigation as potential indicators of kidney injury in children with T1D.

Keywords: xanthine oxidase; oxidative stress; diabetic nephropathy; type 1 diabetes; pediatric; KIM-1; reactive oxygen species; superoxide; diabetic ketoacidosis; prediction; complementary markers

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1. Introduction

Type 1 diabetes mellitus (T1D) is a chronic metabolic disease characterized by absolute insulin deficiency and persistent hyperglycemia, requiring lifelong insulin therapy. Current estimates indicate that in 2025, there were approximately 9.5 million people living with T1D worldwide, of whom 1.85 million were under the age of 20. The number of people

living with T1D is projected to increase to 14.7 million by 2040 [1,2]. These data highlight the significant and growing global burden of T1D, particularly among children and young people.

Prolonged hyperglycemia and metabolic abnormalities can lead to the development of micro- and macrovascular complications. Major chronic complications include diabetic nephropathy, retinopathy, neuropathy and cardiovascular disease. The development of these complications significantly impairs the long-term prognosis and quality of life of patients. Kidney damage is of particular importance, as it is one of the main causes of chronic kidney disease (CKD) and end-stage renal disease (ESRD) in patients with diabetes [2,3].

In children, early changes in T1D may occur before the development of severe albuminuria or a decrease in glomerular filtration rate. Therefore, early detection of subclinical tubular damage is of particular importance for timely assessment of the risk of disease progression. Traditional indicators, such as albuminuria and eGFR, remain the main clinical markers, but they do not always reflect early changes in the renal tubules. This justifies the search for additional biomarkers of early kidney damage in children with type 1 diabetes [3].

Diabetic nephropathy (DN) is one of the most serious microvascular complications of type 1 diabetes and can progress to chronic kidney disease and end-stage renal disease [3,4]. According to previous studies, a number of factors are involved in the pathogenesis of DN, i.e., the formation of advanced glycation end products (AGEs), inflammation, and apoptosis [3]. A common characteristic of all these pathways is an imbalance between the production and degradation of free radicals, which is called oxidative stress. Diabetic hyperglycemia plays an important role in oxidative stress through increased levels of reactive oxygen species (ROS) [5,6].

Excessive production of reactive oxygen species (ROS) is known to be mediated, in particular, by increased activity of Xanthine oxidoreductase (XOR) in plasma [7]. Xanthine oxidoreductase (XOR) exists in two interconvertible forms: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XO is an enzyme involved in the catabolism of purines to uric acid. XO can also generate superoxide ions (O^{2-}) and hydrogen peroxide (H_2O_2). Under physiological conditions, serum XO is present at very low concentrations, mainly due to hepatic cell turnover. Plasma XO levels and activity are increased under hypoxic/ischemic conditions and pathological conditions such as cardiovascular disease and pro-inflammatory states [8,9].

There is evidence that XO contributes to the increased oxidative stress seen under the hyperglycemia, i.e., T1D. Human studies have shown that serum XO activity is increased in patients with T1D [8]. Furthermore, plasma XO activity correlates with insulin resistance (IR). Consistent with these human data, studies of liver and plasma from streptozotocin-induced diabetic Wistar rats have shown higher XO activity compared with non-diabetic rats [9,10].

Kidney injury molecule 1 (KIM-1) is a transmembrane glycoprotein that is upregulated following proximal renal tubular injury [11]. KIM-1-mediated cellular and humoral effects are involved in a variety of physiological and pathophysiological processes. Oxidative stress and inflammatory signaling associated with hyperglycemia may contribute to tubular epithelial cell damage and subsequent KIM-1 expression. Following its upregulation, the extracellular domain of KIM-1 is released into the tubular lumen and can be detected in urine, making urinary KIM-1 a useful non-invasive indicator of proximal tubular injury. Importantly, XO-derived ROS may represent one of the potential mechanisms linking metabolic disturbances in diabetes with tubular epithelial injury and increased urinary KIM-1 levels [12]. Elevated urinary KIM-1 levels are associated with proximal tubular injury and may be detected early in diabetic kidney injury, including before the development of overt albuminuria. KIM-1 levels may also correlate with albuminuria and other markers

of renal dysfunction [11,13]. The aim of this study is to examine the relationship between plasma xanthine oxidase activity and early markers of kidney damage in children with T1D.

2. Materials and Methods

2.1. Study Design and Settings

This study was conducted as a single-center, cross-sectional observational investigation. The study design followed accepted methodological and ethical principles for clinical research in pediatric populations. All clinical assessments, sample collections and laboratory analyses were performed at the Endocrinology Unit of the Pediatric Department of Clinical Hospital No. 18 in Kyiv, Ukraine. Conducting the study within a single institution allowed for the use of uniform diagnostic criteria, standardized follow-up procedures and consistent laboratory protocols. Participants were enrolled over the period 2023–2025 during routine outpatient visits as well as during the scheduled inpatient stay. The cross-sectional nature of the study enabled the concurrent assessment of clinical parameters, biochemical measurements, oxidative stress indices and tubular injury biomarkers.

2.2. Study Population, Sample Size and Sampling

This cross-sectional study included 150 participants between the ages of 10 to 17 years of age. One hundred subjects with previous medical diagnosis of T1D and 50 non-T1D were included in our study. The required sample size was estimated using an online sample-size calculator. The calculation was based on a preliminarily obtained minimum clinically relevant difference of 30% in the primary outcome, with a two-sided significance level of $\alpha = 0.05$ and a statistical power of 80%.

All participants provided signed informed consent before study participation and were evaluated for a complete clinical and family history. Anthropometric and biochemical parameters were determined. Participants were eligible for inclusion according to the criteria specific to their study group. The control group included 50 healthy children without T1D and any acute or chronic illnesses. The T1D group included 50 children aged 10–17 years with an established diagnosis of T1D without diabetic nephropathy. The group called DN included 50 children aged 10–17 years with an established diagnosis of T1D and early/subclinical diabetic kidney involvement, defined by microalbuminuria (30–300 mg/24 h) and an eGFR ≥120 mL/min/1.73 m^2, consistent with renal hyperfiltration. The eGFR was calculated using the 2009 updated bedside the Schwartz equation: eGFR = 0.413 × height (cm)/serum creatinine (mg/dL), with eGFR expressed in mL/min/1.73 m^2 [14].

Exclusion criteria included incomplete clinical history or blood sampling, chronic proinflammatory diseases (arthritis, autoimmune disease, and malignancy), endocrine diseases (hyperthyroidism, hypothyroidism, or Cushing's disease), treatment with anti-inflammatory drugs, or personal rejection of study participation. Patients with hyperuricemia were excluded because elevated uric acid levels are associated with XO hyperactivity in patients with diabetes.

2.3. Data Collection and Study Variables

Using standardized protocols, all participants were evaluated including their individual and family clinical history. Anthropometric, clinical and laboratory parameters were assessed in all participants. Height (meters), weight (kg), and body mass index (BMI) were measured. BMI was calculated as weight/height^2 (kg/m^2). Blood pressure (BP), including systolic and diastolic BP, was measured using a standardized procedure with an appropriately sized cuff after a period of rest for 5 min. Measurements were taken at 1–2-min intervals, and the average of the three readings was used for analysis. Hemato-

logical parameters included red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT) count, and erythrocyte sedimentation rate (ESR) and measured using analyzer Mindray BC-20s (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). Biochemical assessment included serum alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) activities. Glycated hemoglobin (HbA1c) was measured as an indicator of long-term glycemic control measured using private laboratory Dila facilities (Kyiv, Ukraine). HbA1c was measured in a certified clinical laboratory using an automated analytical platform. Venous blood was taken after overnight fasting (10–12 h) between 7 am and 10 am by venipuncture. Blood samples were collected to obtain plasma. Information on the occurrence and frequency of diabetic ketoacidosis (DKA) episodes within the preceding 12 months was collected from patient's medical records and verified during clinical interviews.

The 12_month observation window was selected to provide a standardized assessment of recent DKA history. In addition to collecting information on DKA history during the preceding 12 months, an evaluation of the XO during DKA episodes and during periods without clinical or biochemical evidence of DKA was done. DKA status was established based on clinical and laboratory findings documented in the patients' medical records.

The urine albumin level microalbuminuria (MAU) was assessed in 24-h urine samples using the standard methods. The value less than 30 mg/24 h was considered as normoalbuminuria and 30–300 mg/24 h as MAU. The level of kidney injury molecule type 1 (KIM-1) in the morning spot urine samples was determined using a commercial Human KIM-1 ELISA Kit (Cat. No. EH0210, 96 determinations; Wuhan Fine Biotech Co., Ltd., Wuhan, China). The assay was performed according to the manufacturer's instructions and protocol.

Plasma xanthine oxidoreductase (XOR) activity was assessed spectrophotometrically by measuring uric acid formation from hypoxanthine at 290 nm. For each assay, 1 mL of plasma was incubated with hypoxanthine (50 μM final concentration) in sodium phosphate buffer (50 mM, pH 7.8), with a total reaction volume of 3 mL. The reaction was performed at 37 °C for 5 min, and the increase in absorbance at 290 nm (A290) was monitored continuously. The reaction was linear over the 5-min measurement period, and the reaction rate was calculated from the slope of the absorbance-versus-time curve (ΔA290/min).

Two parallel reaction conditions were analyzed under otherwise identical conditions. In the absence of NAD^+, uric acid formation was used to determine total XOR activity. In the presence of NAD^+ (500 μM final concentration), uric acid formation was used to determine XDH activity. Thus, both XOR and XDH activities were assessed by monitoring uric acid formation at 290 nm; NADH formation at 340 nm was not measured.

A plasma blank containing 1 mL of plasma and sodium phosphate buffer but no hypoxanthine was used to account for background absorbance of the plasma at 290 nm. The blank-corrected change in absorbance was used to determine the reaction rate attributable to uric acid formation.

Hypoxanthine was used at a fixed final concentration of 50 μM. Substrate saturation was not experimentally assessed in the present study; therefore, the measured activities represent reaction rates under the specified assay conditions rather than experimentally determined Vmax values.

The rate of uric acid formation was converted to enzyme activity using the molar extinction coefficient of uric acid at 290 nm ($\varepsilon 290 = 12.2\ mM^{-1} \cdot cm^{-1}$) and a 1-cm optical path length. Enzyme activity was expressed in μU/mL of plasma, where 1 U was defined as the amount of enzyme catalyzing the formation of 1 μmol of uric acid per minute under the assay conditions. No external calibration curve was used; enzyme activity was calculated directly from the blank-corrected ΔA290/min using the extinction coefficient of uric acid, the optical path length, and the assay volume. XO activity was calculated from the

experimentally determined XOR and XDH activities according to the following equation: XO activity = XOR activity − XDH activity [15].

Superoxide production was assessed by measuring the superoxide-dependent reduction of ferricytochrome c spectrophotometrically. Plasma samples were mixed with hypoxanthine (50 μM final concentration) as the substrate, cytochrome c (50 μM final concentration) and sodium phosphate buffer (50 mM, pH 7.8). The total reaction volume was 3.0 mL in a cuvette. The time-dependent increase in absorbance at 550 nm, corresponding to the reduction of cytochrome c, was recorded during incubation of the reaction mixture 37 °C. The rate of cytochrome c reduction, reflected by the change in absorbance at 550 nm over time, was used as an index of superoxide production. Because endogenous SOD activity was not inhibited, the assay was interpreted as an indirect measure of superoxide-generating activity rather than a direct quantitative determination of superoxide concentration [16].

2.4. Data Management and Analysis

The data were processed using the GraphPad Prism 10.5.0 for Mac (San Diego, CA, USA). Normality of continuous variables was assessed using the Shapiro–Wilk test. Non-normally distributed variables were presented as median and interquartile range (IQR). Qualitative parameters were expressed as absolute and relative frequencies (%).

Non-normally distributed data were compared by the use of the Mann–Whitney U test (for two unrelated samples) or the Kruskal–Wallis test with the following Dunn's test for post hoc comparisons (for three unrelated samples). The frequency of binary parameters was compared using the Fisher's exact test (for two unrelated samples) or the Fisher's exact test with Bonferroni correction (for three unrelated samples). Chi-Square test and Fisher's Exact test were used to analyze the relationships between categorical variables.

To explore the underlying structure of the data and reduce multicollinearity, principal component analysis (PCA) was applied. Variables were normalized before analysis to prevent scale differences from influencing the component extraction. Components with eigenvalues exceeding 1 were considered significant, and the scree plot was used to confirm the appropriate number of components. The resulting principal components were used in subsequent analyses to capture the main sources of variability in the dataset.

Spearman's rank correlation analysis we used to assess associations between XO activity, superoxide production and urinary KIM-1. Spearman's correlation coefficients (r_s), 95% confidence intervals (CI) and corresponding *p* values were reported.

Multivariable regression analysis was performed to identify factors independently associated with urinary KIM-1 levels. Gender, age, T1D duration, BMI, HbA1c, XO and superoxide production were included as predictor variables. Regression coefficients (β), 95% CI, t statistics and *p* values were presented. Multivariable logistic regression analysis was used to identify factors independently associated with DN. T1D duration, BMI, HbA1c, urinary KIM-1, XO and production were included in the model. Associations were expressed as odds ratios (ORs) with 95% CI, likelihood-ratio statistics and *p* values.

Receiver operating characteristic (ROC) curve analysis was performed to evaluate the discriminatory ability of XO, superoxide production and urinary KIM-1 for identifying DN. ROC analysis was also used to assess the discriminative performance of the multivariable logistic regression model incorporating T1D duration, BMI, HbA1c, KIM-1, XO and superoxide. Diagnostic performance was expressed as the area under the ROC curve (AUC), SE and 95% CI. p value < 0.05 was considered statistically significant.

2.5. Ethical Considerations

The study protocol was reviewed and approved by the Ethics Committee of Bogomolets National Medical University, Kyiv, Ukraine. All procedures involving human participants

were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from parents or legal guardians, and assent was obtained from children when they are >12 years old, prior to enrollment in the study.

3. Results

3.1. Clinical Characteristics of the Study Participants

A total of 150 participants were included in the study, including 50 healthy controls, 50 children with T1D without DN and 50 children with T1D and DN. The groups differed significantly in age, body weight, BMI, eGFR and HbA1c. As expected, the DN group demonstrated higher eGFR values, consistent with renal hyperfiltration. No significant differences were observed in systolic or diastolic blood pressure, hematological parameters or most biochemical indicators between the groups (Table 1).

Table 1. Basic clinical characteristics of the examined patients.

Parameter	Control ($n = 50$)	T1D ($n = 50$)	DN ($n = 50$)	p
Boys/girls, n (%)	12/38	15/35	12/38	>0.05
Age (years)	15.0 (10.75–17.0)	13.0 (9.0–15.0)	14.0 (9.0–15.0)	<0.05
Body weight, kg	49.0 (33.5–55.75)	34.5 (23.0–45.0)	39.0 (32.75–43.25)	<0.001
BMI (kg/m^2)	19.7 (18.2–21.1)	16.25 (14.88–18.55)	17.9 (16.95–20.33)	<0.0001
T1D duration, years		7.0 (5.0–9.0)	9.5 (8.0–11.0)	<0.01
Systolic BP, mmHg	101.5 (80.0–120.0)	107.5 (96.0–120.0)	104.0 (87.5–120.0)	>0.05
Diastolic BP, mmHg	65.0 (55.0–81.25)	65.0 (60.0–75.0)	65.0 (55.0–77.0)	>0.05
eGFR, mL/min/1.73 m^2	91.0 (87.75–98.00)	87.04 (80.93–93.50)	138.0 (116.5–145.0)	<0.001
RBC, 10^{12}/L	4.5 (3.6–4.6)	4.5 (3.9–4.8)	4.56 (4.1–4.8)	>0.05
WBC, 10^9/L	4.3 (4.1–4.8)	4.5 (4.2–5.2)	4.3 (4.17–4.8)	>0.05
PLT, 10^9/L	240.0 (225.0–286.3)	234.5 (225.0–262.5)	239.5 (225.5–262.5)	>0.05
ESR, mm/h	5.0 (3.0–8.0)	4.5 (3.0–6.25)	4.5 (3.0–6.25)	>0.05
Blood cholesterol, mmol/L	4.1 (3.5–4.3)	4.0 (3.45–4.3)	4.1 (3.5–4.3)	>0.05
ALAT, U/L	14.7 (12.9–17.8)	14.9 (12.78–18.15)	14.8 (12.7–17.88)	>0.05
ASAT, U/L	20.6 (8.0–25.63)	24.25 (21.3–28.83)	20.6 (8.0–25.63)	<0.001
Hb1Ac, %	4.6 (4.27–4.9)	8.3 (6.8–10.2)	8.95 (7.6–10.7)	<0.0001

3.2. Oxidative Stress Markers in Children with T1D and Subclinical DN

Analysis of the measured parameter of oxidative stress across three groups revealed a progressive increase depending on disease status. In healthy controls the median value of plasma xanthine oxidase was 10.25 (9.49–11.12) μU/mL. Children with T1D without DN showed a substantial elevation 27.95 (26.31–25.29) μU/mL ($p < 0.0001$). The highest values were observed in children with DN—54.79 (52.62–57.73) μU/mL ($p < 0.0001$). These data indicate a graduate increase of the XO from healthy individuals to T1D and then to DN (Figure 1).

Analysis of superoxide production revealed a gradual increase across the study groups. Control group exhibited the lowest value 0.6 (0.4–0.76) nmol/(mL*min). Children with T1D without nephropathy showed significantly higher levels as compared to the control value of 1.68 (1.49–1.94) nmol/(mL*min). ($p < 0.0001$). The highest superoxide production were observed in children with DN—2.84 (2.6–3.22) nmol/(mL*min). The DN group demonstrated the highest superoxide production, which significantly exceeded the values of T1D group and the controls ($p < 0.0001$) (Figure 2).

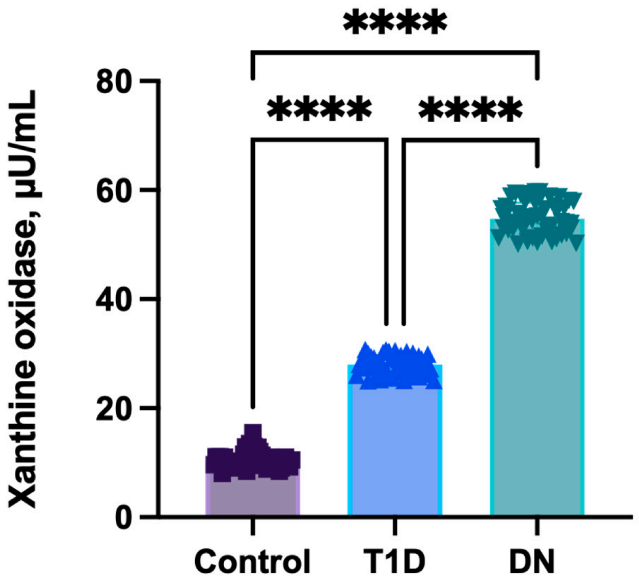


Figure 1. Plasma XO levels in healthy controls, children with type 1 diabetes T1D and children with diabetic nephropathy DN. ****—$p < 0.0001$.

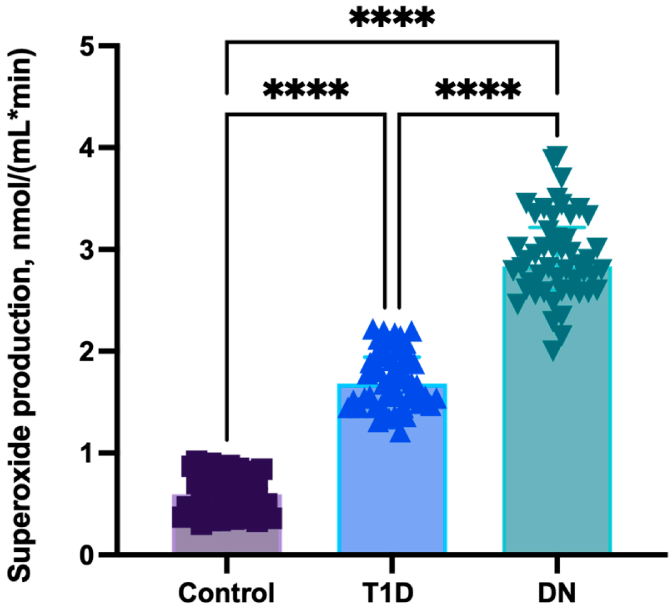


Figure 2. Plasma superoxide production in healthy controls, children with type 1 diabetes T1D and children with diabetic nephropathy DN. ****—$p < 0.0001$.

We analyzed levels of KIM-1 in all children from three groups. The control group exhibited the lowest values—1.68 (1.17–2.35) ng/mL. Children with T1D without nephropathy showed a substantial elevation 4.24 (3.55–5.12) ng/mL ($p < 0.0001$). The highest values were observed in children with DN—10.17 (9.08–12.84) ng/mL ($p < 0.0001$). These data indicate a graduate increase of the XO from healthy individuals to T1D and then to DN (Figure 3).

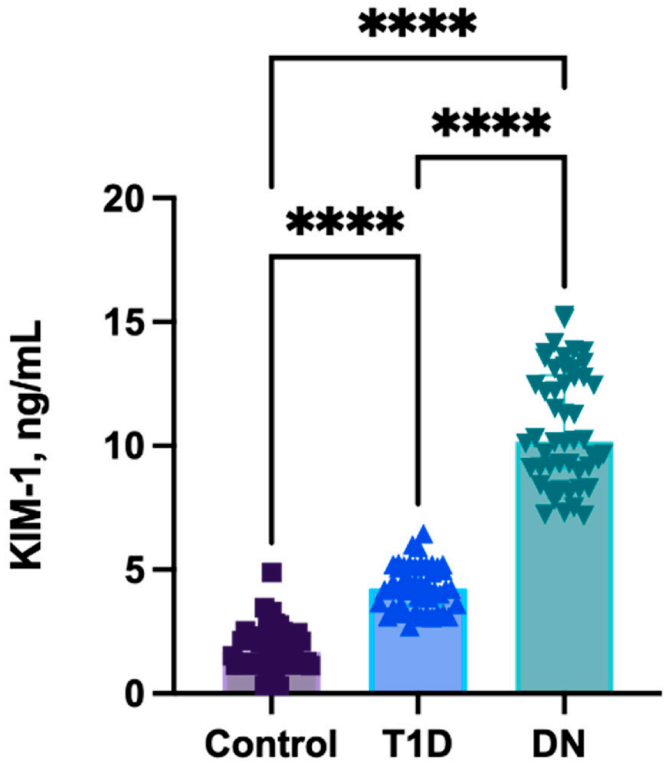


Figure 3. Urinary KIM-1 levels in healthy controls, children with type 1 diabetes T1D and children with diabetic nephropathy DN. ****—$p < 0.0001$.

We analyzed correlations between XO, superoxide and KIM-1 in the T1D and DN groups. In T1D a strong positive association was observed between XO activity and superoxide generation (r = 0.85, 0.74 to 0.91 95% CI, $p < 0.0001$) (Figure 4A). This pattern demonstrates that higher XO activity is closely linked to enhanced superoxide formation, supporting the role of XO as an important contributor to oxidative stress in the studied cohort. Moreover, a strong positive correlation was found between superoxide levels and KIM-1 levels (r = 0.76, 0.6 to 0.86 95% CI, $p < 0.0001$) (Figure 4B).

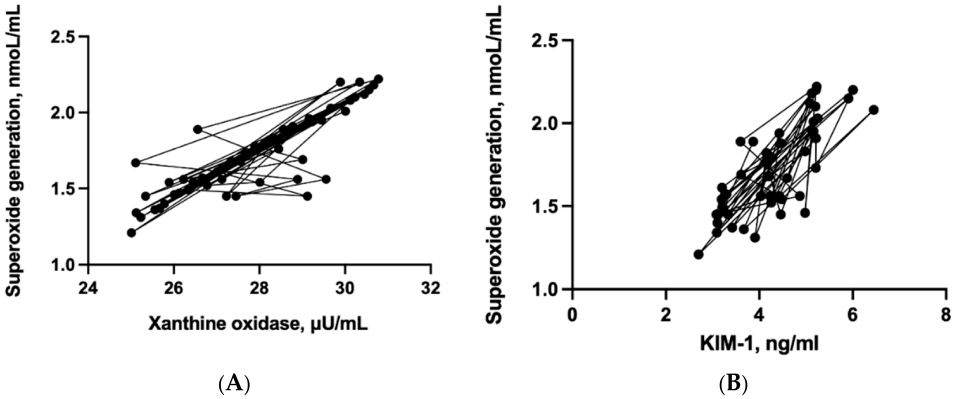


Figure 4. Correlation analysis in T1D group. (**A**) Correlations between superoxide generation and xanthine oxidase. (**B**) Correlations between superoxide generation and KIM-1. Dots represent individual patients; connecting lines indicate paired measurements from the same patient.

In DN group a strong positive correlation found between XO activity and superoxide generation ($r_s = 0.84$; 0.73 to 0.91 95% CI; $p < 0.0001$) (Figure 5A). This pattern demonstrates that higher XO activity is closely linked to enhanced superoxide formation, supporting the role of XO as an important contributor to oxidative stress in the studied cohort. A strong

positive correlation was found between superoxide levels and KIM-1 levels (r_s = 0.77, 0.62 to 0.87 95% CI, $p < 0.0001$) (Figure 5B).

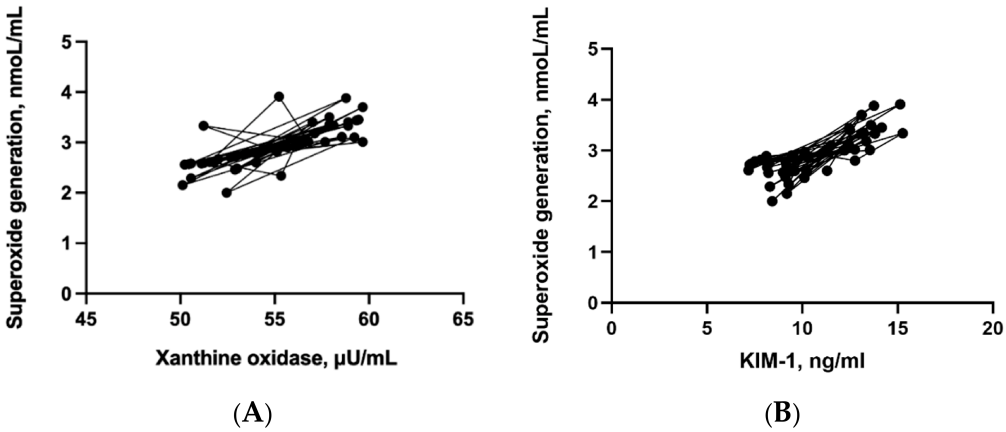


Figure 5. Correlation analysis in DN group. (**A**) Correlations between superoxide generation and xanthine oxidase. (**B**) Correlations between superoxide generation and KIM-1. Dots represent individual patients; connecting lines indicate paired measurements from the same patient.

3.3. Patterns of Clinical, Biochemical, Oxidative and Tubular Injury Biomarker Variables

A PCA was performed to explore the multivariate structure of clinical parameters, traditional biochemical tests and tubular injury biomarkers in children with T1D and DN. The first two principal components (PC1 and PC2) together explained a substantial proportion of the total variance and revealed clear patterns of variable clustering relevant to early renal injury and metabolic status. PC1 and PC2 refer to the first and second principal components derived from PCA. PC1 primarily reflects renal, hepatic, metabolic and oxidative/tubular injury parameters. PC2 predominantly represents anthropometric variables and T1D duration.

Strong positive loadings were observed for traditional renal and hepatic biochemical markers, including creatinine, urea, ASAT and ALAT, together with several metabolic parameters (glucose, cholesterol) and inflammatory/hematological indicators. These variables clustered tightly. The latter suggests that PC1 represents a combined axis of renal filtration load, hepatic metabolic stress and general biochemical background.

Tubular injury biomarkers, oxidative stress markers and MAU showed strong negative loadings on PC1. These variables also aligned with reduced eGFR, reflecting their coordinated relationship with early renal dysfunction. The separation of tubular markers from creatinine and other conventional renal function tests indicates that PC1 effectively discriminates between early tubular pathology and later-stage or systemic metabolic alterations.

PC2 was dominated by anthropometric variables, including age, height, weight, BMI and duration of T1D. These variables displayed strong positive loadings, forming a cluster that was clearly separated from biochemical and injury markers. Lower PC2 loadings were associated with stress-related physiological measures such as heart rate (pulse) and with urea (Figure 6).

3.4. Xanthine Oxidase Levels in Children with T1D and DN in Relation to DKA Episodes

XO oxidase levels exhibited lower values in T1D group at time point with no signs of DKA as compared to those measured where DKA was documented—27.95 (26.31–29.29) µU/mL vs. 30.51 (29.29–31.34) µU/mL, respectively ($p < 0.05$). A similar tendency was observed in the DN group. XO oxidase levels at the time point with no signs of DKA were

lower as compared to those measured where DKA was documented—54.79 (52.62–57.73) µU/mL vs. 60.29 (57.98–62.01) µU/mL, $p < 0.01$ (Figure 7).

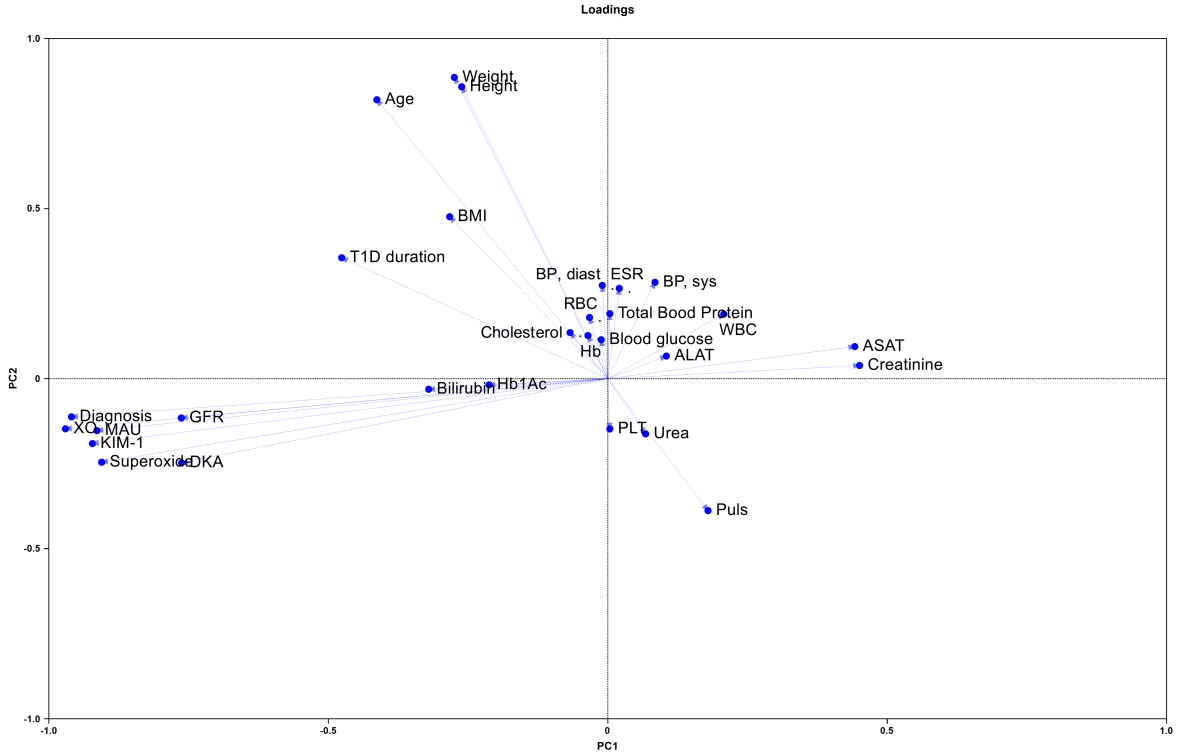


Figure 6. Principal component analysis PCA loadings plot of clinical, biochemical, oxidative and tubular injury biomarker variables.

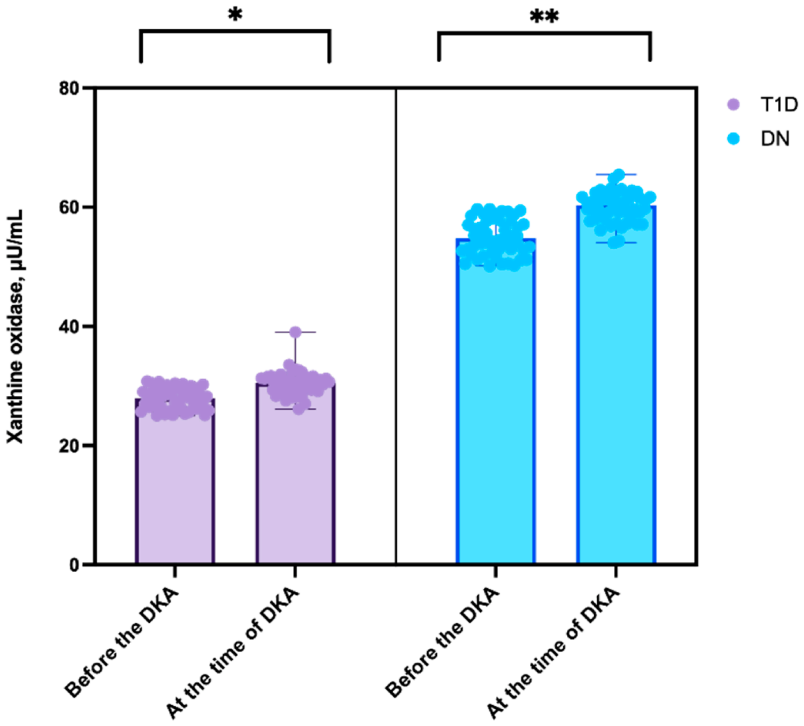


Figure 7. Xanthine oxidase levels in children with T1D and DN depending on DKA occurrence. *—$p < 0.05$, **—$p < 0.01$.

3.5. Independent Predictors of Diabetic Nephropathy and the Role of Oxidative Stress and Tubular Injury

We performed multivariable regression analysis to identify factors independently associated with urinary KIM-1 levels. The model included gender, age, duration of T1D, BMI, HbA1c, XO and superoxide production as predictor variables. Multivariable regression analysis identified XO activity and superoxide production as independent factors associated with urinary KIM-1 levels (both $p < 0.0001$). Other variables, including gender, age, T1D duration, BMI and HbA1c, were not significantly associated with KIM-1 (Table 2).

Table 2. Multivariable regression analysis of factors associated with urinary KIM-1 levels.

Variable	Estimate	95% CI (Profile Likelihood)	|t|	*p* Value
Intercept	−5.044	−6.877 to −3.211	5.465	<0.0001
Gender	0.1375	−0.3643 to 0.6393	0.5443	0.5876
Age	−0.01006	−0.1058 to 0.08564	0.2087	0.8352
T1D duration	0.02980	−0.06471 to 0.1243	0.6262	0.5327
BMI	0.02044	−0.06403 to 0.1049	0.4806	0.6320
Hb1Ac	0.08469	−0.02532 to 0.1947	1.529	0.1297
XO	0.09938	0.05857 to 0.1402	4.837	<0.0001
Superoxide production	3.122	2.342 to 3.902	7.948	<0.0001

Overall, these findings indicate that increased XO activity and superoxide levels are independently associated with higher KIM-1 levels, whereas demographic characteristics, T1D duration, BMI and glycemic control were not significant predictors after multivariable adjustment. Multivariable logistic regression analysis was done in order to identify factors independently associated with DN. Multivariable logistic regression identified T1D duration, BMI, urinary KIM-1, XO activity and superoxide production as those associated with DN. HbA1c was not significantly associated with DN (Table 3).

Table 3. Multivariable logistic regression analysis of factors associated with DN.

Odds Ratios	Variable	OR	95% CI (Profile Likelihood)	Likelihood Ratio	*p* Value
β0	Intercept	$2.345 \cdot 10^{-9}$	$8.727 \cdot 10^{-14}$ to $4.186 \cdot 10^{-6}$	46.83	<0.0001
β1	T1D duration	1.427	1.132 to 1.870	9.477	0.0021
β2	BMI	1.372	1.085 to 1.807	7.135	0.0076
β3	Hb1Ac	1.147	0.8574 to 1.566	0.8412	0.3591
β4	KIM-1	1.656	1.230 to 2.362	11.99	0.0005
β5	XO	1.125	1.012 to 1.273	4.792	0.0286
β6	Superoxide production	3.383	1.273 to 11.10	6.248	0.0124

As the next step we performed an ROC curve analysis to evaluate the discriminative performance of the multivariable logistic regression model incorporating T1D duration, BMI, HbA1c, KIM-1, XO and superoxide for distinguishing patients with DN from those without DN. The model demonstrated good discriminative ability, with an AUC of 0.933 (SE = 0.024, 95% CI: 0.886–0.980) (Figure 8).

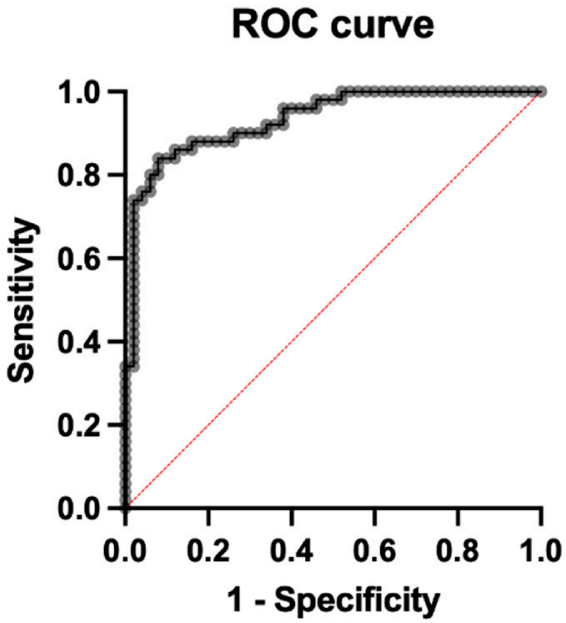


Figure 8. ROC Curve of the multivariable logistic regression model for diabetic nephropathy prediction.

3.6. Diagnostic Value of XO for Early Diabetic Kidney Damage

ROC curve analysis demonstrated that the XO had good discriminatory ability for distinguishing between the study groups. The AUC was 0.7644 (SE = 0.0473, 95% CI: 0.6717–0.8571). For superoxide production, the performed analysis showed AUC 0.7704 (SE = 0.0461, 95% CI: 0.6800–0.8608, $p < 0.0001$). AUC for KIM-1 demonstrated the level 0.7962 (SE = 0.0454, 95% CI: 0.7073–0.8851, $p < 0.0001$) (Figure 9).

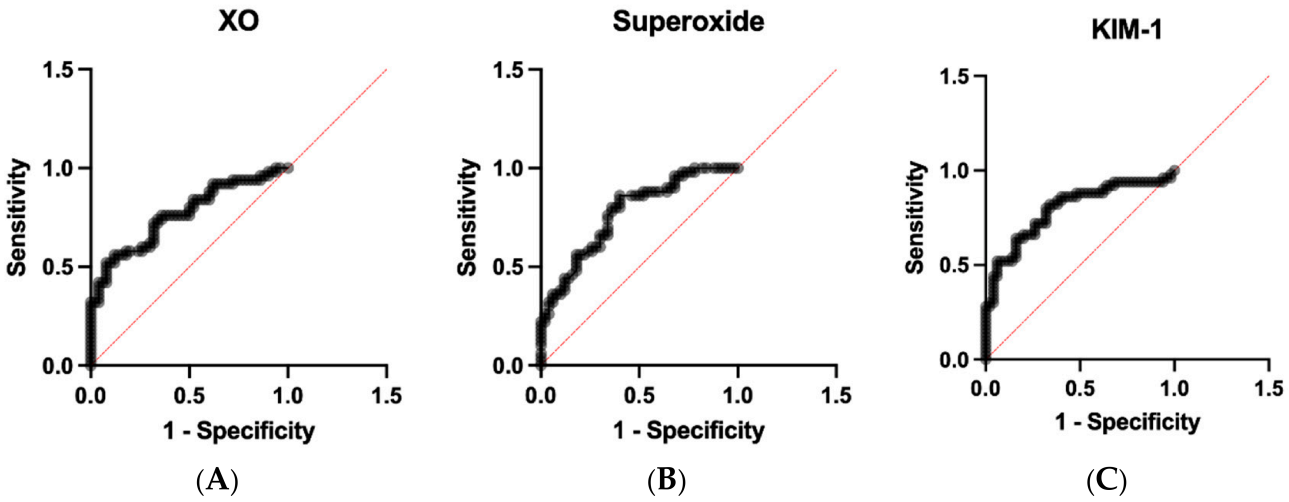


Figure 9. ROC curve of Xanthine Oxidase (**A**), superoxide (**B**), KIM-1 (**C**) for predicting diabetic nephropathy.

4. Discussion

In this study we investigated the relationship between plasma XO activity, oxidative stress effects and early markers of renal injury in children with T1D and subclinical DN. Our results demonstrate a progressive increase in XO activity, superoxide production and urinary KIM-1 levels from healthy controls to children with T1D and, further, the group with DN. These findings highlight the potential role of XO as a key contributor to oxidative stress and early renal injury in pediatric T1D.

XO contributes to oxidative stress by generating ROS. The strong positive correlations observed between XO activity and superoxide production in both T1D and DN groups ($r = 0.85$ and 0.84, respectively) support the hypothesis that XO-mediated ROS production is a major contributor to oxidative stress in pediatric diabetes. Elevated XO activity has previously been documented in adults with diabetes and in animal models of diabetes, further supporting our observations in a pediatric population [17–19]. Superoxide production increased progressively across the groups, with the highest levels in children with DN. The positive correlation between superoxide production and urinary KIM-1 underscores the link between oxidative stress and proximal tubular injury.

KIM-1 is a highly sensitive and relatively specific, noninvasive biomarker of proximal tubular injury that may detect early renal changes. Elevated urinary KIM-1 levels may reflect tubular injury before abnormalities become apparent in conventional measures of renal function. Therefore, KIM-1 may have clinical value for the early identification of children at increased risk of progressive renal injury [16,20].

Previous studies have demonstrated that elevated KIM-1 levels are associated with reduced eGFR and pronounced albuminuria and may also be associated with the progression of chronic kidney disease. In our study, XO activity was positively correlated with urinary KIM-1 levels, further supporting the association between increased XO activity, oxidative stress, and proximal tubular injury in children with early stage of DN [20].

KIM-1 was selected in the present study as a marker of early proximal tubular injury because it is closely associated with injury to proximal tubular epithelial cells. The assessment of additional biomarkers of proximal tubular injury may provide a more comprehensive understanding of early DN. KIM-1 primarily reflects proximal tubular epithelial cell injury and dedifferentiation. Other biomarkers may be involved in complementary pathophysiological processes. Neutrophil gelatinase-associated lipocalin (NGAL) is associated with tubular stress and injury. Liver-type fatty acid-binding protein (L-FABP) reflects metabolic and oxidative stress in proximal tubular cells. Retinol-binding protein 4 (RBP4) is a low-molecular-weight protein normally reabsorbed by proximal tubular cells. RBP4 may serve as an indicator of impaired tubular reabsorptive capacity when its urinary excretion increases. Therefore, simultaneous assessment of KIM-1, NGAL, L-FABP, RBP4 and XO could help distinguish different aspects of tubular injury and provide a broader characterization of early DN. The diagnostic and prognostic value of such a multimarker approach in pediatric T1D requires validation in larger longitudinal studies. These biomarkers were not measured in our study and therefore cannot be directly compared with KIM-1 [21]. However, comparative evaluation of these tubular biomarkers is an important direction for future research. In subsequent studies, we plan to assess NGAL, L-FABP, and RBP4 alongside KIM-1 to determine whether their combined assessment may provide a more comprehensive characterization of early tubular injury and improve the identification of children with T1D at risk of DN.

The parallel trends of XO activity, superoxide production and KIM-1 indicate that XO-mediated ROS generation may be a trigger of early tubular injury in children with T1D. These changes were observed before the onset of overt DN. These results are consistent with previous studies showing that oxidative stress contributes to podocyte dysfunction, tubular apoptosis and the progression of DN [22,23].

The links between XO-mediated ROS and tubular injury involves several pathways. XO generates superoxide, a potent oxidant that damages proteins, lipids and nucleic acids. ROS-mediated damage in proximal tubular cells leads to mitochondrial dysfunction, loss of membrane potential, and activation of apoptotic pathways through caspase-3 and Bax/Bcl-2 signaling. We speculate that these processes contribute to tubular cell apoptosis, epithelial-

mesenchymal transition and interstitial fibrosis [23,24]. All these disorders are hallmarks of early DN.

Additionally, oxidative stress enhances the formation of advanced glycation end-products (AGEs), which further amplify ROS production and activate pro-inflammatory pathways through receptor for AGE (RAGE) signaling. This creates a vicious cycle, where hyperglycemia-induced XO activity elevates ROS, triggers tubular apoptosis and further promotes inflammation, leading to DN progression [25].

Our data obtained with PCA revealed clustering of clinical, biochemical, oxidative and tubular injury biomarkers. PC1 included an axis of renal filtration load, hepatic metabolic stress, and oxidative injury, with strong positive loadings for creatinine, urea, ASAT and ALAT. At the same time there was a negative loading for tubular injury markers, oxidative stress markers and microalbuminuria. These results indicate that early tubular injury and oxidative stress represent a distinct pathological process from conventional biochemical changes and systemic metabolic alterations. Taking these findings together, we suggest that XO activity and tubular injury biomarkers provide additional, clinically relevant information beyond traditional biochemical or demographic measures.

We observed that XO activity was higher during episodes of DKA in both T1D and DN groups. DKA is a T1D complication characterized be acute metabolic stress due to hyperglycemia, ketonemia and dehydration. All of these changes can increase ROS generation. The significant rise in XO during DKA episodes highlights the dynamic nature of oxidative stress in pediatric diabetes. It also highlights the importance of acute metabolic decompensation in renal injury triggering. This finding is in line with prior reports showing elevated oxidative stress markers and worsened renal function during DKA episodes. The increase in XO from T1D without nephropathy to DN during DKA supports the hypothesis that repeated DKA accelerates renal tubular damage [25,26]. Urinary KIM-1 was not serially assessed during DKA and non-DKA episodes. Therefore, the potential association between acute increase in XO during metabolic decompensation and dynamic changes in tubular injury, which is reflected by KIM-1, remains to be investigated. Further longitudinal studies with simultaneous assessment of oxidative stress and tubular injury biomarkers during DKA episodes and recovery are warranted.

Multivariable regression analysis showed that XO and superoxide production were independently associated with urinary KIM-1 levels after adjustment for gender, age, T1D duration, BMI and HbA1c. These data further support an association between oxidative stress and tubular kidney injury in children with early DN.

Our findings are consistent with previous studies showing that increased oxidative stress is associated with early renal tubular injury in diabetes in adults and that urinary KIM-1 may increase before conventional markers of diabetic kidney disease become abnormal [27,28].

Taken together with the multivariable regression findings, these results indicate that the model incorporating clinical factors and biomarkers of tubular injury and oxidative stress provides excellent discrimination of DN status. In particular, T1D duration, BMI, KIM-1, XO and superoxide production were independently associated with DN. The overall model achieved an AUC of 0.933. ROC curve analysis further supported the diagnostic relevance of oxidative stress markers, demonstrating good discriminatory performance of XO, superoxide production and KIM-1 for identifying DN.

These findings suggest that combining conventional clinical characteristics with markers reflecting tubular kidney injury and oxidative stress may provide a useful approach for identifying patients with T1D who have a higher probability of DN. A summarized scheme of our hypothesis is presented in Figure 10.

Clinically, these findings have several implications. XO may serve as a biomarker for early oxidative stress and impending renal injury in pediatric diabetes. Interventions targeting XO activity, such as allopurinol or febuxostat, could reduce oxidative stress and prevent kidney damage. Studies in adults have shown that XO inhibition reduces oxidative stress, improves endothelial function and slows down the progression of kidney disease [23]. Monitoring urinary KIM-1 in parallel with XO and superoxide production can be a potential instrument in early identification of children for DN risk.

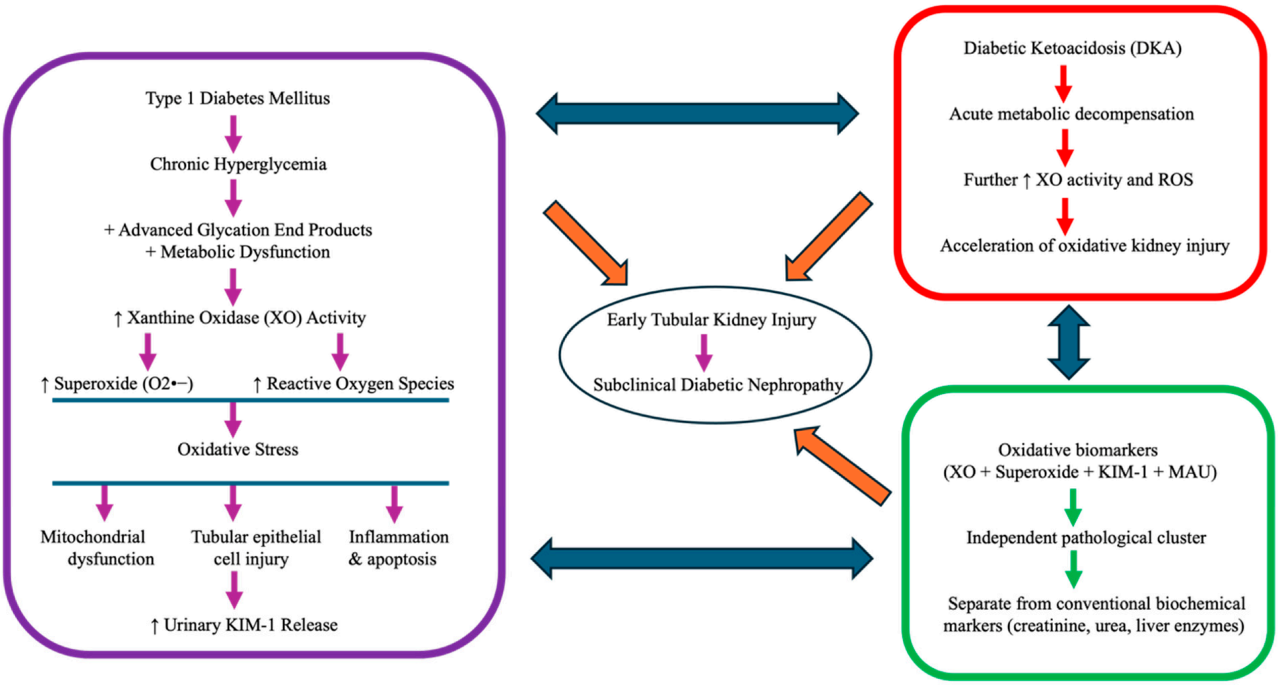


Figure 10. Pathophysiological model of early DN injury in children with Type 1 Diabetes.

Our study has limitations. The cross-sectional design limits our ability to assess the longitudinal impact of XO activity on renal outcomes. Longitudinal studies are needed to determine whether elevated XO predicts progression from subclinical nephropathy to overt DN. Our cohort was recruited from a single center with a relatively small sample size, which may limit generalizability. KIM-1 was measured only once, so we could not assess its dynamics and long-term prognostic value. Finally, although KIM-1 was chosen as a marker that most directly reflects proximal tubular damage, other tubular markers, such as NGAL, L-FABP and RBP4, were not measured in our study. Therefore, we cannot directly state that KIM-1 has a better diagnostic value than these markers. At the same time, the choice of KIM-1 was due to its close association with damage to proximal tubule epithelial cells and its relevance to the purpose of our study. A further limitation is that endogenous SOD activity was not inhibited during the superoxide assay. Therefore, SOD-mediated dismutation of superoxide may have influenced cytochrome c reduction and introduced a potential confounding factor, limiting the interpretation of the assay as a direct quantitative measure of superoxide production. Another limitation of the study is that the urinary albumin-creatinine ratio (ACR) was not measured.

5. Conclusions

Plasma XO activity increases progressively from healthy children to those with T1D and further to children with DN. XO levels show a positive correlation with superoxide production and urinary KIM-1. Episodes of DKA exacerbate XO activity, highlighting acute

metabolic stress as a potential driver of oxidative injury. The regression model has shown that XO and superoxide production were independently associated with urinary KIM-1. Multivariable regression and ROC analyses showed that XO and superoxide production were independently associated with tubular injury. Together with KIM-1 and clinical factors, these markers were associated with DN. These findings indicate that oxidative stress and tubular injury biomarkers may be useful for early identification of diabetic nephropathy in children with T1D. Future longitudinal and interventional studies are needed to validate these observations and to establish XO inhibition as a strategy to prevent or delay renal complications in children with T1D.

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Institutional Review Board Statement: This study was conducted in accordance with the principles outlined in the Declaration of Helsinki, as well as the recommendations established by ethical committee of the Bogomolets National Medical University (No. 4/2024, dated 11 December 2022).

Informed Consent Statement: Prior to the start of the study, all participants received comprehensive information regarding the purpose, methods, potential risks and potential benefits of participation. Participation in the study was based on the principle of informed consent, and all respondents signed an appropriate consent form before data collection began. Participants had the right to withdraw from the study at any time without providing a reason. In the case of research involving minors or individuals with limited legal capacity, informed consent was obtained from their legal representatives. In accordance with confidentiality standards, all data were collected anonymously and processed in compliance with the applicable data protection legislation, including the General Data Protection Regulation (GDPR), to ensure the protection of personal information. All information was used exclusively within the scope of this study and was aggregated for subsequent analysis of the results.

Data Availability Statement: Data are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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