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# SERUM LEVELS OF LIPOCALIN-2/NGAL, KIDNEY INJURY MOLECULE 1 (KIM-1), AND INTERLEUKIN-18 ARE NOT RELATED TO RISK PREDICTIONS FOR CARDIOVASCULAR AND RENAL EVENTS IN TYPE 2 DIABETES

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***Abstract.*** *Type 2 diabetes is currently one of the leading causes of cardiovascular and renal morbidity and mortality. The quest for reliable biologic markers for future cardiovascular and renal events is still ongoing.* ***Objective.*** *This study was designed to explore serum levels of Neutrophil Gelatinase-Associated Lipocalin (NGAL), Kidney Injury Molecule-1 (KIM-1), and Interleukin-18 (IL-18) in type 2 diabetes patients and to describe their relationship with computer-based predictions for future cardiovascular (CV) and renal events.* ***Materials and methods.*** *This was a cross-sectional observational study. One hundred sixty-five patients with type 2 diabetes participated: 1) group A – non-insulin hypoglycemic treatment (84 patients); 2) group B on insulin treatment (with or without added oral agents, 81 patients). The glycemic and metabolic parameters were assessed by routine methods: glycated hemoglobin A1c in % and mmol/mol, lipid profiles (mmol/l), serum creatinine in μmol/l, urinary albumin/creatinine ratio (ACR in mg/mmol/l and μg/mg), and estimated glomerular filtration rate (eGFR in ml/min/1.72 m$^2$) using the MDRD formula. Interleukin-18 and Lipocalin-2/NGAL were measured by ELISA (BioVendor), while Human KIM-1 was measured using the FineTest Kit (Wuhan Fine Biotech Co.). Cardiovascular risks were calculated using the UKPDS version 2.0 and the ADVANCE risk engines. In addition, the ADVANCE risk engine allowed predictions about future renal events. All statistical analyses were performed on an IBM SPSS 19.0 for Windows platform (SPSS Corp., Chicago, IL).* ***Results.*** *The three markers did not differ between patients treated with insulin or non-insulin drugs. We found minor differences in levels of NGAL and IL-18 in patients with CKD grade 4 only, as well as of KIM-1 in CKD grade 1. No intergroup differences based on the presence/grade of albuminuria were detected. No differences in the three kidney markers were detected among the three risk groups for coronary artery disease based on the ADVANCE and UKPDS formulas. No differences were found in the three ADVANCE-based risk groups for renal events or new-onset albuminuria except for the levels of KIM. The correlation analyses were unable to find a relationship between the renal markers and the calculated risk for CV or renal events.* ***Conclusion.*** *Our results show an almost complete lack of relationship between the three markers for kidney damage (IL-18, NGAL, and KIM-1) and the calculations of long-term CV- and renal risks. The presence/absence of CKD and/or albuminuria did not affect the mean levels of the three markers. Therefore, those*

*markers should be regarded as indicators for acute kidney damage only, without any direct relationship to the actual renal or CV-conditions or calculated future risks.*

***Key words:*** *type 2 diabetes, cardiovascular, renal, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, Interleukin-18.*

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## INTRODUCTION

Diabetes mellitus, and type 2 in particular, has evolved as a leading cause of cardiovascular and end-stage renal disease and renal replacement in recent decades. Despite efforts for improvement of metabolic control, the prevalence of chronic kidney disease (CKD) remains unchanged in diabetes. The quest for reliable biologic markers with a good prognostic value for future renal events, such as worsening CKD stage or albuminuria, is still ongoing [1, 2]. At best, these markers could also be of use in the prognosis of future cardiovascular events in diabetes [3].

Lipocalin-2 (LCN2), also called NGAL (neutrophil gelatinase-associated lipocalin), is upregulated in cells under different forms of biological stress (e.g., infection, inflammation, involution to ischemia, or neoplastic transformation). Recent reports suggest that LCN2 might represent a sensitive biomarker for early renal injury and may play a causative role in the pathogenesis of obesity-induced metabolic disorders, such as insulin resistance, Type 2 Diabetes Mellitus, and cardiovascular disorders [4-6].

Interleukin (IL)-18 is a newly discovered cytokine with profound effects on T-cell activation, primarily by its ability to induce IFN gamma production in T cells and natural killer (NK) cells. IL-18 induces gene expression and synthesis of tumor necrosis factor (TNF), IL-1, Fas Ligand, and several chemokines, as well as the differentiation of Th1 or Th2 cells. An important role of IL-18 has been shown in clinical implications, in the role of IL-18 modulation in tumours, infections, and autoimmune and inflammatory diseases, and more recently in the pathogenesis and possibly prediction of vascular events in diabetes and metabolic syndrome [7, 8].

Human Kidney Injury Molecule 1 (KIM-1) is synthesized almost uniquely in the kidney as a response to the accumulation of apoptotic epithelial cells on the basal membrane. Its role as a marker of proximal tubular injury and worsening renal function is undisputed [9-11]. Recent research focuses on its relationship to cardiovascular events in the setting of type 2 DM, heart disease, and chronic kidney disease (CKD) [12-14].

The aim of this study was to measure serum levels of NGAL, KIM-1, and IL-18 in type 2 diabetes patients and to explore their possible relationship with the presence of diabetic nephropathy, as well as with risk-engine predictions for future CV and renal events.

## MATERIALS AND METHODS

This was a cross-sectional observational study. It was approved by the Ethical Committee and the Council for Medical Science at Medical University – Sofia, and was conducted according to the international ethical standards and the Declaration of Helsinki. All participants signed informed consent prior to any procedure.

One hundred and sixty-three patients with type 2 diabetes participated. They were selected from inpatients of our tertiary endocrine clinic referred for an annual check-up of their metabolic status. Inclusion criteria were previously diagnosed type 2 diabetes with at least 6-month duration on stable treatment for the last 3 months, and willingness to participate. Exclusion criteria included acute diabetic complications (such as diabetic ketoacidosis or hyperosmolar conditions), as well as acute illness (including cerebrovascular events in the acute/subacute phase). Exclusion criteria concerning the kidney were end-stage renal disease/kidney transplant, acute infections of the genitourinary tract, and previously diagnosed renal pathology other than inactive chronic pyelonephritis. Acute conditions affecting urinary protein excretion (such as excessive exercise, uncontrolled hy-

pertension, urinary infection, febrile conditions, etc.) were also applied as exclusion criteria. Cardiovascular events in the acute or subacute phase were also used as exclusion criteria.

The participants were divided into 2 subgroups according to their treatment regimen: 1/ group A – non-insulin hypoglycemic treatment; 2/ group B on insulin treatment (with or without added oral agents such as metformin, sulphonylurea, DPP-IV inhibitors).

***Measurements and risk calculations***

Medical history was collected based on the current status and the available previous documentation. Physical examination was performed. Height in cm and weight in kg were measured on a calibrated scale (Tanita Corp., Japan). The BMI was calculated as kg/m$^2$. The waist was measured in cm at the umbilical level, and the hip circumference at the widest part of the buttocks or hip. Waist-to-hip (WHR) was calculated. The blood pressure was measured after a 5-minute rest in the sitting position with an aneroid sphygmomanometer and recorded in mm Hg.

Morning blood samples were collected after a 12-hour overnight fasting, centrifuged at 4,000 rpm for 15 minutes, and the supernatant was refrigerated and transferred to the laboratory.

The glycemic and metabolic parameters were assessed by routine methods: glycated hemoglobin A1c in % and mmol/mol, lipid profiles (total cholesterol, HDL-cholesterol, calculated LDL-cholesterol, triglycerides – all in mmol/l), serum creatinine in μmol/l, urine measurements for albumin/creatinine ratio (ACR in mg/mmol/l and μg/mg) in a fresh morning urine sample. Estimated glomerular filtration rate (eGFR) was calculated in ml/min/1.72 m$^2$ using the Modification of Diet in Renal Disease (MDRD) formula.

***Serum markers related to kidney injury***

Interleukin-18 was measured by ELISA (BioVendor; Cat. No.: RAF143R) with a limit of detection of 9 pg/ml, an overall intra-assay coefficient of variation (CV) of 6.5%, and an inter-assay coefficient of variation 8.1%. The reference range suggested by the manufacturer was from not detectable to 732.7 pg/mL.

Human KIM-1(Kidney Injury Molecule 1) was measured by ELISA. We used the FineTest Kit (Wuhan Fine Biotech Co., Ltd.; Cat. No.: EH0210). The manufacturer-provided expected range was 0.156-10 ng/ml with a sensitivity < 0.094 ng/ml, Intra-Assay CV < 8%, and Inter-Assay CV < 10%.

Lipocalin-2/NGAL (neutrophil gelatinase-associated lipocalin) was measured by ELISA (BioVendor Cat. No.: RD191102200R). The limit of detection of this specific ELISA kit is 0.02 ng/ml. The intra-assay CV% is 7.0-8.4% and the inter-assay CV% 9.8% (data provided by the manufacturer). For men aged 50 years and older, the manufacturer provided a maximum value of 169.2 ng/ml, and for women of the same age, a maximum of 276.0 ng/ml.

Cardiovascular risks were calculated using the UKPDS version 2.0 and the ADVANCE risk engines. The UKPDS and the ADVANCE calculators were selected as very well-validated diabetes-specific tools [15, 16]. All formulas calculated absolute risks in % for a different period of time. The UKPDS risk engine calculated separate 10-year risks for coronary heart disease (CHD) and stroke [15]. The ADVANCE risk engine produced a composite 4-year CV-risk [16]. In addition, the ADVANCE risk engine allowed predictions about future renal events and new-onset albuminuria.

***Statistical analysis***

All analyses were performed on an IBM SPSS 19.0 for Windows platform (SPSS Corp., Chicago, IL). The sample size was selected based on a theoretical minimum of 150 participants divided into two subgroups, allowing correlation and regression analyses. Descriptive statistics and frequency analysis were performed. Data were checked for normal distribution based on their skewness and kurtosis, as well as on Q-Q plots. Correlation analysis was performed, and the corresponding Spearman's Rho was recorded. The regression analyses included 10 curves (linear, quadratic, cubic, power, S, inverse, exponential, log models, growth, compound models), and the best models were selected based on the highest R2 and the lowest p-value. Statistical significance was set as $p < 0.05$. A power analysis was performed post hoc and revealed the significance of the study based on already known correlation/regression coefficients and a confidence interval of 95%.

## RESULTS

Eighty-four patients with type 2 diabetes on oral and non-insulin diabetes treatment and 81 patients on insulin were found eligible for this study during an 18-month enrollment period. Their basic anthropometric and clinical data are summarized in Table 1.

Table 1 shows that the basic anthropometric and clinical data of the two subgroups did not differ significantly, except for the longer diabetes duration, the higher glycated hemoglobin A1c ($p = 0.004$), s-Creatinine ($p = 0.002$), albumin-to-creatinine ratio ($p < 0.001$) and lower estimated GFR ($p = 0.023$) in Group B (on insulin), compared to non-insulin treated patients. The serum levels of the three renal markers did not differ between the two subgroups. Serum Lipocalin 2/NGAL were below the manufacturer-provided upper normal

**Table 1.** The basic anthropometric, clinical, and metabolic data of the participants are displayed (A – on oral and injectable antidiabetic drugs other than insulin; B – on insulin-based therapeutic regimen)

| | Study group | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| Age (years) | A | 58.3 | 9.2 | 33 | 81 |
| | B | 61.8 | 10.3 | 32 | 83 |
| Age at diabetes diagnosis (years) | A | 51.9 | 10.0 | 23 | 73 |
| | B | 48.2 | 11.9 | 27 | 83 |
| Diabetes duration (years)* | A | 6.4 | 6.7 | 1 | 37 |
| | B | 13.6 | 8.7 | 1 | 37 |
| Systolic BP (mm Hg) | A | 134.4 | 18.2 | 70 | 200 |
| | B | 131.4 | 20.0 | 90 | 200 |
| Diastolic BP (mm Hg) | A | 83.2 | 10.8 | 50 | 120 |
| | B | 78.7 | 10.9 | 50 | 100 |
| Body mass index (kg/m$^2$) | A | 33.0 | 5.8 | 19.3 | 53.2 |
| | B | 32.1 | 6.9 | 20.4 | 54.1 |
| Waist (cm) | A | 104.7 | 16.9 | 70 | 139 |
| | B | 102.4 | 16.8 | 66 | 143 |
| Glycated hemoglobin A1c (%) * | A | 8.3 | 2.1 | 5.0 | 15.8 |
| | B | 10.2 | 2.5 | 6.0 | 15.2 |
| Triglycerides, mmol/l | A | 2.45 | 2.11 | 0.46 | 13.97 |
| | B | 2.85 | 2.63 | 0.60 | 13.76 |
| LDL-Chol., mmol/l | A | 2.97 | 1.10 | 0.40 | 5.88 |
| | B | 2.64 | 1.38 | 0.67 | 7.26 |
| eGFR-MDRD ml/min/1.73 m$^2$ * | A | 81.5 | 25.7 | 33.3 | 153.5 |
| | B | 70.4 | 30.5 | 9.3 | 147.4 |
| ACR, mg/mmol * | A | 15.5 | 65.0 | 0.28 | 492.6 |
| | B | 48.5 | 141.9 | 0.22 | 898.7 |
| IL-18, pg/ml | A | 403.3 | 321.6 | 10.3 | 1,866.8 |
| | B | 372.9 | 270.7 | 15.9 | 1,546.6 |
| NGAL, ng/ml | A | 64.3 | 32.5 | 14.0 | 162.2 |
| | B | 64.0 | 34.9 | 14.5 | 161.3 |
| KIM-1, ng/ml | A | 2.01 | 5.12 | 0.01 | 20.00 |
| | B | 2.08 | 5.47 | 0.02 | 20.00 |

* A significant difference is registered between subgroups A and B ($p < 0.05$)

limit (UNL) in all patients, while those of serum KIM-1 and IL-18 exceeded the UNL in 4.8% and 7.1% of patients of group A (non-insulin), and in 2.5% and 16.3% in group B (on insulin), respectively.

The estimated risks for cardiovascular and renal events according to the ADVANCE formula are presented in Table 2. In addition, the estimation of risks for coronary heart disease and stroke according to the UKPDS engine is also listed.

As shown in Table 2, all calculated risks for CV- and renal events were significantly higher in Group B (on insulin).

**Table 2.** ADVANCE- and UKPDS-based calculations of risks for CV and renal events

| Risk subgroups | Study group | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| Overall CV-risk (ADVANCE-based), % * | A | 3.9% | 3.5% | 0.5% | 16.5% |
| | B | 8.3% | 7.1% | 1.1% | 38.3% |
| ADVANCE-based risk for new onset albuminuria, % * | A | 48.5% | 28.4% | 19.7% | 100.0% |
| | B | 67.8% | 33.2% | 16.8% | 100.0% |
| ADVANCE-based risk for major renal events, % | A | 0.5% | 1.1% | 0.0% | 6.0% |
| | B | 4.0% | 8.0% | 0.0% | 31.8% |
| UKPDS-based risk for coronary disease, % * | A | 22.4% | 15.5% | 2.6% | 73.6% |
| | B | 32.5% | 18.8% | 7.4% | 98.9% |
| UKPDS-based risk for stroke, % * | A | 10.6% | 13.6% | 1.1% | 88.1% |
| | B | 20.2% | 20.1% | 1.0% | 89.7% |

* Significant differences were found between subgroups A and B ($p < 0.05$)

Table 3 shows the serum levels of the renal markers stratified by the patients' CKD stage (based on eGFR) and the presence/absence of albuminuria.

The intergroup differences were significant for NGAL and IL-18 in CKD grade 4 (p = 0.009 and p = 0.013, respectively), as well as for KIM-1 in CKD grade 1 (p = 0.003). No intergroup differences based on the presence/grade of albuminuria were detected.

The serum levels of the studied markers for renal damage stratified to the calculated risk for coronary disease (based on the ADVANCE and UKPDS engines), as well as for major renal events and new-onset albuminuria (ADVANCE engine only), are presented in Table 4.

**Table 3.** Serum levels of the three renal markers stratified to the patients' CKD stage (1 to 4, no patients with end-stage renal disease were included) and the presence of albuminuria (ACR, mg/mmol: 0 – < 30 mg/mmol; 1 – 30 до 300 mg/mmol; 2 – > 300 mg/mmol)

| | N | Variable | IL-18, pg/ml | NGAL, ng/ml | KIM-1, ng/ml |
|---|---|---|---|---|---|
| **CKD stage based on eGFR** | | | | | |
| **0** | 25 | Mean | 337.9 | 63.9 | 0.5* |
| | | Std. Deviation | 243.7 | 47.0 | 1.0 |
| **1** | 35 | Mean | 401.7 | 71.0 | 2.7 |
| | | Std. Deviation | 265.9 | 34.4 | 6.3 |
| **2** | 68 | Mean | 372.3 | 60.0 | 1.8 |
| | | Std. Deviation | 300.0 | 30.0 | 4.9 |
| **3** | 30 | Mean | 389.9 | 69.8 | 2.4 |
| | | Std. Deviation | 347.5 | 34.4 | 6.0 |
| **4** | 6 | Mean | 609.2* | 42.9* | 2.5 |
| | | Std. Deviation | 271.8 | 10.8 | 5.2 |
| **CKD based on albuminuria** | | | | | |
| **0** | 108 | Mean | 382.2 | 63.9 | 1.9 |
| | | Std. Deviation | 319.5 | 33.9 | 1.9 |
| **1** | 36 | Mean | 391.8 | 66.2 | 2.0 |
| | | Std. Deviation | 230.6 | 36.1 | 2.3 |
| **2** | 20 | Mean | 443.9 | 64.4 | 3.3 |
| | | Std. Deviation | 288.5 | 28.3 | 3.8 |

* if p < 0.05 for the difference with the next subgroup

**Table 4.** The serum levels of the studied markers for renal damage stratified to the risk for coronary disease, calculated with the ADVANCE and UKPDS engines (upper part), and to the risk for major renal events and new-onset albuminuria – ADVANCE engine only (lower part) are displayed

ADVANCE: 1 – low risk (< 4%); 2 – moderate risk (4-8%); 3 – high risk (>8%);

UKPDS: 1 – low risk (15%), 2 – moderate risk (15-20%), 3 – high risk (20-30%), and 4 – very high risk (≥ 30%).

| The three markers (KIM-1, NGAL and IL-18) stratified to risk-engine and calculated risk | Risk strata | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| **ADVANCE formula - risk for coronary disease** | | | | | |
| **IL-18, pg/ml** | **1** | 365.4 | 295.4 | 10.3 | 1,546.6 |
| | **2** | 467.0 | 358.4 | 10.3 | 1,866.8 |
| | **3** | 344.2 | 208.9 | 15.9 | 783.1 |
| **NGAL, ng/ml** | **1** | 61.5 | 34.7 | 13.9 | 162.2 |
| | **2** | 66.8 | 31.5 | 19.5 | 146.2 |
| | **3** | 67.7 | 33.6 | 20.7 | 161.3 |
| **KIM-1, ng/ml** | **1** | 2.2 | 5.4 | 0 | 20.0 |
| | **2** | 2.4 | 5.8 | 0 | 20.0 |
| | **3** | 1.3 | 4.4 | 0 | 20.0 |
| **Risk level for CV-disease (UKPDS formula)** | | | | | |
| **IL-18, pg/ml** | **1** | 437.2 | 338.1 | 34.7 | 1,546.6 |
| | **2** | 345.6 | 254.5 | 11.1 | 898.9 |
| | **3** | 343.3 | 266.9 | 10.3 | 1,115.4 |
| | **4** | 400.0 | 309.7 | 13.5 | 1,866.8 |

| The three markers (KIM-1, NGAL and IL-18) stratified to risk-engine and calculated risk | Risk strata | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| NGAL, ng/ml | 1 | 61.6 | 31.0 | 13.9 | 162.2 |
| | 2 | 64.0 | 38.5 | 15.1 | 145.2 |
| | 3 | 60.6 | 32.4 | 18.3 | 146.3 |
| | 4 | 69.0 | 34.7 | 14.5 | 161.3 |
| KIM-1, ng/ml | 1 | 2.1 | 5.4 | 0 | 20.0 |
| | 2 | 3.1 | 6.4 | 0 | 20.0 |
| | 3 | 1.3 | 3.9 | 0 | 20.0 |
| | 4 | 1.5 | 4.6 | 0 | 20.0 |
| ADVANCE formula – risk for new onset of albuminuria | | | | | |
| IL-18, pg/ml | 1 | 371.6 | 299.7 | 10.3 | 1,546.6 |
| | 2 | 384.6 | 383.4 | 10.3 | 1,866.8 |
| | 3 | 411.7 | 240.3 | 65.8 | 916.6 |
| NGAL, ng/ml | 1 | 58.8 | 30.4 | 14.0 | 145.2 |
| | 2 | 74.3 | 39.2 | 23.7 | 162.2 |
| | 3 | 65.8 | 33.4 | 16.1 | 161.3 |
| KIM-1, ng/ml | 1 | 2.3 | 5.5 | 0.0 | 20.0 |
| | 2 | 0.5 | 1.1 | 0.0 | 5.1 |
| | 3 | 2.7 | 6.2 | 0.0 | 20.0 |
| ADVANCE formula risk for major renal events | | | | | |
| IL-18, pg/ml | 1 | 382.1 | 304.9 | 10.3 | 1,866.8 |
| | 2 | 363.4 | 259.2 | 69.0 | 874.4 |
| | 3 | 467.5 | 273.0 | 65.8 | 916.6 |
| NGAL, ng/ml | 1 | 65.1 | 34.0 | 14.0 | 162.2 |
| | 2 | 58.6 | 35.6 | 18.3 | 143.9 |
| | 3 | 63.8 | 27.9 | 35.9 | 121.0 |
| KIM-1, ng/ml | 1 | 2.0 | 5.2 | 0.0 | 20.0 |
| | 2 | 1.4 | 5.0 | 0.0 | 20.0 |
| | 3 | 3.7 | 6.6 | 0.0 | 20.0 |

No differences for the three kidney markers were registered among the three risk groups for coronary artery disease based on the ADVANCE formula, as well as for the risk of CV-events based on the UKPDS risk engine. Significant differences were found only in the levels of KIM ($p = 0.030$, both for comparison of low and moderate risk for albuminuria, as well as when comparing moderate- and high-risk subgroups).

The correlation analyses were unable to find a relationship between the renal markers and the calculated risk for CV or renal events (Table 5). Our results were, therefore, negative.

Table 5 underlines the lack of a stable relationship between the three studied renal markers and the calculated risk for CV and renal complications.

## DISCUSSION

We performed a cross-sectional study in type 2 diabetes patients, trying to find differences in three mark-

**Table 5.** Correlation analysis: markers for renal damage are not related to the risks of CV-events (UKPDS and ADVANCE risk engines) and of major renal events (ADVANCE risk engine). The Spearman's rank coefficients are listed below.

| | | ADVANCE-based CV-risk, % | ADVANCE-based risk for new onset albuminuria, % | ADVANCE-based risk for major renal events, % | UKPDS-based risk for coronary disease, % | UKPDS-based risk for stroke, % |
|---|---|---|---|---|---|---|
| IL-18, pg/ml | Spearman's rho | 0.035 | 0.100 | 0.070 | -0.073 | 0.013 |
| | Sig. (2-tailed), p | 0.671 | 0.220 | 0.388 | 0.376 | 0.874 |
| NGAL, ng/ml | Spearman's rho | 0.061 | 0.092 | 0.019 | 0.065 | 0.055 |
| | Sig. (2-tailed), p | 0.442 | 0.249 | 0.807 | 0.415 | 0.494 |
| KIM-1, ng/ml | Spearman's rho | -0.093 | 0.016 | -0.015 | -0.067 | -0.092 |
| | Sig. (2-tailed), p | 0.240 | 0.843 | 0.845 | 0.404 | 0.253 |

ers for kidney damage (Interleukin-18, N-Galectin, and KIM-1) according to the presence/absence of diabetic nephropathy (CKD and/or albuminuria) or according to the treatment regimen (oral agents and GLP-1 analogs versus insulin). In addition, correlations between the three serum markers and different types of calculated risks for cardiovascular and renal events (based on the UKPDS and ADVANCE risk engines) were explored. The three markers did not differ between patients treated with insulin or non-insulin drugs. We found minor differences in levels of NGAL and IL-18 only in patients with CKD grade 4, as well as of KIM-1 in CKD grade 1. No intergroup differences based on the presence/grade of albuminuria were detected. No differences in the three kidney markers were detected among the three risk groups for coronary artery disease based on the ADVANCE formula, as well as for the risk of CV-events based on the UKPDS risk engine. The same lack of differences was found in the three ADVANCE-based risk groups for renal events or new-onset albuminuria, except for the levels of KIM. The correlation analyses were unable to find a relationship between the renal markers and the calculated risk for CV or renal events. In summary, our results show almost complete lack of relationship between the three markers for kidney damage (IL-18, NGAL, and KIM-1) and the calculations of long-term CV- and renal risks. Even the presence/absence of CKD and/or albuminuria did not affect the mean levels of the three markers. Therefore, those markers should be regarded as indicators for acute kidney damage, only without a direct relationship to the actual renal or CV-conditions or calculated future risks.

Of note, the three biomarkers under study (NGAL, KIM-1, and IL-18) have been extensively explored as urinary biomarkers to predict renal progression and end-stage renal disease in type 2 diabetes patients [17-19]. In a study of 257 type 2 diabetes patients, urine KIM-1 and urine NGAL were significantly associated with rapid renal deterioration when compared with the patients in the lowest quartiles (Hazard ratio 2.77 and 2.53, respectively) [18]. In a more recent study, 952 type 2 diabetes patients were screened for nephropathy, and IL-18 emerged as a potent marker to diagnose diabetic nephropathy during pre-albuminuric stages [19]. Older studies were not very optimistic about the diagnostic utility of the novel biomarkers compared to the established ones – eGFR and albuminuria [1, 17]. CKD is a risk factor for CVD per se. This explains the interest in the three biomarkers as indicators of uremic cardiotoxicity [7], or as a link between renal and heart failure or coronary syndromes [12].

NGAL/Lipocalin seems to be most extensively studied as a predictor of worsening kidney function or risk of CV events [6, 13, 20, 21]. A cross-sectional study including 180 hospitalized type 2 DM patients documented an area under the curve for serum NGAL of 0.8 in the detection of early renal damage [20]. In 386 patients who underwent coronary angiography, the area under the curve for discrimination of severe coronary stenosis by NGAL was 0.838 [6]. Urinary NGAL/Lipocalin remained independently associated with ischemic atherosclerotic events in 3,386 participants with a eGFR between 20 and 70 ml/min/1.73 m2 [21]. In the setting of coronary heart disease (633 patients), increased levels of NGAL were independently linked with elevated risk of CV death (HR = 2.62) during a 10-year follow-up period [22].

IL-18 is not so well studied. Its predictive role was explored mainly as a marker of diabetic nephropathy [8, 19]. Its role as a cardiovascular marker is still under debate.

All the above studies documented a link between the three biomarkers with both worsening kidney function and CV events. Of note, they were rather prospective or, if cross-sectional, related the biomarkers to real events. Our study related the biomarker levels with estimated risks coming from risk engine calculations, not with real events. This is a major difference, as calculated risks are quite different from realized events. Another limitation of our study is that we measured serum levels of KIM-1, NGAL/Lipocalin, and IL-18, while their urinary levels are more widely used and better validated, as can be seen from the above-cited studies.

The molecular mechanisms of diabetic kidney disease include a variety of different biological pathways. L. Gaydarski et al. give an excellent overview of the involvement of the apelinergic system, the VEGF/VEGF-receptor, and the nitric oxide metabolism [23]. Of note, NGAL and Interleukin-17 might be key factors in the renal damage caused by Coronavirus infections [24]. In addition, hypertension modulates the renal response through different vascular adaptive changes [25]. Hypertension-induced renal damage was studied in rats and revealed the major contribution of the apelinergic system [26]. This is why it is difficult to discern the influence of a single biomarker. This may be one explanation for our rather negative results.

## CONCLUSION

In conclusion, our study is one of the few ones trying to find a link between serum markers of acute kidney damage and calculated renal and CV risks. The lack of correlation might be interpreted as the inability of markers of acute kidney injury to predict renal and CV events in the distant future. Given the amount of positive studies addressing the issue, further research is well warranted.

***Conflicts of interest:*** *All authors report no conflicts of interest related to this study.*

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