In search of novel uremic toxins: a proteomics-based pilot ELISA study

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Abstract:

Introduction: Uremic toxins are harmful metabolites that accumulate in the body of patients in parallel with the loss of renal function. Based on the results from a previous shotgun proteomics study that identified lumican, matrix remodeling associated 5 (MXRA5), neuropilin 1 (NRP1), and leucine-rich alpha-2-glycoprotein (LRG) as potential uremic toxins, we conducted a small pilot study in order to determine their expression levels in plasma and urine across the stages of chronic kidney disease (CKD).

Materials and methods: We conducted a cross-sectional study in which participants were divided into six subgroups (CKD1-5 and healthy controls). We determined the expression levels of lumican, MXRA5, NRP1 and LRG from blood plasma and expression levels of MXRA5, NRP1 and LRG from urine using enzyme-linked immunosorbent assays.

Results: This study included a total of N=40 participants, divided across 6 subgroups. We found no statistically significant differences in blood plasma expression levels between the subgroups for any of the assessed protein. However, we found that urinary concentration of NRP1 and LRG to be statistically significantly higher in the CKD stages 2-5 group as compared to the healthy + CKD1, with arithmetic mean of NRP1 being 3.2 times higher and arithmetic mean of LRG 19.4 times higher in the CKD stages 2-5 comparing to healthy + CKD1.

Conclusions: We did not find that any of the assessed proteins followed the expected kinetics which would be expected for uremic toxins. However, urinary LRG and NRP1 could potentially be new biomarker candidates for CKD – but further research is needed.

Keywords:

chronic kidney disease, uremic toxins, NRP1, LRG

Sažetak:

U potrazi za novim uremičnim toksinima: ELISA pilot studija temeljena na proteomici

Uvod: Uremijski toksini su štetni metaboliti koji se nakupljaju u tijelu usporedo s gubitkom bubrežne funkcije. Na temelju rezultata prethodno provedene proteomske studije kojom su identificirani lumikan, protein povezan s pregradnjom matriksa (MXRA5), neuropilin 1 (NRP1) i leucinom-bogat

alfa-2-glikoprotein (LRG) kao potencijalni uremijski toksini, proveli smo malu pilot studiju kako bi se odredile njihove razine ekspresije u krvnoj plazmi i urinu pacijenata u svim stadijima napredovanja kronične bubrežne bolesti (KBB).
Materijal i metode: Proveli smo presječnu studiju u kojoj su ispitanici bili podijeljeni u 6 skupina (KBB stadiji 1-5 i zdrave kontrole). Odredili smo ekspresiju lumikana, MXRA5, NRP1 i LRG u krvnoj plazmi te ekspresiju MXRA5, NRP1 i LRG u urinu pomoću *enzimski-povezanog imunosorbentnog testa*.
Rezultati: Ova studija uključila je ukupno N=40 ispitanika podijeljenih u 6 skupina. Statistički značajne razlike u razinama plazmatske ekspresije među analiziranim skupinama pacijenata nisu otkrivene za analizirane proteine. Međutim, otkrili smo da je koncentracija NRP1 i LRG u urinu statistički značajno viša u skupini s KBB stadijima 2-5 u usporedbi sa skupinom zdravih pacijenata I KBB1, pri čemu je aritmetička sredina NRP1 3,2 puta viša, a aritmetička sredina LRG 19,4 puta viša u stadijima KBB2-5 u usporedbi sa zdravima i KBB1.
Zaključak: Niti jedan od odabranih plazmatskih proteina ne slijedi očekivanu kinetiku karakterističnu za uremijske toksine. Međutim, urinarni LRG i NRP1 mogli bi potencijalno biti novi kandidati za biomarkere KBB – no potrebna su daljnja istraživanja.

Ključne riječi: kronična bubrežna bolest, uremijski toksini, NRP1, LRG

1. Introduction

Chronic kidney disease (CKD) is caused by various pathological conditions that lead to progressive and irreversible loss of kidney function. The kidneys play a crucial role in maintaining the body's homeostasis, and their dysfunction affects most organ systems. CKD is also associated with disruption in lipid, amino-acid, mineral, bone and homocysteine metabolism [1]. Impaired kidney function leads to the accumulation of harmful substances, resulting in cellular damage and disturbances in electrolyte metabolism, along with water and sodium retention. These changes can result in hemodynamic instability and secondary hypertension [2]. Despite numerous studies, the molecular mechanisms and key events in the onset and progression of CKD remain elusive, though it is known that inflammatory, angiogenic, fibrotic, and regenerative processes play a significant role [3, 4]. According to Hill et al., the prevalence of CKD is estimated between 11 - 13% of the global population, with an increasing number of cases due to the aging populations and rising risk factors in the developed countries, such as hypertension, obesity, and diabetes [5]. Based on the 2012 Kidney Disease Improving Global Outcomes (KDIGO) organization, the disease is classified into five stages (CKD 1-5) based on glomerular filtration rate (eGFR) and albuminuria, of which CKD5 requires dialysis or kidney transplantation [6]. While there are various drugs and methods available today that can slow the progression of kidney disease in some patients, a method to halt the disease's course, fully prevent its progression, or restore lost kidney function has yet to be found [7, 8, 9].
Uremic toxins elements are a diverse group of molecules that accumulate in the body due to reduced kidney function that have toxic effects on various organ systems [10]. According to the European Uremic Toxin Work Group (EUTox), they can be classified into three categories based on their molecular characteristics and physio-chemical properties: protein-bound uremic toxins, middle molecules (with a molecular mass greater than 500 Da), and small water-soluble molecules (with a molecular mass less than 500 Da) [10]. Vanholder et al. emphasized that not only the presence, but also the resistance of these substances to dialysis removal contributes to their classification as uremic toxins [10]. Due to their toxic effects, it is necessary to develop better methods for identifying and removing these substances in order to improve treatment outcomes for patients with CKD [11]. The presence of uremic toxins is associated with various complications, including cardiovascular disease, immune dysfunction, and neurological impairments, which significantly reduce the quality of life for CKD patients. Early detection of uremic toxins in the initial stages of chronic kidney disease (CKD) presents a promising therapeutic target, potentially enabling more effective intervention strategies. [12].
Biomarkers are substances, structures, or processes that can predict the onset of a disease or its outcome [13]. In medical practice, biomarkers are crucial for early diagnosis, monitoring disease progression, risk assessment, and personalized therapy [14]. We have previously identified a link between the protease, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) and kidney fibrogenesis, proposing ADAMTS-4 as a potential diagnostic CKD marker [7]. This was followed by a proteomic analysis of CKD patients' plasma at distinct stages of disease progression, which revealed CKD2 as a potential turning point in disease progression [15]. Specifically, relative changes in the expression levels of several proteins were identified in correlation with disease progression. These proteins, which may serve as potential uremic toxins, include matrix remodeling-associated

protein 5 (MXRA5), neuropilin-1 (NRP1), lumican, and leucine-rich alpha-2-glycoprotein (LRG). Notably, these molecules are either integral components of the extracellular matrix (ECM) or closely associated with it, contributing to inflammatory, angiogenic, and fibrotic processes.

Building on the observation that these proteins may act as potential uremic toxins, we conducted a small cross-sectional pilot study to assess their plasma and urine expression levels across different CKD stages, comparing these findings with levels observed in healthy individuals.

2. Materials and Methods

2.1. Study Participants and Study Outline

This cross-sectional study involved participants categorized by eGFR into six subgroups: five CKD stages (CKD1-5) and healthy individuals. The study was approved by the Ethics Committee of the University Hospital Center Zagreb (EP-16/106-2). Blood and urine samples from the participants were collected between January 2017 and December 2018 at the Department of Nephrology, Arterial Hypertension, Dialysis, and Transplantation of the University Hospital Center Zagreb. The exclusion criteria for the study included: individuals under 18 years of age, patients with confirmed malignant conditions, systemic autoimmune disorders, rheumatic diseases, or central nervous system disorders, patients on immunosuppressive therapy, and patients suffering from acute cardiovascular or infectious diseases.

2.2. Sample Collection

Blood and urine samples were collected during regular check-ups. Blood was drawn into tubes with 3.8% sodium citrate (1:9 ratio), and plasma was separated by centrifugation at 3000 rpm for 15 minutes at 4 °C. Plasma and urine samples were then stored at -80 °C until analysis.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

Plasma expression levels of MXRA5, NRP1, lumican, and LRG, as well as urinary expression levels of MXRA5, NRP1, and LRG, were quantified by commercial indirect ELISAs (Figure 1), using a plate reader (Molecular Devices-SpectraMax i3x), according to the manufacturer's instructions. The Human MXRA5 ELISA Kit (abx152411) was used for MXRA5 detection, while NRP1 expression level was determined using the Human NRP1 SimpleStep ELISA® Kit (ab227901), with samples outside the ELISA detection range retested after dilution of 5 or 6 times as needed. Lumican expression level in plasma was measured with the Human Lumican ELISA Kit (ab213809), using a twofold dilution. LRG expression level was analyzed using the Human LRG SimpleStep ELISA® Kit (ab260066), with plasma diluted 50,000 times and urine 10 or 100 times as needed. All samples and standards were analyzed in technical duplicates, and the obtained results were evaluated by researchers blinded to clinical patient data. Expression levels of MXRA5, NRP1, and LRG were determined in plasma and urine, while lumican expression level was assessed only in plasma.

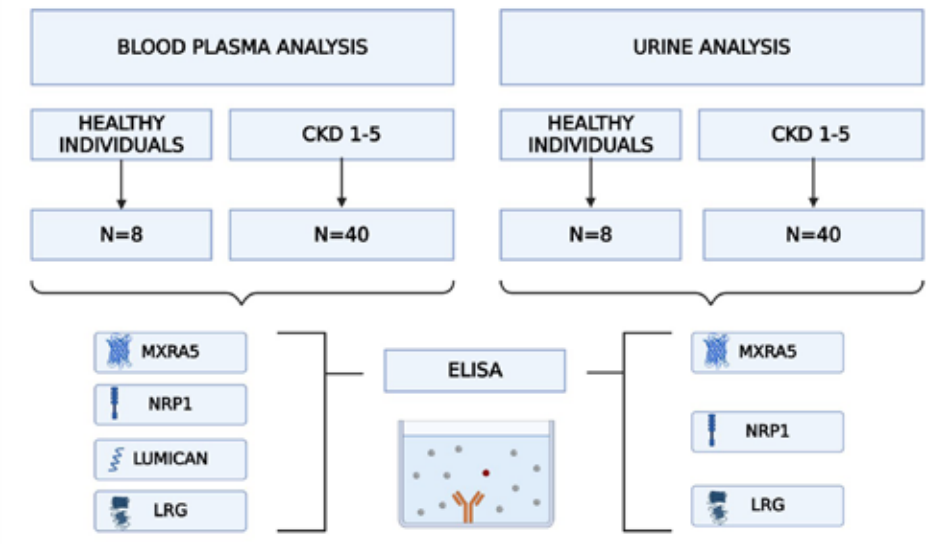

Figure 1. Study outline showing experimental groups and ELISAs conducted during the study. A total of 7 ELISA experiments were conducted on separate 96-well plates, with each plate containing samples from 40 participants divided into 6 experimental groups (8 healthy volunteers, 6 patients in CKD stage 1, 6 patients in CKD stage 2, 8 patients in CKD stage 3, 6 patients in CKD stage 4, and 6 patients in CKD stage 5) in duplicate, as well as standards, also in duplicate. This image was created using BioRender. (https://biorender.com/).

2.4. Statistical Analysis

The study included four groups containing 6 participants and two groups containing 8 participants. Normality was formally tested using Shapiro-Wilk test. Since variables were not normally distributed, the Kruskal-Wallis test was used to assess differences among groups. The Conover test was used to identify significant differences between specific groups. Due to high variability and small sample size, data was grouped into two categories: healthy + CKD1 and CKD stages 2-5, with a rationale that CKD1 patients more closely resemble healthy individuals than patients with more advanced CKD stages. Furthermore, our previous research identified CKD2 stage as a potential tipping point in disease progression [15]. The Mann-Whitney U-test compared protein expression levels between healthy + CKD1 and CKD stages 2-5. Receiver-operating characteristic (ROC) curve analysis was performed using MedCalc software for NRP1, lumican, and LRG to compare CKD1 patients and healthy volunteers with CKD stages 2-5 patients for their sensitivity and specificity in plasma and urine. To evaluate test quality, the area under the ROC curve (AUC) was calculated. An AUC value >0.7 was deemed acceptable for distinguishing between patient groups [16]. Type one error (alpha) was set at 0.05.

3. Results

3.1. Study participants

A total of 40 subjects were included in this study, of which 32 subjects were patients with CKD and 8 subjects were healthy volunteers. The participants were divided in the following groups: a group of 6 patients CKD1, a group of 6 patients in CKD2, a group of 8 patients in CKD3, a group of 6 patients in CKD4, a group of 6 patients in CKD5 and a group of 8 healthy volunteers. Participants' characteristics are shown in Table 1.

Table 1. Participants' characteristics at the time of plasma sampling, categorized according to the stage of chronic kidney disease. Gender, comorbidities, and underlying conditions are presented as the number of participants (percentage). Age, body mass index, and serum creatinine are expressed as the mean ± standard deviation, while proteinuria is reported as the median (with the first and third quartiles).

	Healthy	CKD1	CKD2	CKD3	CKD4	CKD5
N	8	6	6	8	6	6
Age (years)	Unk	35,2 ± 6,0	59,5 ± 7,7	55,4 ± 5, 8	58,8 ± 4,6	74,7 ± 7,9
Gender: N (%) female	2 (25)	4 (67)	2 (33)	3 (38)	5 (83)	2 (25)
BMI (kg/m^2)	Unk	37,7 ± 1,6	51,7 ± 4,5	40,9 ± 3,3	44,5 ± 2,2	45,4 ± 2,8
eGFR (mL/min/1.73 m^2)	Norm	98,7 ± 2,6	69,2 ± 2,8	35,5 ± 1,9	21,3 ± 2,2	9,3 ± 1,1
Proteinuria (g/24 h)	norm	5,03 (0,08 – 8,30)	2,05 (0,22 – 3,05)	2,14 (0,11 – 4,84)	0,89 (0,16 – 3,03)	2,02 (0,48 – 3,20)
Serum creatinine (µmol/L)	norm	72,2 ± 6,5	94,8 ± 2,7	168,8 ± 9,5	231,0 ± 16,3	515,7 ± 59,4
Underlying Disease N (%)						
Primary glomerular KD	0 (0)	4 (67)	6 (100)	5 (63)	4 (67)	0 (0)
Hypertensive / atherosclerotic KD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Autosomal dominant polycystic KD	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)	1 (17)
Diabetic KD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Other specific cause	0 (0)	1 (17)	0 (0)	1 (13)	1 (17)	2 (33)
Unknown	0 (0)	0 (0)	0 (0)	2 (25)	1 (17)	3 (50)
Comorbidities N (%)						
Hypertension	Unk	2 (33)	5 (83)	6 (75)	6 (100)	6 (100)
Diabetes	Unk	0 (0)	1 (17)	2 (25)	1 (17)	1 (17)
Smoker	Unk	1 (17)	1 (17)	2 (25)	1 (17)	2 (33)
Hyperlipidemia	Unk	1 (17)	6 (100)	3 (38)	3 (50)	3 (50)
Atherosclerosis	Unk	0 (0)	2 (33)	2 (25)	1 (17)	3 (50)

BMI – body mass index; CKD – chronic kidney disease, eGFR - estimated glomerular filtration rate (CKD-EPI formula used); KD – kidney disease; N – number of participants, norm – normal range of values; Unk – unknown

3.2. MXRA5 in Plasma and Urine of the Studied Groups

MXRA5 was detected in only one plasma sample from a healthy volunteer, with a concentration of 14.438 pg/mL, while in the other samples this molecule was not detected. We did not detect measurable expression levels of the MXRA5 protein in any urine sample.

3.3. NRP1 in Plasma and Urine of the Studied Groups

NRP1 levels in the participants' plasma showed no statistically significant differences between the experimental groups, and there was no statistically significant difference in NRP1 plasma expression levels between the healthy + CKD1 group and CKD stages 2-5 (Figure 2). However, significant differences in NRP1 urine expression levels were observed among the experimental groups (p = 0.014). Notably, the CKD5 group exhibited significantly higher NRP1 urine levels compared to all other groups: arithmetic mean of CKD5 was 14,9 times higher than in healthy individuals, 5 times higher as compared to CKD1, 7.5 times higher than CKD2, 2.9 times higher than CKD3 and 7.6 times higher when compared to CKD4.

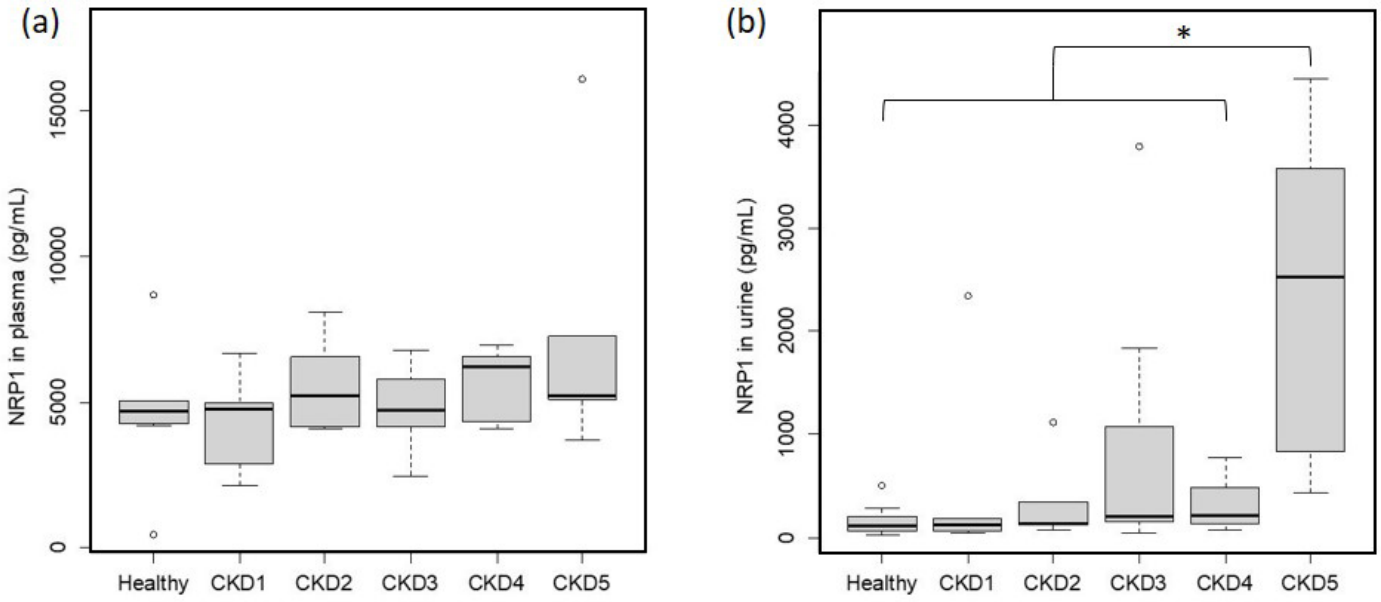

Figure 2. Expression level of NRP1 in the plasma (a) and urine (b) of experimental groups. The middle line in the box represents the median, while the lower and upper edges of the box display the first and third quartiles, respectively, or the interquartile range (IQR). The whiskers indicate the range of data within 1.5 times the IQR, while points outside the whiskers are identified as outliers. Statistical significance is marked by an asterisk (*).

Additionally, Figure 3 shows NRP1 levels in urine when participants are divided in groups healthy + CKD1 and CKD stages 2-5. The urinary concentration of NRP1 differs significantly between the groups healthy + CKD1 and CKD stages 2-5 ($p = 0.009$) with the arithmetic mean of urinary NRP1 in CKD stages 2-5 being 3.2 times higher than in the healthy + CKD1 group.

3.4. Plasma Lumican Levels Across Study Groups

Plasma levels of lumican showed no statistically significant difference in expression between the experimental groups, and there was no significant difference in expression levels between the healthy + CKD1 group and the CKD stages 2-5 group (Figure 4).

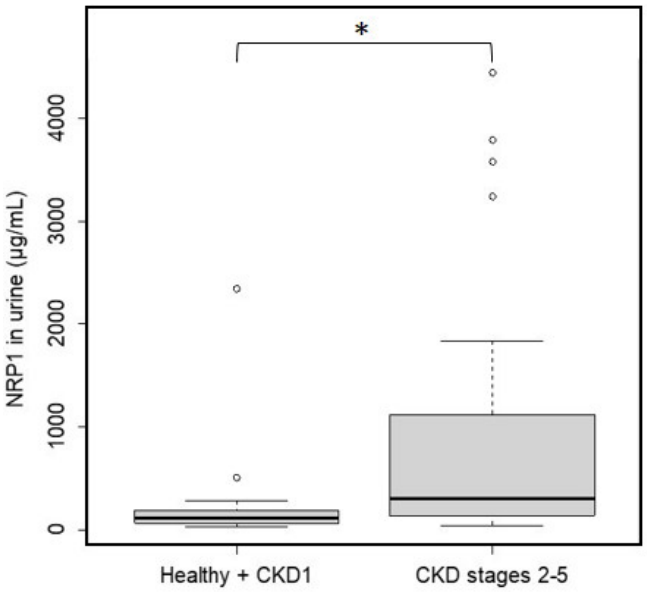

Figure 3. Expression level of urinary NRP1. Patients are divided in two groups: healthy + CKD1 and CKD stages 2-5. The middle line in the box represents the median, while the lower and upper edges of the box display the first and third quartiles, respectively, or the interquartile range (IQR). The whiskers indicate the range of data within 1.5 times the IQR, while points outside the whiskers are identified as outliers. Statistical significance is marked by an asterisk (*).

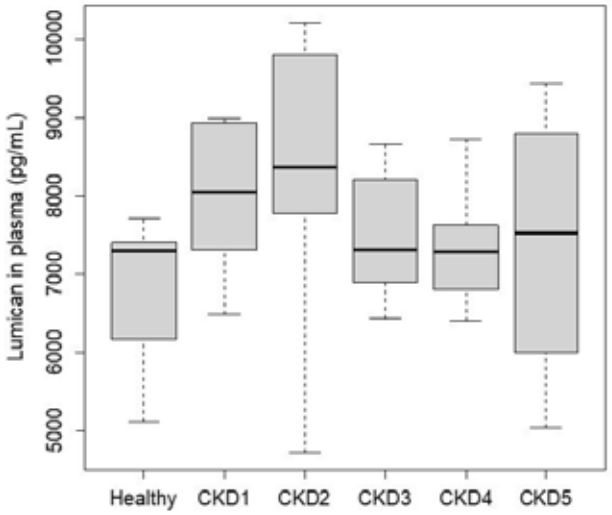

Figure 4. Lumican expression level in the plasma of experimental groups (pg/mL). The middle line in the box represents the median, while the lower and upper edges of the box display the first and third quartiles, respectively, or the interquartile range (IQR). The whiskers indicate the range of data within 1.5 times the IQR.

3.5. LRG in Plasma and Urine of the Studied Groups

LRG expression levels in plasma and urine showed no significant differences in expression levels among the experimental groups (Figure 5).

However, when participants are divided in groups healthy + CKD1 and CKD stages 2-5, a significant difference in urinary LRG expression levels was found (p = 0.011), with the CKD stages 2-5 group showing 19.4 times higher arithmetic mean of urinary LRG levels.

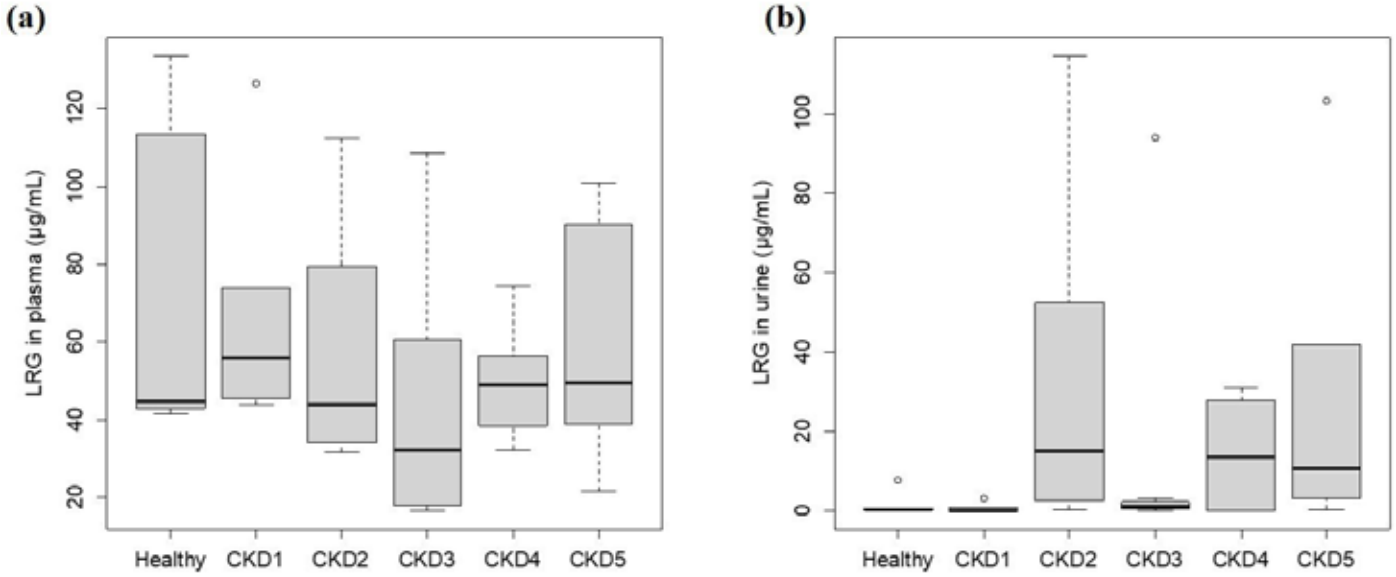

Figure 5. LRG expression levels in plasma (a) and urine (b) of experimental groups. The middle line in the box represents the median, while the lower and upper edges of the box display the first and third quartiles, respectively, or the interquartile range (IQR). The whiskers indicate the range of data within 1.5 times the IQR, while points outside the whiskers are identified as outliers.

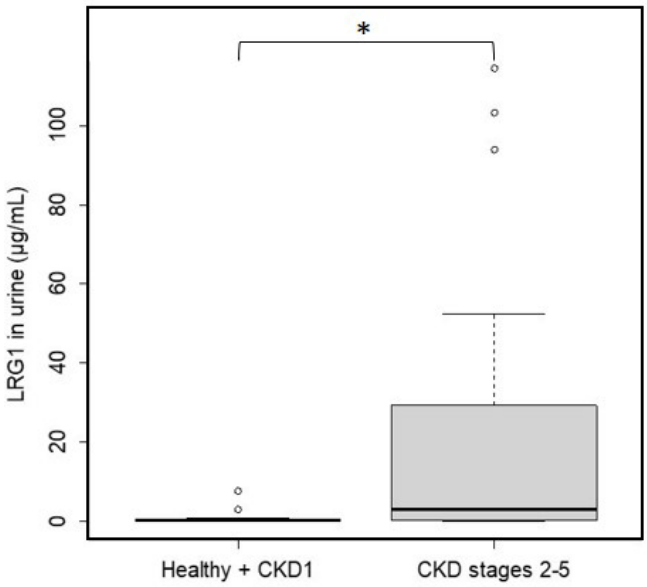

Figure 6. Expression level of urinary LRG when participants are divided into healthy + CKD1 and CKD stages 2-5. The middle line in the box represents the median, while the lower and upper edges of the box display the first and third quartiles, respectively, or the interquartile range (IQR). The whiskers indicate the range of data within 1.5 times the IQR, while points outside the whiskers are identified as outliers. Statistical significance is marked by an asterisk (*).

3.6. ROC Curve Analysis

Plasma NRP1 levels in patients with CKD in stages 2-5 were compared to the control group, i.e. healthy + CKD1. The sensitivity of the analysis was 57.7%, with specificity at 85.7%, and an AUC of 0.64, indicating that plasma NRP1 expression levels cannot distinguish between the healthy + CKD1 group and the CKD stages 2-5 group. Urinary NRP1 had a sensitivity of 73.1% and specificity of 71.4%, with an AUC of 0.75, showing good test quality with a threshold of 141.9 pg/mL. Plasma lumican expression levels had a sensitivity and specificity of 46.2% and 78.6%, respectively, with an AUC of 0.54, suggesting a low reliability and accuracy in distinguishing between the analyzed patient groups. Plasma LRG levels have shown sensitivity and specificity of 38.5% and 100%, respectively, but with an AUC of 0.67, which was not considered reliable. Urinary LRG levels showed a sensitivity and specificity of 70.8% and 81.8%, with an AUC of 0.77, indicating significant expression differences and good reliability in distinction between the two patient groups with the LRG expression levels above 707.4 µg/mL (Figure 7). Based on the obtained results, urinary concentrations of NRP1 above 141.9 pg/mL and of LRG above 707.4 µg/mL could be considered a threshold discerning the groups healthy + CKD1 and CKD2-5.

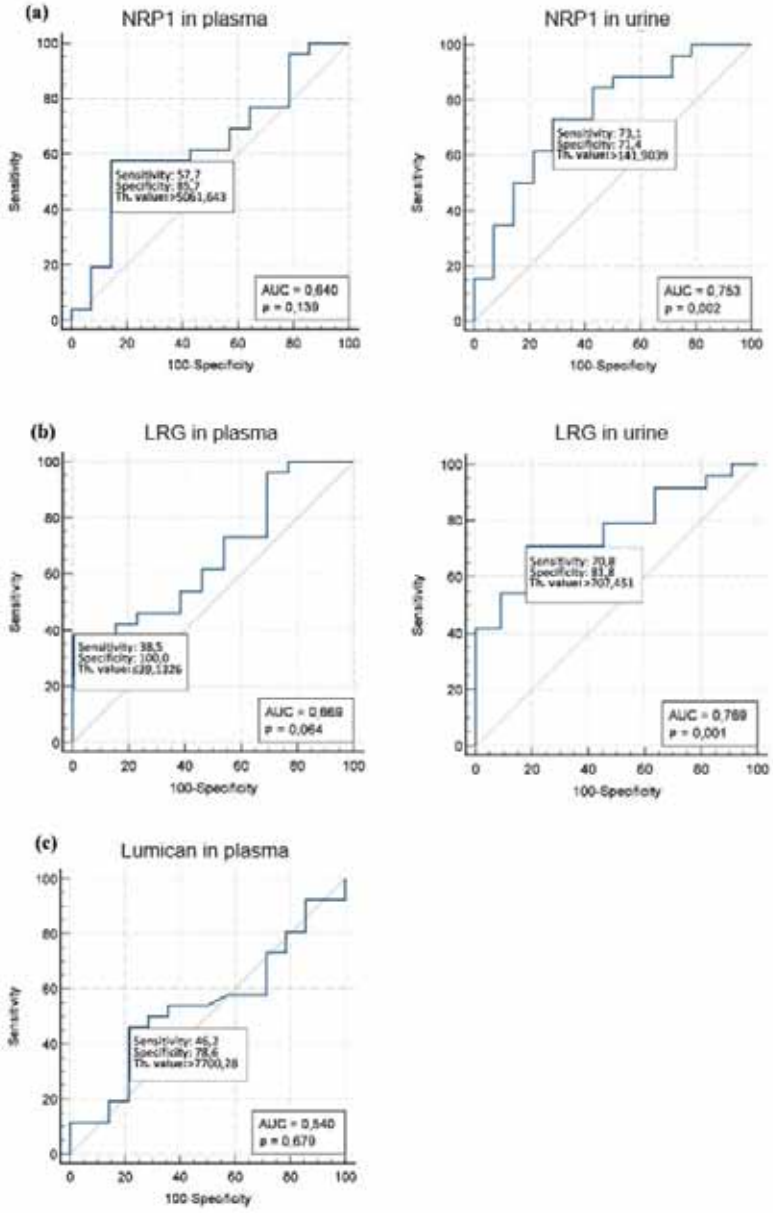

Figure 7. Receiver-operating characteristic (ROC) curves for distinguishing between patient groups (healthy + CKD1 vs. CKD2-5) based on expression levels of plasma and urinary NRP1 (a) and LRG (b), and plasma lumican (c). Proteins with calculated area under the curve (AUC) values >0.7 are deemed acceptable for differentiating patient groups above the threshold urinary concentrations.

4. Discussion

CKD is a worldwide public health problem. Despite its growing frequency, no definite modality for treatment or prevention of further progression of the disease has been established [17]. Recognition of new uremic toxins as harmful compounds that accumulate in the body with the loss of kidney function would enable their eventual removal with the aim of eliminating their negative effects [14]. Our previous proteomics-based research identified CKD stage 2 as a potential tipping point in disease progression, as numerous molecules exhibit the most significant changes in expression specifically at this stage.The results of this research, along with findings from a supplementary literature review, identified four molecules—MXRA5, NRP1, lumican, and LRG—that display notable trends in expression changes correlating with disease progression. We therefore conducted a small cross-sectional pilot study to determine the expression levels of these proteins in blood plasma and urine among healthy individuals and across the five CKD-KDIGO stages.

MXRA5 is a cell surface glycoprotein previously showed significant expression levels in healthy kidney tissue where it participates in ECM remodeling [18]. Although MXRA5 has been previously detected in plasma, a marked plasma increase in MXRA5 expression levels was shown in CKD2. As we detected MXRA5 only in a single healthy individual, we failed to confirm it as a potential uremic toxin, and to the best of our knowledge, its plasma expression was never reliably confirmed [15]. In our research, we did not confirm the presence of MXRA5 in urine samples and, accordingly, we cannot consider it a biomarker for CKD.

NRP1 was considered as another potentially interesting molecule affecting the progression of CKD [15]. Considering its important role in fibrotic and angiogenic processes, we assumed a significant change in the expression level of NRP1 [20]. However, we found no statistically significant difference in the expression level of this protein in plasma between different stages of CKD, as well as between the groups healthy + CKD1 with the group CKD stages 2-5. However, the expression levels of NRP1 in urine show an interesting distribution amongst experimental groups, where a statistically significant increase in concentration was found in the urinary NRP1 in subjects suffering from CKD5 compared to all other groups, which can possibly be interpreted as reduced eGFR with a significant non-specific proteinuria in the final stage of the disease. Furthermore, the two experimental groups were differentiated according to urinary NRP1 expression levels implying its possible use as a late stage CKD biomarker.

A member of the family of small leucine-rich proteoglycans, lumican, is one of the ECM proteins with a potential role in the fibrotic processes that are key to the progression of CKD. Despite previous research that showed elevated plasma lumican expression levels on the course of disease progression, this study did not establish a statistically significant difference in lumican expression level by disease group, nor between healthy + CKD1 vs. CKD stages 2-5 [15]. Although this study did not analyze urinary lumican expression, lumican was found to be specifically expressed in the urine of CKD patients, which is why this calls for further research [21].

LRG is a plasma protein of poorly defined function that belongs to a group of extracellular proteins containing leucine-rich repetitive sequences [22]. Previous studies have identified LRG as a uremic toxin whose expression shows an increasing trend with CKD progression. [11, 15]. Although we found no difference in the expression levels of urinary LRG in the experimental groups, there is a significant increase in urinary LRG expression between the groups healthy + CKD1 vs. CKD stages 2-5. High levels of LRG in the advanced stages of CKD may indicate kidney damage, making LRG a compelling target for further research as a potential biomarker for CKD. This study suggests that urinary LRG could serve as a CKD biomarker, demonstrating a sensitivity of 70.8% and a specificity of 81.8%.

In this research, none of the selected molecules shows the characteristics of a potential uremic toxin since no statistically significant difference was found in the expression levels of molecules in the plasma between the experimental groups. Also, this research showed no significant differences in the expression levels of selected molecules in plasma between the healthy + CKD1 group and the CKD stages 2-5 group, which points to the fact that the selected molecules cannot serve as a marker of CKD in plasma. However, our results do tentatively show promise in two potential urinary markers of CKD – namely, NRP1 and LRG showed significantly higher urinary levels in the CKD stages 2-5 group than in the healthy + CKD1 group. Their urinary levels also showed promising sensitivity and specificity in distinguishing the two groups, when subjected to ROC curve analysis.

This study has several limitations. A small sample size and a large number of research subgroups must be taken into account. Furthermore, CKD patients had markedly different underlying causal disease that led to CKD. Finally a significant interindividual expression level variability was observed in the measured parameters. However, considering the small number and variability of patient samples, the expression patterns of selected molecules in plasma should be further studied on a larger number of samples and preferably focused on distinguishing healthy subjects from early kidney disease to attain maximum clinical yield – which is early diagnosis of progressive CKD, which would allow timely management and better outcomes. Furthermore, this study shows that one should be very careful in drawing conclusions about what can possibly be declared a uremic toxin or a biomarker molecule important in the diagnosis of certain conditions. This should be preceded by an exhaustive validation on a significant number of patient samples with clearly defined characteristics for a particular condition. Our study highlights a small number of many, yet unexplored molecules which might expand our knowledge and enhance our practices in the research and treatment of CKD.

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