REVIEW

by **assoc. prof. Daniela Ivanova Gerova, MD, PhD**

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Internal member of the Scientific Jury

Subject: awarding the Doctor of Philosophy (PhD) degree

Field of higher education: 7. Health and sport

Professional Direction: 7.1. Medicine

Scientific specialty: Clinical Laboratory

of full-time PhD student: Dr. SEVIM AHMED SHEFKET, MD

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TOPIC: PREDICTIVE ROLE OF NGAL AS AN EARLY MARKER OF RENAL IMPAIRMENT IN PATIENTS WITH TYPE I AND TYPE II DM

Scientific adviser: assoc. prof. Yana Dimitrova Bocheva, MD, PhD

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General presentation of the procedure

According to the Order № 109-112/11.03.2022 of the Rector of the Medical University of Varna, Prof. Dr Valentin Ignatov, and to the Protocol of the Faculty Council №61/01.03.2022 I have been elected as internal member of the Scientific Jury for awarding the Doctor of Philosophy (PhD) degree of full-time PhD student: Dr. Sevim Ahmed Shefket, MD. On the basis of the decision taken during the first meeting of the Scientific Jury (Protocol №1/23.03.2022) I have been elected as a Chairman of the jury and reviewer of the scientific work of Dr. Sevim Ahmed Shefket.

The set of materials provided to me on paper/electronic media fully complies with the requirements of the Procedure for acquiring an educational and scientific degree "Doctor" according to the Regulations of MU-Varna.

Brief biography and data on the career development and qualification of the PhD candidate

Dr. Sevim Ahmed Shefket completed her secondary education in her hometown of Shumen in 2004 and her higher medical education in 2010 at the Medical University "Prof. Dr. Paraskev Stoyanov", Varna city, Bulgaria (Diploma No. 000646/2010). Immediately after graduation, she started working as a physician at the Center for Emergency Medica Care – Varna, and soon after as a doctor at Medical Center "South". In 2014 she started a specialization in the field of clinical laboratory at the Medical University "Prof. Dr. Paraskev Stoyanov", Varna city. Since 2016 she has been appointed as an intern in the field of clinical laboratory at Clinical Laboratory of the University General Hospital "Sveta Marina", in Varna, where she successfully completed her specialization and in 2019 she obtained a Certificate of Completion of Specialist Training in the field of Clinical Laboratory (No. 4077/2019). Since 2019 she has also been appointed as a clinical lab assistant at the Department of Clinical Laboratory, Faculty of Medicine, Medical University – Varna. Her main responsibilities as a lecturer are related to the training of students in the educational programs of "Medicine" (taught in Bulgarian language) and "Medical Nurse". At the same time, she was enrolled as a full-time PhD student in the PhD program of "Clinical Laboratory". For the period between 2019 – 2022 she has invested the necessary hard work and efforts, and has presented her dissertation paper within the set timeframe.

For the short period she has worked at University General Hospital "Sveta Marina", Dr. Shefket developed extensive scientific activity by participating in 7 scientific forums in the country and abroad. Her scientific interests are in the field of laboratory diagnosis of chronic kidney disease and diabetic kidney disease. Some of these interests are presented in 4 articles published in Bulgarian and international journals.

Dr. Sevim Ahmed Shefket is a member of the Bulgarian Medical Association and the Bulgarian Society of Clinical Laboratory.

Topic relevance

The dissertation of Dr. Sevim Ahmed Shefket is dedicated to a topical issue of endocrinology, i.e. early detection of diabetic kidney disease (DKD). Diabetes mellitus (DM) is a disease of extremely high social significance. The continuous rise in the incidence of DM is at risk of reaching epidemic proportions and requires comprehensive measures on its prevention, proper control, and management of the risk of severe complications. One of its most severe complications is the DKD, often leading to end-stage renal disease. Latest data in the

literature point to a change in the traditional understanding that diabetes mellitus primarily affects the glomerulus, and secondary the nephron tubular system. The view of the primary role of tubular damage in DKD is becoming increasingly prevalent. It is quite logical to seek new, more indicative than the classic glomerular indicators, whose levels change early enough in tubular damage of the nephron and have the ability to accurately reflect even subclinical renal dysfunction. One of the most promising tubular biomarkers is neutrophil gelatinase-associated lipocalin (NGAL) and the topic of the current dissertation "*Predictive role of NGAL as an early marker of renal impairment in patients with type I and type II DM*", is undeniably relevant and very promising.

General characteristics and structure of the dissertation paper

The dissertation's volume and form meet the requirements. It is printed on 150 standard pages and is illustrated with 47 tables and 36 figures. The dissertation is structured correctly and includes: Title page, Table of Contents – 2 p., Abbreviations – 1 p., Introduction – 1 p., Literary Overview – 40 p., Purpose and Objectives – 1 p., Materials and Methods – 7 p., Results – 37 p., Discussion – 33 p., Conclusions – 2 p., Contributions – 2 p., List of publications and participations in scientific forums related to the dissertation – 1 p., Two Annexes – 4 p. and References – 18 p., including a total of 221 literary sources: 211 in Latin, and 10 in Cyrillic script. Of all the literary sources cited in the dissertation, 160 were published in the period 2010-2021 and represent 72.4% of the entire literary reference, which in turn is a prerequisite for a modern treatise on the issues presented in the dissertation paper.

Assessment of the structural parts of the dissertation paper

The Literature Overview is presented in several sections. A general review of diabetes mellitus is given, including up-to-date data on its prevalence and classification. Attention is drawn to the diagnosis and classification criteria for chronic kidney disease and DKD in particular. There is a discussion on the risk factors for developing DKD, as well as the pathomorphological changes in the tubules and interstitium arising with diabetic nephropathy (DN). The PhD student skilfully navigates the literature and manages to prove that functional and structural changes in tubules and cortical interstitium may precede pathognomonic changes in glomeruli, leading to a change in the *traditional paradigm for the pathogenesis of DKD – from glomerulo-centric to tubulo-centric hypothesis*. Significantly, this section of the overview points to the role of tubular impairment in the development and progression of proteinuria in DM. The end of this section, quite logically, examines the routine laboratory parameters currently used for the diagnosis and classification of CKD and DKD. The shortcomings and

limitations of GFR and ACR/AER, as well as the widely used surrogate markers (eGFR calculation formulae) have been highlighted. This conditions the search and implementation of new markers for diagnosis of DKD in clinical practice.

In the following sections, the PhD student collects and summarizes all available data on the structure, metabolism, and molecular mechanisms of biological action of NGAL, which is the subject of her scientific searches. Its role in the diagnostics of CKD (Chronic Kidney Disease) and DKD has been presented. There has been an exponential growth in evidence of NGAL as a marker for *tubulo-interstitial impairment*, that could identify development of DKD, long before its evidencing by AFR. In addition to its role as an *early marker* in the diagnosis of DKD, plasma and urine values of NGAL are useful indicators for *staging and risk assessment* of developing vascular complications in the progression of DKD. Urinary NGAL (uNGAL) data have been specified, as a *non-invasive marker* with the potential to differentiate DKD from non-diabetic kidney disease (NDKD), as a foundation for affirmative kidney biopsy for which standardised criteria have not yet been established in patients with DM. Data are also presented that refute the above observations. A concept is promoted, that metabolic changes in DM lead to a chronic proinflammatory condition, which in turn determines the increased production of NGAL from extrarenal tissues. Particular attention is also drawn to the fact that urinary tract infections (UTIs), a common complication with DM, are accompanied by established elevated uNGAL values as an acute-phase protein.

The lack of standardisation in the various immunochemical methods that test plasma levels of NGAL (pNGAL) and uNGAL leads to differences in both their reference ranges (RR) and diagnostic cut-off values, highly dependent on the method used, test platform and type of biological material. This makes it difficult to comparatively analyse the results obtained in various studies. Some of the contradictory results in the literature are also due to insufficient information about the sources of analytic interference, the optimal biological material for analysis and the biological variation significantly influenced by the selection and distribution of individuals in the groups studied.

The critical analysis and summary of the scientific data presented in the literary overview allow Dr. Șhefket to accurately articulate the ***purpuse*** of the dissertation, namely, to *determine the diagnostic reliability of NGAL as a marker for DKD in patients with DM I and DM II.* For the successful achievement of this purpuse, ***seven specific objectives*** have been identified and presented in a logical sequence.

The ***Materials and Methods*** section is presented in 7 pages. The study is prospective in type and started after obtaining approval from the Committee on Ethics of Research at the Medical University of Varna. It encompasses a three-year period, which initially included a total of 312 researched individuals. Eighteen of them met the exclusion criteria. Following their drop-out the study remained with *294 individuals*, fully meeting the inclusion criteria. They were distributed as follows: *127 healthy individuals* – 85 adults over 18 years of age and 42 children under the age of 18 *and 167 DM patients* – 92 adult patients with DM II and 75 children with DM I.

Three types of biological material were taken from all participants:

- complete K2EDTA blood for CBC and measurement of HbA1c
- heparinized plasma for measurement of pNGAL, urea, creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides
- first morning urine, examined semi-quantitatively with urine test strips; after centrifugation, the sediment was evaluated, and uNGAL, creatinine and albumin were quantified from the released supernatant.

All samples were tested within two hours of receipt of the material for all the parameters listed, by modern routine laboratory methods, except for NGAL. NGAL (plasma and urine) samples were stored in labelled additive-free vacutainers at -20°C until the analysis was carried out. The analysis of pNGAL and uNGAL was carried out serially on biochemical analyser ADVIA 1800 using the latex-enhanced immunoturbidimetric method by BioPorto Diagnostics A/S, Denmark, authorised for IVD use in the European Union.

Adequate statistical methods have been applied for analysis and processing of the results obtained.

The materials and methods were appropriately selected and in accordance with the defined purpose and primary objectives set to be performed.

The ***Results*** section is laid out in 37 pages, in which the scientific results are correctly described, illustrated with relevant tables and graphs, and presented in a sequence consistent with the set objectives:

1. *Verification of immunoturbidimetric method for determining NGAL.*

A calibration curve check and verification of the analytical measuring range have been carried out. A lower value for the Lower Limit of Quantification (LLOQ) was established than that announced by the kit manufacturer. The set of characteristics describing the analytical reliability of quantitative methods was studied, *strictly adhering to* both the ISO 17025 –

Requirements for Method Verification guidelines, and the criteria for acceptability of analytical characteristics consistent with EMEA/CHMP/EWP/2009 Guideline on bioanalytical method validation. The method was found to have high analytical reliability.

2. *NGAL reference ranges in adult subjects. Biological variation.*

For this purpose, NGAL was measured in plasma and urine from 85 healthy individuals (45 women and 41 men) at an average age of 52.96±8.39 years. Due to non-Gaussian distribution of the results, their normalization is required, after which a percentile method of establishing the RR (2.5 and 97.5 percentile, at a 95% confidence interval) was applied. In strict adherence to all the rules for determining general, gender-independent RR, they were defined as follows: pNGAL: 25-119.49 ng/mL; uNGAL <52.37 ng/mL; uNGAL to creatinine ratio in the urine, indicated as UNC: <5.16 μg/mmol.

With regard to the biological variation of these parameters, the following parameters are examined:

(a) gender influence – a statistically significant gender difference was found only for UNC, which is also supported by the confirmed significant gender difference in creatinine levels in urine. This necessitated extracting gender-dependent RR for UNC, namely <6.34 μg/mmol for women and <3.36 μg/mmol for men.

(b) age influence – the overall group was subdivided into three age subgroups: ≤49 yrs. of age; between 50 and 59 yrs. of age; and ≥60 yrs. of age. It was found that age differentiation of the control cohort leads to a reduction in the number of individuals in the separate subgroups, which limits the possibility for extracting of statistically significant different age-dependent RR for all three parameters. After unifying subgroups one and two, statistically significant difference was proven for the pNGAL for the newly formed two age groups: <60 and ≥60 yrs. of age. Statistically significant correlation dependencies were also studied and identified: age/eGFR (negative) and age/pNGAL (positive). This explains the statistically significant negative correlation between pNGAL and eGFR.

3. *NGAL reference ranges in children. Biological variation*

For this purpose, NGAL was studied in plasma and urine from 42 healthy children (21 girls and 21 boys) at an average age of 12.50±3.69 g. Due to non-Gaussian distribution of the results, normalization is required, after which a percentile method of establishing the RR (2.5 and 97.5 percentile, at 95 % confidence interval) is applied. In strict adherence to all the rules for determining general, gender-independent RR, they were defined as follows: for pNGAL < 96.88 ng/mL; for uNGAL < 47.30 ng/mL and for UNC < 3.48 μg/mmol. No significant

differences between the genders were identified and no significant correlation with age was identified between the three parameters studied.

4. *Diagnostic reliability of NGAL in the diagnostics of DKD in DM II*

To fulfil this objective, the PhD student examined 92 patients (51 women and 41 men) with a confirmed DM II diagnosis, according to the ADA and WHO criteria. They were divided *into three subgroups relative to ACR*: A1 with ACR < 3 g/mol, A2 with ACR 3–30 g/mol and A3 with ACR > 30 g/mol and in *two subgroups according to eGFR*, calculated using the CKD-EPI formula: ≤G2 with eGFR62 ≥ 60 mL/min/1.73m² and ≥G3 with eGFR < 60 mL/min/1.73m² (it was specified that distribution in four groups of G1 to G4 reduces the number of patients in subgroups and limits the possibility of statistical processing of data, and G5 is an exclusionary criterion for the study). In addition, patients are divided into two other subgroups, depending on whether they have or not DKD, as defined in the KDIGO 2020 guidelines.

Correct non-parametric methods of statistical analysis were selected.

With respect to pNGAL, statistically significant differences were found between the control group and subgroups A3 and ≥G3, as well as for the DKD subgroup. The ROC analysis conducted at a cut-off value of 121.65 ng/mL for pNGAL found good diagnostic effectiveness (82%) with AUC = 0.753 for differentiating DM II patients with lowered GFR, from those with maintained eGFR, but poor to sufficient diagnostic effectiveness was found in terms of differentiation of patients with albuminuria and norm-albuminuria.

With regard to uNGAL and UNC, values were found to correlate significantly with changes in albumin excretion, with UNC showing a better dependence on ACR than uNGAL. uNGAL and UNC values increased progressively from A1 to A3, with a significant difference found between A1 vs A2 and between A1 vs A3. Patients with DM II with moderately and highly elevated ACR had statistically significantly higher scores for uNGAL and UNC than the control group, with patients with normalbuminuria (A1) also having significantly higher UNC results. A gender difference in UNC values was also found in the subgroups of patients divided by ACR and eGFR. Women had higher UNC scores than men in each of the subgroups, with the difference being significantly relevant only in subgroup A1 and in the subgroup with eGFR ≥ 60 mL/min/1.73 m² and decreasing with the progression of renal impairment.

Multiple ROC analyses have been performed and diagnostic cut-off values have been set for pNGAL, uNGAL without gender differentiation. For pNGAL a cut-off value was defined to differentiate patients with reduced glomerular filtration, and for uNGAL cut-off values were defined to differentiate both patients with DKD, and with albuminuria. For UNC, gender-

dependent cut-off values were also derived, in order to differentiate both patients with DKD, and with albuminuria. It is summarized that UNC shows better diagnostic effectiveness than uNGAL in distinguishing DM II patients with renal impairment, which gives justification for it to be preferred in assessing the role of the combined application of two markers – pNGAL and UNC. UNC elevations of 1 μg/mmol were found to increase the chance for a DM II patient to have DKD by 1.29 times. Even after separating patients by gender, UNC continued to significantly predict the presence of DKD, where an increase in UNC by 1 μg/mmol in women increased the probability of DKD 1.16 times, and in men – 2.40 times.

5. *NGAL as a marker of DKD progression in patients with DM II*

The PhD student subdivided the patient cohort into 4 groups according to the severity and prognosis of the DKD, determined according to the ACR and eGFR values and according to the KDIGO 2020 guidelines (low, moderate, high and very high-risk patients, respectively). Since poor glycaemic control was a major factor in the development and progression of DKD, the overall patient group was subdivided into two other groups depending on their glycaemic control (with good control – HbA1c $\leq$ 7.5 % and with poor control > HbA1c >7.5 %).

The values of all three studied indicators, pNGAL, uNGAL and UNC, are found to increase progressively with the degree of renal impairment. Statistical significance was achieved only in the tested urine indicators (uNGAL and UNC values in the low-risk group differed statistically significantly from those in all other groups – moderate, high and very high risk). With respect to both groups with good and bad glycaemic control, no significant difference in pNGAL, uNGAL and UNC values was found. The correlation analysis shows a significant positive correlation between pNGAL versus urea and creatinine was found, and negative for eGFR (markers for glomerular filtration evaluation), while for urine indicators significant positive correlations were identified with AER, ACR and negative with eGFR, a weak positive but significant correlation with HbA1c was further identified for UNC. With the help of discriminatory analysis, two markers were examined at the same time and their prognostic value was evaluated. The pNGAL and UNC model was found to be statistically significant in discriminating against groups with DKD and the derived discriminatory function (D=−1.43+UNCx0.068+pNGALx0.08) successfully predicted the distribution of 57% of patients with DM II in the relevant groups. By comparison, the independently used pNGAL, uNGAL or UNC indicators successfully predicted the distribution of 26%, 42.4% and 49% of patients in the relevant groups. In order to assess the probability with which pNGAL and UNC together could successfully predict the presence of high and very high risk for DKD among

patients with DM II, a logistical regression analysis was conducted after patients were redistributed into two main groups (one group – patients at low and moderate risk and another – patients at high and very high risk). The model correctly classifies 75% of patients to the so-defined groups. It was found that an increase in pNGAL by 1ng/mL increases 1.01 times the chance that a patient with DM II has DKD with high and very high risk, and an increase in UNC by 1 μg/mmol increases 1.09 times (women – 1.07 times, men – 1.14 times) the likelihood of a patient having DKD with high and very high risk. By comparison, uNGAL and UNC alone were able to correctly classify patients in both groups at 66% and 65%, respectively.

6. *NGAL marker for diagnosis and prognosis of DKD in patients with DM I*

The PhD student selected 75 patients (33 girls and 42 boys) at an average age of 13.38±2.82 years. The individuals had a proven DM I with a disease duration of 5 to 14 years. Reasoning was given for their distribution into two subgroups only according to ACR (A1 – with ACR < 3 g/mol, A2 with ACR 3 – 3- g./mol), and two subgroups according to eGFR: once according to the formula of eGFR (Bedside Schwartz) and once again according to eGFR (CKD-EPI40), with an eGFR=90 mL/min/1.73m² limit value. Depending on glycaemic control, children were divided into two subgroups, the distinguishing value for HbA1c being 7.5%. It was chosen not to form groups of DM I with and without DKD, since they were identical with A1 and A2 (only in one patient eGFR was identified to be < 60 mL/min/1.73 m2 and pathological albuminuria). Cut-off values derived from this study to identify pathological albuminuria in patients with DM I correspond to cut-off values for DKD. The regression analysis revealed a very good relationship between the two eGFR calculation formulas used, as well as a lack of a statistically significant difference when comparing the results. pNGAL, uNGAL and INC were tested.

When comparing the entire DM I patient cohort with the control group of children, only the UNC results were significantly higher.

With regard to pNGAL, a statistically significant difference was found among the different compartmentalized types of patients, in the groups subdivided according to $eGFR_{(CKD-EPI40)}$. Children with a value of ≥ 90 mL/min/1.73m² had a median pNGAL significantly lower than in the $eGFR_{(CKD-EPI40)}$ group < 90 mL/min/1.00 73m² (53.95 (41.63 – 67.30) ng/mL vs 77.10 (53.90 – 111.60) ng/mL). The correlation analysis conducted between pNGAL and all other indicators showed a statistically significant negative correlation only between pNGAL and eGFR (CKD-EPI40), with a similar trend also observed for the pNGAL/eGFR (Bedside Schwartz) interrelationship. The ROC analysis conducted showed a diagnostic effectiveness of

72% in distinguishing patients with DM I with slightly reduced glomerular filtration at cut-off pNGAL=96.80 ng/mL.

With regard to uNGAL, a significant difference was found for all comparisons between the groups subdivided on the basis of albuminuria, with the exception of that between A1 and the control group. The correlation analysis showed a statistically significant positive correlation between uNGAL/AER, uNGAL/ACR, uNGAL/HbA1c, uNGAL/BMI and uNGAL/TG pairs. The ROC analysis conducted shows a diagnostic effectiveness of 86% in correct identification of DM I patients with albuminuria, with cut-off for uNGAL – 47.85 ng/mL.

With respect to UNC, a significant difference was found for all comparisons between the groups subdivided on the basis of albuminuria except between A1 and the control group, as well as for the groups subdivided on the basis of glycaemic control. The correlation analysis shows statistically significant correlation between the pairs UNC/AER, UNC/ACR, UNC/HbA1c and UNC/TG. The ROC analysis conducted showed a diagnostic effectiveness of 89% for the correct identification of DM I patients with albuminuria at cut-off for UNC=47.85 ng/mL.

In order to determine the importance of pNGAL and UNC for the diagnosis of DKD in patients with DM I, defined as ACR>3g/mol, a logistical regression analysis was conducted that correctly diagnosed 90% of patients with DM I versus available renal impairment (99% of patients without DKD and 67% of patients with DKD). When both markers are reported simultaneously, they significantly predict the presence of DKD, with UNC being more significant for the prognosis – UNC's increase of 1 μg/mmol increases the chance of a patient with DM I having DKD by 2.1 times.

The discussion is well illustrated and shows a respectable ability in the PhD student to discuss her own results, comparing them with the known facts in the relevant literature, and aptly interpreting them to draw important conclusions about the findings and their importance for clinical practice.

1. *Verification of immunoturbidimetric method for determining NGAL.*

Arguments have been presented for the need of verification and validation of the method chosen for determining NGAL. The reliably established lower value for LLOQ=12 ng/mL gives the research team certainty to obtain quality results for samples with low NGAL values. Correct methods are used to analyse the set of parameters characterizing analytical reliability, and after applying adequate statistical processing, the PhD student conclusively proves that the method has very good analytical characteristics.

2. *NGAL reference ranges in adults. Biological variation.*

a) *in plasma* – a comparison has been made with studies in which pNGAL RR are determined using the PETIA/Bioporto method. Despite the use of different types of biological material (EDTA plasma and serum), the results of the dissertation are completely comparable to those of the mentioned studies. Statistically significant age difference has been proven for individuals over and below 60 years of age.

b) *in urine* – the differences in the uNGAL and UNC RR's in the literature are indicated and discussed depending on the types of urine (diuresis urine, first morning urine and spot urine), as well as their dependence on the method of determining NGAL and their presentation in different measurement units, well-illustrated in Tables 30 and 31. Both the choice of biological material (first morning urine, an approved standard for routine urine analysis) and the need to calculate the UNC ratio have been justified. In strict compliance with the requirements, the RR's have been determined for our population. No gender differences have been identified for uNGAL, but with normalization of results (presented as UNC) statistical significance is being reached. The gender-dependent RR's are comparable to those from literature that have used the same method, type of urine and measurement units.

The PhD student also draws attention to the fact that when interpreting results for UNC, conditions in which there is a change in the excretion of creatinine, which does not always follow that of NGAL (e.g., acute kidney injury and kidney transplant) should always be taken into account.

3. *Reference NGAL ranges in children. Biological variation*

(a) *in plasma* – there are few literarure sources on pNGAL reference ranges (RR's) in children, nonetheless, when taking into account the analytical method and the type of plasma, it is clear that there are no significant differences between the RR's identified in this study and those cited in the literature.

b) *in urine* – for uNGAL determined in childhood, there is slightly more data, but the results were obtained with different analytical methods and different types of urine (mainly spot urine), which explains the observed slight differences in RR in different studies. For studies with larger numbers of participants, age- and gender-dependent RR's have been found. UNC expression in different units of measurement is an additional factor, which clarifies differences in RR in childhood. With some studies with a larger number of children it was also possible to determine age- and gender-dependent RRs, which found a decrease in value with age, and higher values in girls, which can be explained with age- and gender-changing of creatinine

values in urine. In view of the negative, yet still statistically insignificant correlation found between UNC and age, quite correctly, the PhD student has drawn a conclusion on the need to include additional children of different ages in order to assess physiological age-related changes in UNC values in children.

4. *Diagnostic reliability of NGAL in the diagnosis of DKD in DM II*

With regard to pNGAL, a reasonable conclusion has been made from the results, that it is a marker for evaluation of glomerular filtration. No correlation relationship with ACR was established, with statistically significantly higher values for patients in A3 compared to those of the control group in line with expected more severe renal impairment and more severely reduced glomerular filtration. The results of other studies have been commented on, but once more it was underlined, that a comparison between them is not appropriate to make, due to methodological and other differences. In the natural course of renal impairment with DM, the decrease in eGFR is most commonly a late indicator of the presence of DKD and biomarkers, which correlate only with the decrease in eGFR, but not with albuminuria, are not suitable for diagnosis of DKD. It has been concluded logically that the independent use of pNGAL does not have the necessary effectiveness for routine diagnostics of DKD in patients with DM II.

With regard to uNGAL and UNC urine parameters, the results of this study are conclusive in terms of their relationship to albumin excretion – their levels are significantly increased in parallel with an increase in albuminuria levels, which is more pronounced for UNC. It also achieved a statistically significant difference even between patients with normalbuminuria (A1) and the control group. Their positive correlation with ACR is logical. These dependencies are consistent with numerous literature sources. Only one of them [177] did not establish a positive correlation relationship between ACR and uNGAL and it would be interesting to explain this fact. One of the main criteria for classifying patients with DM II in two main groups (with or without DKD) was the presence or absence of albuminuria and was the logical result for statistically significant differences for these two urine parameters in the two study groups, as well as the significant difference reached for UNC between the DM without DKD and the control group. The results of this study support the idea that NGAL as a marker of tubulointerstitial impairment can detect the development of DKD *prior to* its diagnosing through the increased AER. The diagnostic effectiveness of these indices for distinguishing between patients with DKD among DM II patients in the so-defined cut-off values shown in the Results section, has been summarized.

5. *NGAL as a marker of DKD progression in patients with DM II*

Evidence from numerous studies on pNGAL, uNGAL and UNC has been provided, which lead to the conclusion that tubular damage plays a crucial role in the progression of DKD and its development to the terminal stage. All this has been skilfully demonstrated in the current dissertation paper through the established correlations between pNGAL and decreased glomerular filtration (one of the criteria for stratification of DKD), the positive correlation between UNC and ACR (the other of the criteria for stratification of DKD), the demonstration that the simultaneous use of the results for pNGAL and UNC significantly predicts the severity of DKD. In line with the perception that cumulative glycaemic exposure is a major risk factor for the development of DKD, data on the significant correlation between UNC and HbA1c were provided, most likely an expression of more severe renal impairment in patients with poor glycaemic control.

6. *NGAL marker for diagnosis and prognosis of DKD in patients with DM I*

Adequate statistical processing of the data shows that pNGAL is an appropriate marker for differentiating patients with DM I with slightly reduced glomerular filtration. Two facts determine the impossibility of examining pNGAL for assessing the role of glomerular hyperfiltration – a small number of patients with eGFR (CKD-EPI40)≥120 mL/min/1.73 m² and expected low pNGAL<LLOQ values of the method used. Thus, the reasons for not assessing the role of the marker for the diagnosis of this earliest stage of renal impairment in the DM I, are correctly specified.

While in several studies of children with DM I, increased pNGAL values were found, even with normalbuminuria and positive correlation with ACR, and in others negative with GFR, only a negative correlation with eGFR (CKD-EPI40) was found in this study. Some of the authors suggest that normal AER in diabetics does not rule out the presence of DKD and point to pNGAL as a more sensitive marker than AER for assessing kidney function in children with DM.

Data on uNGAL and UNC as markers for identifying children with elevated ACR and detecting DBB among patients with DM I, presented in the dissertation paper, are more explicit and are consistent with numerous literature results establishing a positive correlation between uNGAL and UNC with ACR, and pointing to their predictive value for DKD diagnostics. The PhD student demonstrated high diagnostic effectiveness for uNGAL and UNC, at cut-off values of 47.85 ng/ml and 3.86 μg/mmol, respectively.

Through the statistical processing of the data for the five DM II, and three DM I participants, who dropped out of the study, due to existing UTI (urinary tract infection) and

leukocyturia, the PhD student makes a perfectly relevant and reasoned recommendation when interpreting results for uNGAL and UNC to take into account the impact of leukocyturia and UTI.

At the end of the discussion, it is summarized that the results of the dissertation unequivocally present NGAL as a promising marker, valuable for the diagnostics of DKD. Some limitations and limiting factors were also taken into account in the conduct of the scientific study, which proves not only the critical thinking of the PhD student, but also her utter candour and honesty, which should be characteristics of each and every scientist.

There are 13 conclusions drawn on the basis of the results obtained. They are logical and well-articulated, in compliance with the set purpose and objectives.

I fully accept the **theoretical contributions (7 in number) and contributions of a practical nature (7 in number)** pointed out.

The abstract of dissertation is set out in 80 pages and structurally meets the generally accepted requirements. It includes the most important research, results and discussions reflecting the work on the dissertation paper.

Publications related to the dissertation paper

Two full-text original articles related to the dissertation paper topic are published: one in a Bulgarian non-referenced journal and one in Bulgarian referenced journal. The PhD student has participated in two international scientific forums, at which she presented two posters, including part of the results obtained.

Conclusion

By structure, volume and content, the dissertation submitted to me for review, fully complies with the normative requirements. It is associated with a socio-significant disease and is the doctoral student's own development on a topical scientific and medical problem. The tasks assigned, fully tailored to the purpose of the study, have been successfully accomplished. The results obtained are discussed exhaustively and lead to the articulation of precise and clear conclusions. I agree with the stated contributions of a theoretical, practical, and applicable nature.

All this gives me a good reason to express **my positive assessment** with conviction, as to recommend to the esteemed Scientific Jury to award Dr. Sevim Ahmed Şhefket the scientific and academic degree of "Doctor" in the professional field 7.1. Medicine, and in the scientific specialty of Clinical Laboratory.

28.04.2022 Assoc. Prof. Daniela Gerova, MD, PHD: