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URINARY THROMBIN AS A MARKER OF LOCAL DISSEMINATED INTRAVASCULAR COAGULATION IN PATIENTS WITH CHRONIC KIDNEY DISEASE

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Abstract. Urinary thrombin as a marker of local disseminated intravascular coagulation in patients with chronic kidney disease. Mykhaloiko I.S. The aim of this research was to study the diagnostic markers of nonovert local disseminated intravascular coagulation (DIC) syndrome in the urine of patients with chronic kidney disease (CKD). We conducted a prospective study involving 140 patients with CKD, of these patients, 100 patients (71.4%; 95% CI 53.4-76.7) had glomerulonephritis (GN) and 40 patients (28.6%; 95% CI 21.3-36.8) had diabetic nephropathy (DN). We diagnosed overt DIC syndrome on the International Society of Thrombosis and Haemostasis (ISTH) scale (>5 points) in 18.6% of patients. We determined the level of thrombin in the urine of patients who had <5 points on ISTH scale for the diagnosis of local nonovert DIC syndrome in the kidneys. In the urine of healthy individuals, the level of thrombin did not exceed 1 ng/ml, so we found no thrombinuria at a thrombin level <1 ng/ml. In 56.1% of patients, we found urinary thrombin levels >1 ng/ml. The average level of thrombin in the urine of these patients was 6.5 (4.8; 10.6) ng/ml. In our opinion, the presence of thrombinuria indicates the intensity of monocytic-macrophage inflammation in the glomeruli and may be a criterion for nonovert, local DIC syndrome in the kidneys. The association of overt DIC syndrome with decreased blood albumin, reduced glomerular filtration rate (GFR), increased daily protein excretion (DPE) indicates its occurrence in severe underlying disease, in the presence of nephrotic syndrome and in the severe stages of CKD. Early diagnosis of nonovert local DIC syndrome would be more useful, since the process is still reversible and controlled, and timely use of antiplatelet and anticoagulant therapy would affect the course and the progression of CKD.

Реферат. Тромбін у сечі як маркер локального дисемінованого внутрішньосудинного згортання у хворих на хронічну хворобу нирок. Михалойко І.С. Метою цього дослідження було вивчення діагностичних маркерів прихованого локального синдрому дисемінованого внутрішньосудинного згортання (ДВЗ) у сечі хворих на хронічну хворобу нирок (ХХН). Для вирішення поставленої мети нами було проведено одномоментне проспективне обсерваційне дослідження із залученням 140 пацієнтів із ХХН, серед них 71,4% хворих на гломерулонефрит (ГН) і 28,6% на діабетичну нефропатію (ДН). Ми діагностували наявний ДВЗ-синдром за шкалою International Society of Thrombosis and Haemostasis (ISTH) (>5 балів) у 18,6%. З метою діагностики локального прихованого (nonovert) ДВЗ-синдрому в нирках ми визначили рівень тромбіну в сечі хворих, у яких за шкалою ISTH було <5 балів. У сечі практично здорових осіб рівень тромбіну не перевищував 1 нг/мл, тому ми констатували відсутність тромбінурії при рівні тромбіну <1 нг/мл. У 56,1% хворих ми виявили рівень тромбіну в сечі >1 нг/мл. Середній рівень тромбіну в сечі цих хворих становив 6,5 (4,8; 10,6) нг/мл. На нашу думку, наявність тромбінурії свідчить про інтенсивність моноцитарно-макрофагального запалення в гломерулах і може бути критерієм прихованого, локального ДВЗ-синдрому в нирках. Асоціація наявного ДВЗ-синдрому зі зниженням альбуміну крові, зниженням швидкості клубочкової фільтрації (ШКФ), підвищенням добової екскреції білка (ДЕБ) свідчить про його виникнення при тяжкому перебігу основного захворювання, за наявності нефротичного синдрому та в пізніх стадіях ХХН. Рання ж діагностика прихованого локального ДВЗ-синдрому була б більш корисною, оскільки процес є ще зворотнім та контрольованим, а своєчасне застосування терапії антиагрегантами й антикоагулянтами дозволило б вплинути на перебіг та прогресування ХХН.

The problem of stabilizing or slowing the progression of chronic kidney disease (CKD) remains unresolved, despite modern advances in nephrology. This encourages the identification of new links in the pathogenesis of CKD, prognostic markers of progression to develop more effective, individualized treatments [8]. Diagnosis of CKD in the early stages may prolong the pre-dialysis period, and in some patients renal replacement therapy may be effectively prevented [9].

All the above said makes a very important search for early diagnostic markers of disorders in CKD and methods of active influence on them, which would allow to prevent or delay the progression of kidney function loss.

Given the role of activation of the hemostasis system in the progressin of kidney disease, there is no doubt about the importance of detecting early signs of hypercoagulation in patients with CKD to assess the severity and prognosis of the disease [13].

Local, intrarenal changes of hemostasis in nephropathies are still not studied enough. It is not known how changes in the hemostatic system in general circulation in nephropathy correspond to the same processes in the vascular bed of the kidneys. However, the diagnosis and treatment of hemostasiological disorders in CKD is based on the study of coagulogram of total blood flow [11].

In our opinion, it is important to diagnose local latent hypercoagulation, which practically does not change the indicators of the total blood coagulation, that improves treatment and slows the progression of CKD.

Undoubtedly, in nephropathies not only blood hemostatic properties vary but also urine, due to the fact that the kidneys secrete procoagulants, anticoagulants and fibrinolytic substances into the blood and urine [2]. It is shown that the immunological status of urine changes earlier than blood. This shows the idea to diagnose CKD in its onset [8]. It is hard to say, if there are similar patterns in changing the coagulation balance of urine.

In 2001, the International Society of Thrombosis and Haemostasis (ISTH) proposed a definition of DIC (disseminated intravascular coagulation): "DIC is an acquired syndrome characterized by intravascular activation of coagulation without specific localization and occurs for various reasons. It can seriously damage the microcirculatory system and lead to organ dysfunction. ISTH distinguishes overt and nonovert DIC. It is clear that early diagnosis of nonovert, local DIC syndrome is more useful because the process is still reversible and controlled [11].

Thrombin was discovered as an enzyme that converts fibrinogen to fibrin, the structural basis of a

blood clot. Thrombin is a key component of the hemostasis system, it can be produced in all damaged tissues and its receptors are distributed on different cell surfaces [15]. In the area of endothelial damage, thrombin activates platelets and coagulation factors and enhances proinflammatory reactions, this provides local thrombus formation in the area of vascular wall damage [7]. Thrombin may also exacerbate glomerular inflammation by modulating monocyte/macrophage chemotaxis [10]. It is known that thrombin in the blood is rapidly inactivated by antithrombin in endothelial cell heparansulfate, forming a thrombin-antithrombin complex, so thrombin almost does not get into the urine from the blood [15], therefore, the presence of thrombinuria, in our opinion, may indicate the intensity of monocytic-macrophage inflammation in the glomeruli and may be a criterion for nonovert, local DIC syndrome in the kidneys.

The aim of this research was to study the diagnostic markers of nonovert local DIC syndrome in the urine of patients with CKD.

MATERIALS AND METHODS OF RESEARCH

We conducted a study involving 140 patients with CKD who were hospitalized to the Ivano-Frankivsk Regional Clinical Hospital (Ukraine) during 2018-2019. Of these patients, 100 patients (71.4%; 95% CI 53.4-76.7) had glomerulonephritis (GN) and 40 patients (28.6%; 95% CI 21.3-36.8) had diabetic nephropathy (DN). Among the patients, there were more men (n=92, 65.7%; 95% CI 57.2-73.5) than women (n=48, 34.3%; 95% CI 26.5-42.8). The average age of the patients was 46 years (41; 49).

CKD stage I was diagnosed in 36 patients (25.7%; 95% CI 18.7-33.8), CKD stage II – in 21 patients (15.0%; 95% CI 9.5-22.0), CKD stage IIIa – in 24 patients (17.1%; 95% CI 11,3-24,4), CKD stage IIIb – in 31 patients (22.1%; 95% CI 15.6-29.9) and CKD stage IV – in 28 patients (20.0%; 95% CI 13.7-27.6).

In 86 patients (61.4%; 95% CI 52.8-69.5) with GN and DN urinary syndrome was present, and in 54 patients (38.6%; 95% CI 30.5-47.2) – nephrotic syndrome.

In 25 patients (17.9%; 95% CI 11.9-25.2) the diagnosis of GN was confirmed morphologically, as follows: in 11 patients (44.0%; 95% CI 24.4-65.1) mesangioproliferative GN was confirmed, 5 patients (20.0%; 95% CI 6.8-40.7) had membranous nephropathy, 4 patients (16.0%; 95% CI 4.5-36.1) – focal-segmental glomerulosclerosis, 3 patients (12.0%; 95% CI 2.5-31.2) – nephropathy with minimal changes, and 2 patients (8.0%; 95% CI 1.0-26.0) – membrane proliferative (mesangiocapillary) GN.

Also, 40 practically healthy individuals were selected comprising a comparison group and were representative to the main group.

The clinical diagnosis was determined based on standard methods of examination according to the classification of kidney diseases and protocols of management of patients with CKD.

During the study, all patients underwent a standard examination, which included general clinical, biochemical and instrumental research methods. Biochemical tests and enzyme-linked immunosorbent assays (ELISAs) were performed in the laboratory of Ivano-Frankivsk Regional Clinical Hospital.

The research was performed in accordance with international standards for the coordinated participation of respondents, the ethical component of research and biomaterial collection (WMA Declaration of Helsinki – “Ethical principles for medical

research involving human subjects” and “Universal Declaration on Bioethics and Human Rights” (UNESCO)). All patients signed a written informed consent to participate in the study.

The glomerular filtration rate (GFR) was determined using a CKD-EPI calculator (<https://nephrology.kiev.ua/eGFR/gfr.htm>). Daily protein excretion (DPE) in urine collected within 24 hours was determined by colorimetric method (Dialab, Wiener Neudorf, Austria). Urine was stored at a temperature of 2-25°C. DPE reference value: <300 mg/day [6].

According to the ISTH criteria for the diagnosis of overt DIC syndrome in patients with CKD, we determined a number of coagulogram indicators: platelet count, prothrombin time (PT), D-dimer level, fibrinogen level (Table 1) [5].

Table 1

**International Society of Thrombosis and Haemostasis (ISTH)
scale for the diagnosis of overt DIC**

The presence of a clinical disease that can lead to the development of DIC	The main criterion - 1 point
Platelet count ($10^9/L$)	50-100 – 1 point <50 – 2 point
Fibrin-related marker (D-dimer, mg/L)	Moderate increase – 2 point Strong increase – 3 point
Fibrinogen, g/L	< 1 g/L – 1 point
Prolonged prothrombin time	> 3 s - 1 point > 6 s - 2 point
Diagnosis of overt DIC syndrome	≥ 5 point

Blood obtained from the patient’s ulnar vein (on an empty stomach) was used to study D-dimer. Blood serum was separated from erythrocytes by centrifugation (3000 rpm) for 10 min. Serum was stored at a temperature of 2-6°C. The analysis was performed up to 12 h from the time of blood collection. D-dimer was determined in blood serum quantitatively by ELISA using a set of reagents (Getein Biotech, Nanjing, China). In the test D-dimer monoclonal antibodies conjugated to colloidal gold applied in the test area were used. After application of the test sample of blood serum on the pad of the test strip, D-dimer monoclonal antibodies interact with the D-dimer in the sample and form an

antigen-antibody complex. After that, the test cassette was inserted into the ELISA FIA8000 (Getein Biotech, Nanjing, China) to quantify the level of D-dimer. Reference value for D-dimer levels: the concentration of D-dimers in blood serum of healthy people with no thrombotic risk was <0.5 mg/l [16].

Platelet counts in peripheral blood were determined using hematology analyzer Siemens (Berlin, Germany) [12].

The method of determining PT is based on measuring the clotting time of blood plasma in the presence of tissue thromboplastin and calcium chloride. Reference values 11-15 s [3].

Fibrinogen was determined by the Claus method which includes the definition content of fibrinogen by the rate of clot formation adding excess thrombin to the diluted plasma on the Siemens automatic coagulometer (Berlin, Germany). Reference values are 2-4 g/l [4].

Determination of thrombin levels in urine was carried out quantitatively by enzyme-linked immunosorbent assay using a set of reagents MyBioSource (San Diego, USA). For the study, a morning portion of urine was collected, centrifuged (1500 rpm) for 10 minutes and used to determine thrombin 1-2 ml of supernatant. Supernatants were stored at –20°C until use. In this experiment a double-sandwich elisa technique was used in which monoclonal antibodies to human thrombin conjugated to biotin were applied in the test area. After applying the test sample of the supernatant on the pad of the test strip, the antibodies interact with thrombin in the sample and form an antigen-antibody complex. Detection range 0.312-20 ng/ml, sensitivity – 0.06 ng/ml [14].

STATISTICA 8 software (StatSoft, Serial STA862D175437Q) was used for statistical analysis. The frequency of qualitative indicators was presented in absolute (n) and relative (%) frequencies with the indication of the 95% confidence interval (CI) in the form of “n (%; 95% CI)”. When analyzing quantitative data, it was necessary to determine the nature of the distribution of indicator values using Shapiro-Wilk’s test. For quantitative data with a normal distribution, the results were represented as “M (σ),” where M is the mean value and σ is the standard deviation. For quantitative data with an abnormal distribution, “Me (q1; q2)” was used, where Me is the median and q1; q2 are quartiles. Quantitative indicators with normal distribution of values in 2 independent groups were compared using the Student’s criterion. Quantitative parameters with abnormal distribution in 2 independent groups were compared using the MannWhitney method. Comparison of 2 independent groups for the qualitative indicator was carried out according to the exact Fisher criterion [1].

RESULTS AND DISCUSSION

We diagnosed overt DIC syndrome (> 5 points) in 26 of 140 patients (18.6%; 95% CI 12.5-26.0), of whom 16 patients (61.5%; 95% of CI 40.6-79.8) were diagnosed with GN, and 10 patients (38.5%; 95% CI 20.2-59.4) – DN, in 23 patients (88.5%; 95% CI 69.8-97.6) the disease was accompanied by nephrotic syndrome.

We determined the level of thrombin in the urine of patients who had <5 points on ISTH scale for the diagnosis of local nonovert DIC syndrome in the kidneys.

In the urine of healthy individuals, the level of thrombin did not exceed 1 ng/ml, so we found no thrombinuria at a thrombin level <1 ng/ml. In 64 of 114 (56.1%; 95% CI 46.5-65.4) patients, we found urinary thrombin levels >1 ng/ml. The average level of thrombin in the urine of these patients was 6.5 (4.8; 10.6) ng/ml. Thrombinuria was observed in 18 patients with DN (28.1%; 95% CI 17.6-40.8) and in 46 patients (71.9%; 95% CI 59.2-82.4) with GN.

The presence of free thrombin in the urine of patients with GN and DN indicates the possibility of the thrombin being excreted with the urine when it is generated in the affected glomeruli under the influence of tissue factor. The researchers also found that mesangial glomerular cells can secrete tissue factor due to stimulation by cytokines, which can also aggravate thrombinuria [17]. In our opinion, the presence of thrombinuria may be a criterion for nonovert local DIC syndrome in the kidneys. This hypothesis is also confirmed by the data on the absence of thrombin in the urine in patients with overt DIC [7]. There are isolated studies which report the detection of thrombin in bronchoalveolar lavage in patients with pulmonary fibrosis, also thrombin is detected immunohistochemically in brain tissues in Alzheimer's disease [15].

We divided patients into three groups (group I – patients without general and local hemostasis disorders, group II – patients with nonovert local DIC syndrome, group III – patients with overt DIC syndrome and compared them by sex, age, GFR, blood albumin levels and DPE (Table 2).

As can be seen from Table 2, patients with overt DIC syndrome had significantly lower GFR, significantly lower blood albumin levels and significantly higher DPE compared with group without general and local hemostasis disorders and a group with nonovert local DIC syndrome, respectively ($p<0.05$; $p_1<0.05$). Patients with nonovert local DIC had significantly higher DPE compared with the group without general and local hemostasis disorders ($p<0.05$). While there were more men than women in both groups, the difference was not statistically significant ($p=0.437$).

Association of overt DIC syndrome with decreased blood albumin, reduced GFR, increased DPE indicates its occurrence in severe underlying disease and in the late stages of CKD.

Early diagnosis of nonovert local DIC syndrome would be more useful, since the process is still reversible and controlled, and timely use of antiplatelet and anticoagulant therapy would affect the course and progression of CKD.

Table 2

Comparative characteristics of the patients

	I group - patients without general and local hemostasis disorders (n=50)	II group - patients with nonovert local DIC syndrome (n=64)	III group - patients with overt DIC syndrome (n=26)
Sex, male (%; 95% CI)	72.0 (57.5-83.8)	62.5 (49.5-74.3) p=0.828	61.5 (40.6-79.8) p=0.437 p ₁ =1
Sex, female (%; 95% CI)	28.0 (16.2-42.5)	37.5 (25.7-50.5) p=0.828	38.5 (20.2-59.4) p=0.437 p ₁ =1
Age, years Me (q1 ; q2)	43 (40; 45)	45 (43; 47) p>0.05	49 (46; 53) p>0.05 p ₁ >0.05
GFR, ml/min/1.73 m ³ , Me (q1 ; q2)	76 (63; 89)	73 (58; 86) p>0.05	47 (38; 54) p<0.05 p ₁ <0.05
Albumin levels, g/l, Me (q1 ; q2)	34.4 (29.3; 36.7)	33.5 (28.5; 35.6) p>0.05	28.4 (25.3; 30.7) p<0.05 p ₁ <0.05
DPE, mg/day, Me (q1 ; q2)	1232 (923; 1545)	1546 (1012; 1945) p<0.05	3242 (2787; 4324) p<0.05 p ₁ <0.05

Notes: CI – confidence interval, DPE – daily protein excretion, GFR – glomerular filtration rate; Me (q1 ; q2) – median and quartiles; p – the reliability of the difference between the II and III groups in comparison with the I group; p₁ – the reliability of the difference between the III and II groups.

CONCLUSIONS

Determination of thrombin in urine can be a new marker for the diagnosis of nonovert local DIC syndrome in the kidneys, which will allow the

timely use of antiplatelet and anticoagulant therapy and evaluate the effectiveness of this therapy.

Conflict of interests. The authors declare no conflict of interest.

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