Optical method for screening and a new proteinuria focus group

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Abstract. The detection of small quantities of proteinuria has gained significance as multiple studies have demonstrated its diagnostic, pathogenic, and prognostic importance. More than 260 samples of urine taken from the patients suffering chronic kidney disease (CKD), diabetes and hypertension have been analysed in the certified laboratory, with urine analyser H-50 (urine test strips) and with an optoelectronic set-up specially designed for this study. Albumin, protein and creatinine concentrations have been determined in the laboratory and the data thoroughly analysed with the aim to find new approaches to tackle the lowered level proteinuria problems. Special attention has been paid to a particular screening focus group of 16 patients all having normal or slightly abnormal levels of albumin in parallel with enhanced levels of total protein (45% cases) up to 0.4 g/L. A fair correlation between the maxima in the protein, protein/creatinine, protein/albumin values and CKD in the focus group has been observed. The urine test strips method gave 94% negative false results for the focus group whereas the new sensor has shown in all cases the presence of proteins. The sensor signals higher than the mean in this focus group were obtained for the donors with the diagnosed CKD and some other diseases. The new method is based on the optical absorption measurements (285 nm) in the protein fractions received with use of the commercial desalting columns PD-10. The method can be applied in the wide region of protein concentrations from ≤0.1 g/L up to the levels of severe proteinuria (~10g/L).

Keywords: albuminuria, screening, Tamm-Horsfall protein, Bence-Jones protein, diabetic nephropathy.

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References

1. K. Matsushita, M. van der Velde, B. C. Astor, M. Woodwart, A. S. Levey, P. E. de Jong, J. Coresh, and R. T. Gansevoort, "Association of estimated GFR and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis," The Lancet 375(9731), 2073-2081 (2010).
2. KDIGO Clinical Practice Guideline of CKD, Kidney International Supplements 3, (2013).
3. M. Rosenberg, and Ü. Pechter, "Microalbuminuuria – südame- ja veresoonkonnahaiguse riski näitaja," Eesti arst, 86(7), 470-473 (2007).
4. KDIGO Guideline for the Care of Kidney Transplant Recipients.
5. G. Pittman, "Evidence lacking on screening for kidney disease," Reuters Health, 27 August. 2012.
6. R. T. Gansevoort, K. Matsushita, M. van der Velde, B. C. Astor, M. Woodward, A. S. Levey, P. E. de Jong, and J. Coresh, "Lower estimated GFR and albuminuria associated with adverse kidney outcomes. A collaborative meta-analysis of general and high-risk population cohorts," Kidney Int. 80(1), 93-104 (2011).

7. J. A. Simerville, M. C. Maxted and J. J. Pahira, "Urinalysis: A Comprehensive Review," Am. Family Physician 71(6), 1153-1162 (2005).
8. J. Dube, J. Girouard, P. Leclerc, and P. Douville, "Problems with the estimation of urine protein by automated assays," Clin. Biochemistry 38(5), 479-485 (2005).
9. P. L. Lynch, J. Savory, and D. M. Haverstick, "Urine total measurement with the Vitros dry reagent technology: modification of diluent to resolve positive bias of diluted samples," Clin. Chem. 44(3), 674-675 (1998).
10. R. E. Chambers, D. G. Bullock, and J. T. Whicker, "Urinary total protein estimation – fact or fiction?" Nephron 53(1), 33-36 (1989).
11. M. Rosenberg, A. Kuznetsov, and A. Frorip, "Threshold-like dependences in albuminuria/proteinuria," Eesti Arst (Estonian Doctor) 92(Suppl. 2), 1-52 (2013).
12. A. Sünter, A. Kuznetsov, A. Frorip, V. Korsakov, and M. Rosenberg, "Optical methods, devise and statistics of determination of albumin and/or protein concentration in human urine," Saratov Fall Meeting 2015, Biophotonics Internet Invited Lecture.
13. S. G. Guminetsky, O. R. Gauka, G. I. Kokoschuk, P. M. Grigorishin, and N. L. Kirsh, "Absorption spectra of the main organic components of human urine in the absence of proteins," Proc. SPIE 3904, 579 (1999).
14. K. Umimoto, Y. Kanaya, H. Kawanishi, and N. Kawai, "Measuring of uremic substances in dialysate by visible ultraviolet spectroscopy," IFMBE Proc. 25(7), 42-45 (2009).
15. H.A. Schwertner, "Isolation and chromatographic analysis of unidentified fluorescence in biological fluids of patients with chronic renal disease," Nephron 31(3), 209-211 (1982).
16. A. Kuznetsov, A. Frorip, A. Maiste, M. Ots-Rosenberg, and A. Sünter, "Visible auto-fluorescence in biological fluids as biomarker of pathological processes and new monitoring tool," J. Innov. Opt. Health Sci. 8(3), 1541003 (2015).
17. PD-10 Desalting Columns (GE Healthcare), Instructions 52-1308-00 BB, General Electric Company, September 2007.
18. G. Konoplev et al., "Dual-wavelength optical sensor for on-line monitoring of uric acid concentration in spent dialysate during hemodialysis," XI Baltic Nephrology Conference (Tartu), Programme and Abstract Book 48, 20-22 September 2012.
19. Y. Zuo, C. Wang, J. Zho, A. Sachdeva, and V. C. Ruelos, "Simultaneous Determination of Creatinine and Uric Acid in Human Urine by High-Performance Liquid Chromatography," Analytical Sciences 24(12), 1589-1592 (2008).
20. Y. Yue-dong, "Simultaneous determination of creatine, uric acid, creatinine and hippuric acid in urine by high performance liquid chromatography," Biomedical Chromatography 12(2), 47-49 (1998).
21. Urine Anlyzer H-50.
22. E. V. Lerma, T. Desai, and P. Jawa, "Proteinuria: Background, Pathophysiology, Etiology," 10 December 2015.
23. Reference < 200 g/g for protein/creatinine is being issued in the routine praxis of the TUHL.
24. Extinction Coefficients, A guide to understanding extinction coefficients, with emphasis on spectrophotometric determination of protein concentration, Pierce Biotechnology, October 2002.
25. S. C. Gill, and P. H. von Hippel, "Calculation of Protein Extinction Coefficients from Amino Acid Sequence Data," Anal. Biochem. 182(2), 319-326 (1989).
26. D. C. J. Rhodes, "Binding of Tamm-Horsfall protein to complement 1q measured by ELISA and resonant mirror biosensor techniques under various ionic-strength conditions," Immunology and Cell Biology 78(5), 474-482 (2000).
27. SIGMA ProductInformation.
28. I. Björk, F. A. Karlsson, and I. Berggård, "Independant Folding of the Variable and Constant Halves of a Lambda Immuniglobulin Light Chain," Proc. Nat. Acad. Sci. 68(8), 1707-1710 (1971).
29. B. L. Boyanton, and K. E. Blick, "Stability Studies of Twenty-Four Analytes in Human Plasma and Serum," Clin. Chem. 48(12), 2242-2247 (2002).
30. A. Kuznetsov, A. Frorip, M. Rosenberg, and N. Mulina, "Time dependences of concentrations of serum creatinine and uric acid in spent dialysate," X1 Baltic Nephrology Conference (Tartu), Programme and Abstract Book 51, 20-22 September 2012.
31. C. Y. Yang, F. A. Chen, C. F. Chen, W. S. Liu, C. J. Shih, S. M. Ou, W. C. Yang. C. C. Lin, and A. H. Yang, "Diagnostic Accuracy of Urine Protein/Creatinine Ratio Is Influenced by Urine Concentration," PLoS One 10(9), e0137460 (2015).

1 Introduction

Population studies have demonstrated the presence of the chronic kidney disease (CKD) in 10% cases and therefore CKD is an important public health issue. CKD is the common disease but few individuals have strongly reduced glomerular filtration rate (GFR), kidney failure or severely increased albuminuria. However, there is

substantial evidence linking increased albuminuria to outcomes of CKD [1, 2]. The detection and evaluation of small quantities of proteinuria have gained significance as multiple studies have demonstrated its diagnostic, pathogenic, and prognostic importance [1-3]. There exists a large need for the express and cheap methods for the population screening and regular monitoring of albumin and/or protein levels in urine of people suffering the CKD. Albuminuria should be monitored also regularly in patients with transplanted kidney [4]. Screening of healthy people is also an important topic but there is not enough evidence to recommend checking the kidney function of otherwise healthy people for signs of disease [5]. One can think that the development of assay methods will allow the people to perform self-screening even at home. This may be appropriate because the CKD is the common disease and higher albuminuria is associated with adverse kidney outcomes [6].

Urine test strips (or dipsticks) are well known instruments invented for such purposes but the cheap test strips cannot enable sufficient sensitivity and precision. In the KDIGO 2012 guidelines [2] it is stated that "…there is no standardization between manufacturers. The use of such strips should be discouraged in favour of quantitative laboratory measurements of albuminuria or proteinuria". Strips are envisaged mainly for selective detection of albumin and are not sensitive to some other proteins, e.g., Bence-Jones proteins (monoclonal heavy or light chains) and give often negative false results [7].

At the same time the laboratory total protein assays are also commonly flawed and under these circumstances are often still standardized against albumin [2]. Comparison of different automated assays of urine protein was carried out [8]. It was established that the results of different methods (Randox, Beckman, Roche and Vitros) at low, so called, normal protein concentrations (≤ 0.1 g/L in the untimed samples) do not correlate with each other and with the data obtained by the Ponceau S/TCA manual method used by the authors. It was also speculated that the colorimetric (a dye binding; Randox, Beckman, Vitros) and turbidimetric (protein agglutination; Roche) methods usually used in automated technologies can be not neutral for very complex urine environment and can depend on the concentration of one or another urine component, e.g., sodium chloride and other salts [9].

There is one more possible explanation of deviations connected with different reaction of different protein components (albumin, globulin, Tamm-Horsfall, polypeptides) with different reagents under use. For instance, small peptides induced significant signals with the Beckman and Randox assays and were neglected by the Roche and Ponceau S/TCA assays [8].

All circumstances mentioned above manifest that the protein quantification at lowered concentrations can be a challenge [10] not only for the work with the express methods but even for the certified automated laboratories. At the same time it is often impossible to avoid the direct estimation of proteins because there are the diseases when it is necessary to know just the total protein and not only albumin concentration (tubular proteinuria, children diseases etc.) [2].

In this study we have encountered a particular case where by the low (normal or slightly abnormal) levels of urine albumin (≤ 0.03 g/L) the total protein concentrations can be rather high, e.g., by the factor of 10 and much more. Abnormal protein level is correlated with diabetes, hypertension, kidney and urinary failures in these urine donors. This new fact directly points to one more reason why it is important to provide the total protein quantification in urine.

2 Aims

The main aim of the undertaken study was to demonstrate the possibility to exploit the UV absorption of proteins at $\lambda \approx 285$ nm in specially fractionated urine for estimation of their concentration in the whole human urine. The second goal of the project was the comparison of optical results with those obtained in parallel by use of urine test strips and automated (Roche) assays. Additionally, a concise analysis of the obtained results from the clinical point of view is also undertaken. The given research is a continuation of our previous studies [11, 12].

3 Method, patient and samples

3.1 Urine fractionation

We have used the direct UV absorption measurement at $\lambda \approx 285$ nm for the estimation of total protein concentration. In the whole urine the absorption at ~280 nm is very high and is induced mainly by uric acid [13, 14] and, so called, visible (blue) fluorescence substance (VFS) present in all biological fluids [15, 16]. The absorption of proteins is usually totally masked by these components. Therefore urine has to be fractionated to extract the protein fraction envisaged for optical measurements.

For fractionation commercially available and cheap PD-10 desalting columns (GE Healthcare) with the cut-off at M < 5 kDa were used [17]. The urine fraction eluted as the timely first contains the whole pool of the typical urine proteins (albumin, globulin, Tamm-Horsfall, etc.) and, eventually, some large peptides and complexes with the molecular masses M > 5 kDa.

The chronogram of such a fractionation of urine with the starting protein concentration of 5 g/L is represented in Fig.1. The sharp peak at the retention time $t \approx 1.5$ min belongs to the total protein fraction. The other peaks are induced by the VFS (the interval 3.5 – 5 min) and uric acid (7.5 min). Additional analysis has shown that in the protein fraction there are residuals of the abundant metabolites urea and creatinine in a rather high concentration of ~ 10 and ~1 mmol/L, respectively. These metabolites absorb the light in the region $\lambda < 250$ nm and do not disturb our

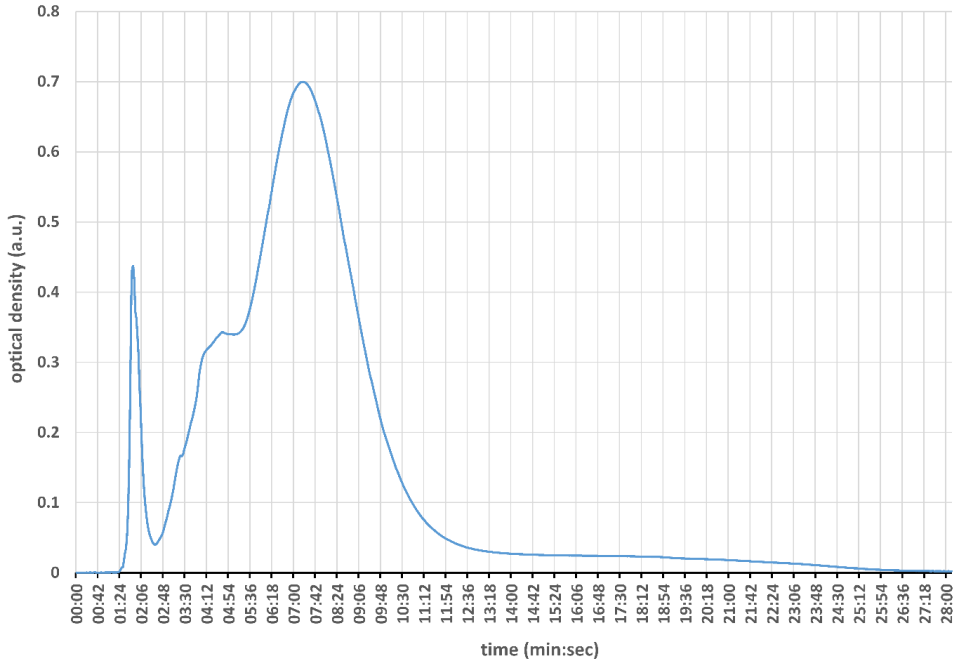

Fig. 1 Fractionation chronogram of albuminuria urine in a desalting column PD-10. The 1.5 min elution peak belongs to proteins with M > 5 Da whereas the hump at 3.5 min to the VFS and the most intensive maximum (6.5 min) to uric acid.

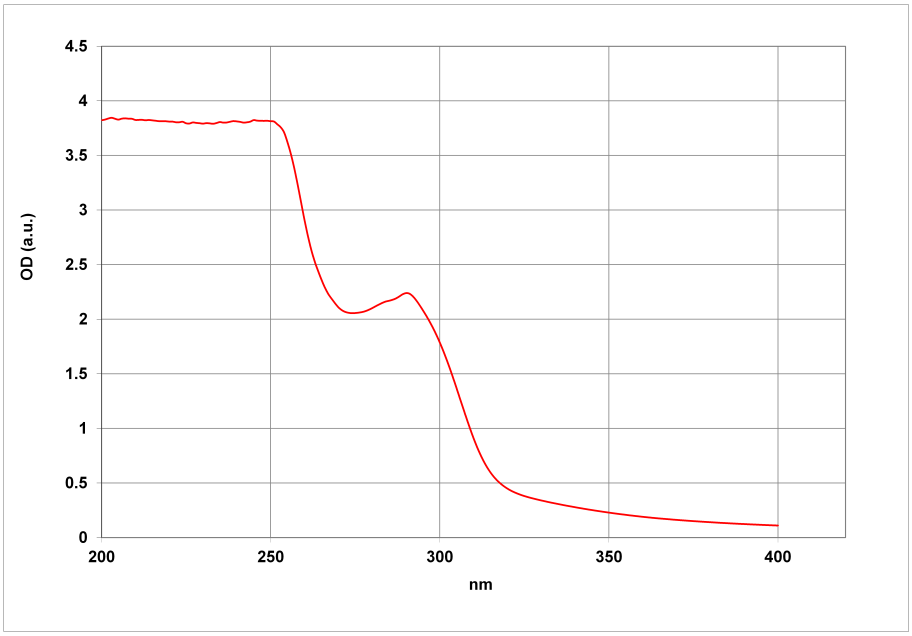

Fig. 2 Absorption spectrum of a urine fraction M > 5kDa in the 5 mm thick layer. The concentration of protein in the whole starting urine was 5 g/L.

measurements (see also [14]). The presence of uric acid could not be established in the protein fraction at the sensitivity level of the Cobas 6000 Roche method (<12 µmol/L). No correlation between the uric acid concentration in the starting urine and the protein peak maximum in the chronograms has been seen.

The absorption spectrum of a protein fraction produced by use of a PD-10 column is given in Fig.2. The spectrum was measured at a spectrophotometer Jasco V-550. One can see that the absorption for the 5 mm layer in the case of protein concentrations in the starting urine material in the range of several g/L (5 g/L in the given case) is high with optical density (OD) of >2. In the special test with water dilution of a high protein level fraction the linearity of OD in the range 2.84 – 0.05 with a very small correlation variation (CV) (R^2 = 0.9996) was established.

It is important to mention that one and the same PD-10 column is suitable for multiple usages up to 100 times and more. This experience has been obtained in

the exploitation mode applied in this study: 200 μL of starting urine plus 50 ml of a buffer for one fractionation procedure. By this protocol strong dilution 1:250 does take place and the variations in concentration (specific density) of starting urines are minimised and could not be taken into account.

3.2 Choose of buffers

We have proved different buffer liquids needed for fractionation with the wide pH range from pH3.5 (e.g., blank dialysate SW 380 A) up to pH9.3 (10 mM TRIS + 150 mM NaCl + 2 mM EDTA). The distilled or salt water with 0.9 wt. % of NaCl (physiological fluid) can be used too. One can obtain higher signals in the chronograms for the protein peaks (M > 5 kDa) with distilled water but the measurement errors, i.e., stdev/average in this case were sometimes higher than with the other buffers, i.e., ≈10% instead of 3.5 – 5% for the more complex buffers. The results represented in this paper are obtained mostly with the basic buffer pH9.3 (10 mM TRIS +150 mM NaCl +2 mM EDTA).

3.3 CKD patients

Urine samples were collected from the CKD or at CKD risk subjects (diabetes and/or hypertension) treated in the nephrology division at the Tartu University Hospital (TUH) or monitored in the ambulatory service at the TUH or at the Family Medicine Providers Centre (FMPC) Mõisavahe (Tartu).

In this study we have paid much attention to the patients with the normal urine albumin (≤ 0.03 g/L) and with abnormal protein (≥ 0.1 g/L) in untimed samples. The FMPC part of them (16 patients) constituted the main focus group for the further scrutinised investigation.

Additionally, 5 urine samples were taken from the healthy people for comparison purposes.

The average age in the main focus group (50% males and 50% females) was 56.75 years, whereas the average age for the controls (4 males and one female) was "tuned" to the close level of 57.6.

3.4 Urine samples

Altogether 261 samples of urine were collected and analysed. All samples were taken one or two times and usually in the first half of the day. No 10-hours or day night collection attempts were undertaken. Approximately 150 different urine samples were assayed in the laboratory of the Tartu University Hospital (TUHL) where the Cobas 6000 Roche systems technology is being used. The albumin, protein, creatinine and, sometimes, uric acid concentrations were determined. The dominant part of urine samples were delivered to the TUHL as soon as possible (mostly within one hour). The samples collected at the FMPC could be transported to the TUHL in several hours.

Many urine samples were stored in the frozen condition at -10^{0}C and some of those were reused for additional or special tests. No remarkable changes in relation to protein optical absorption after the freezing/de-freezing circles have been noted.

A part of urines were centrifuged before fractionation at the angle speeds of 1200-3000 rpm. Sometimes there were precipitates on the bottom of test-tubes with fresh or de-frozen centrifuged urines. We avoided using in experiments the lowest part of the fluids with precipitates but one cannot exclude the influence of the smallest suspension particles which scatter the light and affect the accuracy of absorption measurements. Different scattered light levels were indicated in some samples in the urine transparency region at $\lambda > 300$ nm but they were not quantified and not used in the estimations. This matter should be given more attention in the future (see p. 5.1.).

Another part of urines was filtered with use of the Whatman FP30/0.45 CA-S 0.45 μm filters. More stable results have been obtained with filtered urines than with centrifuged ones.

3.5 Optical cell and chronograms recording

For the measurements a UV optical cell cognate with that used in [18] was connected to the exit of a column PD-10 and the standard chronogram recording on a computer monitor took place (Fig. 1). This approach resembles those used earlier for determination of small molecular mass urine components by the methods of high performance liquid chromatography [19, 20]. We have used the area under the protein peak in the chronograms as the measure of protein absorption (concentration) after the recalculation "*peak area → concentration*". The computer programme stopped the measurement at the first minimum in the chronogram, i.e. approximately at the retention time t ≈ 2.5 min (Fig.1) so that the peak measurement procedure lasted usually less than 2 min. One needs an additional time (~10 min) for the elution of all smaller mass fractions and prepare the column for the next measurement.

The analytical error "stdev/average" in the multiple test measurements (10 up to 20) with one and the same urine was mostly between 3.5 and 5% at the low (0.1-0.2) as well as at the high protein concentrations (3.5 g/L).

There is some inaccuracy in this mode of measurements because of the overlapping of the protein and strong VFS elution peaks (Fig. 1). We have circumvented this difficulty by the use of the empirical correlation "*protein peak area*" versus "*certified protein concentration in the starting whole urine*". This correlation was obtained on the base of the numerous (~ 100) parallel measurements in the TUHL and by our method with all possible urine protein concentrations from 0.03 up to 10 g/L. The obtained linear dependence formula $p = k\ x\ + b$, where p is the protein concentration, x – peak area, k – slope and b – bias, served further for estimation of a newly measured protein concentration (for the value of the parameters k and b see *p.4.1*). This approach does work well. The

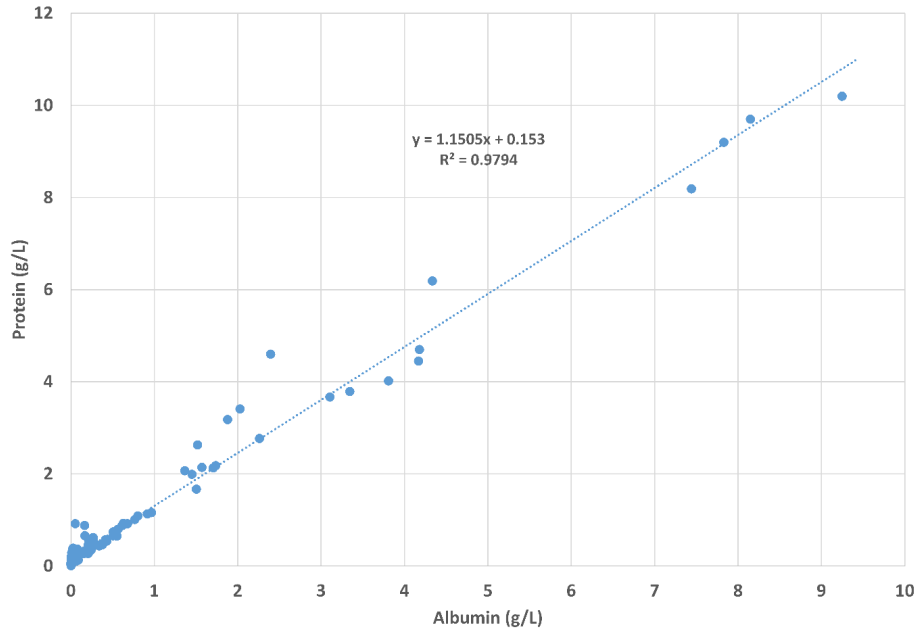

Fig. 3 Correlation between albumin and protein concentrations determined by the Roche assays in the whole urines (133 samples) in the widest available range of concentrations from 0.002 g/L for albumin up to 9.3 g/L for both albumin and protein.

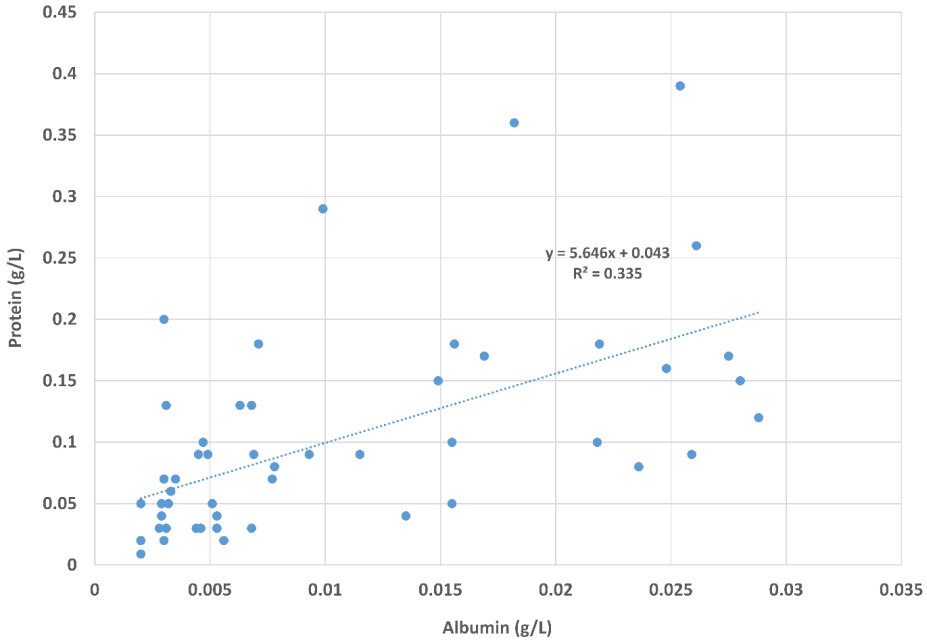

Fig. 4 The part of the lowest albumin vs protein concentrations correlation as a fragment from the united interdependence in Fig.3.

differences sometimes still observable in the results obtained by the proposed method and Cobas 6000 Roche assays can be connected not so much with the technical aspects of measurements but with different and hardly predictable variations in interaction of urine components in the different methods and technologies (see also Introduction and Discussion). Nevertheless, we have used in this study the laboratory data as the reference and designate these as “certified data”.

3.6 Use of urine test strips

We have used a commercial DIRUI urinalysis instrument H-50 for the work with the samples collected at the FMPC. In this photoelectric colorimetry device the urinalysis strips of the type H13 were used for an automated reading of coloured strips at six possible wavelengths 400, 520, 560, 610, 660 and 940 nm [21].

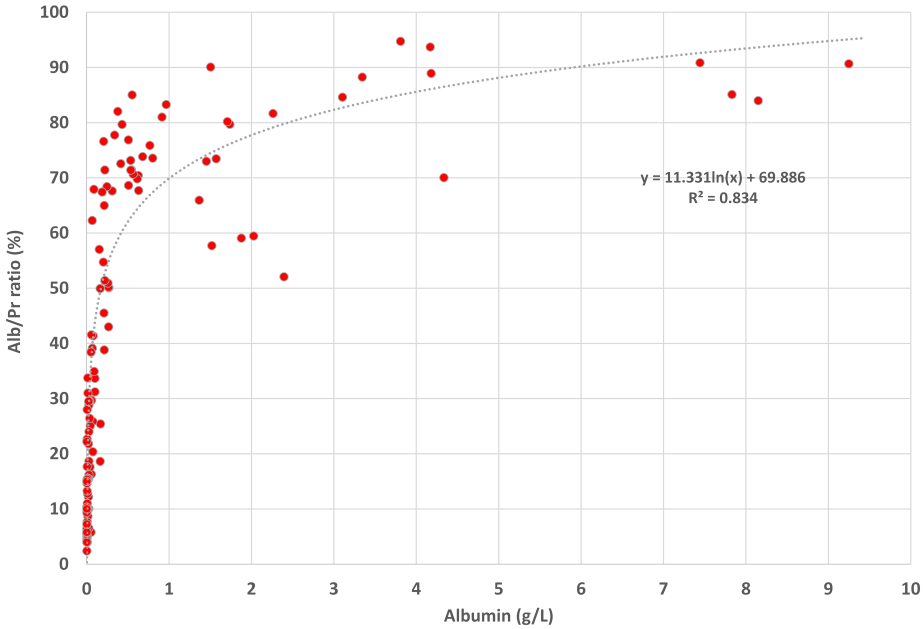

Fig. 5 Albumin/protein ratio Alb/Pr dependence on albumin concentration in 133 samples of the whole urines (compare with Fig. 3).

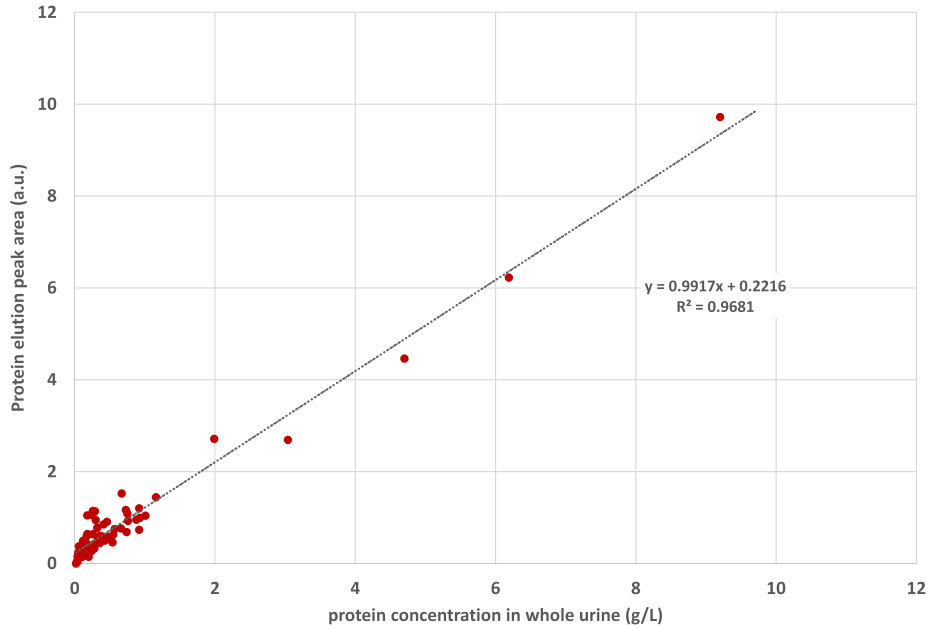

Fig. 6 Correlation between the absorbed light sum designated as the "protein elution peak area" and concentration of protein in the corresponding whole urines established for the widest range of concentrations up to ~10 g/L.

4 Results

4.1 Certified data obtained with the whole urine samples

In Fig.3 we demonstrate the correlation between albumin and protein concentrations in the whole urines using the data obtained by the Cobas 6000 Roche technique for 133 samples. We have tested the urines with the widest available concentration extension from the lowest 0.002 g/L of albumin and 0.01 g/L of protein for healthy people up to ≈10 g/L for both components in the severe proteinuria case. In these wide scales the obtained correlation

$$y = 1.15x + 0.15 \text{ g/L} \quad (1)$$

is perfectly linear (R^2 = 0.9794) and can be used for rough estimation of protein concentration y on the base of known albumin data x or *vice versa*.

It is important to note that in the empirical formula (1) there is the bias parameter b = 0.15 g/L. This would mean that in the albumin-free urines (x=0) there are other proteins at a rather high concentration of 0.15 g/L.

The similar correlation formula holds also for the albuminuria region 0.03 – 0.3 g/L (36 samples in our collection)

$$y = 1.15x + 0.19 \text{ g/L} \quad (2)$$

with the CVs much larger in this case (R^2 = 0.2391).

If we had focused our consideration particularly onto the region of the lowest levels of both albumin and protein, i.e., at the normal albumin concentrations $x \leq 0.03$ g/L (50 donors) we have obtained the essentially different correlation

$$y = 5.65x + 0.04 \text{ g/L}. \quad (3)$$

The corresponding distribution is depicted in Fig. 4. The proportionality coefficient 5.65 before x in (3) is much larger than in (1) and (2) showing the diminished role of albumin in this region. The decrease of the bias down to 0.04 g/L is also remarkable. The scattering of the dots in Fig. 4 is high (R^2 = 0.335) what creates difficulties for precise work in this domain. One can see that there are some points which deviate especially strongly upwards and lie in the range of protein concentrations 0.15 – 0.4 g/L. This region is beyond the normality level of protein determined typically at < 0.15 g /24 hours or translated to the concentration < 0.1 g/L by the daily urine dose 1.5 L [22]. We will pay later on the main attention just to these enhanced protein values (see *p.4.3*).

Fig. 5 demonstrates one more possibility to distinguish three sub-regions in the wide proteinuria region 0.01 – 10 g/L which need to be addressed separately. There is in Fig. 5 the depicted distribution of concentration ratios albumin/protein Alb/Pr in the same family of samples as in Fig. 3. The distribution can be fairly approximated by the logarithmic law

$$y = 11.33\ln(x) + 69.89, \quad (4)$$

where x is the concentration of albumin in g/L and y – ratio Alb/Pr in %. At the largest concentrations (> 2 g/L) the total protein consists mostly of albumin (>80%). The albumin portion asymptotically approaches 100% and one can say that in this region proteinuria is, in essence, albuminuria or *vice versa*. At the curve bend around 1 g/L the domination of albumin is not so strong (~70%) and in the region of lower values (< 0.3 g/L) the ratio Alb/Pr decreases abruptly. In this region the significance of albumin is diminished and it is even doubtful whether the albumin can be used as a standardization agent. In determination of protein by means of measurement of albumin it would be the case of determination of a "large" through a "small". These observation and distribution (Fig. 5) gave us one more impetus to elaborate a method for direct determination of non-albumin proteins.

4.2 Optical sensor data

In Fig. 6 we represent the correlation between the optical sensor readings obtained with the urine protein fractions as described in *p. 3.1.* and protein concentrations (g/L) in the whole urines as assayed by the Roche method in the TUHL. The correlation

$$y = 0.99x + 0.22 \text{ g/L}, \quad (5)$$

where x is the protein concentration in g/L in a whole urine and y is a sensor reading. The formula (5) is valid for estimation of high protein concentrations (> 1 g/L): the difference y – 0.22 g/L gives the protein concentration. Using obtained protein concentration one can estimate also the albumin level with the aid of the correlation (1). The accuracy of these estimations is not very high (±20% or even more) but one can mention, for comparison, that the urine test strips do not typically work selectively in the region of concentrations > 0.3 g/L.

In the region of the lower proteins the correlation (5) transforms into the other formulas. For instance, for the protein concentrations ≤ 0.3 g/L the correlation is:

$$y = 2.284x + 0.047 \text{ g/L}; (R^2 = 0.405) \quad (6)$$

The remarkable CVs coming together with the correlation (6) is a reflection of the fundamental phenomenon that the proportions of albumin and non-albumin proteins are very different in the urines from the range of normal (≤0.03), microalbuminuria 03 – 0.3) and severe albuminuria (> 1 g/L) (Fig 5).

It is important to stress that the determination uncertainties arise not just from the low accuracy of the method or sensor itself. The analytical error "stdev/average" was in some tests with 10 up to 20 repeatedly done fractionations only 3.3% and always below 10%. The optical sensor readings are induced by the absorption of albumin and other proteins altogether. For instance, two urines can be of one and the same concentration of low total protein but with a different proportion protein/albumin (Fig. 4) and the sensor readings can be different since not all total protein components must have the equal absorption coefficients. In fact, the similar problem exists by the biochemical assays since not all particular proteins give the equal yields in reactions with dyes or coagulants [8]. This problem is considered in more details in *p. 5.2*.

4.3 Screening results

We present here the results of a pilot population screening action performed at the FMPS Mõisavahe (Tartu) (see *p.3.3*). The manifestation of our method as a helpful instrument for such purposes is aimed at.

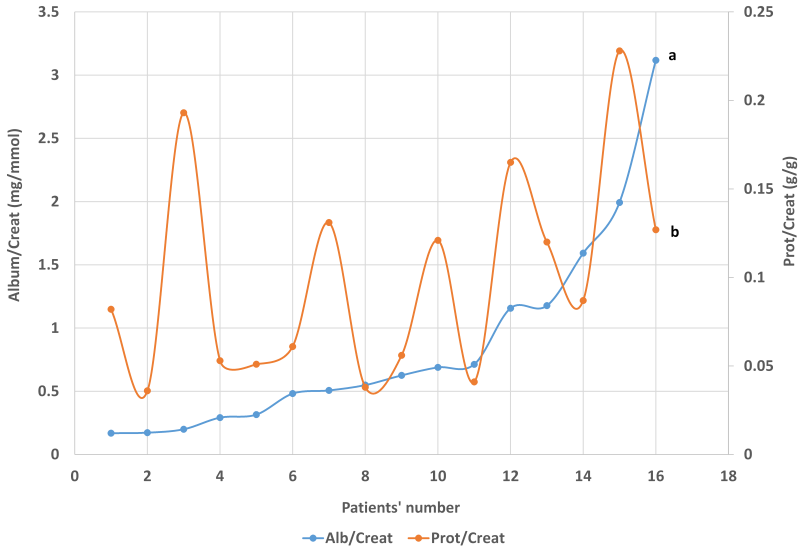

Fig. 7 Markers albumin/creatinine (a) and protein/creatinine (b) in the whole urines from 16 patients of the screening focus group. For other details see Table 1.

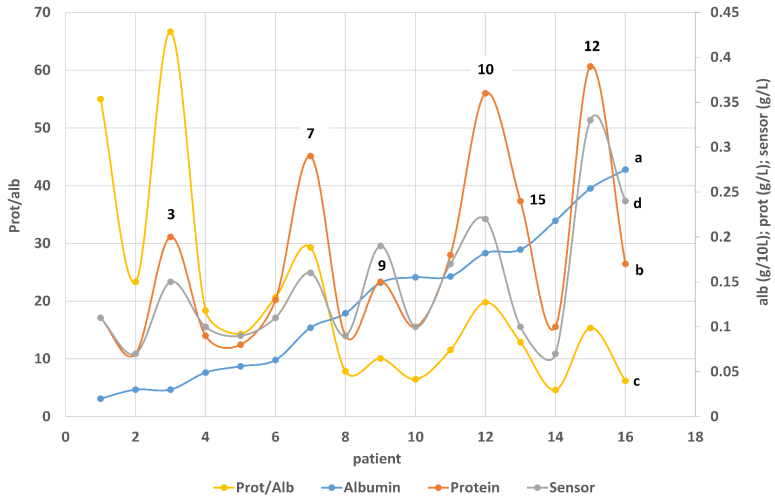

Fig. 8 Distributions of laboratory albumin (*a*) and protein (*b*), ratio protein/albumin (*c*) and readings of the UV sensor (*d*) in the same focus group as in Fig. 7. The inserted numbers 3, 7, 9, 10, 15 and 12 indicate the corresponding positions in Fig. 7. For additional data see Table 2.

Among 41 patients with diabetes or/and hypertension registered at the FMPC there were 16 (39%) patients with the normal or slightly abnormal albumins (≤ 0.03 g/L). These patients formed the focus sub-group for our investigation (see also p. 3.3).

In Table 1 there are the data on the albumin/creatinine (mg/mmol) and protein/creatinine (g/g) parameters in the whole urines which are widely acknowledged in the clinical praxis for searching and monitoring of renal, uremic and other pathologies. Fig. 7 illustrates the specificity in distribution of the given parameters in this focus group. We see a spectacular difference if we depict the distribution of albumin/creatinine values as the gradually growing curve *a* and the corresponding curve *b* for protein/creatinine. Some maxima of the curve *b* point to the patients 3, 7 and 10 with the CKDs as it is noted in Table 1.

One can note, however, that the CKD patient 9 (a young girl) in Table 1 did "remain" without such a particular maximum in the curve b in Fig.7. Her albumin /creatinine (0.626 mg/mmol) and protein/creatinine (0.056 g/g) parameters are remarkably lower than the mean value 0.86 mg/mmol and median ≈ 0.085 g/g in these two rows, respectively. The repeated tests done with the intervals of two-three months gave the similar results for the patients 3, 7, 9 and 10 (Table 1).

It is also noteworthy that only in two cases the values of the parameters albumin/creatinine

(3.117 mg/mmol, patient 16) and protein/creatinine (0.228 g/g, patient 15) exceed the references indicating the probable danger. In both cases no information about additional diseases was available. The control performed with patient 15 two months later gave 0.117 g/g what points to the transient proteinuria in this case.

Table 1 The patients in the screening focus group with low level albumin/creatinine and protein/creatinine parameters.

Patient	Alb/Creat mg/mmol	Prot/Creat g/g	Diseases
1	0.169	0.082	HT
2	0.173	0.036	HT
3	0.2	0.193	CKD
4	0.292	0.053	HT
5	0.316	0.051	HT
6	0.482	0.061	D
7	0.507	0.131	CKD
8	0.549	0.038	HT
9	0.626	0.056	CKD
10	0.689	0.121	CKD
11	0.713	0.041	HT
12	1.157	0.165	HT
13	1.177	0.12	HT
14	1.592	0.087	HT+D
15	1.993	**0.228**	HT
16	**3.117**	0.127	HT

Explanations to Table 1. HT – hypertension, D – diabetes, CKD – chronic kidney disease. The numbers in bold indicate the patients whom belong the maxima in the curve *b* in Fig. 7 as well as the values exceeding the normality references given in [2] for albumin /creatinine (≤3 mg/mmol) and in the Cobas 6000 Roche practical guide for the protein /creatinine marker (< 0.200 g/g) [23].

Unsatisfied with the results obtained with the standard markers albumin/creatinine and protein/creatinine we have tried another approach to the same patients' group and took into account the data on the single albumin and protein concentrations (Table 2). In Table 2 we give also our sensor data in g/L, ratios protein/albumin as well as urine test strips data. We reproduce the information about the diseases in the focus "group 16".

In Fig. 8 by analogy with Fig. 7, the curve *a* is albumin and the curve *b* protein concentration distributions. Again, we see strong fluctuations in the protein curve *b* on the relatively smooth background of the albumin curve *a*. It is remarkable that all patients 3, 7, 9 and 12 with the identified CKD obtain now their own maxima in the curve *b*. The correspondence of the maxima in Fig. 8 to the "clones" in Fig. 7 is given by the numbers placed near the maxima and at the position 13 in Fig. 8. Additionally, we have drawn the curve *c* for the ratios protein/albumin. The curve *d* represents the protein concentration data obtained with our sensor.

We see that the similar behaviour of three distributions *b*, *c* and *d* is obvious: the quantity (5+4) and positions of all extrema coincide sharply. Numerically, the Pearson correlations are: r = 0.79 for the arrays "laboratory protein vs sensor data" and r = 0.086 for "laboratory protein vs protein/albumin". Regardless the latter weak correlation we would consider the parameter protein/albumin as a useful one for its insensitivity to the urine density and other characteristics (time of sampling, is it a 24 h dose or not, is it stored for long or short time etc.). As one can see from Table 2 and Fig. 8 the high values of the protein/albumin ratios (> 10) can serve as indications to the pathology.

The important result seen in Table 2 and in Fig. 8 is that all patients 3, 7, 9 and 12 with the CKD have their own maxima in the characteristics based on the protein (*b*), protein/albumin (*c*) and sensor (*d*) data. This is in the contrast to the situation with the creatinine parameter involvement (Table 1 and Fig. 7) where the CKD patient 9 had no specific maximum. All laboratory and sensor data for the patients 3, 7, 9, 12 and 15 with maxima in Fig. 8 and Table 2 are higher than the mean (0.14 g/L) or median (0.11 g/L) values in the focus group.

In parallel, almost all tests with strips gave negative results, i.e. 15 from 16 or 94%, only for the patient 15 with the laboratory protein 0.39 g/L the reading was positive (0.15 g/L). We take this information as a fact.

At the same time, we see that not all certified laboratory data coincide well with our sensor readings. These deviations are more pronounced for the higher protein concentrations than for the lower ones: in the last case the perfect coincidence is predominant (nine patients 1, 2, 4, 5, 6, 8, 10, 11, 14 or 56% of the cohort).

We can also mention that for the FMPC patients with the albuminuria in the range 0.03 – 0.3 g/L, sometimes called microalbuminuria, (19 persons) 15 tests with strips (79%) gave positive results in the relation to presence of protein. Three tests from these 15 (or 20%) one can name as false positive since the strip readings were 1.5 up to 5 times higher than the Roche assay ones.

The sensor data obtained in this albuminuria group correlated well with the laboratory protein (r = 0.863) and, surprisingly, weakly with albumin values (r = 0.124). It means that our method works preferably for detection of non-albumin proteins even in the albuminuria region, at least, for the hypertension, diabetes and CKD patients.

Table 2 Focus group patients' characteristics with a partial connection to the data given in Table 1.

	Albumin, g/L	Protein, g/L	Sensor, g/L	Prot/Alb	Strip, g/L	Disease
1	0.02	0.11	0.11	55	Neg	HT
2	0.03	0.07	0.07	23.33	Neg	HT
3	0.03	0.2	0.15	66.67	Neg	CKD
4	0.049	0.09	0.1	18.37	Neg	HT
5	0.056	0.08	0.09	14.29	Neg	D
6	0.063	0.13	0.11	20.63	Neg	HT
7	0.099	0.29	0.16	29.29	Neg	CKD
8	0.115	0.09	0.09	7.83	Neg	HT
9	0.149	0.15	0.19	10.07	Neg	CKD
10	0.155	0.1	0.1	6.45	Neg	HT
11	0.156	0.18	0.17	11.54	Neg	HT
12	0.182	0.36	0.22	19.78	Neg	CKD
13	0.186	0.24	0.1	12.90	Neg	HT
14	0.218	0.1	0.07	4.59	Neg	HT
15	0.254	0.39	0.33	15.35	0.15	HT
16	0.275	0.17	0.24	6.18	Neg	HT+D

Explanations to Table 2. HT, D and CKD meaning is the same as in Table 2. Albumin concentration values are multiplied by the factor 10; Neg means a negative result indicated by the strips method.

We have achieved a rather good correlation between the Roche laboratory assays of proteins and our sensor data (p. *4.3* and, particularly, Table 2). This fact gives a perspective for the development and practical use of the proposed method. Nevertheless, the deviations between the laboratory and our data are sometimes remarkable and they need to be explained. One of the discrepancy sources can be the phenomenon established in [8] and, namely, that small peptides do not at all produce signals with the Roche assays and Tamm-Horsfall protein gives a weak reaction. This can, in principle, explain why our sensor values for patients 9 and 16 (Table 2) are higher than the corresponding laboratory ones.

On the other hand, the same logic is valid in another aspect: not all non-albumin proteins must have high and equal absorption coefficients at 285 nm. It is possible that some non-albumin proteins produce strong Roche assay signals and have a lowered optical absorption coefficient (see also *5.2*). The matter has to be clarified in details in the way of concrete identification of all non-albumins actually present in the urine samples under investigation and establishing the correlation between the absorption spectra of the definite non-albumins and their correspondence to the Roche or other automated assays signals. This could be the aim for the further studies which could be also more extensive and embrace a larger screening group. The question about the factor of light scattering in urine fractions should also be scrutinised.

We have calibrated our method in relation to the Roche method by fractionations with pH9.3 buffers with a complex composition (10 mM TRIS +150 mM NaCl +2 mM EDTA) (see *3.2*). For the wide practical use can be, however, more appropriate the standard physiological fluid, i.e., salt water (0.9 wt % NaCl). A number of fractionations with salt waters have been done with positive results (see *3.2*) but the full-scale recalibration should be done in the development of this method.

5.2 Real proteinuria

To our best knowledge earlier the protein/albumin ratio was not used extensively for proteinuria analysis. We have here usefully involved it and can give one more argument to confirm the usefulness of this parameter.

We can suppose that the readings s of our optical absorption sensor depend, primarily, on the concentration of non-albumin proteins p and albumin a, i.e., $s = n\,(p + a)$, where n is the adjusting coefficient. We have in reality the laboratory data on protein / albumin ratios or $(p + a) / a$, what we designate as $(p + a)/a = m$. With use of the empirical m values from Table 2 we get the equation of only one variable p:

$$s = n\,(p + p / (m\text{-}1)). \qquad (7)$$

For our focus group $m_{mean} = 17.8$ and the second member $p / (m\text{-}1)$ in eq. 7 is in the most cases very small. That is rather negligible for the patients 1, 3, 7 ($m > 29$). It means that our sensor is responsive overwhelmingly to the non-albumin proteins. This gives one more argument to say that we are dealing just with proteinuria, especially, in our focus group. Therefore our sensor can serve as a real proteinuria sensor. This

matter needs and deserves further attention to establish direct correlation between the individual non-albumins and sensor readings.

Our method correlates with the well-known and widely used in industry and laboratories method of certification of proteins mixture solutions. The measurement of the UV absorbance at a chosen wavelength from the range 277 – 280 nm is being carried out. It is recommended for 1% solutions to use a single averaged extinction coefficient ε = 10 "for a mixture of many different proteins" [24]. We did the same and considered the mix of abundant urine proteins as a (quasi)homogenous substance. By advancing of this method one should take into account the differences in extinction coefficients for separate proteins: ε = 5.8 (human albumin) [25], 10.8 (Tamm-Horsfall) [26], 13.8 (γ-Globulins) [27], 14.6 (Bence-Jones) [28] and search for distinct correlations between absorbance and the composition of typical urine proteins. This work was out of the scope of the present investigation and can be undertaken in the future.

It seems that the acknowledged reference values for identification of abnormalities can be too high. For instance, all patients 3, 7, 9, 12 with the CKD (Fig.7 and Table 1) had albumin/creatinine values smaller than the low limit of normality range 3 mg/mmol given in the guidelines [2]. The protein/creatinine reference value for indications of abnormalities by the Roche technology is > 0.200 g/g (>22.6 mg/mmol) and that is also too high. In our focus group only the patient 15 had once a higher ratio protein/creatinine (0.228 g/g) and this was, most probably, a transient proteinuria case.

5.3 With creatinine or without it?

We have seen that the predictable power of the markers involving creatinine concentrations has turned out to be weaker than the appropriate protein and protein/albumin parameters free of creatinine (the case of the patient 9 (Fig. 7&8 and Tables 1&2). We acknowledge that our statistics is not rich and needs to be enlarged. Nevertheless, one can draw some additional facts and speculate a little bit why the use of creatinine data is not always profitable, can be excessive and bear unnecessary costs.

It is clear without saying that for a given specimen of some biofluid the ratios albumin/creatinine and protein/creatinine have the larger uncertainties (or standard deviations) than the single albumin and protein magnitudes. Moreover, earlier there were carried out profiled investigations in the respect to the creatinine data stability for the human plasma and serum [29] and hemodialysate [30]. It was found that the creatinine concentration assay data can fluctuate depending on the store conditions and time intervals more than those of the other metabolites. In the recent paper [31] it was established that the diagnostic accuracy of the urine protein/creatinine ratio depends on urine concentration. Therefore we think that such a creatinine-free approach can be plausible and useful. The stabile ratios protein/albumin could be used in parallel or even replace the markers with creatinine involvement at least in the low level proteinuria region.

5 Conclusion

As the result of the performed study we would like to propose a new method of screening and monitoring for the low level proteinuria. This straightforward method can be exploited easily in work "in the field", i.e. outsides of the automated laboratories. The determination of protein concentration is based on the measurement of the UV (285 nm) absorption in fractions of urine eluted using the commercial columns PD-10 and specially designed optical cell. The applied standard urine test strips method failed to indicate in 94% cases any presence of proteins in the urines in the patients' focus group whereas the proposed method, in the parallel comparison, gave the correct positive results. One more advantage of the new method over the strips method is the possibility to use that in the wide region of protein concentrations from the normal proteinuria (≤ 0.1 g/L) up to the levels of severe proteinuria (~10 g/L). The trials in the larger screening groups and clinical tests are under preparation with the aim to elaborate the screening and monitoring algorithms.

6 Ethics

The study has been approved by the Ethics Committee on Human Research of the University of Tartu, Estonia (protocol no 232/M-8(1) 2013 and 244/M-25, 2015).

7 Acknowledgments

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