From the meeting
"Le Proteine: dal Laboratorio alla Clinica"
"Proteins: from the Laboratory to the Clinic"
X Edition - Castrocaro, 24th – 26th October, 2001
Organised by CEFAR – Centro Europeo per la Formazione e la Ricerca in Scienze Sanitarie e in Biotecnologie
Patron: SIBioC – Società Italiana di Biochimica Clinica e Biologia Molecolare Clinica

Proteinuria – Diagnostics and Interpretation with Marker Proteins

Computerising Processing and report *("MDI–LabLink")*

Werner H. Siede [a], Axel Regeniter[b]

a) Presenting Author: Central Laboratory, Klinikum Lippe-Lemgo, Lemgo, Germany
b) Author for correspondence: Central Laboratory, Kantonsspital Basel, Switzerland - Fax +41 61 265 4600
E-mail: aregeniter@uhbs.ch

Index

- Introduction
- Pathophysiology
- Pre-analytical considerations
- Analytical considerations
- Diagnostic strategy
- Data Presentation and Interpretation
- Indications and Applications
- Figures

Non standard abbreviations

BJP	Bence Jones Proteins	ALB	Albumin
BJ	Bence Jones	TRF	Transferrin
FRL	Free Light Chains	A1M	Alfa-1 microglobulin
FRL-K	Free Light Chains kappa	A2M	Alfa-2 macroglobulin
FRL-L	Free Light Chains lambda	IGG	Immunoglobulins IgG
IFE	Immunofixation	IGM	Immunoglobulins IgM
		RBP	Retinol binding protein
		B2M	Beta-2 microglobulin

Key Words

Glomerular proteinuria, Tubular proteinuria, Bence Jones Protein, Kappa Free Light Chains, Lambda Free Light Chains

Introduction

The development of immunological assays for specific proteins in urine has led to considerable improvement in the early detection, prognostic evaluation and therapeutic monitoring of kidney and urinary tract diseases.

Basic urine examination by test strips and/or microscopy should be supplemented with a sensitive and quantitative determination of total protein or principal urinary marker proteins like albumin and alpha-1-microglobulin.

Complete differential diagnosis can be accomplished by measuring only a few marker proteins. This replaces the solely qualitative evaluation of protein patterns with laborious SDS-PAGE.

This article reviews the work-up of proteinuria.

It covers pathophysiology, analytical and pre-analytical considerations, the diagnostic strategy, data presentation and interpretation, medical indications and its applications.

Pathophysiology

The nephron is the functional unit within the kidney. It consists of two main structures, the glomerulum and tubulus. In the glomerulum, filtration produces the primary urine; while the concentration and reabsorption of small proteins takes place in the tubular system.

Functional and structural defects of one or both of these parts lead to various and distinct patterns of proteinuria (Fig. 1).

The principal reason for the different protein handling by glomerulum and tubulus is molecular size (molecular weight). Groups of proteins within a certain molecular weight range are affected similarly.

In SDS-PAGE, the traditional technique to identify patterns of proteinuria, proteins are separated strictly according to their molecular weight. Single proteins can then be identified within these patterns. They serve as marker proteins for typical pathobiochemical conditions (Fig. 2) (1 – 8).

The glomerular filtration of proteins depends on two different functional properties of the glomerular basal membrane. Larger proteins (e.g. IgG, 150 kdal) are restricted from filtration simply by pore width.

Smaller Proteins of a size range that allow them to just pass through the glomerular pores (e.g. albumin, 67 kdal, and transferrin 90 kdal) are repelled effectively by negatively charged molecules in the membrane („anionic filter“) since these proteins are also negatively charged at a physiological blood pH.

Therefore, proteinuria is observed not only in structural changes of the basal membrane (***“unselective glomerular proteinuria”***), but also in potentially reversible conditions with loss of the glomerular anionic filter function.

This condition is characterized by a ***“selective glomerular proteinuria***” pattern (e.g. microalbuminuria, minimal change nephritis).

Proteins with a lower molecular weight of albumin pass freely through the glomerular basal membrane and are physiologically reabsorbed within the tubular system.

However, protein reabsorption is a highly energy-consuming process. In addition, its transport capacity. is limited It is therefore easily disturbed by even minor factors (e.g. fever, medication).

An extremely sensitive indicator for the resulting pattern of "***incomplete tubular proteinuria"*** is alpha-1-microglobulin (33 kdal).
Severe tubular damage leads to further structural changes (nephrotoxicity of drugs, progressive renal diseases) and a disturbed tubular reabsorption of even very small proteins. These proteins, for example retinol-binding protein (21 kdal) or beta-2-micro-globulin (12 kdal) are then measurable in urine:and form the pattern of "***complete tubular proteinuria"***.
Primary glomerular defects effect the tubulo-interstitium and vice versa, since the three dimensional structure of nephrons is closely interwoven. Mixed forms of glomerular/tubular patterns are therefore common in advanced forms of renal disease.
The frequently symptomless and often underestimated involvement of the tubulo-interstitium is the critical component in the final outcome of kidney diseases.
Tubulo-interstitial protein markers reflect these changes and indicate the prognosis (Fig. 10) (9, 24 – 26).
Post-renal (strictly: post-glomerular) affections with or without hematuria add unfiltered serum proteins to the urine. Thus, they mimic glomerular or mixed types of proteinuria. The post-renal addition of proteins can be identified by the urinary presence of very large molecules (e.g. alpha-2-macroglobulin (720 kdal), IgM, Apolipoprotein A) Even in advanced glomerular disease, these molecules are almost completely restricted from glomerular filtration.
Pre-renal (overflow) proteinuria is characterized by a large production of smaller proteins that is excessively contributed to serum. Due to their small size, these molecules pass the glomerulum freely. However, the quantity of these proteins exceeds the protein reabsorption capacity of the tubular part of the kidney. Thus, they appear in urine. The most frequent type of pre-renal proteinuria is the appearance of free monoclonal light chains (Bence Jones protein). A longer existing conditions of overflow proteinuria will damage the tubular system, which can again be assessed by urinary marker proteins effectively (10, 11).

Pre-analytical considerations

Sampling conditions are extremely important for a correct determination of urinary proteins. 24 hour timed urine samples and also samples collected as first morning urine have several drawbacks. The material of choice for the determination of almost all urinary parameters is the second morning urine (Fig. 3)(4, 12 – 16).
Nevertheless, the variations in diuresis have to be corrected. All protein measurements must therefore be related to the creatinine concentration of the sample and are expressed as mg/g creatinine (or alternatively as mg/mmol creatinine).
Also, typical characteristics of individual proteins may influence their value as a marker protein . For instance, beta-2-microglobulin is very unstable at acidic urine pH and therefore not an ideal marker protein (17 – 19).

Analytical considerations

The concentrations of proteins range from just above the reference range limit in early renal damage to extremely high amounts in patients with nephrotic syndrome.
The immunological measurement of marker proteins by nephelometry is well suited for this situation.
The instruments provide the maximum analytical sensitivity required to measure proteins that have upper reference ranges near or even below 1 mg/g creatinine (e.g. transferrin, retinol-binding protein, beta-2-microgloblin, kappa, lambda light chains).
They are also able to correctly handle antigen excess situations, which occur frequently in proteinuria samples.
The correct total protein determination in urine proves to be especially difficult.
The upper reference range limit of healthy individuals is somewhere between 100-120 mg/g creatinine.
Conventional brands of dipsticks are therefore limited to the detection of advanced renal affections. They are not suited for the exclusion of kidney disease. Their detection limit far exceeds the upper normal reference range and lies somewhere between 150 and 300 mg/l. Furthermore, they measure mostly albumin. Smaller molecules, as seen in mainly tubular or prerenal proteinuria (free kappa or lambda light chains) are detected to a much lesser extent or not at all.
The detection limit of the standard photometric protein determination methods (e.g. Biuret) is also too unsensitive.
Methods using pyrogallol-red or benzethonium-choride offer an acceptable compromise (20 – 22) with a detection limit of 40 - 60 mg/l. They are therefore able to measure within the upper reference range of healthy individuals. In addition, they can be automated.

Diagnostic strategy

The diagnostic strategy is outlined schematically in Fig. 4.
It represents the stepwise diagnostics (SCREENING, DIFFERENTIATION, CONFIRMATION) of all types of proteinuria (GLOMERULAR, TUBULAR, POSTRENAL, PRERENAL) with respect to a rational, cost-effective reflex testing strategy.
All patients are screened using dipsticks for erythrocytes/hemoglobin and leukocytes and total protein (pyrogallol-red or benzethonium-chloride methods) is determined by photometer. All measured proteins are referred to the creatinine content of the sample.
If the protein concentration is below the upper reference limit of 100 to 120 mg/g creatinine, a diagnostic relevant proteinuria is most unlikely, provided no other indications for renal diseases or diseases with renal involvement are present.
However, a considerable percentage of patients with risk factors (e.g. diabetes mellitus, hypertension) show

elevated concentrations of either albumin or alpha-1-microglobulin despite a total protein concentration within the reference range.
In these cases as well as in all samples with a total protein concentration in the 100 to 300 mg/g creatinine range, the most sensitive and specific marker proteins for glomerular and tubular function should be measured; albumin and alpha-1-microglobulin.
If one of these proteins is elevated, further differentiation with additional marker proteins is necessary for the complete differentiation and classification of proteinuria
This initial step can be omitted in patients with a total protein above 300 mg/g creatinine. In these samples additional proteins are elevated regularly.
The complete work-up of proteinuria requires the measurement of transferrin and IgG for glomerular proteinuria, retinol-binding protein and/or beta-2-microglobulin for tubular proteinuria.
In samples with hematuria, alpha-2-macroglobulin is used additionally to identify or rule out post-renal contamination (4).
Evidence for pre-renal proteinuria is a larger than usual gap between the total protein concentration and the sum of the main marker proteins (23).
This is seen frequently in Bence-Jones proteinuria, which subsequently can be confirmed or excluded with the ratio of free kappa to lambda light chains or immunofixation electrophoresis.
In general, the determination of free light chains is a very suitable screening test for Bence-Jones protein. Nephelometric assays with sufficient analytical sensitivity (detection limit 5 mg/l) are available. In normal urine the concentration of light chains is below the detection limit. Besides Bence-Jones proteinuria, elevated values are regularly found in tubular proteinuria. Free light chains in tubular proteinuria are of polyclonal origin and their increase is due to the tubular reabsorption defect.
Bence-Jones proteinuria, in contrast, is an "overflow-proteinuria" caused by an overproduction of monoclonal free light chains. The kappa/lambda ratio is either above or below the reference range e of 1 – 3,7 (28).
Immunofixation electrophoresis is used for confirmation in these cases. Its value, however, is limited due to a lack of analytical sensitivity (detection limit variable between 20 – 50 mg/l).).
Concentrating urinary samples cannot be recommended, since artefacts are often produced ("ladder phenomenon")(29, 30).
A free kappa/lambda ratio in the range of 1 – 3,7 rules out Bence-Jones proteinuria almost completely (28), costly immunofixation electrophoresis is unnecessary and can be avoided in a large number of patients.
This diagnostic strategy in the work-up of proteinuria leaves only few samples with rare and unusual constellations for further work-up with SDS-PAGE.

Data Presentation and Interpretation

The interpretation of urinary protein patterns requires the calculation of numerous ratios and formulas, based on the fundamental work by Hofmann and Guder (4, 5).
Knowledge-based systems („expert systems") on a Personal computer, like MDI LabLink, are used with great success for calculation, interpretation of proteinuria patterns and vivid data presentation (Fig. 5) (6, 7).
The graphic presentation of the protein markers needs a common denominator. However, laboratory values referring to the same organ system differ in their units of measurement as well as in their reference ranges.
The values are comparable if they are divided by and expressed as multiple of their upper reference [LM1]limit.
The creatinine adjusted urinary proteins are then ordered according to their molecular weight and plotted against a schematic nephron. Reference range proteins are indicated by a blue bar, elevated proteins with a red one.
The proteins now form distinct patterns displaying the patho-biochemical lesion – Fig. 6: measured proteins in urine, from top to bottom:

i) Alpha-2-macroglobulin (exclusion of post-renal contamination);
ii) IgG, transferrin, albumin (glomerular markers);
iii) Alpha-1-microglobulin, retinol-binding protein (tubular markers).

The graph allows nearly instant recognition („signature pattern") of the underlying type of proteinuria and the localization of the defect. This is most vividly evident in the original color printout (Fig. 7).
The data is also presented numerically and an additional text interpretation classifies the defect.
The interpretation text stems from MDI Lablink's interpretation module. The pattern of the sample is compared with patterns stored in a database. The database is freely accessible, so that the user maintains complete control over program function and output.
Pattern definitions as well as interpretation texts can be changed, deleted or added.
The interpretation texts in the printout can be tailored with little effort for different information needs.
The output always contains text to describe the patho-biochemical classification of proteinuria, proposes additional tests („reflex testing") and comments on the validity of the result according to the built in algorithms for plausibility control (e.g. in samples with extreme creatinine concentrations, unusual IgG concentrations, contamination with erythrocytes or leukocytes). It can be tailored to additionally cite examples of typical diseases observed with the respective pattern.
The follow-up printout (Fig. 8) comments and presents changes in marker protein concentrations tabularly and graphically. It monitors disease progression and effects of therapy .

Indications and Applications

Indications for urinary protein differentiation according to Scherberich are summarized in Fig. 9[LM2]. This includes several laboratory result constellations, clinical conditions, symptoms and diseases with a common kidney involvement. It is supplemented by Fig. 10[LM3]. Here, a review of the principal applications for urinary marker proteins in early detection and therapeutic monitoring can be found.

Besides glomerular proteinuria, e.g. "microalbuminuria" in patients with diabetes and hypertension, tubular proteinuria is of special interest, since the involvement of the tubulo-interstitium is often the critical component in the final outcome of kidney diseases.

Prognostic significance has been emphasized recently for retinol binding protein (24 –26), but also for IgG and Alpha-1-Microglobulin (27).

In conclusion, we would like to emphasize the extraordinary importance of quantitative protein marker determination in urine as a decisive and integral part for diagnosis and therapeutic monitoring of often asymptomatic kidney disease.

References

1. Boesken WH, Kopf K, Schollmeyer P. Differentiation of proteinuric diseases by discelectrophoretic molecular weight analysis of urinary proteins. Clin Nephrol 1973;1:311-8.
2. Boesken WH, Rohrbach R, Schollmeyer P. Vergleich von Histologie und Urinproteinanalyse (SDS-PAA-Discelektrophorese) bei Nierenerkrankungen. Nieren und Hochdruckkrh 1978;5:206-14.
3. Boesken WH. Diagnostic significance of SDS-PAA-electrophoresis of urinary proteins: different forms of proteinuria and their correlation to renal diseases. Curr Probl Clin Biochem 1979;235-48.
4. Hofmann W, Rossmuller B, Guder WG, Edel HH. A new strategy for characterizing proteinuria and haematuria from a single pattern of defined proteins in urine. Eur J Clin Chem Clin Biochem 1992;30:707-12.
5. Hofmann W, Sedlmeir-Hofmann C:, Ivandic M, Schmidt D, Guder WG, Edel HH. Befundung von Urin-Protein-Mustern auf der Basis klinisch gesicherter Patientenkollektive. Typische Beispiele mit Textbefunden. Assesment of urinary-protein patterns on the basis of clinically characterized patients. Typical examples with reports. Lab med 1993;17:502-12.
6. Regeniter A, Siede WH, Seiffert UB. Computer assisted interpretation of laboratory test data with 'MDI- LabLink'. Clin Chim Acta 1996;248:107-18.
7. Regeniter A, Siede WH, Scholer A, Huber P, Frischmuth N, Steiger JU. Interpreting complex urinary patterns with MDI LABLINK: a statistical evaluation. Clin Chim Acta 2000;297:261-73.
8. Regeniter A, Nickeleit V, Dickenmann M, Frischmuth N, Siede WH, Huber P et al. Specific Urine Protein Markers Indicate Histological Changes in Kidney Allografts. XXI. World Congress of Pathology and Laboratory Medicine, (WASPALM) Duesseldorf, November 20-23 2001. Clin Lab 2001;47:622.
9. Bernard AM, Vyskocil AA, Mahieu P, Lauwerys RR. Assessment of urinary retinol-binding protein as an index of proximal tubular injury. Clin Chem 1987;33:775-9.
10. Zappasodi P, Pascutto C, Bosoni T, Corso A. Urinary proteins in multiple myeloma: strong correlation with the indices of tumor burden. Haematologica 2001;86:878.
11. Corso A, Serricchio G, Zappasodi P, Klersy C, Bosoni T, Moratti R et al. Assessment of renal function in patients with multiple myeloma: the role of urinary proteins. Ann Hematol 1999;78:371-5.
12. Torng S, Rigatto C, Rush DN, Nickerson P, Jeffery JR. The urine protein to creatinine ratio (P/C) as a predictor of 24-hour urine protein excretion in renal transplant patients. Transplantation 2001;72:1453-6.
13. Ginsberg JM, Chang BS, Matarese RA, Garella S. Use of single voided urine samples to estimate quantitative proteinuria. N Engl J Med 1983;309:1543-6.
14. Mitchell SC, Sheldon TA, Shaw AB. Quantification of proteinuria: a re-evaluation of the protein/creatinine ratio for elderly subjects. Age Ageing 1993;22:443-9.
15. Bisaz E, Bianchetti MG, Donati R, Peheim E, Colombo JP, Oetliker OH. [Simplified determination of proteinuria in children using a single urine sample]. Klin Padiatr 1994;206:387-91.
16. Steinhauslin F, Wauters JP. Quantitation of proteinuria in kidney transplant patients: accuracy of the urinary protein/creatinine ratio. Clin Nephrol 1995;43:110-5.
17. Davey PG, Gosling P. beta 2-Microglobulin instability in pathological urine. Clin Chem 1982;28:1330-3.
18. Donaldson MD, Chambers RE, Woolridge MW, Whicher JT. Stability of alpha 1-microglobulin, beta 2-microglobulin and retinol binding protein in urine. Clin Chim Acta 1989;179:73-7.
19. Blumsohn A, Morris BW, Griffiths H, Ramsey CF. Stability of beta 2-microglobulin and retinol binding protein at different values of pH and temperature in normal and pathological urine. Clin Chim Acta 1991;195:133-7.
20. Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K, Tokuda K. Urinary protein as measured with a pyrogallol red-molybdate

complex, manually and in a Hitachi 726 automated analyzer. Clin Chem 1986;32:1551-4.
21. Orsonneau JL, Douet P, Massoubre C, Lustenberger P, Bernard S. An improved pyrogallol red-molybdate method for determining total urinary protein. Clin Chem 1989;35:2233-6.
22. Macart M, Forzy G, Gerbaut L, Vekich AJ, Guilbaud JC. Measuring urinary protein with the new BioRad reagent kit: evaluation and comparison with five other methods. Ann Biol Clin (Paris) 1994;52:355-60.
23. Boege F, Koehler B, Liebermann F. Identification and quantification of Bence-Jones proteinuria by automated nephelometric screening. J Clin Chem Clin Biochem 1990;28:37-42.
24. Camara NO, Nishida S, Silva MS, Pestana JO, Pereira AB, Sesso R, Pacheco-Silva A. Monitoring serum beta-2 microglobulin is useful for detecting patients with increased risk of acute rejection during reduction in immunosuppression. Transplant Proc 1998;30:4158-9.
25. Camara NO, Matos AC, Rodrigues DA, Pereira AB, Pacheco-Silva A. Urinary retinol binding protein is a good marker of progressive cyclosporine nephrotoxicity after heart transplant. Transplant Proc 2001;33:2129-31.
26. Camara NO, Matos AC, Rodrigues DA, Pereira AB, Pacheco-Silva A. Early detection of heart transplant patients with increased risk of ciclosporin nephrotoxicity. Lancet 2001;357:856-7.
27. Bazzi C, Petrini C, Rizza V, Arrigo G, Beltrame A, Pisano L, D'Amico G. Urinary excretion of IgG and alpha(1)-microglobulin predicts clinical course better than extent of proteinuria in membranous nephropathy. Am J Kidney Dis 2001;38:240-8.
28. Regeniter A, Siede WH. Determination of free light chains as screening test for Bence-Jones proteinuria (submitted for publication).
29. Harrison HH. The „ladder light chain“ or „pseudo-oligoclonal“ pattern in urinary immunofixation electrophoresis (IFE) studies: a distinctive IFE pattern and an explanatory hypothesis relating it to free polyclonal light chains. Clin Chem 1991;37:1559-64.
30. MacNamara EM, Aguzzi F, Petrini C, Higginson J, Gasparro C, Bergami MR, Bianchi G, Whicher JT. Restricted electrophoretic heterogeneity of immunoglobulin light chains in urine: a cause for confusion with Bence-Jones protein. Clin Chem 1991;37:1570-4.

Figure 1

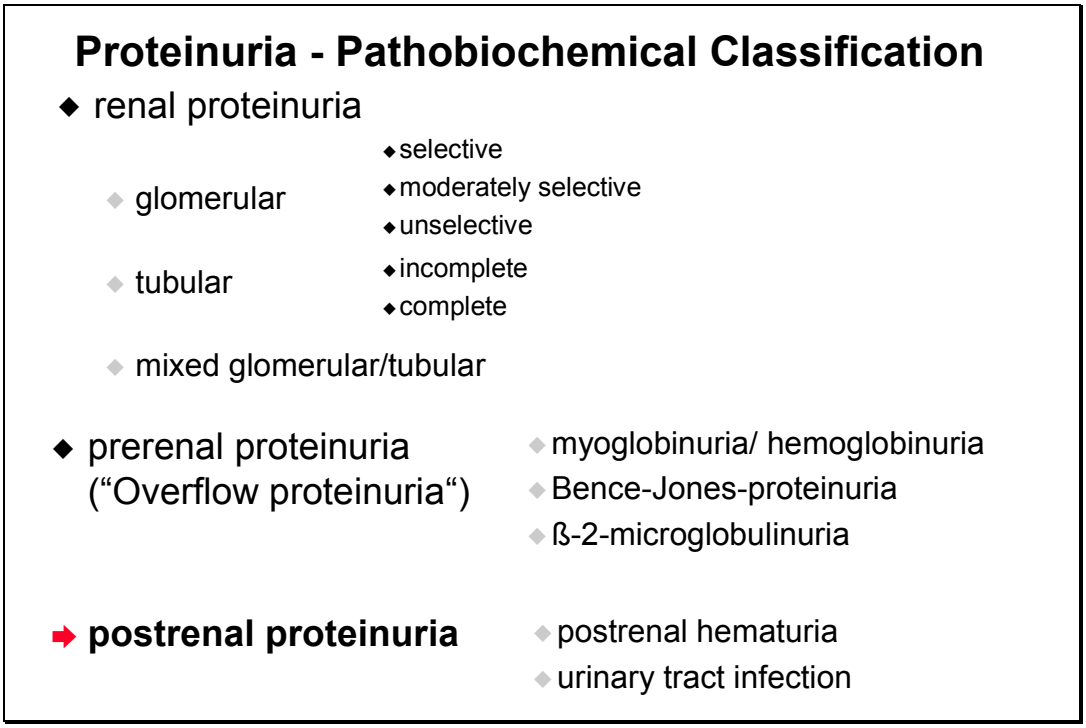


Figure 2

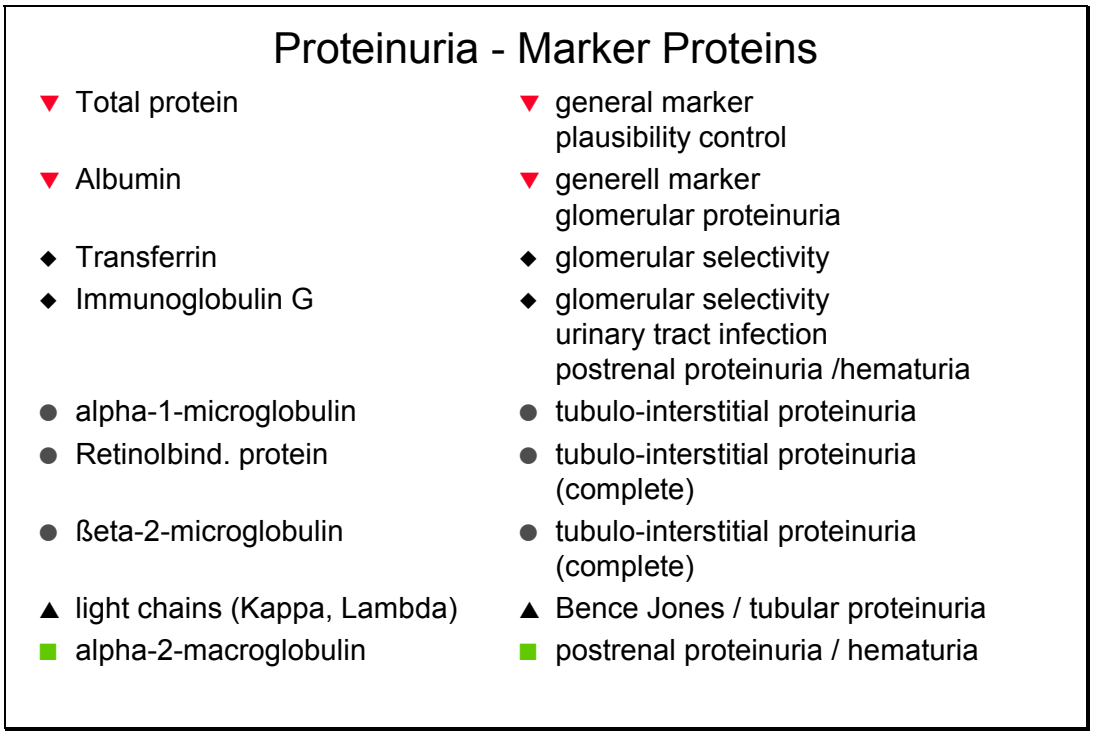


Figura 3

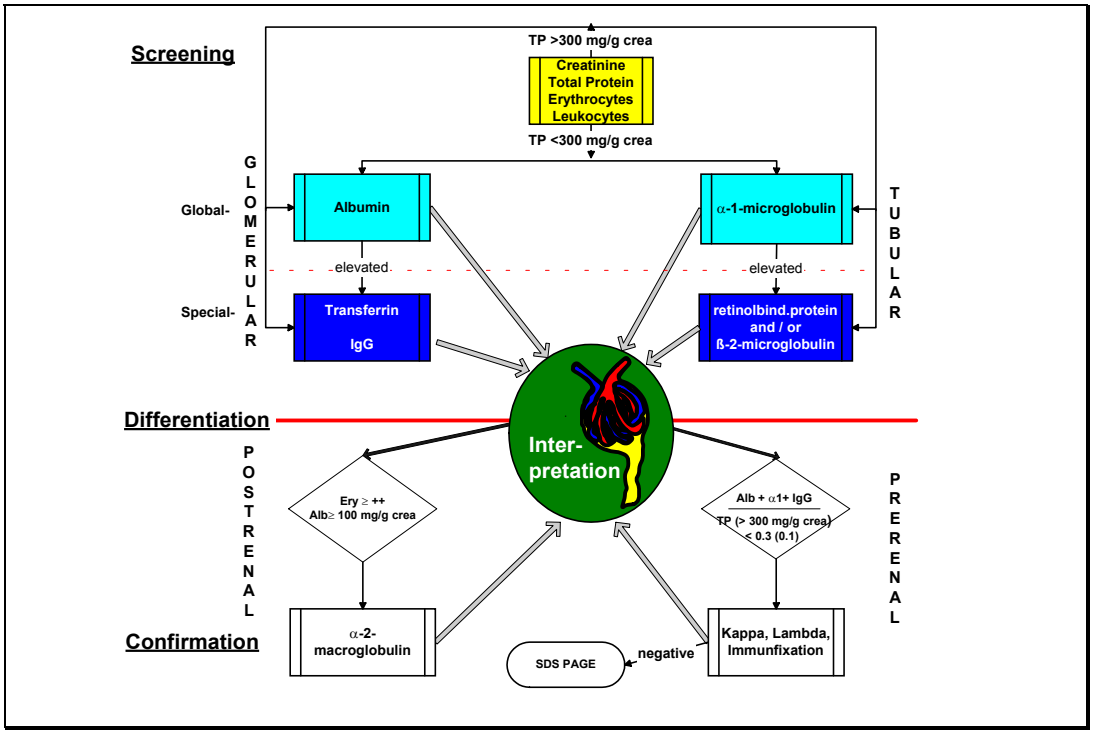


Figure 4

2. *Morning* Urine

➡ *The second urination in the morning, approximately 2 hours after the first*

- **Close correlation to 24h collections (creatinine reference)**
- **Easily and reliably obtainable compared to timed (24h, 12h) samples**
- **Especially suited for a Doctor's office**
- **Suitable nearly for all urinary analytes**
- **Dipstick results (erythrocytes, leucocytes)**

Figure 5

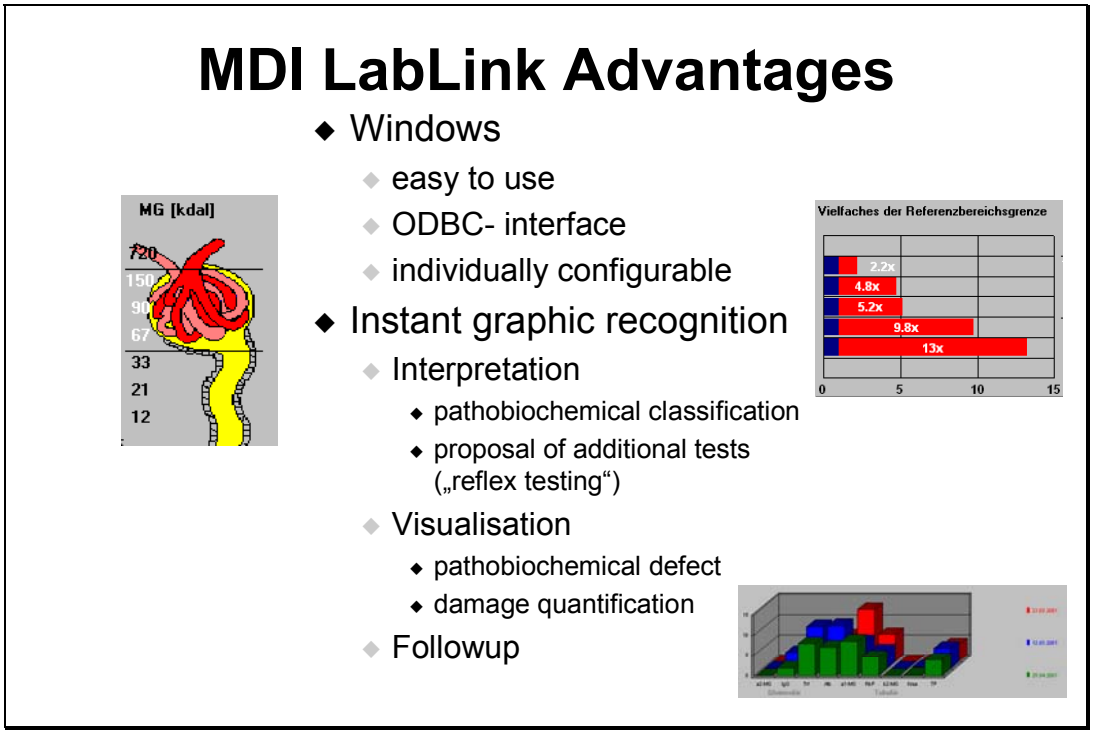


Figure 6

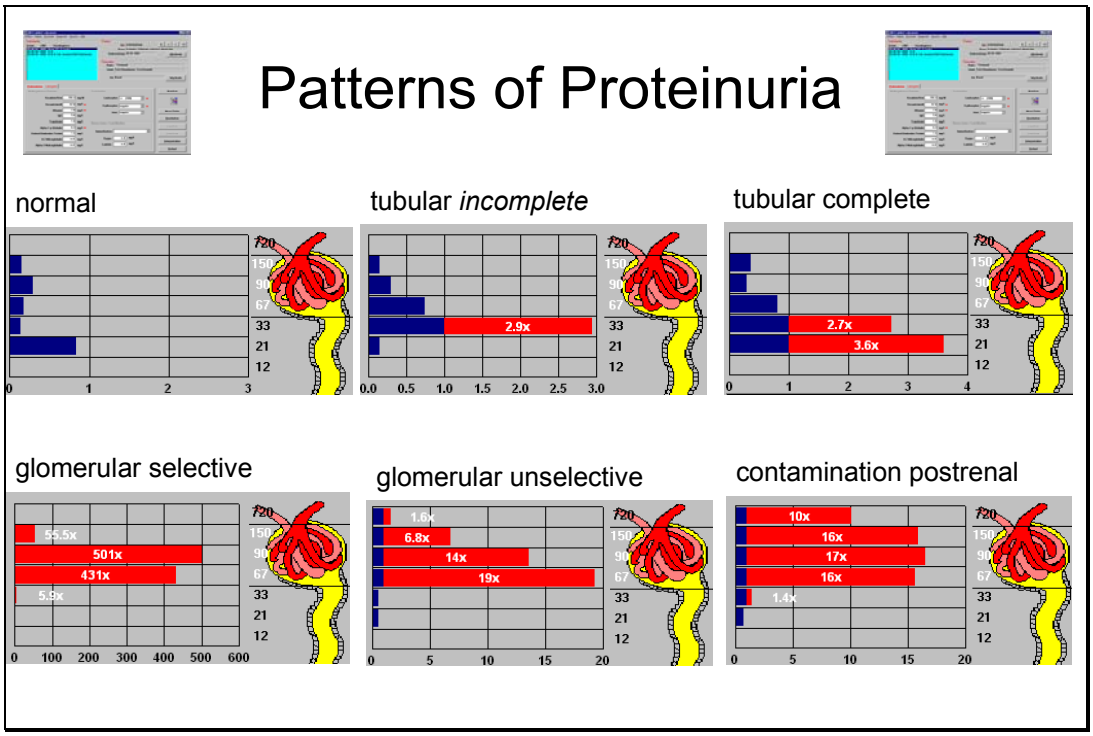


Figure 7

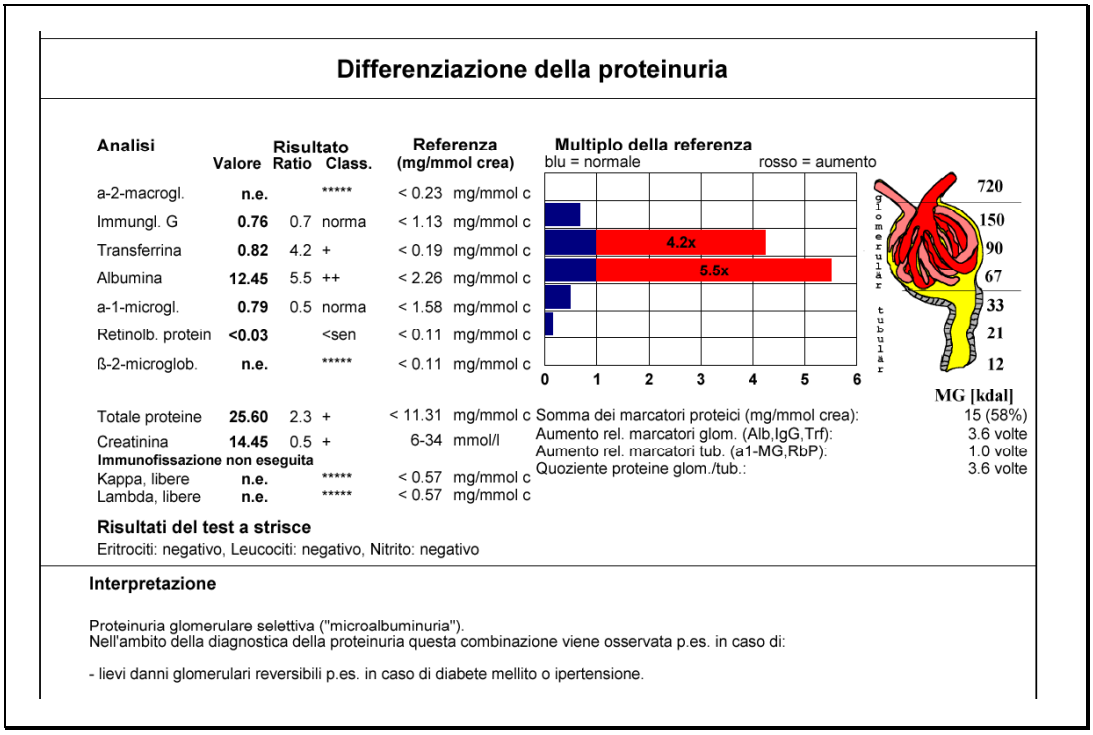
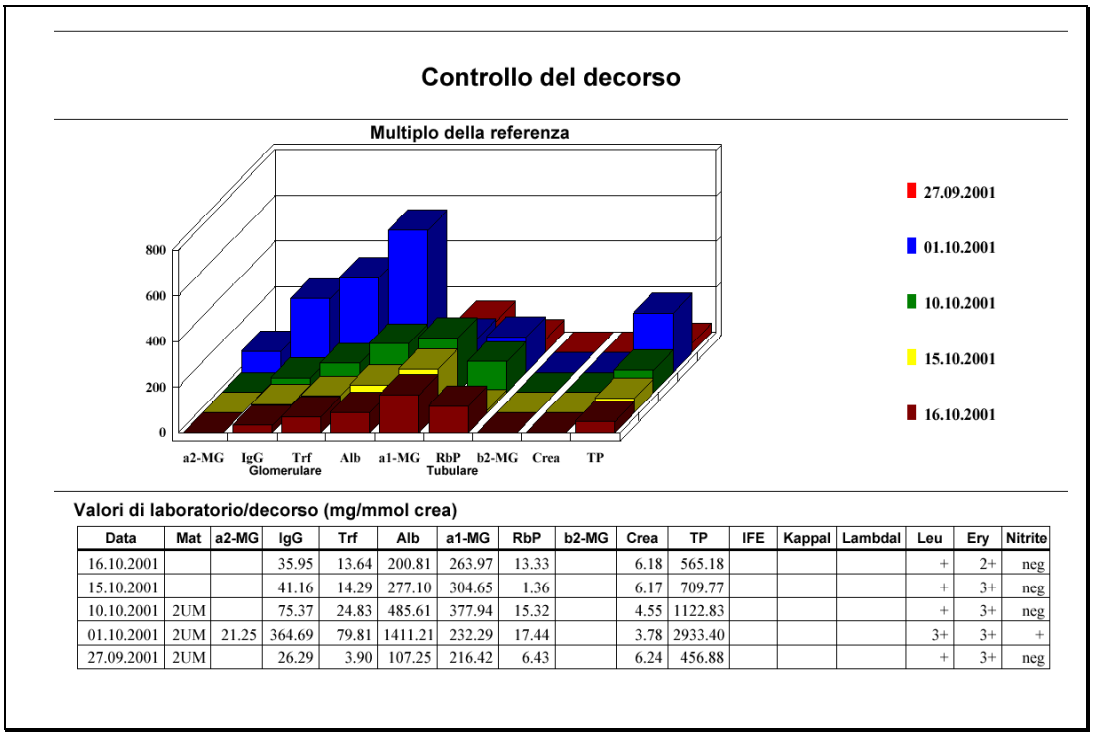


Figure 8



Valori di laboratorio/decorso (mg/mmol crea)

Data	Mat	a2-MG	IgG	Trf	Alb	a1-MG	RbP	b2-MG	Crea	TP	IFE	Kappal	Lambdal	Leu	Ery	Nitrite
16.10.2001			35.95	13.64	200.81	263.97	13.33		6.18	565.18				+	2+	neg
15.10.2001			41.16	14.29	277.10	304.65	1.36		6.17	709.77				+	3+	neg
10.10.2001	2UM		75.37	24.83	485.61	377.94	15.32		4.55	1122.83				+	3+	neg
01.10.2001	2UM	21.25	364.69	79.81	1411.21	232.29	17.44		3.78	2933.40				3+	3+	+
27.09.2001	2UM		26.29	3.90	107.25	216.42	6.43		6.24	456.88				+	3+	neg

Figure 9

The following symptoms and diseases are indicative for a urinary protein differentiation :

- Proteinuria
- Microhematuria
- Leucocyturia with or without bacterial infection
- normoglycemic glucosuria
- inexplainable renal insufficiency with or without proteinuria; serum creatinine >1,4 mg/dl
- hypertonus; " inexplainable edema"
- Systemic rheumatic diseases where renal involvement is common
- Diabetes mellitus
- Nephrotoxic drugs (non-steroidal antiphlogistics, ACE-inhibitors, antibiotics, cytostatica, Cyclosporin A, X-Ray contrast media
- Infections (streptococci, HBV, HIV, malaria etc.)
- Kidney stone carriers; hypopotassemia; hypercalcemia; hypophosphatemia
- EPH-Gestosis
- Renal allograft recipients

Figure 10

Applications for quantitative measurement of urinary marker proteins

- Early detection of
  - glomerular proteinuria (microalbuminuria)
    - diabetes mellitus
    - hypertension
    - systemic rheumatic diseases
    - EPH-Gestosis
  - tubular proteinuria
    - nephrotoxicity
  - prerenal proteinuria
    - monoclonal light chains (Bence Jones protein)
- Therapeutic monitoring
  - Steroids / ACE inhibitors in selective / moderately selective glomerular proteinuria
  - glomerular proteinurias („nephrotic syndrome“)
  - Renal allograft recipients
- Prognostic Significance
  - retinol binding protein (complete tubular proteinuria)
  - alpha-1-microglobulin (cut-off 33.5 mg / g creatinine)
  - IgG (cut-off 110 mg / g creatinine)