OCCASIONAL ARTICLE: REVIEW

Increased albumin excretion in diabetes

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In 1964 Keen and Chlouverakis used a radioimmunoassay to measure the concentration of albumin in the urine of diabetic subjects.¹ Although the urine samples were negative for protein by standard clinical tests, the rate of albumin excretion in the study group was greater than in normal controls. Many studies and review articles have subsequently addressed the clinical implications and pathophysiological mechanisms of these findings. There has been little attempt, however, to assess the consequences of these activities on pathology services.

The aim of this review is to give an historical perspective to the main clinical developments of what is often called "microalbuminuria", outline the various analytical methods available, discuss some of the technical difficulties, and consider how pathologists may best provide a worthwhile service to clinicians.

Terminology

"Microalbuminuria" has been used to refer to albuminuria that is greater than normal (2.5–26 mg/24 hour²) but undetectable by conventional Albustix (less than 250 mg/l). It has been variously defined as an albumin concentration of between 30 and 140 µg/ml³; an albumin excretion rate (AER) of between 15 and 150 µg/minute⁴; 20 and 200 µg/minute⁵; 30 and 300 mg/24 hour⁶; and between 26 and 250 mg/24 hour².

Other terms in common use are "incipient diabetic nephropathy" (between 20 and 200 µg/minute) and "clinical or overt diabetic nephropathy"⁷ (greater than 200 µg/minute). The proposed definitions of both terms rely on excluding other non-diabetic causes of proteinuria and should show an increased albumin excretion rate on two out of three occasions within a six month period. There is no universal agreement on these rigid criteria, which imply definite disease entities rather than evolutionary stages of one disease process.⁸

The terms "microalbuminuria" and "macroalbuminuria" won't be used in this article. Both of these expressions are misleading because they suggest that albumin is present in different molecular weight forms. Although this is true in some circumstances, in nephrotic syndrome^{9,10} where polymeric and fragmentary forms of albumin occur, for example, in diabetes the "micro" and "macro" in fact refer to the amount of albumin excreted. The recently proposed neologism of "paucialbuminuria"¹¹ also fails to provide additional clarification to the simple use of "albuminuria"¹² which is the term used in this article.

Important studies

Three prospective studies of insulin dependent diabetics have shown the prognostic value of a raised urinary albumin excretion rate (AER) with regard to subsequent nephropathy. All the authors arrived at the same conclusion—that there is a threshold rate of albumin excretion which identifies those patients likely to develop diabetic nephropathy. The important difference among these studies, however, is that each identifies a different threshold rate: Mathiesen, 70 µg/minute¹³; Viberti, 30 µg/minute¹⁴; and Mogensen, 15 µg/minute⁴. This disagreement may be explained by differences in the length of study, methods of urine collection and statistical analyses.

In non-insulin dependent patients increased albumin excretion is reported not only to predict subsequent renal disease as evidenced by clinical proteinuria but also early mortality.³ Albumin excretion now seems to be associated with cardiovascular disease in this group of patients.^{15,16} An important recent observation is the association of increased albumin excretion with coronary and peripheral vascular disease in non-diabetic subjects.¹⁷

Studies have shown that control of glycaemia,¹³ blood pressure,^{19,20} and diet²¹ can reduce albumin excretion. In a group of 36 randomised patients strict glycaemic control, judged by glycated haemoglobin and achieved by continuous subcutaneous insulin infusion, resulted in a significant reduction in the rate of progression from an AER of between 30–300 mg/24 hours to clinical diabetic nephropathy (AER > 300 mg/24 hours) over a period of two years.¹⁸

Three questions arise from the above observations. Does a raised albumin excretion actually predict later nephropathy or simply detect disease at an earlier stage? Do manipulations which reduce the rate of albumin excretion really change the progression of renal disease? Should urine albumin be measured routinely? These points have been addressed by Mogensen, who has drawn attention to the six or seven year delay before the results of two large studies (diabetes control and complications trial (DCCT) and the British microalbuminuria collaborative study) are available. Even then the question of whether strict metabolic control will prevent diabetic nephropathy may not be answered. In the meantime he recommends guidelines for patient management based on the measurement of blood pressure, glycated haemoglobin, and urinary albumin.²²

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Mechanisms

The pathophysiology of diabetic proteinuria has received considerable attention.^{2,23} There are, however, certain conflicting reports worthy of discussion. There should be a sound understanding of the mechanisms of albuminuria before attempting to interpret the results of measurements. Albumin in urine may come from renal and non-renal sources. Urinary tract infection has been checked as a source of proteinuria in clinical studies, but other factors including mechanical problems of the ureters, drug effects,²⁴ and especially perineal contamination in women²⁵ have received little attention.

The renal excretion of any substance represents a balance between glomerular filtration, tubular reabsorption, and tubular secretion. It is unlikely that any active secretion of albumin occurs in the kidney²⁶ so that excretion is determined by the processes of filtration and reabsorption. Filtration will be influenced by glomerular filtration rate and the size and electrical charge of the molecule. More than 95% of filtered albumin is reabsorbed by a nearly saturated tubular mechanism (direct evidence from animal studies²⁷).

The case for increased excretion of albumin in diabetic subjects resulting from increased filtration rather than decreased tubular reabsorption² is based on the observation that the excretion of β -2-microglobulin (an indicator of renal tubular damage) is not increased in the presence of increased albumin excretion. The excretion of γ globulin, which differs in electrical charge and molecular size, is also increased, and the relative amounts of albumin and γ -globulin change according to the degree of proteinuria. This suggests a progressive change in glomerular selectivity.

The excretion of β -2-microglobulin, however, may not be the best marker of tubular function. Several studies have investigated the urinary excretion of other low molecular weight proteins (κ light chains, retinol binding protein, and α -1-microglobulin). In 1979 Lopes-Virrelli described a predominant excretion of low molecular weight proteins, suggesting tubular dysfunction, in juvenile diabetics with short disease duration.²⁸ Later work on diabetics without albuminuria showed an increased excretion of κ light chains which was independent of the excretion of β -2-microglobulin.²⁹ Recently the excretion of retinol binding protein, a low molecular weight marker of tubular function, has been shown to be increased in diabetics with normal albumin excretion.³⁰ A study of childhood diabetes concluded that β -2-microglobulin was an unsatisfactory marker of tubular function compared with the excretion of α -1-microglobulin and κ light chains which were significantly increased in diabetic children.³¹ All of this work suggests that there are disturbances of tubular function which precede albuminuria, but it does not resolve the questions of which mechanism is responsible for albuminuria or whether measurement of proteins other than albumin would provide more useful information for predicting nephropathy.

Diabetic subjects have increased concentrations of glycated proteins. Non-enzymatically glycated albumin is more anionic ($pI < 4.7$) than the native protein, and this resulting change of charge could result in altered renal handling of glycated albumin and explain some of the changes in AER associated with improved glycaemic control. Studies of the excretion rate of glycated proteins have used various techniques and have produced contradictory results—namely, a preferential retention or excretion of glycated protein. Gragnoli *et al* reported an increased renal elimination of glycated protein (expressed as nmol 5-hydroxy methyl furfural/mg protein) in diabetics which was related to the degree of proteinuria.³² Ghiggeri *et al*, using isoelectric focusing of chromatographically purified albumin, concluded that glycation was the main determinant of albumin excretion in diabetics with normal or slightly raised albumin excretion.³³ In contrast, Kverneland *et al* reported a preferential retention of glycated albumin (expressed as μ mol furosine/ μ mol albumin), which could be explained by a loss of anionic charge on the glomerular basement membrane.³⁴

There is an increased transcapillary escape rate (TER) of albumin in diabetic subjects.⁶ This was established by following the decline in plasma radioactivity after an intravenous injection of radiolabelled albumin. The TER of albumin is increased by poor metabolic control and hypertension³⁵ and is increased in patients with increased albuminuria. The loss of albumin from the vascular compartment, however, is not explained by the increased loss of albumin in the urine. Long term diabetics without albuminuria do not have increases in TER, suggesting that a common mechanism, probably endothelial dysfunction,³⁶ is responsible for both renal and extra-renal capillary leakage.

Whichever mechanism is responsible for the increased albumin excretion, the mainstays of clinical management will be control of hypertension and glycaemia together with the possible restriction of dietary protein. Recent work investigating renal biopsies emphasises the importance of hypertension by finding that established abnormalities of glomerular structure are only accurately predicted by the presence of increased AER together with hypertension, reduced creatinine clearance, or both.³⁷ A positive family history of hypertension or an increased red blood cell Na^+/Li^+ exchange may also identify patients at risk of nephropathy before any increase in urinary albumin excretion.³⁸

Analysis

To fulfil the requirements of adequate sensitivity, specificity, and reproducibility for urinary albumin in the range of interest (2–200 mg/l) some type of immunological method is necessary. There is a wide choice of available methods but four general approaches predominate. Radioimmunoassay (RIA); non-isotopic immunoassay; nephelometry and

immunoturbidimetry (IT); radial immunodiffusion (RID). Other less common methods include zone electrophoresis,³⁹ liquid chromatography,⁴⁰ and latex particle counting.⁴¹

The idea that diabetic nephropathy can be predicted by a urinary albumin concentration exceeding a critical value has naturally led to the development of semiquantitative tests which can identify urines with more than that critical albumin concentration and are suitable for use in the clinic or ward side-room.

METHODS

Radioimmunoassay

RIA has been established since 1963⁴² and has been used in many of the important clinical studies. Several manufacturers now produce kits specifically for the determination of urinary albumin, typically with analytical ranges in the order of 1–100 mg/l, which may be expanded by sample dilution. The latter requires some prior knowledge of the likely urine albumin concentration. As an increased AER was first shown by RIA there has been a tendency for it to be used as the standard against which proposed methods are compared, although its performance is not always satisfactory.⁴³

Non-isotopic immunoassay

The use of non-isotopic labels overcomes two of the main objections of RIA—the use of radioactive materials and the short shelf life of the labelled reagent. For example, fluorescein has successfully replaced ¹²⁵I as the label in an otherwise identical assay system.⁴⁴

Enzyme linked immunoassay (ELISA) techniques are simple but often require long incubation times with multiple washing and reagent addition steps. Few laboratories have automated equipment capable of washing, dispensing, and plate reading and therefore might consider the method too labour intensive. The method described by Fielding *et al* (an example of the sandwich type) requires a 250-fold sample dilution, five reagent addition steps, five hours of incubations and extra time for plate washing.⁴⁵ Various modifications of this approach have been used⁴⁶ but do not achieve the same large assay range (3–1000 µg/l) described by Fielding. Competitive ELISA methods have been developed either with immobilised antigen^{47,48} or enzyme labelled antigen.⁴⁹ The labelled antigen method is attractive because it is fast (one hour), needs only two washing steps, does not need sample predilution and has a range of 0.9–200 mg/l which is suitable for the concentrations of interest. This approach produces data which can be processed by conventional saturation analysis curve fitting procedures. “Edge effects” are recognised as a problem with microtitre plate methods^{45,47,50} and refer to the discordant results that obtain in the outer wells of the microtitre plate. In practice this means better precision is achieved if only the inner 60 wells of a 96 well plate are used, a considerable

sacrifice. This problem may be explained by inherent plate variability. Considering the increasing use of microtitre plate methods there have been surprisingly few rigorous investigations of these phenomena.⁵¹

Immunoturbidimetry

Spencer and Price investigated kinetic immunoturbidimetry and found its performance comparable with nephelometry and therefore suitable for measuring albumin in urine at low concentrations.⁵² The widespread use of centrifugal fast analysers has allowed many laboratories to have a simple, rapid, and reasonably economical method capable of dealing with large batches. The only requirement for sample pretreatment is a centrifugation step to clear the urine sample of potentially interfering particulate matter. There is no lack of either published^{53–55} or commercially available methods. Most methods cover at least the 5–80 mg/l range and offer within and between batch precision of less than 5 and 10%, respectively. Several aspects of the technique are critical, especially the effect of polymer enhancement and antibody avidity.⁵¹ Other errors may result from the choice of instrument wavelength and the associated urine blank absorbance values.⁵⁶ There is also a report of significantly different results being generated for some urines; depending on the species source of anti-albumin antibody, rabbit anti-serum produced the most reproducible results and the best correlation with an established commercial RIA.⁵⁷

Radial immunodiffusion

Radial immunodiffusion (RID) methods have been used in clinical studies,¹³ and commercial systems are available. RID methods require long incubation times (often overnight) which would not permit a same day or “in clinic” service, but it is unlikely that urine albumin assays would ever be considered as urgent investigations.

Watts *et al* give a comprehensive comparison of RIA, RID, IT and ELISA (sandwich type).⁵⁰ They tested urine samples from diabetic patients in the range 1–120 mg/l and found no systematic difference between RIA and RID. They reported IT and ELISA as giving consistently lower values than RIA with greater random error, the errors increasing at greater albumin concentrations. ELISA gave the poorest between batch performance (CV 8–10%), and a degree of bias (a problem also encountered by others⁵⁸) which was considered to be clinically significant and likely to result in the misclassification of patients. They found IT to be the most expensive method for operating cost but this was assessed against an “in house” RIA method, which is probably many times less expensive than a commercial kit. They concluded that despite the greater technical skill required, given the suitable staff, the attractions of low capital cost and the flexibility of batch size would make RID their method of choice.

Semiquantitative methods

Tests suitable for the clinic or ward side room include latex agglutination of both the direct and indirect types.^{59,60} In the direct test latex particles coated with anti-albumin antibody are mixed with free antibody and test urine sample. This results in visible semiquantitative agglutination if the concentration is between 24 and 166 mg/l. Higher concentrations will give no agglutination and must be distinguished from very low values by a conventional Albustix test.⁶¹ The indirect test uses latex particles coated with albumin added to a mixture of test sample and anti-albumin antibody resulting in visible agglutination at concentrations of less than 40 mg/l.

Another approach is a bromophenol dye binding tablet. Urine is dropped on to the surface of the tablet which is then washed by drops of distilled water. A positive reaction is given by a blue-green colour which is compared with a standard. This simple method has the major disadvantage of not being specific for albumin but reports suggest that it produces few false negative results, when used to detect urinary albumin concentrations greater than 40 mg/l and is therefore a reasonable "screening" method.^{62,63}

A laboratory assay using a colorimetric protein method has been described which gives a similar performance to other semiquantitative methods and has the advantages of speed and low cost.⁶⁴

METHOD INDEPENDENT FACTORS

Potential sources of pre-analytical error include the storage conditions of urine samples. Standards must be stable and accurately prepared. It is also important to perform the analysis on a representative sample and so minimise biological variations.

Albumin adherence

It is known that polypeptides in urine may adsorb on to the plastic of containers⁶⁵ but there is no consensus regarding the behaviour of albumin in urine. Fielding *et al* included 1% (v/v) of rabbit serum in the urine sample before storage to prevent adsorption.⁴⁵ Many reports do not make specific reference to this phenomenon but Feldt-Rasmussen *et al* state that containers were tested for albumin adherence.⁴⁶ Unfortunately, they do not specify which containers, how the adherence was investigated, or what results were produced. Torffvit *et al* also state that albumin adsorbs to plastic tubes and added 0.001% (v/v) of Tween 20 (an ionic detergent) to prevent the phenomenon.⁴⁷ Urine stored with the addition of Tween 20 gave up to 50% greater albumin concentration than if stored without the addition of the detergent.

This problem of adherence has received more attention regarding the preparation of standards. Gosling reported an overestimation of test albumin concentration unless bovine serum albumin was added to the standard solutions to prevent adsorption onto the polystyrene containers.⁶⁶ The interpretation of this

finding was complicated by later work which showed up to 30% cross reactivity between that particular antibody and bovine serum albumin.^{67,68} The problem of "apparent" adherence may be overcome by adding Triton X-100 (a detergent) to the albumin standards.⁶⁹ Unfortunately, there does not seem to have been a definitive study investigating the adherence of albumin to different material. This is of particular importance because the forgoing observations could be explained by the loss of immunoreactivity of the standards. Silver *et al* discuss the possible 20% reduction of immunoreactivity in lyophilised compared with monomeric albumin.⁴⁴ This is because aggregates form during the freeze drying process and there may be batch to batch variation of the amount of aggregate produced.

Storage conditions

There is no standard method either for storing urine or for sample preparation prior to albumin assay. The commonest storage temperature is -20°C , and few studies actually state how long the samples have been maintained at the chosen storage temperature. Some workers have added TRIS buffer to alkalise the urine⁷⁰ while others have added detergents,⁴⁷ proteins,⁴⁵ or sodium azide preservative.³⁹ Some centrifuge urine after thawing^{44,47,71} but many others do not record whether this was done. The relevance of these different approaches is that albumin may precipitate from urine centrifuged after storage at -20°C .⁷² This observation has been endorsed⁷³⁻⁷⁵ and refuted.⁷⁶ There is evidence that such precipitation may occur with other proteins as well as albumin and is prevented by adjusting the urine pH to neutral.^{77,78}

Choice of collection

The easiest sample to obtain is a random (untimed) collection when a patient attends clinic. This sample is likely to show the greatest variations of albumin concentration, influenced by factors like previous exercise,⁷⁹ metabolic state,⁸⁰ and rate of urine production.⁸¹ Short collection periods (20 minutes) have been used to investigate specific effects such as water loading, but the supervision required would not be practicable outside metabolic wards. Overnight collections have shown less variation than companion diurnal collections⁸² and are less inconvenient for patients than 24 hour collections. Coefficients of variation are about 40%, however,⁸³ which are similar to those found for 24 hour collections.⁸⁴ A logical choice would be an untimed first morning collection which combines the convenience of a small sample with the smaller variation of an overnight collection.

Children represent a special case because timed urine collections are so difficult to collect and there are considerable differences of relative age, body mass, and height among subjects. Large (up to 10-fold) within subject variations of albumin excretion are described in normal children, emphasising the need for

repeated determinations before deciding whether albumin excretion is increased.⁸⁵ Marshal *et al* found that AER in both diabetic and normal children was similar (median 2.5 and 2.4 $\mu\text{g}/\text{ml}$, respectively, overnight collection) and less than adult values (median 4.3 $\mu\text{g}/\text{ml}$).⁸⁶ They concluded that cut off values predictive of nephropathy in adults might not be appropriate in children. They also showed a positive correlation between AER and age (girls having higher values than boys). Mathiesen *et al*, however,⁸⁷ did not find any association with age or sex.

Units

A variety of units are used to express albumin excretion. The choice is influenced by the duration of the urine collection: thus for untimed samples albumin excretion may be expressed simply as a concentration or as a ratio of albumin to creatinine concentrations; 24 hour collections are usually expressed as $\text{mg}/24$ hour; timed collections of less than 24 hours are often expressed as $\mu\text{g}/\text{minute}$. In the case of a "rate" two errors are introduced from volume and time estimates and in the case of the "ratio" any error in the creatinine estimation is introduced. The inaccuracies of 24 hour urine collections are well documented and the attraction of expressing albumin excretion as a ratio with creatinine ignores the variation of creatinine excretion both within and among subjects. Both Howey⁸⁸ and Hutchison⁸⁹ found little advantage in the albumin: creatinine ratio and prefer to use the uncomplicated albumin concentration of the first morning urine sample.

Conclusions

Laboratories are likely to receive an increased demand for urinary albumin analyses, and if this is in the form of a service commitment rather than as part of limited research projects pathologists must decide how to provide a satisfactory service. A laboratory's choice of method is bound to be influenced by existing equipment, local expertise, and cost. The fact that over 20 years of experience have not produced one preferred method suggests that any of the main four quantitative approaches is satisfactory, although each has certain disadvantages. Perhaps the more important consideration is whether all patients should have a quantitative assessment if reliable semiquantitative tests are available. This point has major cost implications because of the need to make several determinations to gain a realistic measure of any individual's albumin excretion. In effect a more sensitive "Albustix" approach could identify those patients in whom urine albumin quantitation would influence management.

Whatever analytical method is used, sensible comparisons of the results from different centres will continue to be difficult in the absence of a standard approach. The easiest aspect to control is probably the most important—namely, the choice of urine sample. A first morning, untimed specimen combines the lower overnight variation with the convenience of a random sample. It avoids any need for

timing on behalf of patient or staff and is not inconvenient to repeat should several different determinations be required. Having obtained the sample in a straightforward manner, it seems sensible to express the results as simply as possible—that is, as a concentration. There do not seem to be any clear advantages using the creatinine ratio which requires an extra assay and therefore time and cost. Laboratories usually batch the more specialised tests and if there is a delay in analysis the storage conditions and sample pretreatment may influence the final result, just as may the choice and preparation of standards.

The prevalence of diabetes mellitus and the high incidence of renal failure in the diabetic population ensure a continued interest in the prevention or reduction of diabetic nephropathy. At present the exact role of measuring urinary albumin in patient management is not clear but rests on the expectation that a reduction of albumin excretion will be associated with a reduction of the progression of renal disease.

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