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Tubular proteinuria defined by a study of Dent's (*CLCN5* mutation) and other tubular diseases

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Tubular proteinuria defined by a study of Dent's (*CLCN5* mutation) and other tubular diseases.

Background. The term "tubular proteinuria" is often used interchangeably with "low molecular weight proteinuria" (LMWP), although the former implies a definite etiology. A specific quantitative definition of tubular proteinuria is needed, and we address this by studying five different renal disorders.

Methods. Tubular proteinuria was assessed by measuring urinary retinol-binding protein (RBP), β_2-microglobulin (β_2M), α_1-microglobulin (α_1M), and albumin in 138 patients: 26 affected males and 24 female carriers of the X-linked syndrome "Dent's disease," 6 patients with other Fanconi syndromes, 17 with distal renal tubular acidosis (dRTA), 39 with glomerulonephritis (GN), and 26 with Chinese herbs nephropathy (CHN).

Results. RBP was better than β_2M or α_1M in identifying the tubular proteinuria of Dent's disease. Median urinary RBP levels in mg/mmol creatinine were: affected male Dent's, 18.2, $N = 26$; carrier female Dent's, 0.30, $N = 24$; dRTA, 0.027, $N = 17$; GN, 0.077, $N = 39$; and normal adults, 0.0079, $N = 61$. Elevated urinary RBP (>0.017) and albumin $< (10 \times \text{RBP}) + 2$ identified all patients with the LMWP of Dent's disease and clearly distinguished their LMWP from that of dRTA and GN. This is a quantitative definition of tubular proteinuria. Consistent with this definition, 80% of those patients with CHN who had an elevated RBP had tubular proteinuria. Urinary RBP and albumin in carriers of Dent's disease were strikingly correlated over a 100-fold range ($R = 0.933$).

Conclusion. The combination of elevated urinary RBP (>0.017) and albumin $< (10 \times \text{RBP}) + 2$ (mg protein/mmol creatinine) is a quantitative definition of tubular proteinuria. Furthermore, our findings suggest that a shared defect in tubular RBP and albumin reuptake causes this form of proteinuria.

Key words: low molecular weight proteinuria, Dent's disease, retinol binding protein, albumin, urine proteins, proximal renal tubular disease.

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The term "tubular proteinuria" has been applied to an electrophoretic pattern of urine proteins found in patients with chronic proximal renal tubular disease [1, 2]. This pattern of proteins represents increased excretion of several low molecular weight (LMW) proteins, as well as albumin (molecular weight 65 kd) and β_2-glycoprotein I (molecular weight 50 kd) [2–5]. These LMW proteins include β_2-microglobulin (β_2M; molecular weight 12 kd) [3], α_1-microglobulin (α_1M; molecular weight 30 kd) [6], retinol-binding protein (RBP; molecular weight 21 kd) [7], and urine protein 1 (Clara Cell Protein, molecular weight 20 kd) [8]. "Tubular proteinuria" is due to a failure of protein reabsorption by the proximal tubule [2–4]. The terms tubular proteinuria and LMW proteinuria (LMWP) have usually been used interchangeably [9–11], but tubular proteinuria has no accepted quantitative definition. Here, LMWP is used to mean excretion of one or more LMW proteins above the reference range, and tubular proteinuria is used to mean LMWP in a patient known to have predominantly proximal tubular disease.

Low molecular weight proteinuria is the most consistent laboratory finding among male patients and female carriers of the recently discovered X-linked tubular syndromes caused by mutations of a renal chloride channel, *CLCN5* [12]. These familial tubular syndromes comprise "Dent's disease" [13, 14], "X-linked recessive nephrolithiasis (XRN) with renal failure" [15–17], "X-linked recessive hypophosphatemic rickets" [18], "LMWP with hypercalciuria and nephrocalcinosis" [19, 20], "Japanese idiopathic LMWP" [20, 21], "familial idiopathic LMWP" [22], isolated patients with LMWP [23, 24], and a large kindred having "X-linked renal failure without X-linked recessive hypophosphatemic rickets" [25], previously reported as a form of renal tubular acidosis [26]. As these

syndromes all have similar phenotypic features and share the same genetic etiology of *CLCN5* mutations, they will be referred to as Dent's disease [24].

Studies of individuals at risk of Dent's disease require screening of patients for tubular proteinuria, but this is imprecise without a quantitative definition. Excretion of a single LMW protein above the upper reference level has generally been used to identify tubular proteinuria in such families [12, 25].

Previous, more limited studies showed that female carriers of Dent's disease have a wide quantitative variation of LMW proteins in urine [27]. We have further investigated these patients and affected males with Dent's disease as a clinically homogeneous group for the study of tubular proteinuria. Because Dent's disease can be confused clinically with distal renal tubular acidosis (dRTA) caused by the coexistence of medullary nephrocalcinosis and rickets/osteomalacia [12, 13, 25, 26], it is important to know the prevalence of tubular proteinuria in dRTA. LMWP is common in glomerulonephritis (GN) in both adults and children [8–10], but this form of proteinuria ought to be distinguished qualitatively from tubular proteinuria because the etiology of the LMWP is different [3].

We have developed a new quantitative definition of tubular proteinuria based on the measurement of RBP and albumin in random urine collections. This definition is ideally suited for studies of families at risk of Dent's disease and may substantially improve the specificity of screening for these mutations. It may also be widely applicable to other groups of patients with renal disease.

METHODS

Local research ethical committee approval was obtained for all studies not part of routine care.

Patient selection

Dent's disease. Twenty-eight affected male patients with Dent's disease were studied. Each of these patients had clinical features of the disease, had other affected family members, and had a documented *CLCN5* mutation [16, 24]. These included two atypical male patients with *CLCN5* mutations and a very mild phenotype who are described in detail elsewhere [15, 28]. Patients with a renal transplant or receiving dialysis treatment were excluded. Twenty-four female carriers of Dent's disease were obligate carriers on the basis of being either the daughter or the mother of an affected male. Mothers of affected males also had one other affected first-degree relative. All carriers were shown to be heterozygous for *CLCN5* mutations [16, 24].

Renal Fanconi syndromes. These included four adults (3 males and 1 female) from previously described families with autosomal dominant noncystinotic idiopathic Fanconi syndrome [29, 30] and two males with Lowe's syndrome [31] with severe mental impairment, multiple ocular defects causing blindness, and a renal generalized aminoaciduria, phosphaturia, and glycosuria.

Distal renal tubular acidosis. Urine samples were obtained from 17 patients: six females with maturity onset immune-related disease (all with nephrocalcinosis), eight patients with familial disease (7 males and 1 female, all except one male had nephrocalcinosis), and three patients with sporadic dRTA (2 males with nephrocalcinosis, 1 female without). Full clinical details of the immune-related cases, and all but one of the familial cases, have been published [32]. Seven of the eight familial cases had autosomal-dominant disease and were members of three families with *AE1* mutations [33, 34]. All dRTA cases were acidotic when first studied, except for the youngest familial patients, who had an *AE1* mutation and a urinary acidification defect, but no nephrocalcinosis or systemic acidosis (incomplete dRTA syndrome).

Glomerulonephritis. All 39 patients had renal biopsies confirming glomerular disease. The histologic diagnoses were IgA disease ($N = 6$); focal segmental glomerulosclerosis (6); membranous nephropathy (6); minimal change disease (7); membranoproliferative disease (4); renal amyloidosis (4); diabetic glomerular disease (4); Wegener's granulomatosis and systemic lupus erythematosus (1 each). Twenty-three of these patients were reported previously [11]. Patients with serum creatinine levels of over 200 μmol/L, a level at which raised serum RBP is associated with elevated RBP excretion [35], were excluded.

Unselected patients screened for low molecular weight proteinuria. These comprised specimens of urine from 82 patients submitted over a six-month period for detection of LMWP. These patients were either males ($N = 43$) at risk, by clinical criteria, of having Dent's disease or females at risk of being carriers of Dent's disease ($N = 39$). They were referred from eight different nephrology centers and were unselected with respect to the quality or quantity of any proteinuria.

Chinese herbs nephropathy. We studied 26 female patients with renal failure, a history of intake of Chinese herbal medicine to induce weight loss, and a renal biopsy consistent with CHN. These patients have been reported in detail previously [36].

Normal individuals. Sixty-one adults (age 16.1 to 58 years) without a history of personal or familial renal disease consented to provide specimens.

Urine specimens. We collected random urine specimens into sodium azide, approximately 200 mg/L final concentration. Patients either sent their specimens by mail at ambient temperature, and these were frozen at −80°C within 24 hours of original collection, or specimens were frozen within six hours of collection. We kept

Table 1. Low molecular weight protein and albumin excretion in urine, and serum creatinine concentrations in the subject groups: Dent's disease, Glomerulonephritis, distal RTA, Chinese herbs nephropathy, and normal adults

Protein *mg/mmol creatinine* Mean ± SEM Median (10–90th centiles)	Affected males with Dent's disease	Carrier females of Dent's disease	Glomerulonephritis
RBP	21.4 ± 2.9	0.71 ± 0.22	1.05 ± 0.40
	18.2 (7.8–42)	0.30 (0.13–3.1)	0.077 (0.009–3.0)
	$N = 26$	$N = 24$	$N = 39$
β_2M	12.75 ± 1.3	1.58 ± 1.19	0.54 ± 0.19
	11.6 (4.9–28)	0.23 (0.13–1.9)	0.050 (0.012–2.2)
	$N = 30$	$N = 24$	$N = 39$
α_1M	32.2 ± 5.0	2.75 ± 0.63	4.02 ± 0.82
	23.3 (4.4–62)	1.80 (0.53–8.3)	2.30 (0.44–9.8)
	$N = 32$	$N = 23$	$N = 39$
Albumin	37.9 ± 3.7	3.31 ± 0.79	288 ± 35
	33.2 (14–64)	1.93 (0.47–7.0)	202 (92–710)
	$N = 32$	$N = 24$	$N = 39$
Serum creatinine *μmol/L* Mean ± SEM	192 ± 45 $N = 32$	50.0 ± 9.3 $N = 24$	167 ± 48 $N = 39$

Patients "A1" and "A2" (Fig. 1) are omitted from this Table.

specimens for analysis of albumin, RBP, and α_1M not longer than three months at −80°C and up to one week at −20°C. About one quarter of the specimens for analyses of β_2M were stored up to two years before assay. Among 20 specimens with a wide range of initial β_2M concentrations, β_2M levels did not change significantly for up to two years of storage at −80°C. In contrast, storage of urine specimens for RBP analysis at −80°C for six months, but not two months, caused appreciable loss. Aliquots for analysis were frozen and thawed once only; all protein analyses were performed within four hours of specimens being thawed.

Analytical methods

Creatinine was measured using a Beckman "CX-7®" analyzer with a kinetic alkaline picrate method (Beckman Instruments, High Wycombe, U.K.; protocol 443-340). Albumin and α_1M were measured by rate nephelometry on a Beckman "Array 360®" analyzer; RBP was assayed by a sensitive double-antibody enzyme-linked immunosorbent assay using a generally available urine RBP standard [37]. β_2M was measured by fluoroimmunoassay on an Abbott "IMx®" analyzer (Abbott Diagnostics, Maidenhead, UK). Measurements of albumin, α_1M, and β_2M with these methods yield a level below the lower limit of detection in approximately 5, 3, and 9% of normal individuals, respectively. For presentation of results, these individuals were scored as having protein concentrations at the lower limit of detection; this was 2, 4, and 0.05 mg/L, respectively. Urine specimens were tested for hematuria using a routine "dipstick" method ("Multistix"®; Bayer Diagnostics, Newbury, UK).

Presentation of the results

All protein levels are expressed as mg protein/mmol creatinine. SYSTAT version 7.0® software (SPSS Inc., Chicago, IL, USA) was used for the following: cumulative centile plots to calculate the 10th and 90th centiles of protein measurements (Table 1) and Pearson correlation coefficients on logarithmically transformed protein measurements and sample confidence ellipses (95%). We state the exact number of patients studied when, for technical reasons such as an insufficient specimen, not all of the protein measurements could be made on a small number of patients.

RESULTS

Familial tubular syndromes and glomerulonephritis

Initially, three groups of patients were studied and compared with normal individuals: males affected with Dent's disease and other forms of the Fanconi syndrome, female carriers of Dent's disease, and patients with GN. The excretion of the three LMW proteins, RBP, β_2M, α_1M, and albumin, is shown in Table 1 and Figure 1. The marked LMWP demonstrated for all three proteins, RBP, β_2M, and α_1M and the albuminuria of males affected by Dent's disease were indistinguishable from that of the six patients with other forms of the Fanconi syndrome (4 patients with the idiopathic adult Fanconi syndrome and 2 patients with Lowe syndrome; Fig. 1). Furthermore, these results show that discrimination between female carriers and normal individuals is best with a measurement of RBP and that discrimination was in the order RBP > β_2M > albumin > α_1M. The range of these proteins excreted among these groups of patients was approximately 10^5-, 10^4-, 10^3-, and 10^3-fold, respectively. RBP is therefore the optimum protein for the detection of tubular proteinuria.

Excretion of all three LMW proteins as well as albumin was abnormal in the affected males with Dent's

Table 1. Continued

Protein *mg/mmol creatinine* Mean ± SEM Median (10–90th centiles)	Distal RTA	Chinese herbs nephropathy	Normal adults
RBP	0.048 ± 0.018 0.027 (0.005–0.1) *N* = 17	2.52 ± 0.82 1.24 (0.003–9.5) *N* = 26	0.0085 ± 0.00045 0.0079 (0.004–0.011) *N* = 61
β_2M	0.58 ± 0.35 0.065 (0.02–2.4) *N* = 16	—	0.0138 ± 0.0021 0.0094 (0.004–0.32) *N* = 36
α_1M	2.03 ± 0.56 1.10 (0.2–7.0) *N* = 17	9.55 ± 2.94 5.15 (0.47–17) *N* = 26	0.678 ± 0.052 0.556 (0.29–1.3) *N* = 61
Albumin	18.2 ± 7.1 6.60 (0.5–42) *N* = 17	10.3 ± 1.6 9.7 (0.3–20.5) *N* = 26	0.595 ± 0.038 0.54 (0.28–0.96) *N* = 61
Serum creatinine *μmol/L* Mean ± SEM	120 ± 9.6 *N* = 17	316 ± 43 *N* = 26	82 ± 17

Patients "A1" and "A2" (Fig. 1) are omitted from this Table.

disease. However, for all three proteins, there was substantial overlap between female carriers with Dent's disease and patients with GN; when α_1M was measured, many affected males and patients with GN had similar results (Fig. 1C). Unlike LMW proteins, measurement of albumin gave good discrimination between female carriers of Dent's disease and patients with GN. These findings suggested that it is essential to employ measurements of albumin as well as an LMW protein to discriminate the tubular proteinuria of patients with Dent's disease.

Measurements of the three LMW proteins RBP, β_2M, and α_1M are displayed against albumin excretion in the bivariate graphs of Figure 2. Discrimination between female carriers of Dent's disease and normal individuals is best when RBP and albumin are measured, and there is a striking correlation between RBP and albumin excretion in carrier females of Dent's disease (correlation coefficient, $R = 0.933$ and $R^2 = 0.871$, $N = 24$). To a lesser degree this is also true of β_2M ($R = 0.698$, $R^2 = 0.487$, $N = 24$) and α_1M ($R = 0.719$, $R^2 = 0.517$, $N = 23$). Results of protein measurements in the two atypical patients belonging to the large North American kindred with XRN [27] are labeled "A1" and "A2" in Figure 1.

Because RBP was the best marker among the LMW proteins, graphical results for this protein only are presented further, although similar measurements were made of both β_2M and α_1M (Table 1).

Distal renal tubular acidosis

Patients with autoimmune, familial, and sporadic distal RTA frequently had elevations of RBP excretion (13 out of 17) and therefore LMWP, although the increases were often small (Table 1 and Fig. 3). The degree of albuminuria among these patients was highly variable, ranging from normal to elevated approximately 30-fold above the upper reference limit (Fig. 3).

Effect of hematuria on low molecular weight protein excretion

Hematuria is common among patients with nephrolithiasis, which is a frequent feature of Dent's disease. We therefore used a model system to determine whether hematuria might cause apparent LMW protein excretion. RBP and albumin were measured in normal urine samples after the addition of small quantities of blood (0.001 to 0.1% by volume). Apparently elevated excretion of RBP was found if as little as one part blood in 10,000 parts urine by volume were added; this corresponded to just detectable ("+") hematuria on dipstick testing of fresh urine without added preservative (Table 2). Control experiments demonstrated that sodium azide at the concentration (200 mg/L) used for routine collection of specimens of urine did not affect measurements of RBP or albumin but inhibited positive testing for blood (results not shown). The addition of azide was essential for specimen stability at ambient temperature. Hematuria may therefore confound detection of LMWP, although, as anticipated, the ratio of albumin to RBP in this model (Table 2) is quite different to that in patients with tubular proteinuria.

Quantitative definition of tubular proteinuria

Because males and carrier females with Dent's disease should be groups with qualitatively similar "tubular proteinuria," we explored combinations of RBP and albumin measurements to find a function that separates the LMWP of these patients from the LMWP of the other patients studied. All affected males and carrier females had an albumin excretion $< (10 \times \text{RBP}) + 2$ where the units for albumin and RBP are mg/mmol creatinine. The line of this function is shown in Figure 4. Measurements within the confidence ellipses for carriers and patients

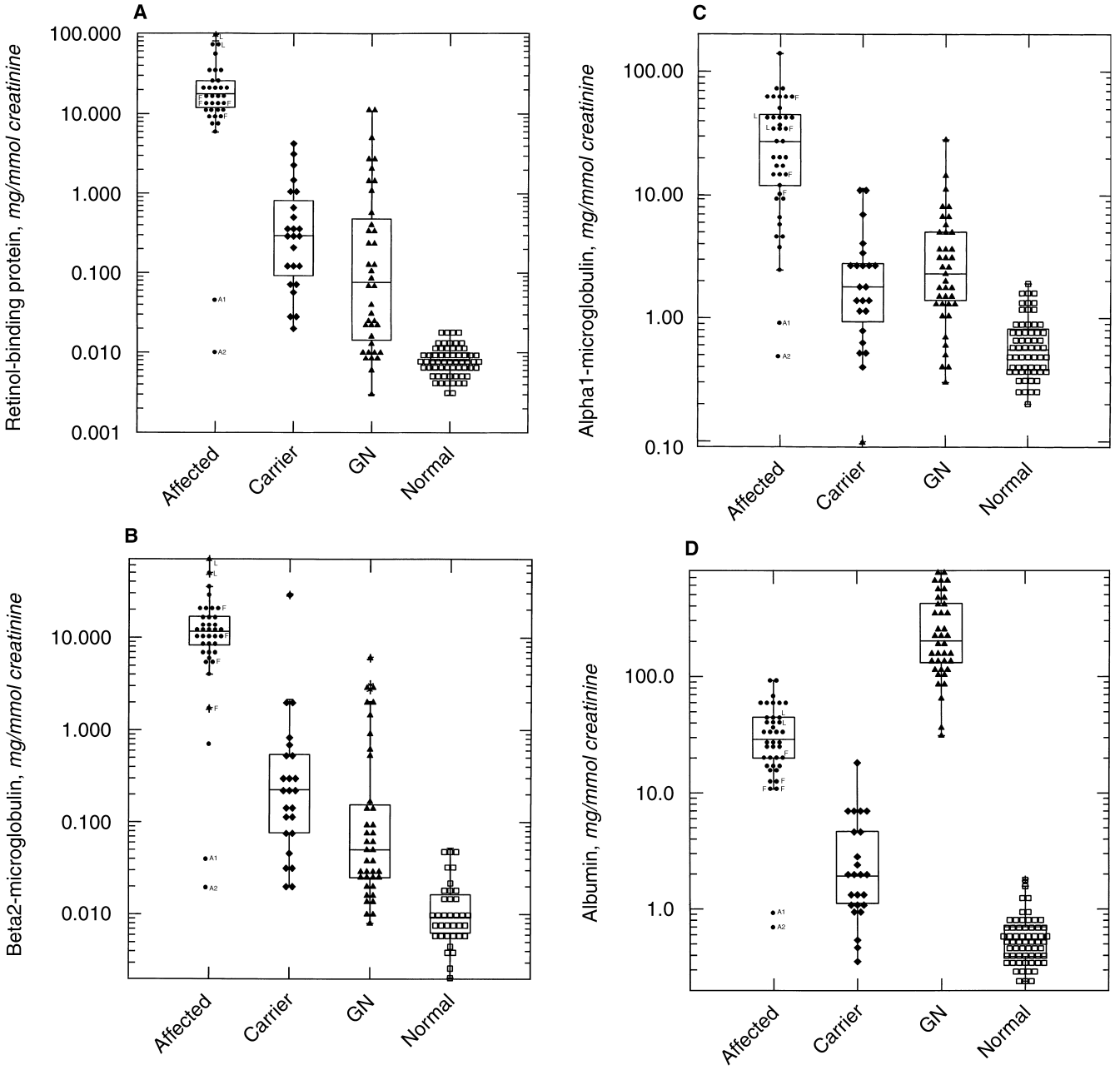

Fig. 1. Excretion of (*A*) retinol-binding protein, (*B*) β_2-microglobulin, (*C*) α_1-microglobulin, and (*D*) albumin in four groups of subjects: "Affected," affected males with Dent's disease and autosomal dominant Fanconi ("F"), and Lowe ("L") syndromes; "Carrier," female carriers of Dent's disease; "GN," patients with glomerulonephritis; and "Normal" adults. Each point is the result for one patient, and the number of patients studied is given in Table 1. Results for the two atypical Dent's disease patients (discussed in the **Results** section) are labeled "A1" and "A2" in the "affected" group. The median is shown by the line that bisects each rectangular box. This box identifies the middle 50th centile of results and the "whiskers" extending from each box identify the lower and upper 25th centiles.

affected by Dent's disease mutations, who have elevated RBP excretion, virtually all lie within the boundary defined by this simple function. Results found in the hematuria model (Table 2) correspond to the specific location labeled in the bivariate graph (Fig. 4).

We applied this definition to results from 10 patients (4 males and 6 females) selected from 82 patients (43 males and 39 females) whose urine specimens were submitted over a six-month period for detection of tubular proteinuria as a screen for Dent's disease. These 10 patients had relatively minor abnormalities of RBP (up to 3 times the upper limit of normal) or albumin excretion (up to 15 times the upper limit of normal) or both (results not shown). Such findings could not be interpreted by reference to previous results on patients with tubular proteinuria [28]. None of these 10 patients were subse-

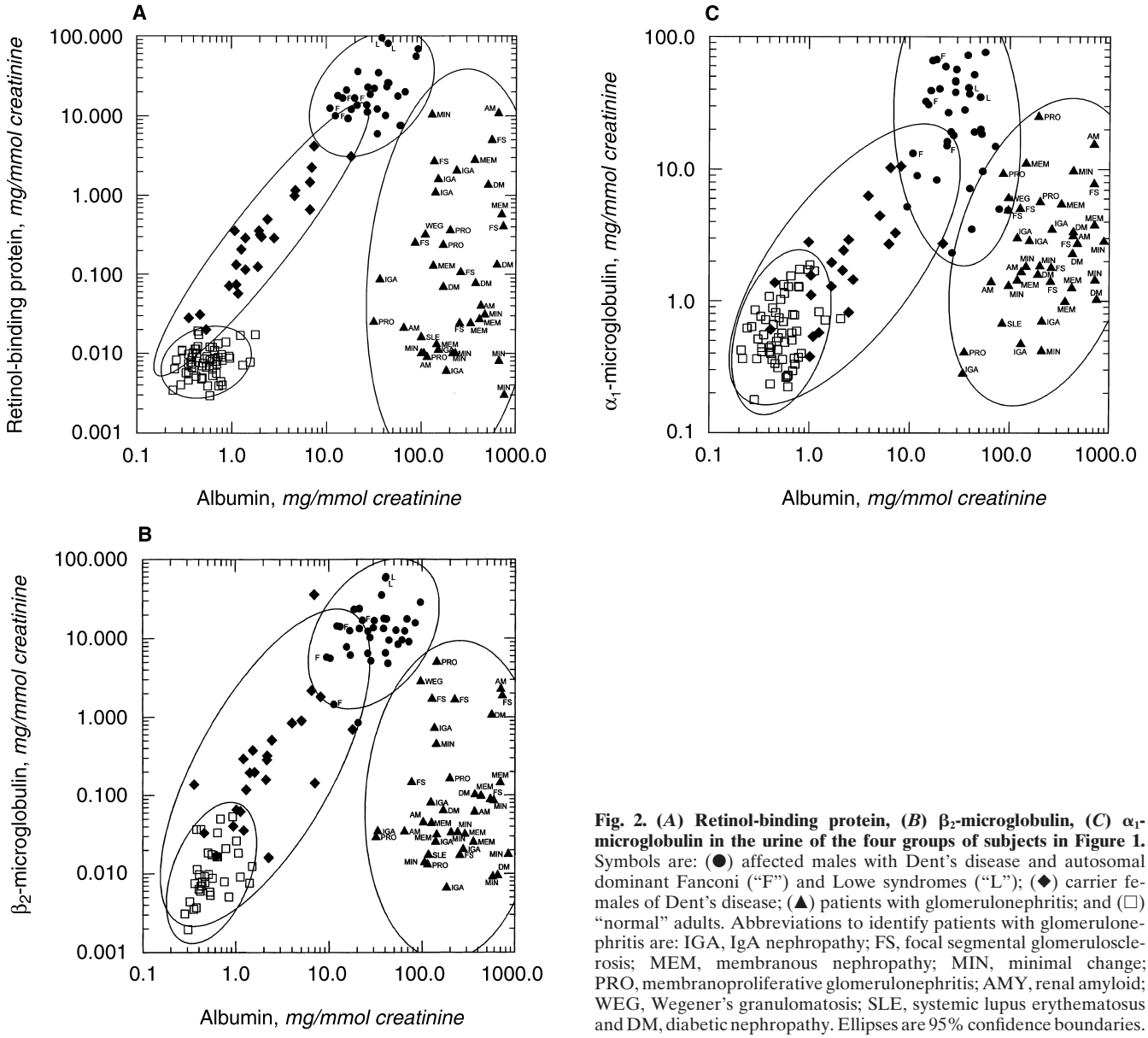

Fig. 2. (*A*) Retinol-binding protein, (*B*) β_2-microglobulin, (*C*) α_1-microglobulin in the urine of the four groups of subjects in Figure 1. Symbols are: (●) affected males with Dent's disease and autosomal dominant Fanconi ("F") and Lowe syndromes ("L"); (◆) carrier females of Dent's disease; (▲) patients with glomerulonephritis; and (□) "normal" adults. Abbreviations to identify patients with glomerulonephritis are: IGA, IgA nephropathy; FS, focal segmental glomerulosclerosis; MEM, membranous nephropathy; MIN, minimal change; PRO, membranoproliferative glomerulonephritis; AMY, renal amyloid; WEG, Wegener's granulomatosis; SLE, systemic lupus erythematosus and DM, diabetic nephropathy. Ellipses are 95% confidence boundaries.

quently found to have molecular genetic evidence of *CLCN5* mutations, and none met our quantitative definition of tubular proteinuria. Application of a quantitative definition in this group of patients with equivocal urine protein abnormalities therefore gave results consistent with molecular genetic data.

Chinese herbs nephropathy

This definition of tubular proteinuria was further examined in a group of patients with a tubulopathy that almost certainly has a different etiology to Dent's disease [36]. To do this, we studied patients with CHN, as the tubulopathy of these patients has been attributed to injury from toxic aristolochic acids in herbal preparations. Twenty-four out of 26 patients with CHN had elevated RBP excretion, and in 79% of these (19 out of 24 patients), RBP and albumin excretion fell within the boundary defining tubular proteinuria (Fig. 5).

DISCUSSION

Identification of tubular proteinuria is necessary to diagnose a male patient affected by one of the X-linked renal syndromes referred to as Dent's disease. LMWP is the most consistent laboratory finding among this group of patients, and a variety of LMW proteins, most usually β_2M, has been measured; substantial elevations of RBP and α_1M have also been found [12, 17, 27]. β_2M

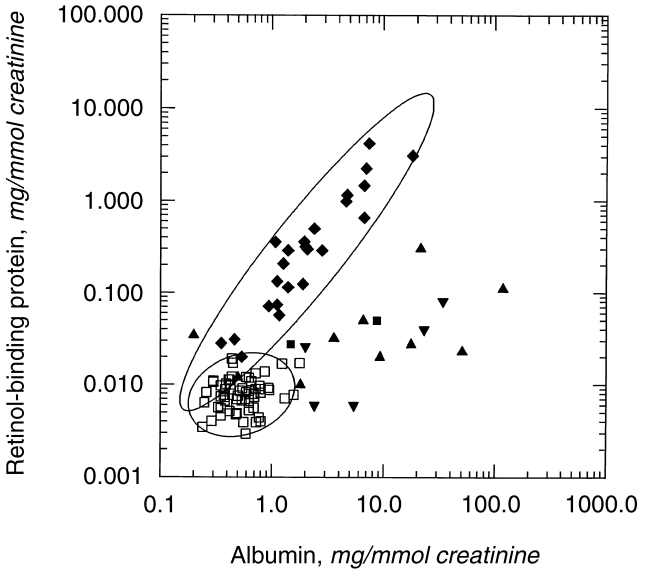

Fig. 3. Retinol-binding protein and albumin in the urine of patients with (▼) autoimmune, (▲) familial, and (■) sporadic forms of dRTA; (◆) carriers of *CLCN5* mutations and (□) normal individuals. Ellipses are 95% confidence boundaries.

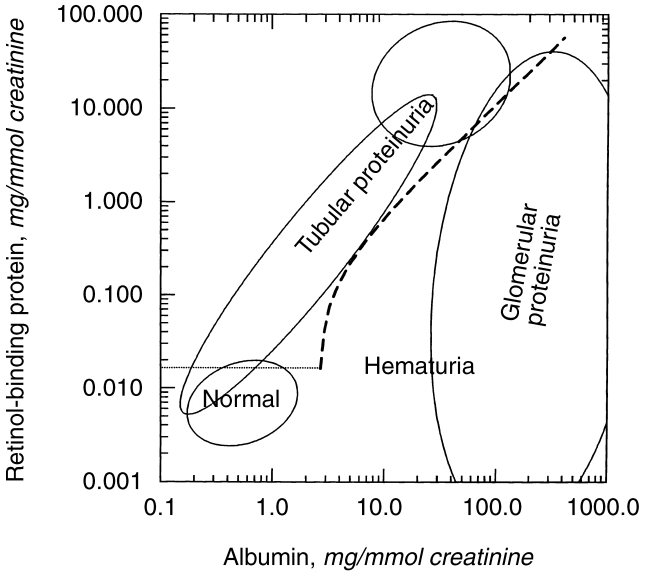

Fig. 4. Plot of the function: albumin = (10 × RBP) + 2 (– – –). The boundary of the upper limit of the adult normal excretion of retinol-binding protein (---) is superimposed on the 95% confidence ellipses for affected male patients with Dent's disease, female carriers of Dent's disease, patients with glomerulonephritis, and normal individuals (Fig. 2A shows identity of confidence ellipses).

Table 2. Apparent retinol-binding protein and albumin concentrations when blood is added to normal urine *in vitro* (protein concentrations are expressed in mg/mmol creatinine)[a]

	Ratio of blood to urine *vol/vol*			
	1:10^5	1:10^4	1:10^3	Control without addition of blood
RBP	0.008 (0.004–0.012)	0.009 (0.005–0.013)	0.020 (0.008–0.12)	0.007 (0.004–0.012)
Albumin	0.58 (0.20–0.9)	1.63 (1.2–1.95)	12.3 (3.3–18.1)	0.48 (0.22–0.8)
Semiquantitative blood estimation by dipstick testing	+	++ (++ to +++)	+++	Negative

[a] Results are the mean of experiments with 5 different specimens of blood and urine (range in parentheses). Blood, 0.01 mL was added to 10 mL of fresh urine (no added sodium azide), left for 30 minutes at room temperature, further diluted 10-fold and 100-fold in the same urine and frozen at -80°C overnight. Assays performed the next day were as described in the **Methods** section.

has been widely used but is known to be unstable in acidic urine [7]. Previous reports and the results here (Table 1, Figs. 1 and 2) suggest that any of the three LMW proteins studied, except perhaps α_1M (Fig. 1), might be used to screen for LMWP in affected males, although, as discussed later in this article, the combination of RBP and albumin is optimal (Fig. 4). LMW protein findings in urine are indistinguishable among Dent's disease and patients with Lowe syndrome and Adult Idiopathic Fanconi syndrome (Figs. 1 and 2), both well-recognized causes of tubular proteinuria [2].

Low molecular weight proteinuria in the female carriers of Dent's disease is less marked than in affected males, and its detection is more difficult [12, 27]; occasionally, it cannot be demonstrated in women proved to carry a *CLCN5* mutation [13, 27]. However, our results show that in carrier females, it may occasionally even exceed that found in an affected male (β_2M and α_1M in Fig. 1 B, C) or at the other extreme may be normal (Fig. 1). Female patients with *CLCN5* mutations and clinical abnormalities of Dent's disease have been reported [13, 17, 21, 25], and one of these female patients had a urinary β_2M excretion in the range of an affected male [21]. RBP appears to be the most sensitive LMW protein for the detection of female carriers, although even with measurements of this protein, a small number of individuals (3 out of 24) were misclassified as normal (Fig. 1A). The cause of this variation is probably varying degrees of X-inactivation among the female patients [12, 38].

Levels of excretion of LMW proteins by patients with GN and female carriers of Dent's disease were virtually indistinguishable, although the degree of albuminuria

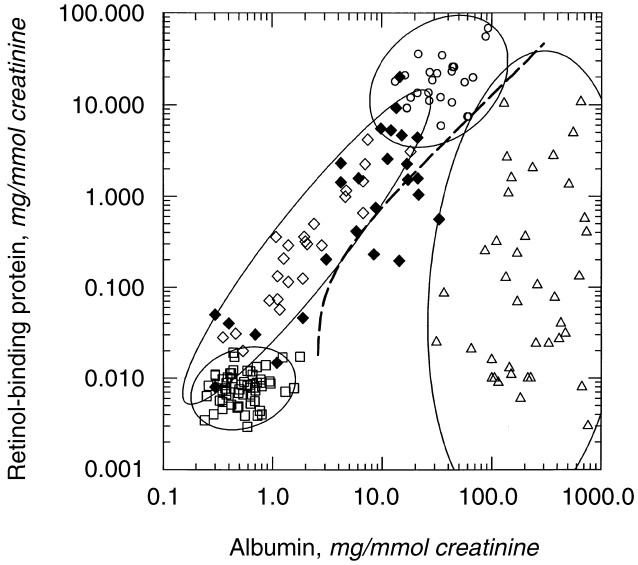

Fig. 5. Retinol-binding protein and albumin in the urine of patients with Chinese herbs nephropathy (CHN). Results for the CHN patients (solid symbols, ◆) are superimposed on the 95% confidence ellipses for male patients with Dent's disease (○), female carriers of Dent's disease (◇), patients with glomerulonephritis (△), and normal individuals (□). The identity of the confidence ellipses is as for Figure 2A.

was quite different (Fig. 1D). This suggested to us that the use of a combination of RBP and albumin as a bivariate distribution (Fig. 2) would give the best discrimination. Such distributions have been used before [10, 11, 39], but areas of the distribution have not been interpreted as in Figure 4. The patients with tubular proteinuria—males with Dent's disease, other patients with a Fanconi syndrome—and carriers of Dent's disease fall predominantly on the upper left of the graph. They have relatively more urinary RBP than albumin. Patients on the right hand of the graph range from those with substantial RBP and albumin excretion (many with forms of GN) to those with almost pure albuminuria and hematuria.

Using an iterative approach, we found that the combination of RBP excretion above the reference range (0.017 mg/mmol) and an albumin excretion less than (10 × RBP) + 2, with units in mg/mmol creatinine, defines the boundary shown in Figure 4 and includes all patients who are affected males (26 out of 26) and most carrier females (21 out of 24) of Dent's disease. This function is simple enough to be applied readily to measurements of RBP and albumin while providing good discrimination. LMWP, defined as RBP excretion above the reference range, is a necessary but not sufficient condition for a patient to be defined as having tubular proteinuria. In a report of studies confined to patients with primary GN, Hofmann et al "corrected" α_1M measurements for urinary albumin using a hyperbolic function to calculate a figure for α_1M excretion due to "tubulointerstitial" disease [39]. In the patients studied here, with predominant proximal tubular disease, α_1M gave poor discrimination (Figs. 1C and 2C).

Thirteen out of 17 patients with dRTA showed slight elevation of RBP excretion, and albuminuria was common but very variable (Fig. 3). However, of these 13 patients, only one patient had a combination of urinary RBP and albumin, demonstrating tubular proteinuria by the previously mentioned definition. In contrast to the other 12 patients, this one patient had no evidence of nephrocalcinosis when studied and, unlike the other dRTA patients who were adults, this patient was 11 years of age. This definition therefore provides results almost fully consistent with the predominantly distal tubular lesion of these patients [32]. The degree of either LMWP or albuminuria was unrelated to the severity of nephrocalcinosis or nephrolithiasis in these patients (results not shown).

Hematuria is, of course, common in patients with nephrolithiasis [40], and abnormal LMW protein levels were found in the hematuria model used. The use of sodium azide preservative inhibited the peroxidase activity of heme and made dipstick testing for hematuria unreliable, so retrospective analysis of the patients studied could not be performed confidently by this method. The *in vitro* model of hematuria suggests that hematuria may cause at least some of the LMWP found, but in combination with albumin measurement, this is easily distinguished from tubular proteinuria.

We found a high incidence of relatively minor abnormalities of RBP or albumin excretion in urine samples from patients at risk of Dent's disease by clinical criteria screened for LMWP prior to *CLCN5* genotyping. The quantitative definition of tubular proteinuria allowed rigorous selection of those patients who had LMWP caused by tubular disease from the others and also avoided possible interference from hematuria. This particularly applies with the use of a sensitive RBP assay [37] and enhances the value of urine protein screening prior to genotype determination.

Chinese herbs nephropathy is a rapidly progressive interstitial fibrosis of which proximal tubular atrophy is a major histologic feature [36]. CHN is probably caused by ingestion of nephrotoxic aristolochic acids. It was important to apply the above quantitative definition to a different group of patients from those in which it was originally derived (Dent's disease patients), and CHN patients were chosen for this task. The bivariate distribution of RBP and albumin and the superimposed function we have used to define tubular proteinuria show that some 80% of patients with CHN who have elevated RBP excretion do indeed have tubular proteinuria (Fig. 5). The five CHN patients without clear tubular proteinuria by this definition were not different from the other 20 patients with respect to residual renal function measured

by creatinine clearance (results not shown). Transient microscopic hematuria occurs in a significant number of these patients and may confound the presence of tubular proteinuria.

Figure 2A demonstrates a striking correlation between excretion of RBP and albumin in the female carriers of Dent's disease. This clinical finding indicates that transport of RBP as well as albumin may, at least in part, share a common tubular reabsorption mechanism. A common LMW protein pathway has also been suggested by *in vitro* findings of competitive binding of cultured proximal cells to immobilized LMW proteins and albumin [41]. However, other clinical and *in vitro* studies differ on whether LMW proteins and albumin compete for reuptake [42, 43].

CLC-5, the *CLCN5* gene product that is absent or defective in Dent's disease, is expressed at multiple sites in the human nephron, and these include the proximal tubule, the thick ascending loop of Henle, and some intercalated cells of the collecting duct [44]. CLC-5 is found in the early endosomes that form part of the albumin transporting receptor-mediated endocytic pathway [44].

It is thought that CLC-5 plays a role in endosomal acidification, and colocalization of CLC-5 with H^+-ATPase and β_2M has been shown [44, 45]. Thus, defective CLC-5 function resulting in abnormal vesicle acidification in Dent's disease may underlie the tubular failure to reabsorb LMW proteins and calcium.

Our results show that RBP is the optimal LMW protein to screen for tubular proteinuria. We suggest that tubular proteinuria is defined by the combination of (*a*) excretion of RBP above the upper reference limit (in adults 0.017 mg/mmol creatinine) with (*b*) simultaneous excretion of albumin less than $(10 \times \text{RBP}) + 2$ mg/mmol creatinine. Molecular studies are needed to examine whether the observed close relationship between RBP and albumin excretion in carriers of the *CLCN5* mutation is indeed due to a common pathway for LMW protein and albumin reabsorption in the proximal tubule.

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