Urinary excretion of fatty acid-binding proteins in idiopathic membranous nephropathy

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Abstract

Background. It is suggested that proteinuria contributes to progressive renal failure by inducing tubular cell injury. The site of injury is unknown. Most studies have used markers of proximal tubular cell damage. Fatty acid-binding proteins (FABPs) are intracellular carrier proteins with different expression in the kidney. Liver-type FABP (L-FABP) is found in the cytoplasm of proximal tubules, whereas heart-type FABP (H-FABP) is localized in the distal tubules. We evaluated the urinary excretion of L-FABP and H-FABP in patients with idiopathic membranous nephropathy (iMN).

Methods. We have studied 40 patients (27 males, 13 females) with iMN. The mean age was 48 ± 15 years, serum creatinine concentration 89 ± 17 µmol/l and proteinuria 8.9 ± 5.0 g/24 h. Urinary L-FABP and H-FABP were measured by ELISA. Renal failure was defined as an increase in serum creatinine > 25% from baseline with a serum creatinine > 135 µmol/l or an increase > 50% from baseline. Urinary L-FABP excretion was detectable in all but one patient. The median (range) level was 3.29 (0.7–165.6) µg/mmol creatinine (normal < 0.38 µg/mmol Cr). Urinary H-FABP was undetectable in nine patients. Median level was 1.53 (0.1–90.5) µg/mmol Cr (normal < 0.1 µg/mmol Cr). Both L- and H-FABP correlated with urinary β2-microglobulin, urinary α1-microglobulin and IgG. Urinary H-FABP paralleled L-FABP.

Results. After a mean follow-up of 75 ± 32 months, 16 (40%) patients have reached the predefined end point of renal failure. Both urinary L-FABP and H-FABP predicted renal outcome, with the calculated sensitivity and specificity of 81 and 83% for both.

Conclusions. Urinary L-FABP and urinary H-FABP are increased in patients with iMN. There was a high correlation between L-FABP and H-FABP, suggesting the concurrent development or existence of proximal and distal tubular cell injury. Both L-FABP and H-FABP predicted prognosis in patients with iMN. These markers may be of interest as research tools; however, they are not superior to more conventional marker proteins.

Keywords: fatty acid-binding protein; H-FABP; L-FABP; membranous nephropathy; renal outcome

Introduction

Proteinuria is the hallmark of glomerular disease and an important independent predictor of progressive renal injury. Proteinuria is not only a reflection of glomerular injury but contributes to renal damage by inflicting toxic injury to the renal tubular epithelial cells. Indeed, chronic progressive renal failure correlates better with the extent of tubulo-interstitial (TI) injury than with the severity of glomerular damage [1,2].

The recognition that tubular injury is important in progressive renal injury has stimulated the interest in urinary markers of tubular cell injury. Proximal tubular cell injury is reflected by an increased urinary excretion of low molecular weight proteins such as beta-2-microglobulin (β2m) and alpha-1-microglobulin (α1m), brush border enzymes such as alkaline phosphatase, or cytosolic and lysosomal cellular enzymes such as N-acetyl-beta-glucosaminidase (β-NAG). We and others have shown that the urinary excretion of proximal tubular cell markers can predict prognosis in patients with glomerular diseases with a reasonable accuracy [3–5].

The above-mentioned proteins are rather non-specific markers of proximal tubular cell injury. Little is known on the role of distal tubular injury in progressive renal disease. In addition, more specific or pathogenetically relevant markers may offer advantages in evaluating various patient groups.

Fatty acid-binding proteins (FABPs) are intracellular carrier proteins. Two types of FABPs are localized in human renal tubular cells. Liver-type FABP (L-FABP) is found in the cytoplasm of proximal tubules, whereas heart-type FABP (H-FABP) is localized in the distal tubules [6,7]. An increased urinary excretion of FABPs may simply result from release by structurally damaged tubular cells. However, it
is suggested that tubular L-FABP expression is upregulated by hypoxia and increased excretion may thus occur before the occurrence of the actual structural damage [8,9].

In this present study we evaluated the urinary excretion of L-FABP and H-FABP in patients with idiopathic membranous nephropathy (iMN).

Material and methods

In our centre, patients with recently diagnosed membranous nephropathy are evaluated using a standardized protocol as described [3]. In brief, blood samples and timed urine samples are collected for the measurement of serum creatinine, albumin, cholesterol, β2m, IgG and transferrin and urinary excretion of creatinine, β2m, albumin, IgG, transferrin and α1m. In addition aliquots of urine are centrifuged and the supernatant is stored at −70°C. Patients are prospectively followed throughout the years, and data on treatment, remission and survival are obtained.

For the present study we evaluated urinary samples of 40 consecutive patients with a biopsy-proven iMN who fulfilled the following inclusion criteria: baseline serum creatinine ≤135 µmol/l, proteinuria ≥2.0 g/day and serum albumin ≤30 g/l and a follow-up of at least 12 months. Patients were excluded when they had been treated with immunosuppressive agents other than oral corticosteroids before the baseline measurements.

Methods for the determination of serum and urinary proteins have been described [3]. Urinary L-FABP and H-FABPs were measured with ELISA assays, using human L-FABP and H-FABP ELISA kits (Hycult biotechnology B.V., Uden, The Netherlands).

Calculations

Endogenous creatinine clearance (ECC) was calculated from creatinine measured in 24-h urine samples. Because 24-h urine samples were not regularly collected during the follow-up, we calculated GFR by applying the MDRD formula [10]. Urinary excretion is expressed as milligrams or micrograms per millimol of urinary creatinine (mg/mmol Cr or µg/mmol Cr).

Renal biopsies

To evaluate the correlation between FABP excretion and the extent of TI damage, renal biopsies were reviewed. We selected those biopsies that were obtained within 6 months of the measurement of the urinary markers. The biopsies were reviewed by the renal pathologist (E.J.S.) without knowledge of the amount of FABP excretion or the clinical outcome. In light microscopy, cross sections were evaluated for the extent of TI damage (atrophy, fibrosis) and scored semi-quantitatively using a 4-point scale: nil (0), mild (1), moderate (2) or severe (3). Furthermore, the tubules themselves were assessed for epithelial damage (loss of brush border, loss of continuity of the epithelium, activated appearing cell nuclei) in a similar semi-quantitative manner. A distinction was made between proximal and distal tubules and both were scored separately. This resulted in three different scores per biopsy: a TI score, a ‘proximal tubular damage’ score and a ‘distal tubular damage’ score.

Statistical analyses

The correlation between two parameters (non-parametric distributions) was analysed by Spearman’s rank coefficient of correlation. To clarify the relation between urinary FABPs and other urinary parameters, we performed multiple regression analysis. This analysis was performed in a forward stepwise fashion. The following variables were tested: β2m, α1m, IgG, proteinuria and eGFR. Variables with evidence of multicollinearity (r > 0.8) were not put in the analysis at the same time. For comparison between two groups (non-parametric distributions) we used the Mann–Whitney U-test.

Renal survival was calculated by using Kaplan–Meier curves. Renal failure was defined as an increase in serum creatinine >25% from baseline with a serum creatinine >135 µmol/l or an increase > 50% from baseline. Survival was calculated using the date of the baseline measurements as t = 0. We compared renal survival using log-rank tests. The predictive accuracy was assessed by making receiver operating characteristics (ROC) curves. We determined the area under the curve (AUC) and calculated the sensitivity and specificity by using the most discriminative threshold values. In this analysis lowest measurable level of the FABPs was taken as value for patients with the undetectable levels, in order to make them suitable for risk stratification.

All data are presented as means (±SD) or medians (range) when appropriate. All statistics were performed using SPSS software, version 12.0.1 (Chicago, IL, USA). P <0.05 was considered significant.

Results

Urinary FABP excretion was studied in 40 patients with iMN. Baseline characteristics of the patients are shown in Table 1. Thirty-six patients had nephrotic range proteinuria, and four patients had a serum albumin <30 g/l and proteinuria ≥2.0 g/day. The GFR was <60 ml/min/1.73 m² in five patients.

Urinary L-FABP excretion was measured and detectable in all patients but one. Median L-FABP was 3.29 µg/mmol Cr, range 0.7–165.6. In a small cohort of non-proteinuric volunteers L-FABP was undetectable in one sample, the median level was 0.14 µg/mmol Cr, range 0.13–0.38.

Urinary H-FABP was undetectable in 9 out of 40 patients. Median H-FABP was 1.53 µg/mmol Cr, range 0.1–90.5. In the healthy volunteers H-FABP was below the detection limit.

Correlation between FABP excretion and other urinary proteins

We observed a significant correlation between urinary excretion of L-FABP and β2m (Figure 1, r = 0.76). However, in 15 out of 16 patients with completely normal levels of β2m excretion (<24 µg/mmol Cr) L-FABP was elevated.
Using multiple linear regression analysis H-FABP excretion was significantly dependent on β2m excretion \((P = 0.001)\) and L-FABP excretion \((P = 0.03)\).

**FABP excretion as a predictor of outcome**

Patients have been followed for 75 ± 32 months. Thus far, 16 (40%) patients have reached the predefined end point of renal failure. Overall renal survival was 85% at 6 months, 75% after 1 year and 60% after 3 years. The reason for renal failure was a rise of serum creatinine >25% with a serum creatinine > 135 µmol/l in seven patients and a rise of >50% in nine patients. We also assessed the extent of deterioration of renal function. In the 16 patients who reached the predefined end point of renal failure, serum creatinine had increased by an average of 58% from 100 ± 17 to 157 ± 26 µmol/l. Estimated GFR was 60 ± 19 ml/min/1.73 m² at baseline and 33 ± 7 ml/min/1.73 m² at the end point. The absolute decrease of GFR averaged 50 ml/min/1.73 m²/year. For comparison, in the non-failure group, the average change of GFR was 0.3 ml/min/1.73 m²/year.

ROC curves were made to assess the predictive accuracy of L-FABP and H-FABP. From the coordinate points of the ROC curves the sensitivity and specificity for both parameters were calculated, using the best discriminative thresholds (Table 2).

Renal survival curves using the risk stratification for L-FABP and H-FABP are depicted in Figures 3 and 4. Renal survival was 45% at 1 year in patients with high urinary L-FABP (>5.5 µg/mmol Cr) and 96% in patients with low urinary L-FABP. At the end of the follow-up the survival rates were 16% and 87%, respectively. Survival rates at 1 year in high (threshold >3.0 µg/mmol Cr) and low urinary H-FABP groups were identical to the high and low L-FABP groups. At the end of the follow-up the renal survival was 15% in patients with high urinary H-FABP and 87% in patients with low urinary H-FABP. All differences in survival were highly significant (log-rank test). Calculated sensitivity and specificity were respectively 81 and 83% for both L-FABP and H-FABP.

### Table 1. Baseline characteristics of patients with iMN (n = 40)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD or Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>27/13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48 ± 15</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>99 ± 16</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>25 ± 5.3</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>8.0 ± 1.9</td>
</tr>
<tr>
<td>Follow-up (mo)</td>
<td>75 ± 32</td>
</tr>
<tr>
<td>ECC-24 h (ml/min/1.73 m²)</td>
<td>87 ± 23</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>70 ± 20</td>
</tr>
<tr>
<td>Interval Bx-urine sample (mo)</td>
<td>2 (0–33)</td>
</tr>
<tr>
<td>Timing sample</td>
<td></td>
</tr>
<tr>
<td>Albumin excretion (mg/mmol Cr)</td>
<td>550 (50–1650)</td>
</tr>
<tr>
<td>IgG excretion (mg/mmol Cr)</td>
<td>18.0 (1.7–228)</td>
</tr>
<tr>
<td>β2m excretion (µg/mmol Cr)</td>
<td>44.5 (8.9–6234)</td>
</tr>
<tr>
<td>α1m excretion (mg/mmol Cr)</td>
<td>3.8 (1.1–38.1)</td>
</tr>
<tr>
<td>L-FABP excretion (µg/mmol Cr)</td>
<td>3.29 (0.7–165.6)</td>
</tr>
<tr>
<td>H-FABP excretion (µg/mmol Cr)</td>
<td>1.53 (0.1–90.5)</td>
</tr>
<tr>
<td>Proteinuria (mg/mmol Cr)</td>
<td>856 (57–2606)</td>
</tr>
</tbody>
</table>

Data are mean ± SD or medians (range).

IMN: idiopathic membranous nephropathy; MAP: mean arterial pressure; ECC-24 h: creatinine clearance calculated from 24-h urine; eGFR: estimated GFR calculated using the MDRD formula; Bx: renal biopsy; mo: months; β2m: beta-2-microglobulin; α1m: alfa-1-microglobulin; L-FABP: liver-type fatty acid-binding protein; H-FABP: heart-type fatty acid-binding protein.

Urinary protein excretion is expressed per mmol of urinary creatinine (mmol Cr).

(median 1.98 µg/mmol Cr, range 0.71–17.2). Urinary L-FABP also correlated significantly with urinary IgG \((r = 0.81)\), α1m \((r = 0.77)\) and total protein \((r = 0.71)\). Using multiple linear regression analysis L-FABP excretion was dependent on β2m excretion \((P = 0.02)\) and H-FABP excretion \((P = 0.04)\). Because of multicollinearity \((r = 0.81)\) β2m and α1m were not analysed at the same time.

Urinary H-FABP was strongly correlated with L-FABP (Figure 2, \(r = 0.88\)). As depicted in the figure, there were no ‘outliers’ among these patients, i.e. no patients with low L-FABP levels but high H-FABP, nor patients with high range L-FABP levels without detectable H-FABP. Urinary H-FABP correlated with urinary β2m as well \((r = 0.7)\). There were five patients with elevated urinary H-FABP but completely normal urinary β2m. H-FABP also correlated significantly with urinary α1m \((r = 0.78)\), IgG \((r = 0.73)\), β2m \((r = 0.7)\) and total protein \((r = 0.58)\). Using multiple linear regression analysis H-FABP excretion was significantly dependent on β2m excretion \((P = 0.01)\) and L-FABP excretion \((P = 0.03)\).
Table 2. Sensitivity, specificity, PPV and NPV of the most discriminative thresholds of urinary proteins in the prediction of renal failure in patients with iMN

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AUC</th>
<th>Threshold</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-FABP</td>
<td>0.844</td>
<td>5.7 µg/mmol Cr</td>
<td>81%</td>
<td>83%</td>
<td>76%</td>
<td>87%</td>
</tr>
<tr>
<td>H-FABP</td>
<td>0.810</td>
<td>3.1 µg/mmol Cr</td>
<td>81%</td>
<td>83%</td>
<td>76%</td>
<td>87%</td>
</tr>
<tr>
<td>β2m excretion</td>
<td>0.858</td>
<td>95 µg/mmol Cr</td>
<td>81%</td>
<td>88%</td>
<td>81%</td>
<td>88%</td>
</tr>
<tr>
<td>IgG excretion</td>
<td>0.820</td>
<td>26 mg/mmol Cr</td>
<td>75%</td>
<td>88%</td>
<td>80%</td>
<td>84%</td>
</tr>
<tr>
<td>α1m excretion</td>
<td>0.896</td>
<td>4.0 mg/mmol Cr</td>
<td>94%</td>
<td>79%</td>
<td>75%</td>
<td>95%</td>
</tr>
</tbody>
</table>

PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve; L-FABP: liver-type fatty acid-binding protein; H-FABP: heart-type fatty acid-binding protein; β2m: beta-2-microglobulin; α1m: alpha-1-microglobulin.

Discussion

Our study demonstrates that urinary excretion of L-FABP and H-FABP is increased in patients with iMN. Both L-FABP and H-FABP predicted prognosis with a high accuracy. Admittedly, in this study neither L-FABP nor H-FABP was superior to known proximal tubular markers like β2m in predicting progressive disease.

To put these results in perspective, other known predictive markers were plotted into ROC curves in the same way. We calculated the sensitivity and specificity of β2m, α1m and IgG excretion in this cohort (data shown in Table 2).

Archival biopsy material of 21 patients could be retrieved. The biopsies were coming from five different pathological laboratories and had been prepared according to local protocol. Median interval between the biopsy and measurement of the urinary markers was 1.5 months (range 0–4.1). The majority of patients (62%) had only mild TI injury, and few had nil or severe TI-injury (Figure 5). There was no significant correlation between L-FABP or H-FABP and the TI score (Figure 5). Next we analysed more specifically damage of the proximal and distal tubules. This analysis also did not reveal a correlation between L-FABP and proximal tubular damage and H-FABP and distal tubular damage, respectively.

Correlation between FABP excretion and TI changes in renal biopsies

In 30 patients the interval between the renal biopsy and the measurement of the urinary markers was <6 months.
serum creatinine, we used an increase of 25% in combination with a fixed value of serum creatinine of 135 μmol/l as the end point for defining renal failure. We do not consider it justified to withhold treatment with immunosuppressive therapy in patients with higher serum creatinine values. From the literature it is well established that a serum creatinine > 135 μmol/l or a deterioration of renal function is a powerful predictor of ESRD [11–14]. Furthermore, our analysis shows that GFR was severely decreased in the patients who met our end point criterion as compared to the non-failure group.

FABPs are intracellular carrier proteins that bind and transport free fatty acids (FFAs) to mitochondria or peroxisomes. The family of FABPs presently contains nine distinct types of 15 kDa cytoplasmic proteins, which are expressed in tissues with an active fatty acid metabolism [15]. Each type is named after the tissue in which it was first identified and shows a characteristic tissue distribution.

The primary function of FABP is the facilitation of intracellular long-chain fatty acid transport. Other functions include putative protection against detrimental effects of locally high concentrations of long-chain fatty acids. FABP may act as an endogenous antioxidant by promoting FFA metabolism and by binding long-chain fatty acid oxidation products [16]. The cellular expression of FABP is primarily regulated at the transcriptional level. Gene upregulation can occur in response to changes in lipid metabolism as caused by (patho-)physiological stimuli that induce oxidative stress, like diabetes, hypertrophy, endurance training and ischaemia [15].

In humans L-FABP is localized in the liver, the small intestine and in proximal renal tubular cells, whereas H-FABP is widespread in the heart, the brain, the small intestine, skeletal muscles and distal renal tubular cells.

Kamijo et al. observed elevated levels of L-FABP in the urine of 48 patients with non-diabetic chronic kidney disease (CKD) [17,18]. Urinary excretion of L-FABP increased with deterioration of renal function. In these patients with various renal diseases urinary L-FABP proved to be more sensitive in predicting progression of CKD than proteinuria. Serum levels of L-FABP in patients with CKD did not influence the urinary L-FABP excretion [19].

As serum H-FABP is known to be a marker of myocardial ischaemia and is used as an indicator of myocardial damage, Nayashida et al. studied the influence of renal function on serum and urinary H-FABP levels in patients undergoing a primary coronary artery bypass [20]. In this small group of 19 patients, the creatinine clearance correlated inversely with the peak levels of serum H-FABP, but correlated with the peak levels of urinary H-FABP. It was concluded that serum H-FABP, which is deriving from myocardial cells, is cleared by the kidney and that this clearance can be impaired when creatinine clearance is decreased, resulting in lower urinary levels of H-FABP. To our current knowledge, urinary excretion of H-FABP has never been studied in patients with renal disease.

In our study urinary L-FABP correlated well with well-known markers of proximal tubular injury such as β2m and α1m. However, while urinary L-FABP was elevated in all but one of the patients, excretion of β2m was normal in about one quarter of patients. These observations lent support to the suggestion of Kamijo et al. that L-FABP may be elevated before structural tubular damage occurs, due to cellular upregulation of L-FABP gene expression in reaction to cellular oxidative stress [8,18]. L-FABP could therefore be an early sign of tubular stress and its urine excretion may increase before other markers do. Studies of the early rise in the urinary excretion of L-FABP may be relevant and contribute to our understanding of the evolution of tubular stress and injury. However, from a clinical point of view our data clearly indicate that measurement of urinary L-FABP is not a better marker of prognosis than more conventional markers such as β2m and α1m. In retrospect, this may not be a surprise since urinary L-FABP may be increased in the early phase in response to stress, before the actual ‘point of no return’ is reached.

Urinary H-FABP levels were elevated in most patients. This suggests that in patients with iMN and proteinuria both proximal and distal tubular cells are involved in the process of TI injury. We cannot exclude that urinary H-FABP, like L-FABP, is increased due to upregulation in the distal tubulus in response to stress rather than cell damage. The fact that there are patients with increased H-FABP excretion but normal β2m levels may support this idea. Thus, the increase of urinary H-FABP may not necessarily reflect injury.

Urinary H-FABP correlated well with the urinary excretion of other proximal tubular markers, including L-FABP. However, H-FABP was never elevated in the absence of elevation of L-FABP, while there were patients with elevated L-FABP in the absence of an increased H-FABP. Based on this finding one could speculate that tubular stress or damage accompanying primal glomerular lesions occurs in a sequential way, starting in the proximal tubules and then progressing to the distal parts. The elevation of the distal tubular marker H-FABP would then be a sign of more extensive disease. While the exact mechanisms of the pathogenesis of TI injury in glomerular diseases remain unclear, further investigation in differences between proximal and distal tubular markers like L-FABP and H-FABP may bring new insights to the pathogenesis and process of TI damage [21].

We did not observe a correlation between the level of FABP excretion and histological characteristics. At first sight this may seem strange. Obviously, the study has limitations. The number of biopsies was limited. In most patients the renal biopsy and the measurement of the FABPs were not performed at the same moment. We studied biopsies that were performed within 6 months of the urine measurement (n = 21). Limiting this analysis to biopsies performed within 3 months (n = 18) did not change the results. Furthermore, the renal biopsies were retrieved from five different centres, which differ in quality of staining techniques, adding bias. Also, evaluation of injury at the cellular level and the distinction between proximal and distal tubules is difficult. Lastly, only few biopsies had evidence of moderate and severe TI injury. This is no surprise since our study was intended to evaluate prognostic markers and thus excluded patients with renal insufficiency. In the latter group of patients high concentrations of urinary FABPs might have been present. However, we have measured urinary L-FABP and H-FABP in some patients with IgA nephropathy and severe renal insufficiency. In these patients the
excretion of the urinary FABPs was increased compared to controls; however, values were comparable to those in the patients with membranous nephropathy. Although the above-mentioned arguments may explain the lacking correlation between urinary FABP excretion and TI injury, an alternative explanation is more likely. Indeed, our data are in line with the notion that FABP expression is upregulated by renal tubular cells even before serious injury is present.

In conclusion, urinary L-FABP and urinary H-FABP are increased in patients with iMN. There is a high correlation between L-FABP and H-FABP, suggesting the concurrent development or existence of proximal and distal tubular cell injury. Both L-FABP and H-FABP predicted prognosis in patients with iMN with the same accuracy. Although they are not superior to more conventional marker proteins, measurements of urinary L-FABP and H-FABP may be of interest as research tools to determine pathogenetic mechanisms.

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Conflict of interest statement. None declared.

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