Cumulative Excretion of Urinary Podocytes Reflects Disease Progression in IgA Nephropathy and Schönlein-Henoch Purpura Nephritis

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Recent studies have revealed that podocytopenia leads to glomerular scarring and that the loss of podocytes into the urine may be a cause of podocytopenia. The purpose of this study was to examine whether serial examinations of urinary podocytes (u-podo) could be a useful predictor of disease progression in children with glomerulonephritis. Urine samples and renal biopsy specimens from 20 patients (10 males and 10 females; mean age 11.8 yr; range 4 to 24 yr) with IgA nephropathy (n = 17) and Henoch-Schönlein purpura nephritis (n = 3) were analyzed. Forty-four renal biopsies were performed on 20 patients. Proteinuria (g/d per 1.73 m²), hematuria (score), and u-podo (cells/ml) were examined twice a month in 24 intervals between two biopsies (mean 16.7 mo; range 4 to 58 mo) and average and cumulative values were determined for the intervals. Renal histologic changes were scored on the basis of acute intracapillary, acute extracapillary, acute tubulointerstitial, chronic intracapillary, chronic extracapillary, and chronic tubulointerstitial lesions, as well as glomerulosclerosis. It was found that hematuria, proteinuria, u-podo, and acute lesion scores decreased during the intervals examined, whereas chronic lesion scores increased. Changes in acute histology scores correlated well with hematuria, proteinuria, and u-podo excretion, whereas chronic histology scores and glomerulosclerosis both correlated well with cumulative u-podo excretion. Patients with severe histologic progression of disease also had persistent u-podo excretion. These findings provide additional data to support a potential causative role for prolonged urinary loss of podocytes in disease progression in children with IgA nephropathy and Henoch-Schönlein purpura nephritis.

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on the surface of podocytes (14–16,23). A significantly higher number of urinary podocytes (u-podo) were found in the acute state of glomerular diseases, including IgAN and Henoch-Schönlein purpura nephritis (HSPN) (16). Numerical determination of the podocyte number in urine was found to be useful clinically as a diagnostic tool for glomerular versus nonglomerular diseases, for inflammatory versus noninflammatory diseases, and as a marker of the severity of active glomerular injury. The purpose of this study was to examine whether the serial examination of u-podo could be a useful diagnostic tool and predictor of disease progression in children with glomerulonephritis.

Materials and Methods

Patients and Urine samples

Urine samples and renal biopsy specimens from 20 patients (10 males and 10 females; mean age 11.8 yr; range 4 to 24 yr) with IgAN (n = 17) and HSPN (n = 3) were analyzed. Forty-four renal biopsies were performed on 20 patients.

Proteinuria, hematuria, and u-podo determination were examined twice a month in 24 intervals between two biopsies (mean 16.6 mo; range 4 to 58 mo). The degree of proteinuria was expressed as g/d per 1.73 m²; hematuria was scored as 0 for 0 to 5 red blood cells/high-powered field (RBC/HPF), 0.5 for 5 to 10 RBC/HPF, 1.0 for 10 to 30 RBC/HPF, 2.0 for 30 to 100 RBC/HPF, 3.0 for >100 RBC/HPF, or 4.0 for numerous RBC/HPF; and u-podo were expressed as cells/ml. These three parameters were expressed both as average values (av-proteinuria, av-hematuria, and av-u-podo) during the intervals and cumulative values (cum-proteinuria, cum-hematuria, and cum-u-podo), representing the total of all monthly values during the study periods.

Ul-Podo

Fresh urine samples that were obtained immediately after the first urination in the morning were used. U-podo were stained by an immunofluorescence technique as reported previously (14,16). The investigators who scored the u-podo numbers are medical technicians in an outside diagnostic laboratory center in Tokyo. The u-podo test is listed as one of the laboratory test items in this site. We ordered this test without providing any clinical information about the patients, ensuring an absence of observer bias. Several medical technicians were involved in this test, and two of them were responsible for validation of all test results, with the reported result being the mean value of the numbers counted by both examiners for each report. The two examiners who were responsible for this test were random, depending on the work schedules of this laboratory. The laboratory supervisor for this test checked the procedures for the u-podo test routinely, including the antibody quality, the indication for evaluating positive podocytes, and the evaluation of differential findings among technicians. In this assay, we confirmed the reproducibility in separate experiments. Two urine samples with different u-pod numbers (samples A and B) were examined by seven observers. The coefficients of variation among the seven observers were 8.8% (mean 5.6 cells/ml) for urine sample A and 9.7% (mean 12.3 cells/ml) for urine sample B. Samples C and D were examined by one observer on five different occasions, and the coefficients of variation among the five occasions were 8.6% (mean 4.8 cells/ml) for the urine sample C and 10.2% (mean 13.6 cells/ml) for urine sample D. Factors that might influence the assay, such as the temperature for conservation and the duration of conservation, were checked previously. All had minimal influence on the assay. Urine samples from 200 healthy children and adolescents (range 3 to 29 y) were used as normal controls (0 u-podo/ml from 146 samples; median and range of the other 54, 0.2 [0 to 0.8]; normal range is 0 to 0.8).

The change in u-podo numbers over time for each patient was classified into two groups, A and B. In group A, the excretion of u-podo gradually decreased to undetectable levels (<1.0 cells/ml for at least 2 mo), whereas in group B, the excretion of u-podo persisted.

Histologic Examinations

Histologic examinations of renal biopsy specimens were performed by a single pathologist without knowledge of any clinical information about individual patients. The sections were stained by at least three stains, including hematoxylin–eosin, periodic acid–Schiff, and periodic acid-silver methenamine. The histologic evaluation for activity and chronicity was performed according to the method proposed by Shigematsu (24). In this method, the histologic evaluation for IgAN is expressed semiquantitatively for both histologic activity and chronicity, with the aim of having a more precise understanding of the extent of disease progression. The histologic activity was estimated by the occurrence of acute intracapillary (a-IN), acute extracapillary (a-EX), and acute tubulointerstitial (a-TI) lesions. The histologic chronicity was estimated by the occurrence of chronic intracapillary (c-IN), chronic extracapillary (c-EX), and chronic tubulointerstitial (c-TI) lesions. The extent of the tissue injury was evaluated in four grades (0, 1, 2, and 3). Regarding the glomerular lesions, this evaluation was applied to all of the glomeruli in the biopsy specimens, and the average of the scores was taken. These semiquantitative evaluations were processed to statistical analyses.

The degree of GS was evaluated by estimation of the podocalyxin-negative glomerular area after immunostaining. Immunoperoxidase staining was performed according to a protocol using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and a mAb against human podocalyxin (PHM5; Australian Monoclonal Development, Ar-тармон, Australia). The scoring system described by Saito et al. (25) was used as follows: 0, normal glomerulus; 1, sclerosis involving <25%; 2, sclerosis involving 25 to 50%; 3, sclerosis involving 50 to 75%; and 4, sclerosis involving 75 to 100% of glomerular tuft area. The representative immunostaining results are shown in Figure 1. The average of the scores of the total glomeruli on the immunostained sections from each patient was expressed as the GS index. A total of 831 glomeruli were examined by one observer on five different occasions, and the coefficients of variation among the five occasions were 8.6% (mean 4.8 cells/ml) for the urine sample C and 10.2% (mean 13.6 cells/ml) for urine sample D. Factors that might influence the assay, such as the temperature for conservation and the duration of conservation, were checked previously. All had minimal influence on the assay. Urine samples from 200 healthy children and adolescents (range 3 to 29 y) were used as normal controls (0 u-podo/ml from 146 samples; median and range of the other 54, 0.2 [0 to 0.8]; normal range is 0 to 0.8).

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(mean ± SEM 18.9 ± 1.5; range 8 to 50) from 44 renal biopsies were evaluated for these semiquantitative histologic analyses.

The study, including initial and repeat renal biopsies, was approved by the ethical committee of our hospital. Informed consent was obtained from patients or from parents in the case of children who were younger than 15 yr.

**Statistical Analyses**

Relationships among the three urinary parameters and the scores of histologic examinations were analyzed by calculation of the Spearman rank correlation coefficients. Comparisons between groups were made with the paired t test and Fisher exact probability. Results were considered significant at \( P < 0.05 \).

**Results**

Laboratory profiles of the patients are summarized in Table 1.

**Change in U-Podo over Time**

The change in u-podo over time for each patient is shown in Figure 2. The 24 intervals were divided into two groups, A and B. In group A (\( n = 10 \)), the excretion of u-podo gradually decreased to undetectable levels, whereas in group B (\( n = 14 \)), the excretion of u-podo persisted.

**Degree of Urinalyses Improved during the 24 Intervals Examined**

The results of urinalyses at the start and end of the intervals are shown in Table 2. The degree of proteinuria, hematuria, and u-podo improved during the 24 intervals examined.

**Scores of Acute Renal Lesions Decreased, whereas Those of Chronic Lesions Increased**

The renal histologic activity and chronicity scores first and second biopsies, and the change of histologic findings between two biopsies is shown in Table 3. The scores for a-IN decreased significantly between the two biopsies (\( P = 0.0021 \)), whereas the scores for c-IN, c-EX, c-TI, and GS increased significantly (\( P = 0.0085 \) for c-IN, \( P < 0.0001 \) for c-EX, \( P = 0.0064 \) for c-TI, and \( P < 0.0001 \) for GS).

**Scores of Urinalyses Correlated Well with Acute Renal Lesions**

The results of correlations between urinary studies and the renal histologic activity and chronicity scores at each renal biopsy are shown in Table 4. We found that hematuria, proteinuria, and u-podo all had significant correlations with each of the three acute renal lesions, including a-IN, a-EX, and a-TI, with the highest correlations occurring between a-Ex and each of the urinary studies. In contrast, there was no significant correlation between the urinary studies and any of the renal chronicity scores.

**Cum-U-Podo Excretion Correlated Well with Disease Progression**

Analyses of the correlations between the average and cumulative urinary studies, as well as the change in renal histologic scores, and each of the renal histologic activity and chronicity scores are shown in Table 5. Significant positive correlations were found between chronic extracapillary lesions (c-EX) and av-proteinuria (\( P = 0.0028 \)), av-hematuria (\( P = 0.0119 \)), av-u-podo (\( P = 0.0006 \)), cum-proteinuria (\( P = 0.0090 \)), and cum-u-

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**Table 1. Clinical profile**

<table>
<thead>
<tr>
<th>Disease</th>
<th>IgAN (( n = 17 )), HSPN (( n = 3 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr; mean [range])</td>
<td>11.8 (4 to 25)</td>
</tr>
<tr>
<td>Proteinuria (g/d per 1.73 m(^2))</td>
<td>2.28 ± 0.55</td>
</tr>
<tr>
<td>Hematuria (score 0 to 4.0)</td>
<td>2.50 ± 0.18</td>
</tr>
<tr>
<td>U-podo (cells/ml)</td>
<td>10.13 ± 3.39</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td>Renal histology</td>
<td></td>
</tr>
<tr>
<td>mild</td>
<td>2</td>
</tr>
<tr>
<td>moderate</td>
<td>10</td>
</tr>
<tr>
<td>severe</td>
<td>8</td>
</tr>
<tr>
<td>Treatment</td>
<td>Steroids + anticoagulant therapy (( n = 20 ))</td>
</tr>
</tbody>
</table>

*Data are means ± SE. HSPN, Henoch-Schönlein purpura nephritis; IgAN, IgA nephropathy; u-podo, urinary podocytes.
In addition, positive correlations were found between GS and av-proteinuria ($P = 0.0227$), av-hematuria ($P = 0.0330$), av-u-podo ($P = 0.0065$), cum-proteinuria ($P = 0.0045$), cum-hematuria ($P = 0.0337$), and cum-u-podo ($P = 0.0001$). In contrast, there were essentially no correlations between any of the acute renal lesions and any of the average or cumulative urinary studies.

Cases with Severe Progression in Histology Had Persistent U-Podo Excretion

Each of the 24 intervals was divided into three groups on the basis of the changes in GS score during the study period. Grade 1 (none or mild progression) was defined by a change in GS score of $0.2$, grade 2 (moderate progression) by a change in GS score of $0.2$ to $0.5$, and grade 3 (severe progression) by a change in GS score of $0.5$. Among these groups, comparisons were made with average and cumulative urinary studies. In addition, the number of patients for each grade with decreasing versus persisting u-podo results during the study period was evaluated. As shown in Table 6, we found that all of the grade 3 (severe progression) patients had persisting u-podo values (group B), whereas all of the grade 1 (mild progression) patients had decreasing u-podo values (group A).

Cumulative U-Podo Value Was the Best Predictor of Disease Progression

The sensitivity, specificity, and validity of each of the urinary studies in predicting histologic disease progression also were...
evaluated. The cutoff values for each urinary study were defined by the maximum value of the grade 1 (mild progression) group, and the cutoff value for disease progression was defined as an increase in GS score of >0.5. Using these cutoffs, we found that both av-u-podo and cum-u-podo showed were very accurate predictors of the development of GS (Table 7).

Discussion

Podocyturia is a common result of podocyte injury in both human and experimental glomerular diseases (14–16,26). In this study, we asked whether serial examination of podocyturia might provide clinically relevant information on disease progression.

Table 6. Urinalysis and u-podo profile in three groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade 1 (Mild Progression; &lt;0.2; n = 9)</th>
<th>Grade 2 (Moderate Progression; 0.2 to 0.5; n = 5)</th>
<th>Grade 3 (Severe Progression; &gt;0.5; n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av-proteinuria (g/d per 1.73 m²)</td>
<td>0.56 ± 0.10</td>
<td>2.81 ± 1.33</td>
<td>1.31 ± 0.17</td>
</tr>
<tr>
<td>Av-hematuria (scores)</td>
<td>1.29 ± 0.13</td>
<td>2.00 ± 0.37</td>
<td>2.19 ± 0.32</td>
</tr>
<tr>
<td>Av-u-podo (cells/ml)</td>
<td>2.08 ± 0.41</td>
<td>8.19 ± 2.85</td>
<td>18.63 ± 6.89</td>
</tr>
<tr>
<td>Cum-proteinuria (× mo)</td>
<td>7.06 ± 1.06</td>
<td>21.28 ± 5.49</td>
<td>23.18 ± 4.97</td>
</tr>
<tr>
<td>Cum-hematuria (× mo)</td>
<td>19.41 ± 3.87</td>
<td>26.53 ± 10.88</td>
<td>35.68 ± 6.44</td>
</tr>
<tr>
<td>Cum-u-podo (× mo)</td>
<td>25.32 ± 5.00</td>
<td>87.94 ± 33.93</td>
<td>203.34 ± 46.64</td>
</tr>
<tr>
<td>GFR (ml/min per 1.73 m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>start of interval</td>
<td>116.6 ± 3.4</td>
<td>114.1 ± 3.4</td>
<td>108.9 ± 3.2</td>
</tr>
<tr>
<td>end of interval</td>
<td>112.4 ± 3.8</td>
<td>112.4 ± 3.8</td>
<td>90.6 ± 9.5</td>
</tr>
<tr>
<td>GS</td>
<td>0.03 ± 0.01</td>
<td>0.39 ± 0.04</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>U-podo profile</td>
<td>A = 9, B = 0^b</td>
<td>A = 1, B = 4^c</td>
<td>A = 0, B = 10^d</td>
</tr>
</tbody>
</table>

^aU-podo profile: Group A, u-podo values decreasing; group B, u-podo values persisting. Significant difference between ^b and ^c (P < 0.05) and ^b and ^d (P < 0.0001).

Table 7. Urinalysis as predictor for disease progression

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of Intervals</th>
<th>Cutoff Values for Urinalyses</th>
<th>Cutoff Value for Progression</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Validity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av-proteinuria (g/d per 1.73 m²)</td>
<td>24</td>
<td>1.035 (M = 15/L = 9)</td>
<td>0.5 (M = 11/L = 13)</td>
<td>0.6</td>
<td>0.79</td>
<td>1.39</td>
<td>0.1345</td>
</tr>
<tr>
<td>Av-hematuria (scores)</td>
<td>24</td>
<td>1.692 (M = 14/L = 10)</td>
<td>0.5 (M = 11/L = 13)</td>
<td>0.7</td>
<td>0.79</td>
<td>1.49</td>
<td>0.05</td>
</tr>
<tr>
<td>Av-u-podo (cells/ml)</td>
<td>24</td>
<td>4.0 (M = 12/L = 12)</td>
<td>0.5 (M = 11/L = 13)</td>
<td>0.9</td>
<td>0.79</td>
<td>1.69</td>
<td>0.0038</td>
</tr>
<tr>
<td>Cum-proteinuria (× mo)</td>
<td>24</td>
<td>13.34 (M = 13/L = 11)</td>
<td>0.5 (M = 10/L = 14)</td>
<td>0.7</td>
<td>0.71</td>
<td>1.41</td>
<td>0.1112</td>
</tr>
<tr>
<td>Cum-hematuria (× mo)</td>
<td>24</td>
<td>42.0 (M = 20/L = 4)</td>
<td>0.5 (M = 13/L = 11)</td>
<td>0.3</td>
<td>0.93</td>
<td>1.23</td>
<td>0.3545</td>
</tr>
<tr>
<td>Cum-u-podo (× mo)</td>
<td>24</td>
<td>52.0 (M = 11/L = 13)</td>
<td>0.5 (M = 11/L = 13)</td>
<td>1</td>
<td>0.79</td>
<td>1.79</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

^aL, less than; M, more than.
This study clearly showed that persistent podocyturia during glomerulonephritis is associated with GS and may have a causative role in the development of GS, because the cum-u-podo correlated well with the histologic progression of glomerular disease. Recent information supports the concept that podocyte number may be a key factor dictating the progression of GS in the Pima Indian population with type 2 diabetes (5). In these studies, Pagtalunan et al. (5) showed that individuals who had more advanced proteinuria and glomerular matrix accumulation (diabetic GS) had fewer glomerular podocytes than did individuals who had experienced diabetes for the same length of time but did not have proteinuria or GS. In contrast, other glomerular cells did not decrease in number in the same glomeruli. In a follow-up study, Pima Indians with a depleted podocyte population tended to progress toward macroalbuminuria faster than did those who had a greater complement of podocytes (27). Meanwhile, Lemeley et al. (3) showed that podocyte loss occurs concomitantly with increasing disease severity in IgAN. In that study, the patients with the most severe glomerular dysfunction had reduced numbers of podocytes per glomerulus. The degree of podocytopenia was related to the extent of GS and the degree of impairment of permselectivity and GFR. In contrast, there were no corresponding correlations between these indices of injury and the number of mesangial and endothelial cells. Our study therefore provides additional evidence to support the hypothesis that loss of podocytes from the glomeruli into urine may either cause or contribute to the GS.

In this study, the cum-u-podo was related better to the degree of GS than the av-u-podo. This suggests that continuous excretion of a significant number of u-podo increases the risk for development of GS and that the total number of podocytes that detached from the GBM during the period examined is important in the development of GS. Even when the initial u-podo excretion is very high, our data suggest that if this is transient, then the risk for development of GS is lower than if it persists. Similar results were reported in experimental nephriti,
in which Yu et al. (28) reported u-podo loss in three different experimental models and noted continuous podocyte excretion in puromycin aminonucleoside nephrosis and five-sixths nephrosis, in contrast to transient excretion in Thy-1.1 nephritis. These findings suggest that podocyte injury is a main cause of progressive GS in the former two models in which podocyte injury persists, whereas podocyte injury may be a secondary cause of injury in the Thy-1.1 nephritis in which podocyte injury is transient. That study and our study together strongly support the concept that continuous podocyte injury and urinary loss are critical for disease progression.

We also found a strong correlation between renal histology and urinary studies, with the strongest correlation between u-podo and acute extracapillary lesions. These results indicate that u-podo reflect acute glomerular injury, as shown in our previous studies (29,30). Prolonged excretion of u-podo was observed in the cases with disease progression, indicating that acute glomerular injury persisted in such cases. Although IgAN generally is defined as a chronic form of glomerulonephritis, progressing to ESRD over a prolonged time (31), recent studies have reported that repeated episodes of acute glomerular injury are involved in the progression of disease (32,33). Thus, disease progression in IgAN can be associated with both prolonged excretion of u-podo and repeated episodes of acute glomerular injury. In distinction from IgAN, HSPN usually is defined as an acute form of glomerulonephritis (31), because the onset of the disease is apparent, often being associated with skin or abdominal symptoms. Therefore, prolonged excretion of u-podo in HSPN suggests either that the initial acute glomerular injury is severe enough to persist after acute injury or that the acute glomerular injury can persist for prolonged periods.

This study suggests the presence of a threshold of podocyte injury that might discriminate the risk for progression of GS. When the cutoff values of cum-u-podo were defined by the maximum value of the grade 1 group in which histologic progression was mild or absent, the cases above this cutoff showed significantly higher risk for development of GS. Consistent with this, previous studies also suggested the presence of a threshold that might discriminate the progression of GS (4,5). Together these studies suggest that once a threshold of podocyte depletion is reached, additional loss of podocytes greatly increases the risk for development GS.

U-podo may represent several populations of cells. Multiple reports have identified the presence of live podocytes in the urine in glomerular diseases (26,28). Some podocyte have been found to be bi- or multinucleated (13). The presence of bi- or multinucleated podocytes also was noted in our previous studies (data not shown), although there is no evidence as yet that such podocytes are alive and can be cultivated. In addition to the live and multinucleated podocytes, there are some populations with apoptotic characteristics, as defined by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (13). In one study, u-podo were double positive for TUNEL staining and immunostaining by anti-podocalyxin mAb, confirming that the apoptotic podocytes still expressed the podocalyxin cell surface protein. The presence of several subpopulations of u-podo suggests the possibility of multiple simultaneous pathogenetic mechanisms that might induce detachment of podocytes from the GBM.

In this study, the cum-u-podo was an excellent predictor for histologic disease progression among children with glomerulonephritis. Moreover, both this and previous studies showed that the appearance of podocytes in the urine indicates ongoing acute glomerular inflammation (14–16,29). In a study of several experimental models of glomerulonephritis, it was shown that podocyturia is a more specific marker of ongoing podocyte injury than proteinuria (28). Therefore, determination of podocyturia may be able to provide tremendously important noninvasive clinical information to nephrologists to guide the care of patients with glomerulonephritis. Given the growing evidence to suggest a strong correlation between u-podo and glomerular disease progression, reduction of u-podo could become a major goal of therapy for these diseases. Serial monitoring of u-podo may provide valuable information about patients’ response to therapy, as well as their risk for development of progressive GS.
Conclusion
This study provides strong additional evidence correlating u-podo loss and subsequent development of GS and suggests that prolonged and/or significant loss of podocytes into the urine during glomerulonephritis may have a causative role in the development of GS.

Acknowledgments
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Disclosures
None.

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28. Yu D, Petermann A, Junter U, Rong S, Shankland SJ, Floege J: Urinary podocyte loss is a more specific marker of


Obviously, noninvasive diagnostic techniques to diagnose and hopefully better manage glomerular diseases has been a goal of basic and clinical research over the past 25 years. Urinary podocyte excretion as outlined in this paper by Hara et al. may be a marker of disease progression in IgA and Henoch-Schoenlein purpura nephritis. In this month’s issue of *JASN*, Varghese et al. provide data regarding the use of urine biomarkers to predict the cause of glomerular disease (pages 913–922). This should be of interest to readers of both journals.