

Cell-Specific Biomarkers in Renal Medicine

Martin Shaw of Argutus Medical explores the potential benefits of using cell-specific biomarkers in human renal studies

Histopathology is the gold standard for defining renal injury, but it is invasive, time-consuming and expensive, plus it is seldom used in subjects with mild renal injury. Using biomarkers linked to distinct, defined cell types and tissues provides a direct link to histopathology without its drawbacks, and provides increased sensitivity and specificity. The nephron consists of several sections, each with its own specific biomarkers; therefore, through the use of a battery of tests, injuries can be localised to distinct areas.

Cell-specific biomarkers have been known about for over 40 years, but they are still underused in renal medicine and research. In particular, there are few studies where they have been used to guide therapy or been linked to quantitative changes in the kidney. This article will discuss how using biomarkers with a known cellular origin may help renal effects to be found earlier and at lower levels of injury. Their potential uses in renal medicine and clinical research will then be presented.

Renal diseases are an expanding problem; half a million Americans could be on dialysis in 2010 at a cost of \$46 billion dollars a year (1). Even so, renal injury may be an under-recognised problem due in part to the ability of the kidneys to regenerate, as well as their great reserve capacity. The most common test of renal function is serum creatinine. Creatinine is produced by the muscles at a relatively constant rate and mainly removed from the blood by glomerular filtration; therefore, the serum level is approximately inversely related to the glomerular filtration rate (GFR) – that is, the rate at which plasma is filtered by the glomeruli. However, serum creatinine is affected by many non-renal factors and, furthermore, it is a late biomarker since there must be a considerable loss of glomerular function before significant increases occur (2). Finally, renal tubular injury is important even in diseases of predominantly glomerular nature (3). As a result, the measurement of biomarkers of renal tubular injury is important in the understanding and monitoring of renal

effects. Their assay offers the opportunity for earlier diagnosis of renal injury with the preservation of long-term renal function.

CELL-SPECIFIC BIOMARKERS

By measuring a selection of biomarkers with known origins, injury can be localised to distinct cell types. Furthermore, since different biomarkers

come from different compartments of the cell, it is also potentially possible to localise injury to distinct sub-cellular compartments. The absence of a biomarker from the urine also provides valuable information in that it shows where injury is *not occurring*, and serves as a negative control. A selection of renal cell-specific biomarkers with their locations is shown in Table 1.

Table 1: A selection of cell-derived biomarkers with their main locations

The proximal convoluted tubules make up the bulk of the kidney (see Figure 1, page 86) and they are the most studied with regard to renal injury. They are the most commonly affected part of the kidney, but injury to other parts of the nephron may be underestimated due to the shortage of convenient biomarkers. The predominance of proximal tubular tissue makes it more difficult to find and utilise biomarkers for other parts of the kidney. Responses for biomarkers that are found in other parts of the kidney will be obscured if those biomarkers are also found in the proximal tubules, albeit at much lower levels.

	Cytoplasm	Lysosomes	Brush border
Proximal tubule	Lactate dehydrogenase (LDH) isoforms 1 and 2 Kidney/liver type fatty acid binding protein Alpha glutathione S-transferase	N-acetyl β-D glucosamidase (NAG) β galactosidase Acid phosphatase	Intestinal alkaline phosphatase Malate dehydrogenase Leucine amino peptidase Gamma glutamyl transferase
Loop of Henle	Renal papillary antigen 2 (in rats)		
Distal tubules	Pi glutathione S-transferase (GSTYb1, in rats) Heart fatty acid binding protein		
Collecting duct/renal papilla	LDH isoforms 4 and 5 Renal papillary antigen 1 (in rats)		

The proximal convoluted tubules make up the bulk of the kidney (see Figure 1) and they are the most studied with regard to renal injury (4). Though they are the most commonly affected part of the kidney, injury to other parts of the nephron may be underestimated due to the shortage of convenient biomarkers. The predominance of proximal tubular tissue makes it more difficult to find and utilise biomarkers for other parts of the kidney. Responses for biomarkers that are found in other parts of the kidney will be obscured if those biomarkers are also found in the proximal tubules, albeit at much lower levels.

URINARY ENZYMES AS INDICATORS OF RENAL INJURY

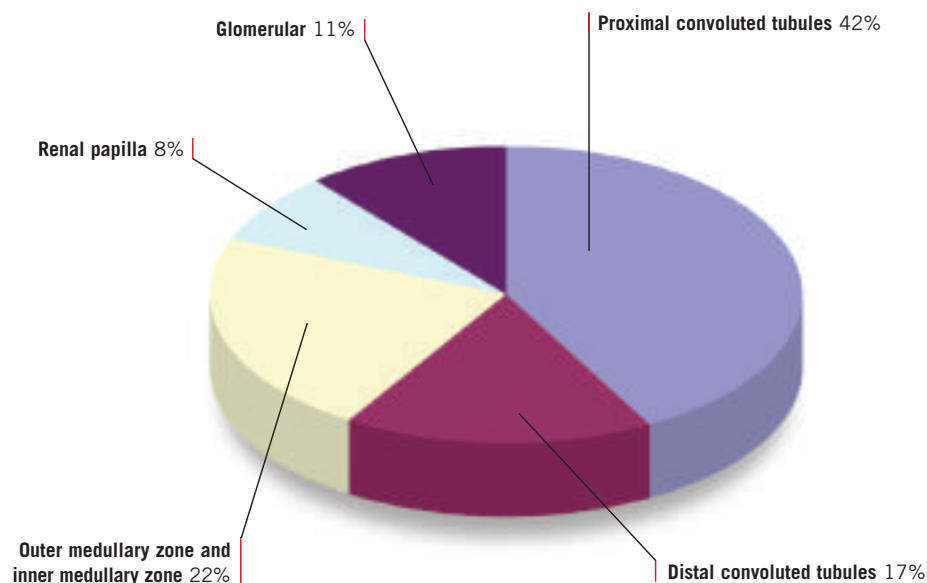
Over 40 enzymes have been identified in the urine but only a handful have been broadly used as renal biomarkers. Urinary enzymes have the advantages of being rapidly and easily measurable using standard colourimetric or fluorometric techniques. The most widely studied are N-acetyl β -D glucosamidase, a variety of brush border enzymes and lactate dehydrogenase. The main problems in measuring enzymes in general are their lack of stability, interference from factors in the urine and that their release varies according to diuresis (5).

N-acetyl β -D Glucosamidase

N-acetyl β -D glucosamidase (NAG) is predominantly found in the proximal tubules, but it is also found elsewhere in the nephron (6). Its drawbacks as a biomarker are that it is up-regulated in response to proteinuria and that it is not totally cell-specific (7). There are three isoforms of NAG, of which the A and B

Figure 1: Kidney composition

Source: *Mattenheimer et al, 1968 (4)*



forms are the most important. The A form may be released during the normal process of exocytosis. The B form is found on the membrane of the lysosome and is released when there is injury to it (8). The B form comprises a relatively larger proportion of the NAG present in the proximal tubule, potentially making it a more specific biomarker. Large increases in the B form can be masked by only modest increases in the total urinary NAG activity.

Brush Border Enzymes

There are a number of enzymes located on the brush border villi of the proximal tubular cells, such as alanine amino peptidase (AAP), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT). Brush border enzymes may be found in the urine in two forms; either soluble, or bound to cell membrane fragments. The finding of the cell membrane bound form may indicate more severe injury (9).

Cytosolic Enzymes

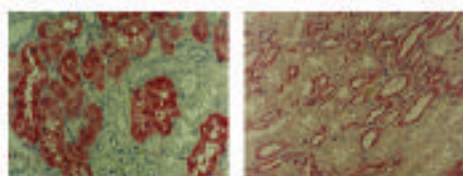
The most widely studied is lactate dehydrogenase (LDH). Different isoforms of LDH are found in the cortex (forms 1 and 2) and in the medulla (4 and 5), enabling renal injury to potentially be localised to different parts of the kidney (10). The need to perform electrophoresis to separate the isoforms has limited the use of LDH for this.

CELL-SPECIFIC PROTEINS

The most studied of these are the glutathione S-transferases (GSTs). The GSTs are a family of Phase II enzymes with distinct distributions along the nephron (11). They are found in high concentrations (two per cent of the soluble protein) in the cytosol and are rapidly released into the urine in the event of injury to the tubular cells. The alpha form of GST (α GST) is localised to the proximal convoluted tubule, and other

Over 40 enzymes have been identified in the urine but only a handful have been broadly used as renal biomarkers. Urinary enzymes have the advantages of being rapidly and easily measurable using standard colourimetric or fluorometric techniques. The most widely studied are N-acetyl β -D glucosamidase, a variety of brush border enzymes and lactate dehydrogenase.

Figure 2: Immunohistochemical localisation of GST isoforms in the human kidney



αGST in proximal tubules πGST in distal tubules

forms are distributed in the distal tubules (see Figure 2). In man, the pi form (πGST) is localised to the distal tubules while in the rat a mu form of GST (GSTYb1) has a similar distribution (12)

CELL-SPECIFIC BIOMARKERS IN RENAL DISEASE

The kidney has great powers of recovery and a large reserve capacity; therefore, apparently mild renal injury may be accepted since one can expect full recovery, as judged by near normalisation of serum creatinine. However, this normalisation may be achieved at the expense of future reserve capacity. Nenov *et al* presented the concept that chronic renal injury may result from “multiple hits” of acute injury that progressively decrease the number of nephrons and increase the load on those remaining (13). Furthermore, even very limited renal capacity can be important for the wellbeing of subjects with renal failure (14). The assay of cell-specific biomarkers offers the potential to detect adverse renal effects of drugs or procedures early, and preserve remaining renal function.

CHRONIC RENAL DISEASE

Chronic renal diseases – such as diabetic nephropathy and IgA nephropathy – are followed by measuring changes in serum creatinine and proteinuria. This provides information regarding long-term changes

in renal function, but little information as to how much destruction of the kidneys is occurring at that moment. Cell-specific biomarkers are complementary in that they are markers of disease activity.

In subjects with glomerulonephritis, enzymuria was associated with a more

serious outcome (15). The most widely-used enzyme biomarker is NAG, but it is up-regulated in response to protein load and, therefore, in cases of mild renal injury, it may not be showing renal injury; rather renal adaptation.

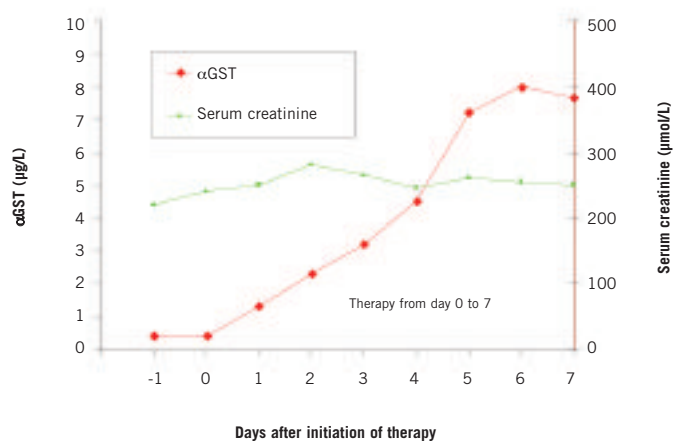
Studies using cell-specific biomarkers – the GSTs – can provide more details regarding the site and progression of the chronic renal disease. Relatively mild chronic renal injury seems to be mainly associated with proximal tubular injury (increased αGST), but as the disease progresses, πGST increases relatively faster, indicating increasing involvement of the distal renal tubules (16).

Renal Transplantation

Whilst one-year survival of renal transplants is approaching 95 per cent, the long-term half-life of grafts is only about eight years (17). Better biomarkers – enabling earlier and better diagnosis and improved monitoring of therapy – could improve graft survival. In transplantation, immunosuppressive (and other) drugs, lead to the risk of nephrotoxicity while too little immunosuppression leads to the risk of rejection. In both instances, serum creatinine increases. Cell-specific biomarkers have the potential for early detection and discrimination between these conditions. Earlier detection and therapy of acute rejection could lead to a reduced risk of chronic rejection, while the reduced nephrotoxicity will help to maintain renal capacity.

Immediately post-transplantation, enzymuria is found, which then declines rapidly. Renal problems are associated with the reappearance of enzymuria days in advance of an increase in serum creatinine (18). However, urinary enzymes

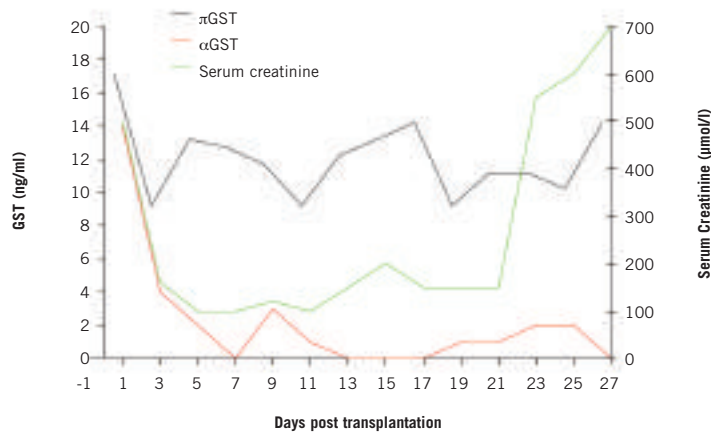
Figure 3: Proximal tubular injury – aminoglycoside therapy



Source: Bäckman et al, 1988

The kidney has great powers of recovery and a large reserve capacity; therefore, apparently mild renal injury may be accepted since one can expect full recovery, as judged by near normalisation of serum creatinine. However, this normalisation may be achieved at the expense of future reserve capacity. Nenov et al presented the concept that chronic renal injury may result from “multiple hits” of acute injury that progressively decrease the number of nephrons and increase the load on those remaining (13).

Figure 4: Renal biomarkers in acute rejection



Source: Sundberg et al, 1994

provide little information as to their cause. Cell-specific proteins can provide further diagnostic assistance.

Most nephrotoxic drugs affect the proximal tubules; therefore, specific biomarkers for the proximal tubule are good early indicators (see Figure 3) (19). Rejection starts initially in the distal tubules; therefore, distal tubular biomarkers such as π GST are potentially sensitive biomarkers (see Figure 4) (20). Simultaneous assay of proximal and distal tubular biomarkers, therefore, offers the potential to diagnose the cause of declining graft function earlier than using serum creatinine, and reduce the need for biopsies. For example, considering the commonest causes of transplant injury, urinary biomarkers could provide the following assistance:

- Rejection – relative elevation of distal tubular biomarkers
- Nephrotoxicity – relative elevation of proximal tubular biomarkers
- Ischaemia – elevation of both types of biomarker

Furthermore, cell-specific biomarkers fall much faster than serum creatinine following therapeutic intervention, providing the physician with confidence in his diagnosis and shortening courses of therapy. Shorter, better-timed and more appropriate therapy could improve the long-term survival of renal grafts.

Renal Infection

Renal infections lead to increases in urinary enzymes and cellular proteins (21). Bacteria are often initially localised around the distal tubules suggesting that distal tubular biomarkers could be especially sensitive indicators (22). In support of this, π GST has been shown to be a sensitive indicator of renal infections (23). A problem in the monitoring of renal infections is that the drugs used could be nephrotoxic. Most nephrotoxins affect the proximal tubules; therefore, using a combination of proximal tubular biomarkers (nephrotoxicity) and distal tubular biomarkers (renal infection) offers the theoretical possibility to independently monitor the effects of the infection and the therapy on the kidney.

Acute Renal Failure

Acute renal failure is a condition that occurs in around 10 to 30 per cent of subjects in intensive care, and has a high mortality rate (24). Elevated serum creatinine is a risk factor as it indicates subjects with already compromised renal function and less renal reserve as a result. However, there is a need for biomarkers that can predict renal problems in individual subjects. A study by Cressey *et al* on subjects undergoing major vascular surgery indicated that this could be possible (25). They showed that there was a close correlation between urinary GSTs during surgery increase in serum creatinine two days later (see Figure 5).

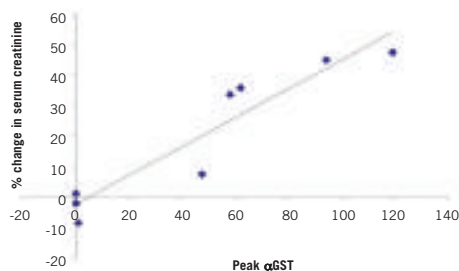
Nephrotoxicity

Many drugs are potentially nephrotoxic – for example, antibiotics, chemotherapeutics, immunosuppressive drugs and contrast media (26). The proximal tubules are most frequently affected and these toxicities are associated with increases in urinary enzymes and cell-specific proteins (27,28). Urinary levels of these biomarkers may be predictive of the extent of injury. For example, Donta and Lembke found that the subjects who had the most rapid increase in urinary NAG following Gentamycin therapy showed the largest later increases in serum creatinine (29). Fewer drugs are recognised as affecting the distal tubules, but this could be due to the paucity of biomarkers for this part of the kidney. However, π GST is released in response to drugs known to affect the distal tubules, for example Foscarnet (see Figure 6) and Amphotericin B (19,27).

Human Clinical Trials

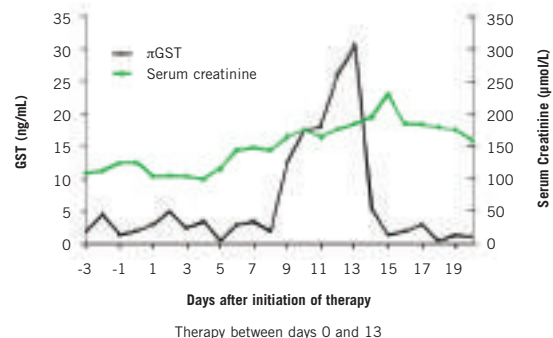
Histological validation of renal injury is seldom possible in clinical trials;

Figure 5: α GST release during vascular surgery



Source: Cressey et al, 2002 (25)

Figure 6: Distal tubular injury – foscarnet therapy



Source: Sundberg et al, 1994

therefore, the use of histologically-proven biomarkers is valuable in that it can provide a window to look into the kidney and a means of comparing animal and human responses.

Published human studies include antibiotics Cyclosporin, contrast media and anaesthetics (27,30,31,32). Combining biomarkers can also provide information as to the mechanism of renal injury. For example, Amphotericin caused the release of α GST and π GST in human subjects, while urinary NAG rose little (27). This matches the toxicological mechanism of Amphotericin which is to affect cell membranes, resulting in the release of cytosolic biomarkers, but not lysosomal biomarkers (at the doses given). Furthermore, only male volunteers showed increases in urinary GSTs, which agreed with the greater frequency of reactions in male subjects.

Specific biomarkers also proved the opportunity to compare the renal effect of different dosage regimes. For example, Ahlmén *et al* demonstrated that the addition of a calcium blocker to cyclosporin, reduced the release of α GST into the urine (30). Similarly Goldberg *et al* (16) showed that urinary α GST correlated with the dose of toxins administered. This agrees with results from animals, where it is further possible to link the mass of α GST released to the extent of histologically-graded renal injury (33).

Cell-specific biomarkers offer the opportunity for closer comparisons between animal and human studies. Knowing how the release of cell-specific biomarkers in animal and *in vitro* studies correlates with renal injury simplifies and improves the results from human studies. The biomarkers are translational and enable similar experimental models to be used in drug development, preclinical and clinical studies.

'HISTOMICS' – THE USE OF IMMUNOHISTOLOGY TO IDENTIFY NOVEL BIOMARKERS

There is a need for novel biomarkers of renal injury, especially for the distal tubules and collecting ducts. Histomics® is a means of searching for novel biomarkers using the sensitivity and specificity of immunohistology.

Histomics involves the development of monoclonal antibodies against biomarkers associated with organ or cellular injury, and the use of these as probes to locate their origin. For example, proteins were separated from urine samples from rats treated with toxins known to cause injury to defined parts of the renal tubule, and used to generate monoclonal antibodies. Antisera demonstrating binding to distinct parts of the nephron are used to develop immunoassays. A novel biomarker for the collecting ducts biomarkers for renal papillary necrosis in rats (renal papillary antigen 1 – RPA-1) has been developed, and studies are now continuing utilising urine samples from human subjects with renal pathologies (34). Eventually, it could be possible to obtain a complete picture of histological injury to the nephron from a urine sample.

CONCLUSION

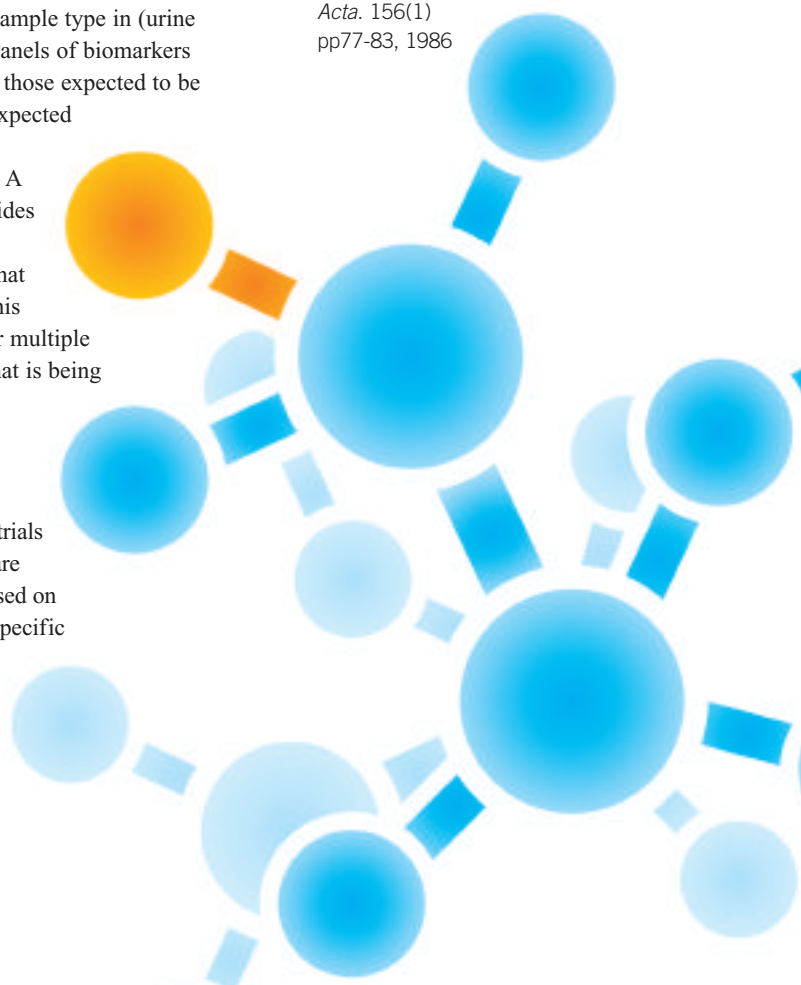
Cell-specific biomarkers offer the potential to improve both patient care and drug development. The main problem lies in their practical introduction. Current clinical procedures were developed based on – and with regards to – the weaknesses of traditional techniques, especially serum creatinine. For example, introducing novel biomarkers could involve changes in patient management, sample collection times (earlier) and sample type in (urine instead of serum). Panels of biomarkers should include both those expected to be positive and those expected to be negative if the diagnosis is correct. A negative result provides very valuable information as to what is not happening. This leads to the need for multiple assays, a problem that is being addressed by the development of multiplex assays.

Prospective clinical trials are needed to compare patient outcomes based on traditional and cell-specific biomarkers. Studies on the use of biomarkers in drug development such as the FDA C-path initiative and the

HESI/ ILSI project on nephrotoxicity will provide information that can be used to speed their introduction in man (35,36). The new biomarkers are more expensive than traditional biochemistry testing, but with the costs of renal failure amounting to billions of dollars every year, using more expensive, but more accurate, biomarkers can easily be justified if it leads to better decision making.

References

1. Lysaght MJ, Maintenance dialysis population dynamics: Current trends and long term implications, *J Am Soc Nephrol*, 13:S37-S42, 2002
2. Price CP and Finney H, Developments in the assessment of the glomerular filtration rate, *Clin Chim Acta* 297, pp55-66, 2000
3. D'Amico G and Bazzi C, Urinary protein and enzyme excretion as markers of tubular damage, *Curr Opin, Nephrol Hypertens*, 12(6), pp639-643, 2003
4. Mattenheimer H, The enzymology of renal tissue, *Enzymes in Urine and Kidney* (Dubach UC ed): pp119-145, 1968
5. Jung K *et al*, Diuresis-dependent excretion of multiple forms of renal brush-border enzymes in urine, *Clin Chim Acta*. 156(1) pp77-83, 1986



About the author

Martin Shaw is Senior Scientific Officer at Argutus Medical. At Argutus, Martin has been involved in the development and application of a series of novel renal and hepatic biomarkers. Particular emphasis has been on the development and use of novel biomarkers in drug discovery and development.

Email:

martin.shaw@argutusmed.com

6. Bourbouze R *et al*, Distribution of N-acetyl- β -D-glucosamidase isoenzymes along the rabbit nephron, *Kidney International* 25: pp636-647, 1984
7. Bosomworth MP *et al*, Urine N-acetyl- β -D-glucosamidase – a biomarker of tubular damage? *Nephrol Dial Transplant* 14: pp620-626, 1989
8. Sanchez-Bernal C *et al*, Variations in the isoenzymes of N-acetyl- β -D-glucosamidase and protein excretion in aminoglycoside nephrotoxicity in the rat, *Cell Biochemistry and Function* 9: pp209-214, 1991
9. Scherberich JE, Urinary proteins of tubular origin: Basic immunological and clinical aspects, *Am J Nephrol* 10(Suppl 1): pp43-51, 1990
10. Ohata H *et al*, Urinalysis for detection of chemically induced renal damage (2) – Changes in urinary excretions of enzymes and various components caused by p-aminophenol, puromycin aminonucleoside and hexadimethrine, *J Toxicol Sci* 12(4): pp357-372, 1987
11. Harrison DJ *et al*, Glutathione S transferase isoenzymes in the human kidney: Basis for possible markers of renal injury, *J Clin Pathol* 42: pp 624-629, 1989
12. Rozell B *et al*, Glutathione transferases of classes alpha, mu and pi show selective expression in different regions of rat kidney, *Xenobiotica* 23(8): pp835-849, 1993
13. Nenov VD *et al*, Multi-hit nature of chronic renal disease, *Current Opinions in Nephrology and Hypertension* 9: pp85-97, 2000.
14. Wang A-YM and Lai K-N, The importance of residual renal function in dialysis patients, *Kidney International* 69: pp1,726-1,732, 2006
15. Cameron JS, Tubular and interstitial factors in the progression of glomerulonephritis, *Pediatr Nephrol* 6: pp292-303, 1992
16. Branten AWJ *et al*, Urinary excretion of isoenzymes of glutathione S-transferase alpha and pi in patients with proteinuria, Reflection of the site of tubular injury, *Nephron* 85: pp120-126, 2000
17. Hariharan S *et al*, Improved graft survival after renal transplantation in the United States, 1988 to 1996, *N Engl J Med* 342: pp605-612, 2000
18. Bornstein B *et al*, Urinary enzymes and detection of graft problems, Cyclosporine nephrotoxicity and rejection crisis: diagnosis by urinary enzyme excretion, *Nephron* 72(3): pp402-406, 1996
19. Sundberg AGM *et al*, Urinary π class glutathione transferase as an indicator of tubular damage in the human kidney, *Nephron* 67: pp308-316, 1994
20. Ivanyi B *et al*, Segmental localisation and quantitative characteristics of tubulitis from patients undergoing acute rejection, *Transplantation* 56(3): pp581-585, 1993.
21. Mengoli C *et al*, Contributions of four markers of tubular proteinuria in detecting upper urinary tract infections, A multivariate analysis, *Nephron* 32: pp234-238, 1982
22. Ivanyi B *et al*, Acute human pyelonephritis: Leukocytic infiltration of the tubules and localization of bacteria, *Virchows Archiv A Pathol Anat* 414: pp29-37, 1988
23. Bouissou F *et al*, Urinary glutathione-S-transferase: excretion in normal children and children with pyelonephritis, Presented at the meeting of the French Society of Infectious Diseases in Paediatrics, Limoges, France, May 1998
24. Westhuyzen J *et al*, Measurement of tubular enzymuria facilitates early detection of acute renal impairment in the intensive care unit, *Nephrol Dial Transplant* 18: pp543-551, 2003
25. Cressey G *et al*, Renal tubular injury after infrarenal aortic aneurysm repair, *J Cardiothoracic Vasc Anesth*, 16(3): pp290-293, 2002
26. Choudhury D and Ahmed Z, Drug-associated renal dysfunction and injury, *Nature Clinical Practice Nephrology* 2(2): pp80-91, 2006
27. Pai MP *et al*, Assessment of effective renal plasma flow, enzymuria, and cytokine release in healthy volunteers receiving a single dose of Amphotericin B desoxycholate, *Antimicro Agents Chemother* 49(9): pp3,784-3,788, 2005
28. Behrends-Steins B *et al*, [Article in German], The evaluation of renal tolerance for roentgen contrast media by determination of urinary kidney-specific proteins, *Wien Med Wochenschr*, 141(8): 164: pp166-170, 1991
29. Donta S and Lembke LA, Comparative effects of Gentamicin and Tobramycin on excretion of N-acetyl- β -D-glucosaminidase, *Antimicrobial Agents and Chemotherapy*, 28(4): pp500-503, 1985
30. Ahlmén J *et al*, Decreased nephrotoxicity after the use of a microemulsion preparation of cyclosporin A compared to a conventional solution, *Transplantation Proceedings*, 27(6): pp3,432-3,437, 1995.
31. Sherman RA *et al*, A prospective study of urinary ligandin in patients at risk of renal tubular injury, *Uremia Invest* 8(2): pp111-115, 1984-1985
32. Goldberg ME *et al*, Dose of compound A, not Sevoflurane, determines changes in the biochemical markers of renal injury in volunteers, *Anesth Analg* 88: pp437-445, 1999
33. Kharasch ED *et al*, Role of renal cysteine conjugate β -lyase pathway in inhaled compound A nephrotoxicity in rats, *Anesthesiology* 88(6) pp1,624-1,633, 1998
34. Kilty C *et al*, Identification of renal papillary necrosis using an EIA for urinary Renal Papillary Antigen-1 (RPA-1); A new biomarker of collecting duct pathology, *Poster presented at the 41st Society of Toxicology meeting, New Orleans, USA, March 7-9 2005*
35. <http://www.fda.gov/cder/genomics/default.htm>
36. <http://hesiglobal.org/committees/technicalcommittees/biomarkers/default>