SAFE-T consortium

Summary Data Package
Novel clinical biomarkers of Drug-Induced Kidney Injury

The Drug induced kidney injury work package of Innovative Medicines Initiative SAFE-T Consortium

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1. Overview of SAFE-T

The Safer and Faster Evidence-based Translation (SAFE-T) Consortium is a non-profit, public-private partnership set up in the Innovative Medicines Initiative-Joint Undertaking (IMI-JU). The objective of the IMI-JU is to support projects for the development of tools and methodologies to address key "bottlenecks" in the pharmaceutical research and development process. The overall objective of the IMI SAFE-T consortium is the regulatory qualification of clinical safety biomarkers of drug-induced injury to three organs; kidney (DIKI), liver (DILI) and vasculature (DIVI) in humans using peripheral samples such as blood and urine. The consortium started in 2009 and completed its work in 2015.

2. DIKI work package objectives

The main aim of the DIKI work package was to address the current gaps in sensitive and specific clinical tests to diagnose, predict and monitor drug-induced injury to the kidney. After an initial assessment of glomerular damage biomarkers, the work package focused on clinical biomarkers of drug-induced renal tubular injury. The primary objective was to identify biomarkers with improved sensitivity and specificity relative to conventional measures. A secondary aim was to determine which biomarkers allow an earlier detection of a renal tubular injury event. Other objectives included identifying prognostic markers and markers of repair; these require large scale studies and may be addressed in future consortia.

3. Proposed context of use

"Individual novel urinary biomarkers corrected for urine creatinine including alpha glutathione-S-transferase (uα-GST), clusterin (uCLU), cystatin-C (uCysC), kidney injury molecule 1 (uKIM-1), Neutrophil Gelatinase Associated Lipocalin (uNGAL), albumin (uALB), total protein (uTPRO), and osteopontin (uOPN) are qualified safety biomarkers of renal tubular injury response for use in clinical studies of human volunteers supporting early drug development."

SAFE-T consortium posits that in this qualification procedure both established renal biomarkers (serum cystatin C [sCysC], uALB,and uTPRO) and proposed novel biomarkers (uαGST, uCLU, uCysC, uKIM-1, u NGAL and uOPN) are relevant for inclusion. Even though serum cystatin C and creatinine corrected urinary albumin and urinary total protein are used in renal safety monitoring, there are no clear guidelines as to what changes should be deemed as indicating an acute kidney injury event. The upper limits of normal for serum cystatin C, urinary albumin/creatinine ratio and urinary protein/creatinine ratio are typically used as thresholds irrespective of baseline value. The SAFE-T consortium recommends that there is value in utilizing injury thresholds for these established markers based upon percent changes from baseline rather than on reference ranges. The SAFE-T consortium, therefore, proposes to include CysC, uALB, and uTPRO in this letter of support.

4. Letters of Support (description of what is supported)

From The European Medicines Agency (14 December 2016) and the United States Food and Drug Administration (23 December 2016)

The European Medicines Agency (EMA) and United States Food and Drug Administration (FDA) have both issued separate Letters of Support to encourage the further development and exploratory use of percent change from baseline of the following urinary markers: alpha-glutathione S-transferase (α-GST), clusterin (CLU), cystatin C (CysC), kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), osteopontin (OPN), albumin (ALB) and total protein (TPRO) as biomarkers of drug-induced renal tubular injury in early clinical trials. The EMA also supports the use of serum cystatin C utilising the percent change from baseline approach proposed by the DIKI group.

The EMA and FDA reinforce that these novel urinary biomarkers should always be used alongside conventional renal safety monitoring (e.g. serum creatinine (sCr)), blood urea nitrogen (BUN), and urinalysis) in the same subjects. Furthermore, the novel urinary biomarkers could be included in preclinical safety studies in addition to clinical testing to expand the knowledge base. Sponsors should discuss prospectively any proposed use of novel urinary biomarkers in an early clinical study with the relevant Competent Authority.

The Letters of Support do not endorse any specific test system or assay validation process the novel biomarkers. The analytical performance characteristics (e.g., quantitative range, limits of the detection, precision, reproducibility, linearity, interference) for each assay should be established in advance of use. The sample stability for each of the biomarkers should be validated for its intended storage, shipping and use conditions.

EMA and FDA encourage the exploratory use of these biomarkers in early clinical development to further assess the sensitivity and specificity of each individual biomarker. The Agencies point out that the performance characteristics of these biomarkers have not been fully determined and, therefore, biomarker findings should be interpreted in the context of results for traditional biomarkers and clinical and non-clinical findings. The EMA and FDA support data sharing and integration of these novel biomarkers across multiple clinical trials. If sponsors intend to include analyses of this panel of urinary biomarkers to support regulatory decision-making for a given development program, they should prospectively discuss the approach to these analyses. For EMA discussions, these should be with the European National Authorities responsible for clinical trial authorisation, and with SAWP/CHMP. FDA discussions should be with the OND division in CDER.

5. Limitations of current tools for renal safety monitoring

In clinical practice, acute kidney injury is defined using the Kidney Disease Improving Global Outcomes (KDIGO)[1] criteria that are based on serum creatinine and urine output changes. The

KDIGO system incorporates elements of the Risk, Injury, Failure, Loss, End-stage (RIFLE) and the Acute Kidney injury Network (AKIN) kidney injury classification systems. These diagnostic and staging criteria are based on acute increases in serum creatinine and/or decreases in urine output and have been extensively validated in large populations. However, despite these diagnostic criteria, kidney damage may not be detected until kidney function is profoundly impaired and any rise in serum creatinine may be delayed by days due to the kinetics of its production from muscle turnover and accumulation secondary to reduced glomerular filtration.

Serum creatinine is influenced by multiple non-renal factors, such as age, gender, muscle mass, muscle metabolism, diet, medications, and hydration status. In acute kidney injury, the serum creatinine level can take several hours or days to reach a new steady state and thus does not reflect the structural changes that occur in the kidney during the early stage of injury and the corresponding decrease in glomerular filtration rate in the acute setting. Serum cystatin has a shorter half-life, and may be more dynamic in reflecting functional loss, but this has not yet been shown to be useful in clinical practice[2].

Besides the kidney injury classification systems described above, blood urea nitrogen, serum cystatin C, and urinalysis are also used to monitor renal safety and to detect kidney injury. However, all these measures lack sensitivity and specificity and are subject to a variety of renal and non-renal influences. Serum creatinine and BUN show relatively little change until at least 50% of renal function has been lost, as evidenced by live kidney donors in whom there are only modest changes in these measures despite the acute loss of 50% of functioning renal mass. Furthermore, sub-acute changes in renal function can be blunted or hidden by compensatory hyperfiltration of non-injured nephrons.

Besides the relative lack of sensitivity of current conventional markers to detect acute kidney injury, changes in serum creatinine or estimated glomerular filtration rate (eGFR) may occur over weeks or months. Thus, clinical recognition of sub-acute or chronic renal impairment due to slowly accruing injury to renal parenchyma may be delayed.

Over the past few years, experts in the field have initiated a search for biomarkers of kidney damage, rather than dysfunction, to detect kidney injury (including DIKI) at earlier time points when damage is less severe and more readily reversible[3]. Global kidney function markers such as serum creatinine or cystatin C do not differentiate between the various nephron segments which may be damaged by a specific nephrotoxin. This information may be important to detect and fully characterize nephrotoxicity in drug development and in clinical practice[4].

6. Brief description of each biomarker and its potential functional/ pathological role including preclinical biomarkers

6.1 Biological Rationale for Each Candidate Biomarker Selection

The following section is a critical review of the literature describing the biological mechanism and toxicological utility of each biomarker across non-clinical species and in humans. See Table 6-1 for a tabular listing of the novel biomarkers.

Table 6-1 Selected Novel Biomarkers

Selected Biomarker*	Main Biological Significance
Alpha GST	Proximal tubular necrosis marker
Clusterin	Cell adhesion
Cystatin C	Proximal tubule re-absorbtion function
KIM-1	Renal tubular inflammatory signaling molecule, regeneration
NGAL	Renal tubular inflammatory signaling molecule
Osteopontin	Distal tubular injury marker

*Urinary biomarker unless otherwise specified

6.1.1 Urinary Alpha-Glutathione S-Transferase (α-GST)

α-GST derives its name from its role as a phase II detoxification enzyme where it couples glutathione to activated toxins to make them more soluble and aid their excretion into the bile or urine[5]. α-GST is found in high concentrations in the liver, intestinal mucosa and the kidney plus in lower concentrations elsewhere in the body[6,7]. In human and rat kidneys, α-GST is localised to the proximal convoluted tubules[8,9] where it forms about 4% of the soluble protein and from which it is readily released in response to injury. The mass of α-GST released into the urine is related to the extent of proximal tubular necrosis[10]. The rapid rise and fall of urinary α-GST (uα-GST) makes it a useful biomarker to study toxicokinetics and other causes of acute renal injury.

Studies on the use of uα-GST to monitor renal injury associated with contrast media induced nephrotoxicity and transplantation date back to 1979[11]. Since then, uα-GST has been a valuable biomarker in studies on proximal tubular injury in nephrotoxicity[12], transplantation[13] and acute kidney injury[14]. α-GST has been used to study proximal tubular injury in *in-vitro* cultures[15] and rats[16] making it a translational biomarker. UαGST has been included in pre-clinical rodent studies performed by ILSI[17] and found to be valuable. Investigations in toxicology, transplantation and surgery–related AKI support the utility of uαGST to predict patient outcome[13,14]. Over 100 articles have been published[18].

Compared to other proximal tubular biomarkers, uα-GST is a very sensitive and rapid biomarker of tubular necrosis and, therefore, complements the other biomarkers in this study that monitor other pathological processes.

6.1.2 Clusterin (CLU)

CLU has a secreted and a nuclear isoform. Only the secreted isoform, is considered relevant in the context of kidney injury. CLU is constitutively expressed at high levels during early stages of renal development and later in response to kidney injury in the proximal and distal tubules, glomerulus, and collecting duct. Secreted CLU has been suggested to play an anti-apoptotic role and to be

involved in cell protection, lipid recycling, cell aggregation and cell attachment[19]. Expression of CLU mRNA is induced by different types of kidney injury in glomeruli, tubules and papilla of rats and dogs as a result of drug nephrotoxicity[20,21,22,23,24], surgery and ischemia[25,26,27,28] and in animal models of different renal diseases[29]. Changes in CLU protein levels have been measured in kidney and in the urine of many rat and dog studies[20,24,26,28,29,30,31,32,33] as well as non-human primates treated with a triple re-uptake inhibitor[34].

6.1.3 Cystatin-C (CysC)

CysC is a protein that is freely filtered at the glomerulus and then reabsorbed by the renal tubular epithelium. In addition to the potential role of serum CysC as a biomarker of glomerular filtration, urinary CysC can be utilized as a biomarker of tubular dysfunction. An impairment of re-absorption in proximal tubules can lead to a several hundred fold increase in urinary levels of CysC in humans and rats[35,36]. uCysC is becoming more commonly used as a biomarker for both AKI and chronic kidney disease (CKD). UCysC has also been characterized in the context of different kidney diseases affecting glomerular integrity and proximal tubular re-absorption in humans[37,38,39].

6.1.4 Kidney Injury Molecule-1 (KIM-1)

Urinary KIM-1 is a type I cell membrane glycoprotein. KIM-1 mRNA levels are elevated after initiation of kidney injury more than any other known genes[40,41.] After injury, the ectodomain of KIM-1 is shed from proximal tubular kidney epithelial cells *in-vitro*[42] and *in-vivo* into urine in rodents[40,41,43,44,45] and humans[46,47,48,49]. Following cisplatin treatment, KIM-1 protein levels in renal tissues and urine were highly correlated[20]. Data from the PSTC across 16 rat studies using well established nephro- and hepatotoxicants conducted across multiple sites, showed that urinary KIM-1 (uKIM-1) significantly outperformed sCr and BUN, using ROC AUC analyses[50]. These results have been repeatedly confirmed in similarly sized rat datasets[30,51,52,53].

uKIM-1 has proven to be one of the most promising biomarkers to monitor AKI[54], CDK and non-diabetic renal disease[55] in humans. It shows high sensitivity and specificity for the detection of various forms of tubular injury earlier than current diagnostic standards. Furthermore uKIM-1 is highly stable and translational between different species.

6.1.5 Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL, also known as lipocalin-2 (LCN2) or Siderocalin, is expressed in various tissues at low levels, but induced in epithelial cells following inflammation or other types of injury including malignancy[56]. In kidney injury, NGAL is upregulated in the thick ascending limb of the loop of Henle, distal tubule and collecting duct, and is secreted into the urine as well as plasma[57]. In mouse models, strongly increased NGAL mRNA and protein levels in the kidney parenchyma and urine are observed shortly after cisplatin administration or renal ischemia and precede changes in sCr[58,59.] NGAL is rapidly upregulated following kidney tissue injury making it is a highly attractive biomarker for the sensitive monitoring of DIKI in clinical trials. In addition, DIKI can cause increased expression and release of NGAL as a protective mechanism, as has been shown for other "tubular stress" proteins such as KIM-1[60]. As a consequence, the utility of NGAL as a kidney biomarker in the context of drug development may be shown in conditions which lead to either the saturation or impairment of the re-absorption and/or to increased *de novo* expression of NGAL[56,58,59,61].

6.1.6 Osteopontin (OPN)

In the kidney, OPN has divergent roles. OPN is a protective agent against oxidative stress and ischemia[62]. OPN, also has pro-inflammatory and profibrotic activity. In normal mouse, rat and human kidney, OPN is expressed at low levels in the distal nephron (thick ascending limb of the loop of Henle and distal convoluted tubules)[63]. With tissue injury, OPN expression has been demonstrated throughout the kidney, and OPN has proven to be a very sensitive and inducible indicator of different forms of AKI[64]. Increased OPN mRNA and protein levels have been reported in the kidney in numerous animal models of renal disease and injury including after gentamicin administration[65,66,67]. Investigations of OPN in renal transplant and critically ill patients support its utility for predicting patient outcome[68,69].

6.1.7 Urinary Total Protein

Increased urinary total protein indicates increased glomerular permeability to high molecular weight proteins and/or decreased re-uptake of proteins by the proximal tubules.

Random or spot urinary protein-to-creatinine ratios (UPCR) have been broadly utilized in clinical patient care more than two decades[70]. Although UPCR may be less frequently utilized than urinary albumin excretion (UAE), UPCR remains in use as a screening tool for kidney disease and as a marker of progression of underlying renal disease due to diabetes, hypertension, nephrotic syndrome, glomerulonephritis, nephrotoxic drug exposure, and more.

Urinary proteins detected by UPCR (heretofore referred to as urinary total protein (uTPRO)) are not limited to albumin (e.g. immunoglobulins and microglobulins).

6.1.8 Urinary Albumin

Increased urinary total protein indicates increased glomerular permeability to high molecular weight protein and/or decreased re-uptake of proteins by the proximal tubules. Urinary albumin (uALB) detection may also be referred to as urinary microalbumin, UAE, or urinary albumin-to-creatinine ratio (UACR)[70]. While, historically, the gold standard for measuring urine albumin levels is a 24-h urine collection, the expedience of the albumin (μg)/creatinine (mg) ratio (ACR) measured in a random urine specimen is well recognized. The use of spot UACR obtained under standardized conditions (first voided, morning, midstream specimen) to detect microalbuminuria is recommended by disease specific advocacy groups (e.g. American Diabetes Association, National Kidney Foundation). The UACR is a more convenient test for patients and may be less prone to errors due to improper collection methods and variations in 24-h protein excretion and may quite sensitively identify people who are at high risk for cardiovascular events and the progression of kidney disease.

7. Biomarker assays/ bioanalytical methods used for generation of DIKI biomarker data

The overall primary focus of the IMI SAFE-T consortium was the clinical qualification of soluble blood and urine protein biomarkers for different organ injuries. When available, commercial assays were used for evaluation and measurement of biomarker concentrations. If required, assay development and validation was performed by the SAFE-T screening sites. Multiplex immunoassays were provided by Rules Based Medicine (now Myriad RBM) and performed by the SAFE-T partners EDI and NMI, which allowed the determination of several analytes from

small sample volumes. The multiplexed assays were first compared to commercial singleplex ELISAs and were considered to be adequate and equally well performing tests. The individual assays with the respective assay platforms used for measurement of the SAFE-T DIKI sample set are listed in Table 7-1; these were all sandwich immunoassays.

For ensuring assay quality, a generic SAFE-T standard validation procedure (SVP) was developed based on the fit-for-purpose concept[71,72] where technical performance was evaluated against the predefined purpose and consequently, the stringency of performance verification varied with the foreseen use. For all assays used here, a basic assay validation adequate for the intended assay applications was done prior to their use in sample measurement ensuring principal assay performance (full SVP is available as download from the SAFE-T website). Validation criteria were set following common assay validation standard procedures. The validation procedures were based on guidelines issued by the regulatory authorities (EMA 2009, FDA 2013), but also considered the guidelines available from the Clinical and Laboratory Standards Institute (CLSI; https://clsi.org/resources/). During assay validation the following parameters were tested: limit of detection, limit of quantification, intra-/inter-assay precision, parallelism and/or dilutional linearity, analyte stability, assay dynamic range, and spike-in recovery, whenever possible.

All assays were used in an exploratory setup and validated following the fit-for-purpose approach and were thus considered suitable for the measurement of a sample set like the one used here for evaluation of biomarker performance. In principal all immunoassays, being biological assays, do per se not have the precision of chemical assays (i.e., sCr). In addition, the added variance of 10-20% introduced by assay imprecision should have minimal impact upon the total variance of the population (often over 50% (see section 13.2, table 13-2) and, in particular, the variance observed between healthy and treated patients for those analytes presented here. Assay validation was coordinated, overseen and approved by a dedicated group of experts within IMI SAFE-T (WP5).

Appropriate Quality Control (QC) controls were applied during the sample screening procedure (defined in the SAFE-T QC guidance document) to ensure data reliability and data comparability over the different phases of SAFE-T.

It should be noted that urine microalbumin, urine total protein, BUN, serum cystatin C, and serum creatinine were measured using well-established routine laboratory tests on clinical analyzers and were not validated further.

Table 7-1 Immunoassays used for generation of biomarker data

Biomarker	method	Provider, order number
Alpha GST	ELISA	Argutus Medical, Human Alpha GST EIA *now*: Teco Medical Human Alpha GST EIA, # TE1056
Clusterin	Sandwich-Immunoassay	Rules Based Medicine (now Myriad RBM) Assays
Cystatin C	Sandwich-Immunoassay	Rules Based Medicine (now Myriad RBM) Assays
KIM-1	Sandwich-Immunoassay	Rules Based Medicine (now Myriad RBM) Assays
NGAL	Sandwich-Immunoassay	Rules Based Medicine (now Myriad RBM) Assays
Osteopontin	Sandwich-Immunoassay	Rules Based Medicine (now Myriad RBM) Assays

8. Overview of the clinical studies supporting the renal tubular injury data

Three studies were completed and results are included in this data summary. Another two studies were conducted by the DIKI work package but are not included here. One was a clinical study in

acute glomerulonephritis patients aimed at investigating biomarkers of glomerular injury. The second was a confirmatory study in patients receiving contrast media. The latter study was not completed in time and the data could not be analyzed.

The three completed studies that are included in this data summary are (1) a study in cancer patients receiving cisplatin chemotherapy, (2) a study in patients being administered contrast medium for a radiological procedure (included in the appendix), and (3) a study to collect samples in healthy volunteers.

8.1 Cisplatin study design

This study enrolled cancer patients with normal renal function who were scheduled to receive a 1st cycle of high dose (>65mg/m^2/cycle) cisplatin chemotherapy. A total of 114 patients had blood and urine samples collected at the following time-points: pre-dose (baseline) and within 12 hours and 1, 2, 4, 7, 14 and 21 days after cisplatin administration. A control group of 21 patients with similar cancers receiving non-nephrotoxic treatment for the malignancy were also enrolled. The control patients had blood and urine samples collected at two separate visits.

8.2 Contrast Media study design

The study enrolled 167 patients who received ≥100 mL iodinated contrast medium. These patients had blood and urine samples collected at the following time-points: pre-injection, 4-6 hours, 24 hours and 48 hours after contract injection. Patients with serum creatinine elevation at 48 hours returned to give blood and urine samples at 7, 14 and 21 days post-injection.

The study also included 20 control patients. This control group consisted of patients with similar medical conditions as the contrast injection group. These patients had blood and urine samples collected at three separate visits.

8.3 Healthy volunteers

Healthy subjects were recruited in two different studies. In one study, healthy male and female subjects attended for three separate visits 1 week apart to have blood and urine samples collected (N=25 subjects). In a second sample collection study, healthy male and female subjects had samples collected during three study visits (N=39 subjects) over a 2-4 week period.

9. Standards of truth: treatment vs. adjudicated decision

There is no gold standard of AKI. To assess BM's accuracy using ROC curves in the context of DIKI, we used two different standard of truth for each study separately:

- The first standard of truth used is the treatment administered. Treated subjects were designated as belonging to the event group and non-treated subjects as belonging to the non-event group.

- The second standard of truth used is adjudicated AKI patients. Patients identified as having AKI by a panel of expert nephrologist adjudicators (see section 9.1) were designated as belonging to the event group and non-treated patients belonging to the non-event group.

Using treatment administered as standard of truth corresponds to a gold standard ROC analysis because the treatment administered is known without any error, therefore, it enables the analysis of biomarker accuracy without bias. The drawback of this analysis is that the thresholds (and estimates of sensitivity and specificity) derived do not necessarily correspond to a case of AKI.

To estimate thresholds (and sensitivity/specificity) corresponding to diagnosis of AKI, the second standard of truth involving an AKI adjudicator was used. The drawback to using adjudicated AKI is the potential misclassification of subjects, thus it cannot be considered a gold standard.

9.1 Adjudication Using Standard Clinical Biomarkers and Novel Urinary Biomarkers

A panel of three expert nephrologists each independently assessed BM data consisting of serial values for standard clinical biomarkers (sCr, BUN, sCysC, and eGFR) and novel urinary biomarkers normalized to urinary creatinine. Blood and urine samples were obtained at Day 0 (prior to dosing), Day 0 (8-12 hours post-dose), Day 1, Day 2, Day 3-5, Day 6-8, Day 11-17, and Day 14-21. Raw standard biomarker values and their percent change from baseline were provided to each adjudicator. Novel urinary biomarker values normalized to urine creatinine and their percent change from baseline was also provided. Adjudicators were not aware of each other's clinical conclusions which were limited to either probable AKI, probable non-AKI, or uncertain. Adjudication consensus was tabulated and consensus was declared if 2 or 3 of the three adjudicators' conclusions concurred.

10. Statistical analysis approaches for ROC curves and for threshold setting

Statistical analysis was conducted separately for the cisplatin study and for the contrast media study. Results of the cisplatin study are presented in Section 11. The contrast media study results are summarized in the appendix.

To assess biomarker accuracy (individual and combination of biomarkers), ROC analyses based on each biomarker's maximum post-baseline values (except eGFR for which the minimum was used) were determined for the following 4 endpoints:

- Maximum post-baseline raw data
- Maximum post-baseline urinary creatinine corrected values
- Maximum percent change from baseline
- Maximum percent change from baseline of urinary creatinine corrected values.

For each individual and combination of biomarkers and endpoint, empirical ROC curves were estimated by comparing treated versus non-treated patients. From these ROC curves, the area under the ROC curve (AUROC) was estimated along with their 95% confidence intervals. All estimates and confidence intervals were bootstrap estimates.

Similarly, AUROC and the biomarker best threshold (biomarker value corresponding to the closest point of the ROC curve to 100% sensitivity and 100% specificity) and its related sensitivity and specificity were estimated by comparing AKI positive patients as determined by an adjudication committee versus the control non-treated group.

Additionally, the kinetics of BM concentrations changes were assessed using time profiles plots of mean BMs values (±SEM) for the treated patients, the AKI-positive patients and the control non-treated group. The median, min and max of peak BM values have also been provided for treated patients and the AKI-positive patients.

11. Results

The main analysis of DIKI urinary biomarkers is based on maximum percent change from baseline of urinary creatinine corrected values. The results described below are from the cisplatin study. Although the DIKI Group also completed a study in patients receiving contrast media, these data are not included in the main analysis because patients with impaired baseline renal function were enrolled and therefore this study does not represent the intended use of the novel biomarkers in subjects with normal baseline renal function. See Section 13.1 for a description of the contrast media study and a summary of results for interest only.

The performance of novel urinary biomarkers was compared to that of standard measures such as serum creatinine, serum cystatin C and BUN. Two separate types of analysis are reported here. Data from Treated vs. Non-treated patients was used to determine the AUROC of each novel biomarker and standard measure and compare the relative performance of individual markers;

results are summarized in Table 11-1. In order to define exploratory thresholds for each biomarker, data from patients that were adjudicated as AKI positive were compared to non-treated control patients. The exploratory thresholds with corresponding sensitivity and specificity performance are summarized in Table 11-2.

The time course of observed biomarker changes is presented in two ways: time to reach maximum change from baseline (Table 11-3) and time to reach the exploratory AKI threshold (Table 11-4). We feel that both sets of information could be useful in the design of clinical studies by helping to define the follow up period needed to see biomarker change in most individuals.

11.1 Performance results of DIKI biomarkers

103 treated patients had baseline and post-dose biomarker or conventional marker data and could be included in the AUROC analysis. Of these patients, 70 were adjudicated as AKI positive using a combination of conventional and biomarker data. This AKI rate of approximately 70% is higher than would be expected for cisplatin-treated patients using a conventional definition of AKI.

Based on maximum percent changes from baseline, serum creatinine showed relatively good performance in the AUROC analysis (Table 11-1). It should be noted that this differs from the conventional definition of AKI (e.g. RIFLE, AKIN and KDIGO classifications for serum creatinine). In this same analysis, serum cystatin C and BUN also showed good performance. Urinary osteopontin, albumin, KIM-1 and total protein all had AUROC values that appeared to be better than that of serum creatinine. Urinary alpha-GST was comparable to serum creatinine but urinary cystatin C, clusterin and NGAL showed relatively poor performance. Please note the overlap of the 95% confidence intervals for AUROC values that point to the overall uncertainty of this analysis.

Based on these results, we conclude that urinary osteopontin, albumin, KIM-1, total protein and possibly alpha-GST can all be used as individual markers in monitoring for acute nephrotoxicity. Other urinary markers require further study to further characterize their performance under different nephrotoxicity conditions.

Table 11-1: Biomarker accuracy based on maximum percent change from baseline of urinary creatinine corrected values comparing treated patients versus non-treated control patients (Cisplatin study)

	Number of subjects		Biomarker performance
Biomarker	**Treated**	**Non treated**	**AUROC and 95% CIs**
BUN	102	17	0.95 [0.89;0.99]
u. osteopontin	88	17	0.95 [0.90;0.98]
u. albumin	92	17	0.95 [0.90;0.98]
u. KIM-1	88	17	0.95 [0.87;0.99]
u. total protein	95	17	0.93 [0.84;0.99]
s. cystatin C	103	16	0.92 [0.85;0.97]
s. creatinine	103	17	0.84 [0.74;0.92]
estimated GFR	103	17	0.84 [0.73;0.92]
u. α-GST	88	17	0.84 [0.71;0.93]
u. cystatin C	88	17	0.79 [0.64;0.90]
u. clusterin	88	17	0.78 [0.60;0.90]
u. NGAL	88	17	0.71 [0.52;0.85]

Creatinine-corrected values were used for urinary biomarkers.

Exploratory thresholds were defined for the novel biomarkers. These thresholds were based on a ROC analysis of adjudicated positive patients vs. non-treatment controls. The sensitivity and specificity of each biomarker at the defined threshold is also presented below. The width of the 95% confidence intervals gives an indication of the uncertainty of these thresholds so these should be used with caution in prospective clinical studies. Studies with larger sample size would be useful to confirm these thresholds.

Table 11-2: Biomarker exploratory thresholds based on maximum percent change from baseline for the comparison of AKI patients determined by adjudication committee versus non-treated control patients (Cisplatin study)

	Number of subjects		Threshold performance			
				Median BM		
Biomarker	Treated	Non treated	AUROC and 95% CIs	Threshold and 95% CIs	Threshold sensitivity	Threshold specificity
u. albumin	62	17	0.97 [0.93;1.00]	112.33% [57.98;284.09]	0.94 [0.82;0.98]	1.00 [0.88;1.00]
u. KIM-1	58	17	0.96 [0.89;1.00]	55.56% [55.56;95.25]	0.97 [0.91;1.00]	0.94 [0.82;1.00]
u. osteopontin	58	17	0.96 [0.92;0.99]	74.69% [31.19;128.80]	0.90 [0.79;0.97]	0.94 [0.88;1.00]
BUN	68	17	0.95 [0.90;0.99]	15.15% [4.76;36.59]	0.88 [0.78;0.97]	0.94 [0.82;1.00]
u. total protein	63	17	0.95 [0.88;1.00]	51.11% [37.75;58.23]	0.97 [0.89;1.00]	0.88 [0.71;1.00]
s. cystatin C	70	16	0.92 [0.85;0.97]	12.35% [10.44;35.29]	0.91 [0.73;0.97]	0.88 [0.75;1.00]
s. creatinine	69	17	0.88 [0.79;0.95]	21.55% [4.11;24.44]	0.78 [0.67;0.91]	0.88 [0.76;1.00]
estimated GFR	69	17	0.87 [0.78;0.95]	-5.84% [-16.99;-5.09]	0.84 [0.68;0.93]	0.88 [0.71;1.00]
u. α-GST	58	17	0.84 [0.71;0.94]	114.58% [33.33;217.07]	0.79 [0.64;0.93]	0.82 [0.65;0.97]
u. cystatin C	58	17	0.82 [0.68;0.93]	21.07% [-7.38;234.55]	0.90 [0.66;0.97]	0.76 [0.53;0.88]
u. clusterin	58	17	0.81 [0.66;0.92]	240% [27.72;280.89]	0.81 [0.67;0.95]	0.82 [0.59;0.94]
u. NGAL	58	17	0.73 [0.52;0.89]	21.65% [-23.74;121.03]	0.84 [0.59;1.00]	0.65 [0.41;0.82]

Creatinine-corrected values were used for urinary biomarkers.

Thresholds represent percent change from baseline

Threshold sensitivity is the sensitivity for detecting AKI of the marker at the given threshold

Threshold specificity is the specificity for AKI changes of the marker at the given threshold

11.2 Time course of biomarker change

In defining the time course of biomarker changes vs. standard measures such as BUN and serum creatinine, we assessed both Treated and Adjudicated Positive populations separately. It was felt that adjudicated AKI cases represent the most useful analysis and the results shown below are based on this population.

Table 11-3: Biomarker time to maximum change from baseline in AKI patients determined by adjudication committee (Cisplatin study)

		Time to maximum change from baseline (days)			
Biomarker	**N**	**25th percentile**	**Median**	**75th percentile**	**90th percentile**
u. α-GST	70	1.0	1.0	4.0	7.0
s. cystatin C	70	2.0	2.0	4.0	7.0
u. KIM-1	70	2.0	2.0	7.0	7.0
u. osteopontin	70	1.0	3.0	7.0	7.0
u. clusterin	70	1.0	4.0	7.0	7.0
u. cystatin C	70	1.0	4.0	7.0	7.0
u. total protein	69	2.0	4.0	7.0	7.0
u. NGAL	70	1.0	4.0	7.0	14.0
BUN	70	2.0	5.5	7.0	7.0
u. albumin	68	4.0	7.0	7.0	7.0
s. creatinine	70	4.0	7.0	7.0	14.0

Creatinine-corrected values were used for urinary biomarkers.

From the Table 11-3 above, it can be seen that most novel urinary biomarkers reach peak change from baseline a median of 2 to 4 days after nephrotoxin exposure. Serum creatinine reaches maximum change from baseline a median of 7 days after exposure.

Table 11-4: Biomarker time to threshold change from baseline in AKI patients determined by adjudication committee (Cisplatin study)

			Time to percentage change from baseline (days)			
Biomarker	**N**	**Threshold (% change from baseline)**	**25th percentile**	**Median**	**75th percentile**	**90th percentile**
s. cystatin C	63	12.35	1.0	1.0	2.0	2.0
u. cystatin C	54	21.07	0.5	1.0	1.3	4.0
u. α-GST	40	114.58	0.6	1.0	3.5	7.0
BUN	60	15.15	1.0	1.0	2.0	7.0
u. osteopontin	51	74.69	1.0	1.0	2.0	7.0
u. KIM-1	56	55.56	1.0	1.0	2.0	7.0
u. NGAL	55	21.65	1.0	1.0	2.0	7.0
u. albumin	54	112.33	1.0	1.0	4.0	7.0
u. total protein	50	51.11	1.0	1.0	4.0	7.0
u. clusterin	41	240.00	1.0	2.0	4.0	7.0
s. creatinine	51	21.55	2.0	2.0	7.0	12.6

Creatinine-corrected values were used for urinary biomarkers.
Thresholds represent percent change from baseline

Given that the thresholds used in the time course analysis are exploratory in nature and that their sensitivity and specificity differ between BMs, the results of this analysis should be used with some caution, especially that some AKI patients never reach BM's threshold for BMs with low sensitivity (e.g. clusterin) whereas all AKI patients reach BM's threshold for BMs with high sensitivity (e.g. KIM-1). Further data will be required to confirm these thresholds as well as the time courses of each novel biomarker. From this exploratory analysis, it can be seen that the 90 centile to reach the threshold for AKI was 7 days for most of the novel urinary biomarkers. Hence, clinical study designs should consider 1 week follow up periods to ensure the detection of AKI patterns in the majority of subjects tested.

11.3 Summary of results

1. Approximately 70% of patients treated with cisplatin were adjudicated as AKI positive using a combination of conventional and novel markers.
2. With the exploratory thresholds applied, serum creatinine, serum cystatin C and BUN showed good performance.
3. Urinary osteopontin, albumin, KIM-1, and total protein showed good AUROC performance and were better than serum creatinine. Alpha-GST showed AUROC performance that was similar to serum creatinine but changed much more rapidly.
4. Exploratory thresholds have been estimated to aid the use of novel biomarkers in clinical studies.
5. The time course of changes in biomarkers showed that AKI patterns demonstrated maximal change as well as reaching threshold values within 7 days in 90% of patients developing AKI and reaching BM's threshold. Serum creatinine reached threshold value in 12.6 days and demonstrated maximal change in 14 days.

12. Recommendations on how to use the novel biomarkers

12.1 Recommended minimum performance criteria for assays that Sponsors should use post-LOS

12.1.1 Limits of quantitation

In choosing a suitable assay for a given biomarker, the Sponsor should consider the measurement range of the assay relative to the normal range in the target study population. Since the biomarkers' thresholds for detecting kidney injury are set based on percent change from baseline, it is important to be able to measure basal levels in the majority of subjects participating in a clinical study. The chosen assay should have a lower limit of quantitation (LLoQ) that allows reliable baseline measurements in most healthy volunteers.

To simplify the assay procedure and reduce the need for serial dilutions to provide quantitative measurements, the measurement range should cover the fold change above baseline in the majority of participating subjects. To achieve this, the chosen assay should preferably have an upper limit of quantitation (ULoQ) that is near to the upper end of the normal range multiplied by the injury threshold for that biomarker.

Table 13-2 provides a summary of the criteria for the assays used in the SAFE-T program. Whilst different assays may provide different absolute values, this table gives an indication regarding required assay performance relative to the normal range and response criteria for each biomarker.

12.2 Single biomarker vs. using groups together – statistical implications based on decision rules (e.g. "one out of three" vs. "three of three" biomarker rules).

All individual BM thresholds for sensitivity and specificity correspond to the probabilities of correctly identifying uninjured patients (= true negative rate = 1- false positive rate) and correctly identifying AKI patients (true positive rate). In cases where one uses several BMs with together with a decision rule based on their combinations (i.e. use BMs as a panel), the probabilities of correctly classifying patients change. As an example, in the case of using a panel of 3 BMs each with 95% sensitivity and 95% specificity we would have:

- if no injury; we would have for uncorrelated BMs (what would be the usual case in the non-event group)
    - a false positive rate = 1 - 0.95^3 = 14.26% for a decision rule being that at least one BM reaches the threshold
    - a false positive rate = 0.05^3 = 0.0125% for a decision rule being that all 3 BMs reach their thresholds
    - a false positive rate between the two if the decision rule is to have at least 2 BMs reaching thresholds.

- if injury; we would have for uncorrelated BMs (what should **not** be the usual case in the event group, the correlated case having same properties as the individual BM case so that it is useless to measure more than one BM)
    - a true positive rate = 1 - 0.05^3 = 99.9875% for a decision rule being that at least one BM reaches its threshold
    - a true positive rate = 0.95^3 = 85.74% for a decision rule being that all 3 BMs reach their thresholds
    - a true positive rate between the two if the decision rule is to have at least 2 BMs reaching thresholds

Therefore, in the above example, using a panel of BMs may cost more in terms of a loss in specificity (- 9.26%) than it improves sensitivity (= 5%).

Using DIKI BMs assessed within the Cisplatin study and assuming that these are uncorrelated (which may not be the case in subjects with AKI), a panel of urinary osteopontin, KIM-1 and alpha-GST may have a sensitivity of 99.94% and a specificity of 72.46% for a decision rule being that at least one of these 3 BM reaches the threshold. On the other hand, if the decision rule is that all 3 BMs reach their threshold, then the sensitivity of the BM's panel would be equal to 68.97% and the specificity equal to 99.93%.

12.3 Time course of testing

The time course analysis of changes in biomarkers is presented in Section 11.2. Table 11-3 summarizes the time to maximal change of each biomarker. Table 11-4 summarizes the time to threshold change for individual markers.

The study design and time course of testing for biomarkers used in a clinical study assessing the renal effects of a potential nephrotoxic drug will need to factor in a number of considerations. These include:

- choice of biomarker(s) to be used for renal monitoring,
- time to threshold/ time to maximal change of the chosen biomarkers,
- pharmacokinetics of the drug,
- time course and pattern of injury seen in preclinical studies etc.

These considerations will determine the optimal time points of urine sample collections as well as duration of follow up following drug administration.

12.4 Recommendations for how to choose Biomarker/s

As these novel exploratory biomarkers have not been broadly applied across drug development, it is important that the biomarkers are evaluated in preclinical safety studies before they are incorporated in a clinical trial design. The preclinical studies will not only allow the drug development sponsor to link the response of the biomarker to histopathological lesions in the kidney, but also to the timing of the injury and the biomarker's response. This knowledge should be used to determine whether or not the biomarkers should be used in clinical trials with the candidate drug, plus guide the relative timing for the collection of urine and evaluation of biomarker levels. Although drug development sponsors can include any biomarker they choose, a defined panel of biomarkers may provide a more robust evaluation of kidney injury. It is important to state that even though these biomarkers have been thoroughly evaluated in nonclinical species (Reference to nonclinical qualifications and LOS's), only limited information is available on the equivalence of the response of the biomarkers across non-clinical species and humans.

The selection of biomarker(s) for use in clinical trials should be based on the marker(s) response in preclinical nephrotoxicity studies. When multiple biomarkers can be used, combinations should include suitable markers in different functional groups (e.g. albumin [reabsorbtion function] and KIM-1 [inflammatory signaling, regeneration]).

12.5 Limitations of our data

The studies conducted were exploratory in nature and confirmatory studies have not been conducted to validate these findings. The limited number of studies conducted, the small sample size, and study subject demographics (predominantly Caucasian males) are relevant limitations of the data presented. While definition of AKI for adjudication was standardized (AKIN criteria), systematic analysis of individual and aggregate adjudicator decision-making has not been presented.

13. Appendices

13.1 Exploratory contrast media study

The DIKI group conducted two studies in patients receiving contrast media. The exploratory study was completed and results are summarized below. The confirmatory study was not completed and the main AKI analysis (measured GFR) could not be conducted for technical reasons.

The DIKI WP concluded that the contrast media study is not representative of renal tubular injury in subjects with normal renal function because (1) the contrast media patients had renal impairment at baseline and their biomarker values at baseline were different from contrast patients without baseline renal impairment and healthy volunteers, and (2) the renal injury caused by contrast media is a combination of vaso-active effect (an effect accentuated in patients with renal impairment) that results in ischemia as well as a potential direct cytotoxic effect. However, a summary of biomarker performance in the contrast media study are presented for completeness. We are not presenting a threshold analysis for the contrast media study since this may not be relevant for biomarker use in subjects with normal renal function). A threshold analysis would be available from the DIKI team upon request.

Study design

The study enrolled patients with abnormal baseline renal function (CKD Stage 3 or 4 defined by eGFR 15-59 mL/min/1.73 m^2) who were scheduled to receive iodinated contrast medium as part of a planned radiological investigation. Subjects also had one additional risk factor for AKI such as atherosclerosis (coronary artery disease, aortic aneurysm, or peripheral arterial disease), presence of diabetes mellitus, or congestive heart failure.

The study enrolled 167 patients who received ≥100 mL iodinated contrast medium. Patients had blood and urine samples collected at the following time-points: pre-injection (baseline), 4-6 hours, 24 hours and 48 hours after contract injection. Subjects with serum creatinine elevations at 48 hours also had blood and urine samples collected at 7, 14 and 21 days post-injection. The study included a control group of 20 patients with similar medical conditions as the contrast injection group. These patients had blood and urine samples collected at three separate visits.

Results

The performance of novel urinary biomarkers, based on maximum percent change from baseline of urinary creatinine corrected values, was compared to that of standard measures such as serum creatinine, serum cystatin C and BUN. Data from Treated vs. Control patients was used to determine the AUROC of each novel biomarker and standard measure and compare the relative performance of individual markers; results are summarized in Table 13-1.

164 treated patients enrolled (121 with biomarker data), 18 control patients (17 with biomarker data). Of these patients, 41 were adjudicated as AKI positive using a combination of conventional and biomarker data.

Based on maximum percent changes from baseline, most of the tested novel biomarkers showed good performance relative to serum creatinine in the AUROC analysis. The biomarkers ranked in descending AUROC values are shown in Table 13-1. Please note the overlap of the 95% confidence intervals for AUROC values that point to the overall uncertainty of this analysis.

Table 13-1: Biomarker accuracy based on maximum percent change from baseline of urinary creatinine corrected values comparing treated patients versus non-treated control patients (Contrast media study)

	Number of subjects		Biomarker performance
Biomarker	**Treated**	**Non treated**	**AUROC and 95% CIs**
u. α-GST	41	17	0.84 [0.71;0.92]
u. total protein	118	17	0.72 [0.59;0.82]
u. cystatin C	116	17	0.72 [0.44;0.88]
u. NGAL	116	17	0.69 [0.57;0.81]
u. clusterin	116	17	0.66 [0.52;0.81]
u. KIM-1	116	17	0.66 [0.51;0.79]
u. osteopontin	116	17	0.61 [0.48;0.74]
estimated GFR	121	17	0.58 [0.49;0.69]
u. albumin	118	17	0.58 [0.46;0.69]
s. creatinine	121	17	0.57 [0.48;0.69]
s. cystatin C	42	17	0.57 [0.38;0.74]
BUN	121	17	0.53 [0.43;0.63]

Creatinine-corrected values were used for urinary biomarkers.

13.2 Summary of assay performance criteria

Table 13-2 Summary of Assay Performance Characteristics

Analyte	**Type of Assay**	**Sample Matrix analyzed**	**Calibration curve range [µg/L]** (in sample at usual assay dilution)	**Normal Range:**	**LoD [µg/L]** (in sample at usual assay dilution)	**LLoQ [µg/L]** (in sample at usual assay dilution)	**ULoQ [µg/L]** (in sample at usual assay dilution)	**intra-assay precision** (% CV)	**inter-assay precision** (% CV)	**dilutional linearity** (% recovery)	**Spike-in recovery (%)**	**short term stability (24 h at RT and 4°C)**[+]	**F/T stability, 3 cycles**[+]
α GST	ELISA	Urine	2.5 - 80 (6.25-200)	5.7 (2.8 – 11.5)	1.9 (4.75)	2.5 (6.25)	80 (200)	2 - 3	3 - 4	95 - 100*	81 - 120*	ND	yes*
Clusterin	Luminex	Urine	0.0791 - 400 (0.158-800)	19.5 (9.87 - 38.5)	0.435 (0.87)	0.625 (1.25)	363.5 (727)	0 - 17	11 - 28	100 - 116	72 - 90	yes	yes
Cystatin C	Luminex	Urine	0.00723 - 36.2 (0.723 -3620)	17.8 (8.46 - 37.4)	0.038 (3.8)	0.38 (3.8)	29 (2910)	1 - 9	4 - 5	100 - 110	120 -131	yes	yes
Cystatin C	ELISA	Serum	200-10000	760 (590-980)	13.4 (13.4)	200	10 000	1 - 6	10 - 16	74 - 142	112 - 118	ND	yes[#]
KIM-1	Luminex	Urine	0.002 – 10 (0.004-20)	0.103 (0.051 - 0.206)	0.002 (0.004)	0.0085 (0.017)	9.5 (19)	1 - 10	7 - 16	93 - 101	87 - 119	yes	yes
NGAL	Luminex	Urine	0.01-50 (1-5000)	26.0 (11.8 - 57.3)	0.046 (4.6)	0.067 (6.7)	4.6 (4600)	0 -10	7 - 11	100 - 108	90 - 124	yes	yes
Osteopontin	Luminex	Urine	0.00047 - 2.36 (0.0474-236)	396 (199 - 788)	0.24 (24)	0.029 (2.9)	198 (19800)	0 - 15	4 - 11	95 - 102	66 - 204	yes	yes

*Abbreviations: limit of detection (LOD) , lower limit of quantification (LLoQ),upper limit of quantification (ULoQ), room temperature (RT), freeze/thaw (F/T), not determined (ND), coefficient of variability (CV), Glutathione S-transferase A (α-GST), Kidney Injury Molecule 1 (KIM-1), Neutrophil Gelatinase-Associated lipocalin (NGAL); [+]recovery 80 - 120 %, *for stabilized urine; [#]recovery 136 - 172 %*

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