Review of Qualification Data for Biomarkers of Nephrotoxicity

Submitted by the ILSI-HESI Nephrotoxicity Working Group

Biomarker Qualification Review Team

The following people participated in writing this review document: Melanie Blank, Fang Cai, Albert DeFelice, Patricia Harlow, Elizabeth Hausner, Aliza Thompson, Li Zhang

The following people participated in at least some team discussions: Melanie Blank, Fang Cai, Sushanta Chakder, Albert DeFelice, Monica Fiszman, Patricia Harlow, Elizabeth Hausner, Federico Goodsaid, Ying Mu, Shona Pendse, Aliza Thompson, Shen Xiao, Nancy Xu, Lynne Yao, Li Zhang

Center for Drug Evaluation and Research

U.S. Food and Drug Administration

September 13, 2010

Table of Contents

1. Executive Summary **7**

a. Background 7
b. Sources of Data and Major Findings 7
c. Data Considerations 7
d. BQRT Conclusions and Recommendations for Future Research to Address Gaps in Understanding of the Performance of Urinary Clusterin and RPA-1 10

2. Background **12**

a. Overview of the problem 12
b. Biomarkers of Drug-Induced Nephrotoxicity Proposed by HESI 12
c. Context Claims Submitted by HESI for the Qualification of Proposed Biomarkers of Drug-Induced Nephrotoxicity 15

3. Summary of the Supporting Data Submitted by HESI for the Qualification of Proposed Biomarkers of Drug-Induced Nephrotoxicity **15**

a. Overall Summary of Results 15
b. Summary of Studies Conducted and Biomarkers Measured 17
c. Histopathology Lexicon and Scoring 18
d. Summary Tables of ROC Curves 19
e. Statistical Analysis 24
i. Superior diagnostic value 24
ii. Incremental diagnostic value 27
f. Individual Animal Data 28
i. Individual results by histopathology grade 28
ii. Individual animal data by study 29
g. Pathology incidence by rat strain 35
h. Recovery Studies 37

4. Reviewer Discussion of Qualification Data **37**

a. The Qualification Process 37
b. Analytical Validation 37
i. Immunoassays 38
ii. Standards/calibrators and alternative methodology 38
iii. Cross reactants/Assay specificity 38
iv. Matrix interference 39
v. Other matrix interference issues 39
vi. Stability 39
vii. Intra-assay reproducibility 40
viii. Inter-assay reproducibility 40
ix. Inter-site reproducibility 40
c. Correlation between Histopathology and Biomarker Data 41
i. Blinding of Histopathology Analysis 41

ii. Basis for Histopathology evaluation 42
iii. Exclusion of Animals from ROC Analysis 42
iv. Generation of ROC curves 43
d. Performance of Proposed Biomarkers Compared with Accessible Biomarkers in Current Use 44
i. Collection of Samples 44
ii. Blinding of Biomarker Assays 45
iii. Urinary creatinine Normalization 45
iv. Background (Control Range) Biomarker Levels 45
v. Control animal variability 46
vi. Threshold Definition 47
vii. Secondary Organ Effects 48
viii. Specificity 49
vii. Unusual Findings 50
e. Format Issues 53
i. Initial submission 53
ii. Subsequent submissions 53

5. Qualification Conclusions 53

a. BQRT Conclusions 53
b. Limitations of Submitted Data 54
c. BQRT Recommendations for Qualification 56
d. Recommendations for Future Research to Address Gaps in Understanding of the Performance of These Urinary Biomarkers 56

6. Appendices 60

a. Background Information on Proposed Biomarkers 60
i. GST isoforms 60
ii. Clusterin 60
iii. RPA-1 63
b. Additional Data Supporting Qualification of Proposed Biomarkers 64
i. Summary of Individual Studies 64
ii. Standardized Kidney Histopathology Lexicon 65
iii. Comparison of AUC_{ROC} values for the Excluded (Table 7) and Included (Table 8) datasets 67
iv. Pair-wise statistical comparisons for all pathologies 68
v. Incremental statistical analyses 74
vi. Box-plots – biomarker versus histopathology grade 76
vii. Pathology incidence by strain and nephrotoxicant 77
viii. Reviewer's pathology incidence by study and strain 78
ix. Summary of analytical validation 80
x. Summary of inter-laboratory data by site 82
xi. List of excluded animals with missing biomarker data 83

7. References 84

List of figures

Figure 1: Reviewer's compilation of HESI figures and data from various tables for the three claimed diagnoses 16

A. PT degeneration or necrosis - α-GST versus BUN, sCr, NAG, and urine protein 16
B. CD degeneration or necrosis - RPA-1 versus BUN, sCr, NAG, and urine protein 16
C. CD degeneration or necrosis - α-GST versus BUN, sCr, NAG, and urine protein 17
D. Cortical tubular regeneration/basophilia - clusterin versus BUN, sCr, NAG, and urine protein 17

Figure 2: Reviewer's graph of the AUC_{ROC} values for pooled data by selected diagnosis 20

Figure 3: Reviewer's graph – Comparison of AUC_{ROC} values in the Sprague Dawley (SD) and Han Wistar (HW) rat strains for selected diagnoses 23

Figure 4: Sponsor's figures – scatterplots from December submission 29

A. Scatterplot of α-GST fold-change versus histopathology grade for PT degeneration or necrosis 29
B. Scatterplotplot of RPA-1 fold-change versus histopathology grade for CD degeneration or necrosis 29
C. Scatterplot of clusterin fold-change vs histopathology grade for cortical tubular regeneration/basophilia 29

Figure 5: HESI figures showing biomarker values for individual animals by study 30

A. Wistar/cisplatin with histopathology grade for tubular degeneration/necrosis of s3 segment 30
B. Sprague Dawley/cisplatin with histopathology grade for tubular degeneration/necrosis of s3 segment 31
C. Sprague Dawley/Gentamicin with histopathology grade for proximal tubular degeneration/necrosis 32
D. Wistar/NPAA with histopathology grade for degeneration/necrosis of papilla collecting duct 33
E.Sprague Dawley/NPAA with histopathology grade for collecting duct degeneration/necrosis 34

Figure 6: Sponsor's figure - Reproducibility (Inter-lab variability) shown as percent difference of lab mean from global mean for blinded samples 41

Figure 7: Reviewer's analysis of the distribution of histopathology scores 44

List of Tables

Table 1: Characteristics of exploratory biomarkers of nephrotoxicity 13

Table 2: HESI claims: Accessible and qualified biomarkers for regulatory decision making that enable drug development 15

Table 3: Reviewer's summary of studies conducted 18

Table 4: HESI summary tables - Biomarkers measured at each site 18

Table 5: Sponsor's table - Histopathology diagnoses summary 19

Table 6: Summary of kidney histology sections in ILSI-HESI submission 19

Table 7: HESI summary table of ROC Curves – AUC_{ROC} estimates (standard error) for pooled data based on animals which had a value for each individual biomarker 20

Table 8: AUC_{ROC} estimates (standard error) based on all available animals 21

Table 9: HESI summary table of AUC_{ROC} estimates (standard error) for Sprague-Dawley animals 22

Table 10: HESI Summary table of AUC_{ROC} estimates (standard error) for Wistar Animals 22

Table 11: Sponsor's table comparing AUC_{ROC} estimates for Sprague-Dawley and Han-Wistar animals for selected pathologies and biomarkers 23

Table 12: Reviewer's compilation comparing AUC_{ROC} values for the claimed pathologies in the two strains and in the pooled sample 24

Table 13: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for α-GST versus reference biomarkers for the claimed pathologies 25

Table 14: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for RPA-1 versus reference biomarkers for the claimed pathologies 26

Table 15: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for clusterin versus reference biomarkers for the claimed pathologies 26

Table 16: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for μ-GST versus reference biomarkers for the claimed pathologies 26

Table 17: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for NAG versus BUN and SCr for the claimed pathologies 26

Table 18: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for total protein versus BUN and SCr for the claimed pathologies 27

Table 19: Reviewer's compilation of HESI Statistical analysis - Summary of incremental analysis from Appendix 21 initial submission 28

Table 20: Overall incidence of negative and positive animals by pathology and strain 35

Table 21: Reviewer's compilation from sponsor's tables - Pathology incidence by study and strain 36

Table 22: Summary of HESI submissions and meetings with the BQRT 37

Table 23: Reviewer's summary of biomarker immunoassays 38

Table 24: Reviewer's summary of HESI recovery data 38

Table 25: Reviewer's summary of statements in product inserts 40

Table 26: Reviewer's summary of HESI Intra-assay reproducibility 40

Table 27: Reviewer's summary of HESI Inter-assay reproducibility 41

Table 28: Reviewer's summary of HESI inter-site reproducibility data 42

Table 29: Reviewer's summary of excluded animals 42

Table 30: From sponsor's tables - Frequency distribution of animals by pathology 43

Table 31: Reviewer's compilation from sponsor's tables – NPAA study in Wistar rats 45

Table 32: Reviewer's summary - Fold-change ranges for control animals across all studies by rat strain 46

Table 33: Reviewer's summary of biomarker urine creatinine-normalized values 46

Table 34: Sponsor's table - Intra-animal and inter-animal %CV estimates for Sprague-Dawley and Han-Wistar control animals in the NPAA studies 46

Table 35: Sponsor's thresholds corresponding to estimated 95% specificity for indicated pathology and diagnostic likelihood ratios 47

Table 36: Sponsor's overview of assessment of hepatotoxicity in dose-range-finding or definitive studies by compound and strain – From December submission 48

Table 37: Reviewer's examination of α-GST values relative to histopathology scores 52

Biomarker Qualification Review

1. EXECUTIVE SUMMARY

This is a review by the Biomarker Qualification Review Team (BQRT) of a submission by the ILSI-HESI Nephrotoxicity Working Group (HESI) for the nonclinical qualification of three urinary biomarkers of nephrotoxicity: clusterin, renal papillary antigen-1 (RPA-1), and alpha-glutathione S-transferase (α-GST).

a. Background

The Biomarker Qualification Process at the FDA evaluates proposals for biomarker qualification submitted by scientists from multiple organizations. This document reviews the data contained in the 2008-2009 HESI submission supporting their request for qualification of the use of three biomarkers of nephrotoxicity in nonclinical drug development studies. This is the second qualification package submitted for nephrotoxicity biomarkers; the first qualification package was submitted in 2007 by the Predictive Safety Testing Consortium (PSTC). At present, the detection of drug-induced kidney toxicity is limited by the lack of sensitive and specific biomarkers for the detection of mild and/or early injury. Biomarkers with greater sensitivity and specificity than blood urea nitrogen (BUN) and serum creatinine (sCr) have the potential to address an unmet need in drug development.

b. Sources of Data and Major Findings

HESI submitted a data package to support the nonclinical use of three urinary biomarkers of drug-induced kidney toxicity in male rats: clusterin, RPA-1, and α-GST, . In studies conducted at five independent sites, each novel biomarker and several comparator biomarkers were measured in the urine from male Sprague Dawley and Han Wistar rats following administration of three model nephrotoxicants (gentamicin, cisplatin and N-phenylanthranylic acid) and no non-nephrotoxicants. Each biomarker was measured using a commercially available singleplex enzyme immunoassay from Biotrin. The analytical validation data including measuring range, limit of detection, linearity, recovery, intra-assay reproducibility, inter-assay reproducibility, recovery, evaluation of some potentially interfering substances, and inter-laboratory variability for each novel biomarker assay, suggest that the assays were generally acceptable.

The performance of each biomarker was compared to that of sCr and BUN against the reference standard of histopathology using Receiver Operating Characteristic (ROC) analysis. Comparisons of the area under the ROC curve (AUCroc) showed the performance of clusterin, RPA-1 and α-GST was statistically superior to sCr and BUN in these studies for the diagnosis of specific kidney pathologies.

c. Data Considerations

Clusterin was previously qualified by the FDA in 2008 based on data reported in a PSTC submission. The HESI data support the qualification of urinary clusterin as a more sensitive biomarker of drug-induced nephrotoxicity as evidenced by an AUCroc value for clusterin that was significantly greater ($p<0.001$) than the AUCroc values for sCr and BUN for the diagnosis

of tubular toxicity (nonspecific with respect to location). The HESI submission provided data not only for the male Han Wistar rat (provided in the PSTC submission), but also for the male Sprague Dawley rat. The HESI submission also provided inter-laboratory validation data on the clusterin assay to support the decision to pool data from different laboratories (data not contained in the PSTC submission). The HESI submission provides additional support for the use of urinary clusterin in nonclinical toxicity studies in the male rat when drug related tubular pathology changes, particularly in the presence of tubular regeneration, are observed. Therefore the HESI data support clusterin as qualified for the following context of use:

> **Urinary Clusterin** is a qualified biomarker for voluntary use in the detection of acute drug-induced renal tubule alterations, particularly when regeneration is present, in male rats when used in conjunction with traditional clinical chemistry markers and histopathology in GLP toxicology studies for drugs for which there is previous preclinical evidence of drug induced nephrotoxicity or where it is likely given the experience with other members of the pharmacologic class.

RPA-1 is a novel biomarker not previously qualified by the FDA. The HESI data show that the AUCroc value for RPA-1 diagnosis of collecting duct injury was significantly greater ($p<0.001$) than AUCroc values for sCr and BUN. In addition, the curves did not cross each other at different levels of specificity. The significant increase in AUCroc values without crossing of the curves indicate that RPA-1 is a more sensitive biomarker of collecting duct injury at all levels of specificity. Furthermore, the AUCroc value for RPA-1 remained high whereas the AUCroc values for clusterin and α-GST decreased when distinguishing between histopathology scores of zero (no pathology) and one (minimal pathology). Therefore the HESI data support RPA-1 as qualified for the following context of use:

> **Urinary RPA-1** is a qualified biomarker for voluntary use in detecting acute drug-induced renal tubule alterations, particularly in the collecting duct, in male rats when used in conjunction with traditional clinical chemistry markers and histopathology in GLP toxicology studies for drugs for which there is previous preclinical evidence of drug induced nephrotoxicity or where it is likely given the experience with other members of the pharmacologic class.

HESI also proposed α-GST as a novel biomarker proposed for use in drug-induced kidney toxicity; however, the BQRT does not recommend α-GST for qualification at this time. The HESI data show that the AUCroc value for α-GST was significantly greater ($p<0.001$) than AUCroc values for sCr and BUN for the detection of proximal tubule and collecting duct injury. However, increases in urinary *α*-GST showed greater sensitivity than sCr and BUN for the detection of proximal tubule injury and decreases in urinary α-GST showed greater sensitivity than BUN and sCr for the detection of collecting duct injury. The opposing effects of proximal tubule and collecting duct injury on *α*-GST levels may confound the interpretation of urinary *α*-GST measurements, particularly for compounds for which there is limited mechanistic information.

The BQRT also considered the following limitations of the HESI submission:

1. The amount of data used to construct the ROC curves is limited by three main concerns:
    i. No non-nephrotoxins and only three nephrotoxins, two of which induce similar proximal tubule injury, were used. It is unclear how well clusterin and RPA-1 will perform in rats for the evaluation of new compounds without nephrotoxicity (i.e., false positive rate) and new compounds that have mechanisms of toxicity different than the compounds studied by HESI. Therefore, the BQRT recommends that traditional clinical chemistry markers (sCr and BUN) and histopathology assessments should also be made when clusterin and RPA-1 are used in a preclinical development program.
    ii. Only male rats were used. It is therefore unclear how well clusterin and RPA-1 will perform in female rats. Although the mechanisms of toxicity should be similar in both genders, differences in basal biomarker levels and the extent and timing of response to injury may differ in males and females. Therefore, the BQRT recommends that the nonclinical qualification of urinary clusterin and RPA-1 should be limited to use in male rats.
    iii. The temporal relationship between changes in histopathology and changes in urinary clusterin and RPA-1 levels was minimally examined with two or three timepoints defining the evolution of injury and no timepoints examining reversibility of the drug-induced renal injury. Therefore, uncertainty exists as to how well clusterin and RPA-1 will perform at different time points post injury, particularly early time points, and whether repair of injury will be reflected by changes in clusterin or RPA-1 levels. Although this information would be needed for a qualification with a context of use that excludes the need for accompanying histopathology, it is not essential for a qualification with a context of use that requires accompanying histopathology.
2. While data pooled across rat strains were used to support the qualification of these biomarkers, there were differences between rat strains in the performance of individual biomarkers. These differences raise concern about the appropriateness of pooling data across strains. Confidence in a biomarker's performance is increased when both rat strains show higher sensitivity and specificity than sCr and BUN as was observed for clusterin for cortical tubular regeneration/basophilia and RPA-1 for collecting duct degeneration/necrosis. For this reason, the BQRT feels that it is important to limit the qualification of clusterin to the detection of cortical tubular regeneration/basophilia and the qualification of RPA-1 to the detection of collecting duct degeneration/necrosis.
3. Since knowledge of the treatment group may have introduced bias into the study results, the BQRT would be more confident of the results if the pathologists had been fully blinded to all information. The initial pathologist, a peer-review pathologist, and a subsequent HESI Pathology Working Group (PWG) were unblinded to treatment group, but were blinded to novel biomarker results. Although the PWG harmonized terminology and severity grading and arrived at a consensus opinion, the BQRT believes that fully blinded readings of histopathology are needed in future qualification studies.

4. A few animals had positive urinary clusterin and RPA-1 values in the absence of positive histopathology. Whether this finding reflects the ability of these biomarkers to detect injury even before there are visible histopathology changes, a non-specific change in biomarker levels (i.e., a false positive), or inadequate tissue sampling resulting in underdetection of an existing histopathology finding cannot be determined. In the submitted studies, only a single section per kidney per animal was examined microscopically. The minimum number of tissue samples needed in biomarker qualification studies to adequately characterize renal injury, particularly low levels of injury, remains unknown and should be better characterized. At this time, however, we do not have sufficient information to conclude that positive urinary clusterin and RPA-1 values in the absence of histopathology changes are predictive signs of injury and are unable to completely describe the optimum implementation of these biomarkers.

d. BQRT Conclusions and Recommendations for Future Research to Address Gaps in Understanding of the Performance of Urinary Clusterin and RPA-1

Despite the aforementioned limitations, the BQRT concludes that the data contained in the HESI submission support the qualification of

- urinary clusterin for voluntary use in rat safety assessment studies for the detection of acute drug-induced tubular injury and tubular regeneration/basophilia.
- urinary RPA-1 for voluntary use in rat safety assessment studies for the detection acute drug-induced collecting duct injury.

We recommend that urinary clusterin and RPA-1 should be used along with traditional clinical chemistry markers and histopathology for the detection of acute drug-induced nephrotoxicity in toxicology studies. Specifically, sponsors may use these biomarkers in GLP toxicology studies in the development of drugs for which evidence of drug induced nephrotoxicity already exists or is likely based on prior experience with the pharmacologic class of the drug being developed to determine more conservative NOAELs (i.e., values below those that would be based on observed histopathology or sCr elevations) for estimating starting doses in the initial human clinical trial of a drug. As indicators of injury, these biomarkers could be used to obtain a NOAEL below levels that show histopathology changes and allow safe initiation of clinical trials.

The BQRT has the following recommendations and suggestions for future research:

1. The BQRT recommends that urinary clusterin and RPA-1 be qualified as acceptable biomarkers for voluntary use along with traditional clinical chemistry markers and histopathology for the detection of acute drug-induced nephrotoxicity in GLP toxicology studies in *male* rats, but not in *female* rats. Testing of these biomarkers should be done in the female rat and should be extended to other animal species when appropriate assays become available.
2. The BQRT recommends that additional studies comparing the performance of each biomarker to that of sCr and BUN against the reference standard of histopathology should be done with a wider array of nephrotoxicants and non-nephrotoxicants to confirm the findings from the HESI submission, to aid in the determination of optimal

biomarker thresholds for acute drug-induced renal tubule alterations, and to assess the presence of false positives (i.e., positive findings with non-nephrotoxicants).

3. The BQRT recommends that nonclinical studies be conducted to characterize better the correlation of the evolution of drug-induced injury (as determined by histology) with changes in biomarker levels by testing throughout the evolution of injury. It is also recommended that studies be conducted to demonstrate whether reversibility of injury (determined by histopathology) can be related to timing, extent, or duration of biomarker changes.

4. The BQRT recommends that future studies address the issue of the minimum number of tissue sections needed in biomarker qualification studies to detect adequately the presence or absence of renal injury, particularly low levels of injury. Such studies will be needed to support any claims concerning the ability of these biomarkers to detect injury prior to histopathology changes.

5. The submission contains some immunohistochemistry data in a limited number of animals as an adjunct (secondary) to histopathology to confirm localization of nephrotoxic injury. These data suggest urinary clusterin may be useful for the detection of acute drug-induced renal tubule alterations in male rats, particularly when regeneration is present, while urinary RPA-1 may be useful for the detection of acute drug-induced renal tubule alterations in male rats, particularly in the collecting duct. The BQRT recommends the collection of additional immunohistochemistry data to support claims concerning the ability of the biomarker to report localization of injury to particular segments of the nephron. Immunohistochemistry or other appropriate techniques should be used to define the temporal relationships among changes in histopathology, changes in tissue levels of the biomarkers and changes in urinary biomarker levels.

6. The opposing behavior of urinary α-GST levels in response to proximal tubule and collecting duct injury raise uncertainty about the usefulness of α-GST for the detection of early and/or mild renal injury; hence the BQRT does not currently recommend the qualification of urinary α-GST. Given the limited amount of data on the specificity of the α-GST biomarker assay, future studies should address the effect of potential interfering substances as well as dilutional effects and the cross-reactivity of other GST isoforms as possible explanations for the decrease in urinary α-GST observed with collecting duct injury. Studies utilizing immunohistochemistry to localize the expression of various GST isoforms before and after collecting duct injury should be conducted to clarify the response of α-GST to different areas of renal injury and provide a better understanding of the mechanistic basis for the observed decreases following collecting duct injury. Additional nephrotoxicants should also be studied to explore the effect of isolated collecting duct injury as well as the effect of concomitant proximal tubule and collecting duct injury on α-GST levels.

7. It is the BQRT's opinion that blinded histopathology readings are needed in biomarker qualification studies to ensure unbiased assessments of the utility of novel biomarkers in detecting early drug-induced injury. The histopathology readings in the HESI submission were conducted by pathologists blinded to the novel biomarker levels, but with knowledge of treatment group, study design, and standard clinical pathology data.

This knowledge may have introduced bias into the assessment of the histopathology resulting in an overly favorable estimate of the diagnostic performance of the novel biomarkers. For this reason, in addition to the other limitations discussed in section 1c., the BQRT recommends that the qualification of urinary clusterin and RPA-1 be limited at this time to voluntary use along with traditional clinical chemistry markers and histopathology. Blinded assessment of histopathology should be the standard in future biomarker qualification studies.

8. The BQRT recognizes the need for biomarkers that can reliably predict injury in both the preclinical and clinical setting. With respect to the clinical use of urinary clusterin and RPA-1, the BQRT recommends the exploration of these novel renal biomarkers in humans when and if sufficiently validated assays become available. However, urinary clusterin and RPA-1 are not currently qualified as primary renal injury monitoring tests or to define dose-stopping criteria in clinical drug development studies. For the time being, sponsors and regulatory divisions should decide on a case-by-case basis how best to explore and/or make use of these biomarkers in a clinical development program.

2. BACKGROUND

a. Overview of the Problem

Biomarkers are used as indicators of physiologic, pathologic and pharmacologic processes. Many commonly used chemical biomarkers lack sufficient sensitivity and specificity for detecting early and mild to moderate drug-induced organ damage. In particular, drug development has been hampered by a lack of accessible markers of renal injury which does not cause overt dysfunction. Although sCr, BUN, and creatinine clearance have traditionally been used to monitor drug-induced renal toxicity, these biomarkers are poor predictors of drug-induced renal damage because they lack sensitivity and specificity for early or sub-critical renal injury and provide little information on the region of the kidney affected by the drug and/or the mechanism(s) by which this injury occurs. As a result, much research has focused on the development of novel biomarkers of early and/or milder renal toxicity.

To improve efficiency of drug development, the Critical Path Opportunities Report (http://www.fda.gov/downloads/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/UCM077254.pdf) called for the identification of new safety biomarkers to (1) identify early toxicity in animal studies, (2) aid in initial dose selection in clinical studies, and (3) improve safety monitoring in phase 1 and 2 clinical trials. Under the FDA Critical Path Initiative, biomarkers will be qualified on the basis of data that support their proposed use within a specified context. The FDA seeks to facilitate the development of biomarkers of renal toxicity by establishing a clear and rigorous process for biomarker qualification.

b. Biomarkers of Drug-Induced Nephrotoxicity Proposed by ILSI-HESI

The ILSI-HESI Nephrotoxicity Working Group submitted data to support the nonclinical qualification of three pre-clinical urinary biomarkers of drug-induced acute kidney toxicity. Table 1 provides an overview of key characteristics of these three claimed biomarkers: α-GST, RPA-1 and clusterin. Additional background information for each biomarker is provided in Appendix 6a.

Table 1: Characteristics of Exploratory Biomarkers of Nephrotoxicity

Urinary marker	General attributes	Proposed mechanism by which increased urinary levels seen during kidney injury	Background data cited by Sponsor
α-GST	An isoform of a phase II detoxifying enzyme that exist in the kidney in the proximal tubule in both rat and human (Beckett & Hayes 1993; Campbell et al 1991; Harrison et al 1989; Rozell et al 1993; Sundberg et al 1993, 1994;). A high concentration of α-GST (~2% of soluble protein) exists in proximal tubule cells. Also, α-GST is highly expressed in liver (Derbel et al 1993)	The increased presence of GSTs in the urine after nephrotoxic injury to rats (Bass et al 1979) and humans (Branton et al 2000) is attributed to leakage from the cells into the lumen of the tubule secondary to epithelial cell damage (Harrison et al 1989). Expression of GST isoforms may be up-regulated after exposure to some xenobiotics and renal toxins (Daggett et al 1997; Derbel et al 1993),	Studies of the effects of volatile anesthetics on the kidney of rats (Kharasch et al (1997) and human volunteers (Eger et al 1997) reported that urinary excretion of α-GST was a sensitive biomarker of tubular injury Urinary levels of specific isoforms GST have been proposed not only as markers of renal tubular damage in general but also as indicators of the location of the injury along the nephron (Eger et al 1997; Sundberg et al 1994; Harrison et al 1989).
RPA-1	A rat collecting duct antigen named renal papillary antigen-1 (RPA-1) was measured using a murine monoclonal antibody PapX 5C10 identified through a process of immunohistochemical screening to confirm the nephronal origin of the released proteins (Falkenberg et al 1996; Hildebrand et al 1999).	RPA-1 is induced in rats by NSAIDs or 2-bromethanamine and results in differential release of RPA-1. Using Western blots, a RPA-1 positive signal is only in kidney, except for faint staining in ileum. Using rat tissue microarrays PapX 5C10 specific binding was in urothelium of the renal pelvis and ureter; collecting ducts from the cortex, medulla and papilla plus some epididymal granular epithelial cell staining in the testis. Expression of RPA-1 is localized on the collecting duct luminal membrane. Experiments with trypsin suggest epitope on RPA-1 is not a linear epitope.	RPA-1 has been shown experimentally to be a specific marker for the rat collecting duct and is an early predictive and sensitive urinary biomarker for renal papillary necrosis, including effects of NPAA (Hardy and Bach, 1984) and other toxicants such as 2-bromethanamine and propyleneimine.(Hildebrand et al 1999).

Clusterin* (sulfated glycoprotein [SGP-]))	Widely distributed heterodimeric glycoprotein Highly expressed during embryonic development (French et al 1993), during kidney development (Harding et al 1991) and following glomerular, tubular and papillary injury in animals (Hidaka et al 2002; Yamada et al 2003; Eti et al 1993)	In mature kidney, basal expression of clusterin is low, with localization in tubular basement membranes and glomerular mesangium (Yamada et al 2003). Clusterin is expressed in response to injury and may be involved in tissue remodeling and repair (Pearse et al 1992).	Clusterin induction has been observed following ureteral obstruction (Pearse et al 1992) and ischemia-reperfusion injury (Witzgal et al 1994). Elevations in the levels of clusterin have also been observed following subtotal nephrectomy (Correa-Rotter et al 1992) and in animal models of hereditary polycystic kidney disease (Cowley and Rupp 1995). Marked increases of clusterin released in urine have also been recorded in animal models of aminoglycoside (Aulitzky et al 1992; Eti et al 1993), sevoflurane (Kharasch et al .2006) and cisplatin-induced nephrotoxicity (Silkensen et al 1997) as well as in dogs with renal papillary necrosis induced by nefiracetam (Tsuchiya et al 2005). Increased expression of clusterin is seen in humans with a variety of renal disorders (Dvergsten et al 1994; Rosenberg and Silkensen 1995); however, few clinical studies have been performed with clusterin as a diagnostic.

*For the purposes of this review, clusterin refers to the secreted isoform of clusterin and not the nuclear isoform.

c. Context Claims Submitted by ILSI-HESI for the Qualification of Proposed Biomarkers of Drug-Induced Nephrotoxicity

The ILSI-HESI Nephrotoxicity Working Group (HESI) makes the following claims for the biomarkers submitted for qualification:

- *α-GST is superior to all of the reference markers for detection of PT injury*
- *RPA-1 is shown to be a very specific marker of CD injury and superior to all of the reference markers for detection of injury to this segment.*
- *The data support the use of clusterin to monitor tubular injury, particularly when regeneration is present.*

Table 2 summarizes the HESI claims.

Table 2: HESI Claims: Accessible and Qualified Biomarkers for Regulatory Decision Making that Enable Drug Development

Urinary biomarker	Qualified pre-clinical	For specific diagnosis		Analytically validated assay	Assay available	Claim:
		Add inform to sCr/BUN	Outperform sCr/BUN			
α-GST	Yes	Yes, both	Yes, both	Yes	Yes (R,H)	Increases with PT degeneration or necrosis.
		Yes, both	Yes, both			Decreases with CD degeneration or necrosis
RPA-1	Yes	Yes, both	Yes, both	Yes	Yes (R)	Increases with CD degeneration or necrosis.
Clusterin	Yes, exten-sion of PSTC	Yes, both	Yes, both	Yes	Yes (R, H)	Increases with Cortical tubular regeneration/basophilia.
R = rat; H = human						

3. Summary of the Supporting Data Submitted by HESI for the Qualification of Proposed Biomarkers of Drug-Induced Nephrotoxicity

a. Overall Summary of Results

The data from short term rat GLP toxicology studies conducted at AstraZeneca, Bayer, Biotrin, BMS, GSK, and sanofi-aventis were evaluated through the joint FDA/EMEA pilot qualification process in an iterative manner. The results from studies supporting this qualification are summarized using Receiver Operating Characteristic (ROC) curves, which are plots of true positives (sensitivity) against false positives (1-specificity). This is the method of choice to characterize the performance of diagnostics (Metz, 1978). In such analyses, the "area under the curve" (AUC) for an ideal biomarker has a value of 1, while the AUC for a biomarker yielding random values is 0.5.

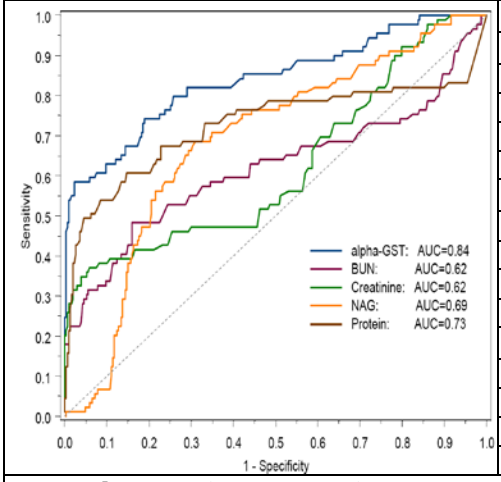
The performance of each new biomarker for specific diagnoses compared to the accepted biomarker standards (BUN, sCr) and two other biomarkers (NAG, total protein) was evaluated by comparing the AUC from the ROC analysis for each new biomarker with similar data for each of the comparison biomarkers. Histopathology was used as the reference standard for providing evidence of toxic injury as a binary endpoint. All animals with histopathology score greater than 0 were defined as 'Positive' animals and all animals with histopathology score equal to 0 defined as 'Negative' animals. Statistical comparison of the ROC curves is described in Section 3e below. ROC curves were generated both for data merged across rat strains from positive histopathology scores (i.e., true positive vs. false positive) for all studies by diagnosis as well as for data from subset ranges of these scores. ROC data generated for all included animals and different

histopathology ranges of the complete datasets are summarized in the figures below (Figure 1 A, B, C, D). The ROC curves on the left are shown for one novel biomarker versus the reference biomarkers. Ideally, all the novel biomarkers should be plotted against each other. However, the novel biomarkers can be compared based on the AUC_{ROC} values in Table 7 (see section 3d). The plots on the right of AUC_{ROC} values as a function of histopathology grade for each specific diagnosis show the AUC_{ROC} value for the specific novel biomarker was greater than the AUC_{ROC} value for sCr and BUN for all comparisons. HESI did not evaluate the results using a composite or maximum histopathology score. Note that data for collecting duct (CD) degeneration/necrosis are derived only from the NPAA studies, while data for proximal tubule (PT) degeneration/ necrosis are derived from the cisplatin and gentamicin studies.

Figure 1: Reviewer's compilation of HESI figures and data from various tables* for the three claimed diagnoses

A. PT degeneration or necrosis - α-GST versus BUN, sCr, NAG, and urine protein

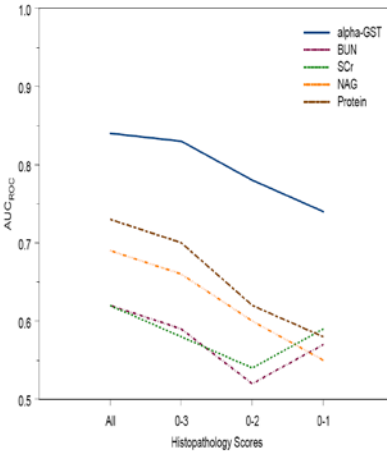
Overall ROC curves - PT degeneration or necrosis



Animals used in calculation

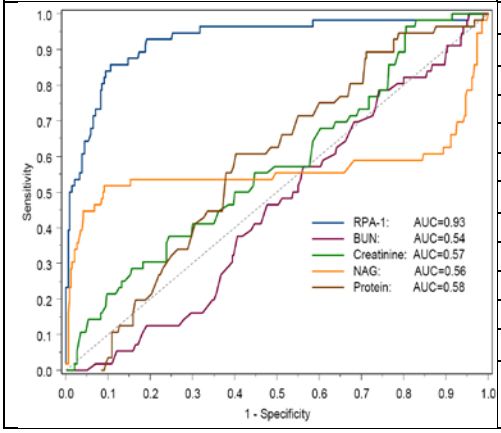
	SD	HW	Tot.
Overall			
Neg	170	137	307
Pos	34	55	89
Total	204	192	396
%	16.7	28.6	22.5
Cisplatin			
Neg	83	64	147
Pos	11	55	66
Total	94	119	213
%	11.7	46.2	30.9
Gentamicin			
Neg	27	ND	27
Pos	23	ND	23
Total	50	ND	50
%	46	ND	46

AUC_{ROC} values vs. histopathology range used for subset.



B. CD degeneration or necrosis - RPA-1 versus BUN, sCr, NAG, and urine protein

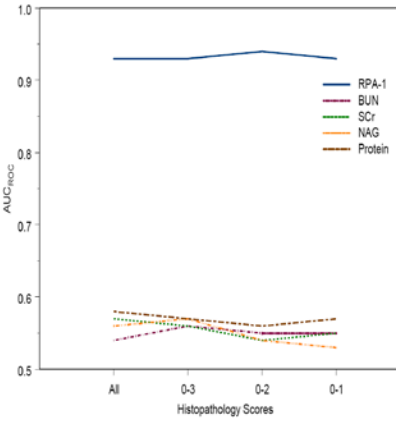
Overall ROC curves - CD degeneration or necrosis.



Animals used in calculation

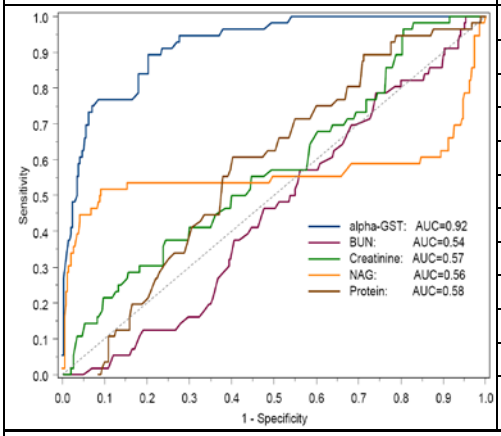
	SD	HW	Tot.
Overall			
Neg	179	161	340
Pos	25	31	56
Total	204	192	396
%	12.3	16.1	14.1
NPAA			
Neg	35	42	77
Pos	25	31	56
Total	60	73	133
%	41.6	42.5	42.1

AUC_{ROC} values vs. histopathology range used for subset.



C. CD degeneration or necrosis - α-GST versus BUN, sCr, NAG, and urine protein

Overall ROC curves - CD degeneration or necrosis.



Animals used in calculation

	SD	HW	Tot.
Overall			
Neg	179	161	340
Pos	25	31	56
Total	204	192	396
%	12.3	16.1	14.1
NPAA			
Neg	35	42	77
Pos	25	31	56
Total	60	73	133
%	41.6	42.5	42.1

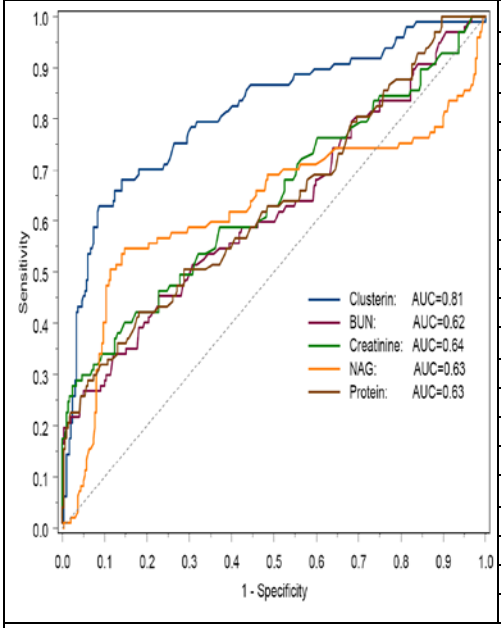
AUC_{ROC} values vs. histopathology range used for subset.

ROC analysis where range of histopathology score is limited was not provided

[Note: In contrast to PT pathology where α-GST values increased, α-GST values decreased in response to increased CD pathology alone.]

D. Cortical tubular regeneration/basophilia - clusterin versus BUN, sCr, NAG, and urine protein

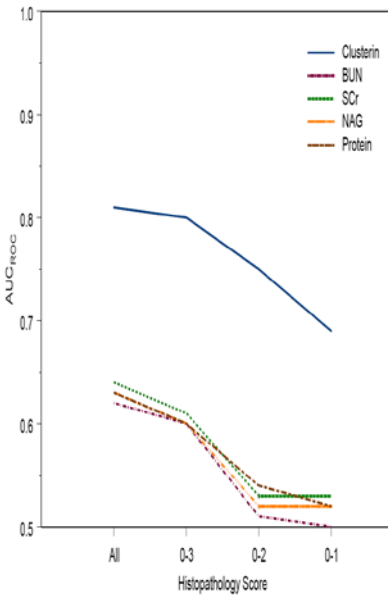
Overall ROC curves – Cortical tubular regeneration /basophilia.



Animals used in calculation

	SD	HW	Tot.
Overall			
Neg	153	146	299
Pos	51	46	97
Total	204	192	396
%	25.0	24.0	24.4
Cisplatin			
Neg	89	99	188
Pos	5	20	25
Total	94	119	213
%	5.3	16.8	11.7
NPAA			
Neg	37	47	84
Pos	23	26	49
Total	60	73	133
%	38	35.6	36.8
Gentamicin			
Neg	27	ND	27
Pos	23	ND	23
Total	50	ND	50
%	46	ND	46

AUC_{ROC} values vs. histopathology range used for subset.



SD = Sprague Dawley; HW = Han Wistar; Neg. = negative; Pos. = positive; ND = Not done
* HESI tables in Appendices

b. Summary of Studies Conducted and Biomarkers Measured

The studies conducted at each site are summarized in Tables 3 and 4 below. Additional details for each study are provided in the HESI summary tables found in Appendix 6bi. The data provided are only from male rats in a total of five studies, one at each of five independent sites. These studies used only three nephrotoxins, two of which induce similar proximal tubule injury, and no non-nephrotoxins.

Table 3: Reviewer's summary of studies conducted		
Rat strain	Han Wistar	Sprague Dawley
Sex	Male	Male
Animal number/group	10-30	10-15
Number of timepoints/study	1-3	1-3
Number of nephrotoxicants	2	3
Common nephrotoxicants	cisplatin NPAA	cisplatin NPAA gentamicin
Number of non-nephrotoxicants	0	0
Biomarkers used	BUN, sCr, GGT, clusterin, total protein, NAG, μ-GST, α-GST, RPA-1	BUN, sCr, GGT, clusterin, total protein, NAG, μ-GST, α-GST, RPA-1

Table 4: HESI Summary tables - Biomarkers measured at each site :

Table 2 – Summary of the biomarkers measured in each of the laboratories

Laboratory	**Bayer**	**BMS**	**AZ**	**GSK**	**sanofi**
Strain of rat/test compound	**Wistar/cisplatin**	**SD/gentamicin**	**Wistar/NPAA**	**SD/NPAA**	**SD/cisplatin**
Urine TP	+	+	+	+	+
Cr	+	+	+	+	+
GGT	+	+	+	+	+
NAG	+	+	+	+	+
α-GST	+	+	+	+	+
μ-GST	+	+	+	+	+
Clusterin	+	+	+	+	+
RPA-1	+	+	+	+	+
albumin	-	-	-	-	+
others	+	+	+	+	+
Serum BUN	+	+	+	+	+
CR	+	+	+	+	+
TP	+	+	+	+	+
others	-	+	+	-	+
Number of animals/group	10	10	13-30	10-15	10
Number of timepoints	3	1-2*	1-2*	1-2*	3
Day of necropsy	2, 3 or 5	8 or 15	8 or 15	8 or 15	2, 3, or 5

* Two for control and high dose; one for low and mid dose

Cisplatin directly alkylates DNA and generates cellular stress. In addition to renal toxicity, cisplatin induces myelosuppression, anemia, ototoxicity, liver damage and neurologic damage (Goodman &Gillman). Gentamicin produces lysosomal phospholipidosis and cochlear, vestibular and renal toxicity like other aminoglycosides (Goodman &Gillman). N-phenylanthranylic acid (NPAA) produces accumulation of acid mucopolysaccharide and renal papillary necrosis (Hardy and Bach, 1984).

c. Histopathology Lexicon and Scoring

The HESI pathologists agreed upon standardized vocabulary of terminology and grading for evaluating renal injury by histopathology. The lexicon used the primary

histopathology processes in Table 5 below. The full lexicon in Appendix 6bii also lists secondary histopathology lesions and structural elements. Because HESI wanted to assess the relationship between changes in urinary markers and injury to specific segments of the rat nephron, histopathology data were further combined to remove redundancies and ensure that each animal had only one histopathology diagnosis per pathologic process. At the top of each boxed set of diagnoses below, the italicized diagnoses represent diagnoses used in the ROC analysis formed by combination of the underlying diagnoses.

Table 5 - Sponsor's table - Histopathology diagnoses summary

	Tubular cell degeneration/ necrosis	Tubular cell regeneration/ basophilia	Other
Proximal tubule	***PT degeneration/ necrosis*** Tubular cell degeneration/ necrosis, proximal tubule, S1/S2 Tubular cell degeneration/ necrosis, proximal tubule, S3	***Cortical tubular regeneration/ basophilia*** Tubular cell regeneration/ basophilia, cortical Tubular cell regeneration/ basophilia, PCT, s1-s2	Inflammation, interstitial, chronic Intratubular casts, granular
Collecting duct	***CD degeneration/ necrosis*** Tubular cell degeneration/ necrosis, collecting duct, medulla Tubular cell degeneration/ necrosis, collecting duct, papilla	***Medullary tubular regeneration/ basophilia*** Tubular basophilia, medulla	Intratubular casts, hyaline
Distal tubule	Tubular cell degeneration/ necrosis, distal tubule		
Diagnoses omitted from the ROC analysis			***Tubular cell alteration, vacuolation*** ***Tubular dilation, cortex*** ***Mineralization, papilla***

For each animal, one section was evaluated per kidney according to Table 6 below. Only one histopathology score was provided per animal for each major diagnosis. The sponsor indicated the most severe score was used for ROC analyses. No information was provided concerning the consistency of the histopathology score between the two kidneys of the same animal.

Table 6 - Summary of kidney histology sections in ILSI-HESI submission

Nephrotoxicant	Rat Strain	Kidney	Type of section	Number of sections
Cisplatin	Wistar	Left	longitudinal	1
		Right	transverse	1
Cisplatin	SD	Left	transverse	1
		Right	transverse	1
Gentamycin	SD	Left	longitudinal	1
		Right	transverse	1
NPAA	Wistar	Left	transverse	Up to 6; only 1 scored
		Right	transverse	Up to 6; only 1 scored
NPAA	SD	Left	longitudinal	Up to 6; only 1 scored
		Right	longitudinal	Up to 6; only 1 scored

Based on response in Dec 08 submission

d. Summary Tables of ROC Curves

HESI summarized the AUC_{ROC} values calculated across both rat strains for all histopathology grades in Table 7 below. This HESI table excluded animals for which a biomarker value was missing. As indicated previously in section 3a, the AUC_{ROC} value for an ideal biomarker has a value of 1, while the AUC_{ROC} for a biomarker yielding random values is 0.5. For the pathology of PT degeneration/necrosis, the highest AUC_{ROC} value was for α-GST, while the AUC_{ROC} values for μ-GST, RPA-1, clusterin and the traditional biomarkers were lower. For the pathology of cortical tubular

regeneration/basophilia, the highest AUC_{ROC} values were for clusterin and RPA-1. For the pathology of medullary tubular regeneration/basophilia, the highest AUC_{ROC} value was for RPA-1. For the pathology of collecting duct degeneration/necrosis, the highest AUC_{ROC} values were for RPA-1 and α-GST. The reviewer's graph in Figure 2 helps visualize the relationships among biomarkers.

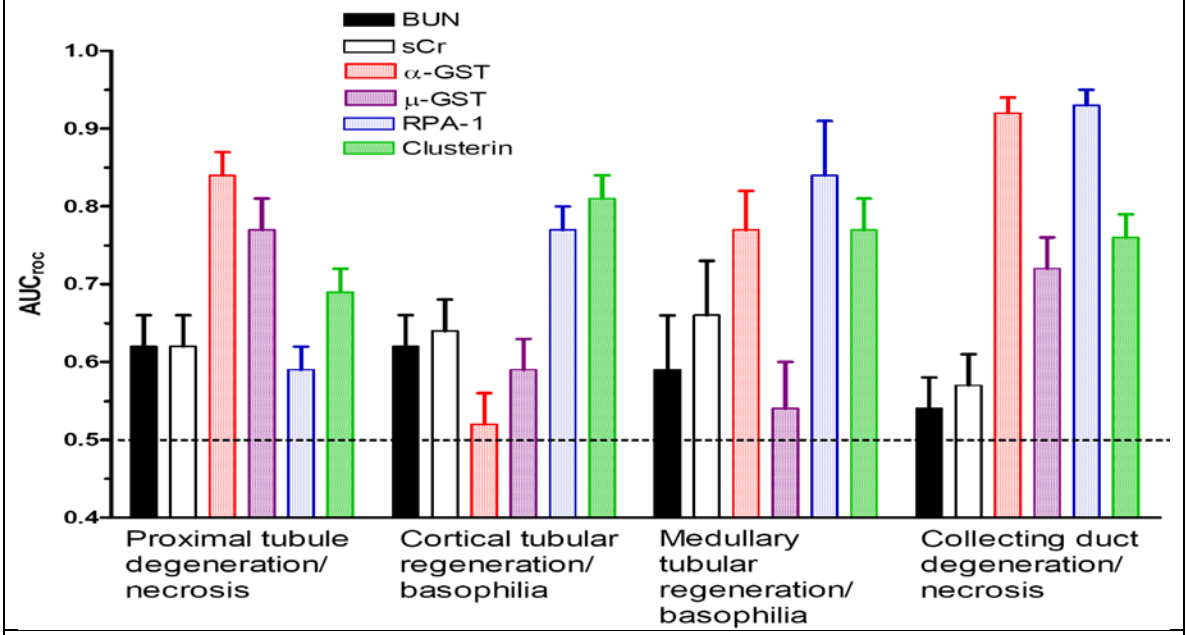
Table 7: HESI Summary Table of ROC Curves – AUC_{ROC} estimates (standard error) for pooled data based on animals which had a value for each individual biomarker

Yellow highlights the principal pathologies claimed. Red text indicates a pathology for which the number of positive animals is <20.

Pathology	BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	# pos.
PT degeneration or necrosis	0.62 (0.04)	0.62 (0.04)	0.69 (0.03)	0.73 (0.04)	0.84 (0.03)	0.77 (0.03)	0.59 (0.03)	0.69 (0.03)	**89**
PT deg/nec with no regen	0.56 (0.05)	0.58 (0.04)	0.52 (0.04)	0.53 (0.06)	0.74 (0.04)	0.62 (0.04)	0.57 (0.04)	0.57 (0.04)	48
PT deg/nec with regen	0.79 (0.05)	0.82 (0.05)	0.87 (0.02)	0.89 (0.03)	0.87 (0.03)	0.87 (0.03)	0.76 (0.04)	0.94 (0.02)	41
Cortical tubular regeneration/basophilia	0.62 (0.04)	0.64 (0.04)	0.63 (0.04)	0.63 (0.03)	0.52 (0.04)	0.59 (0.04)	0.77 (0.03)	0.81 (0.03)	**97**
DT degeneration or necrosis	0.52 (0.06)	0.67 (0.04)	0.89 (0.03)	0.73 (0.05)	0.94 (0.03)	0.87 (0.04)	0.85 (0.02)	0.63 (0.06)	20
CD degeneration or necrosis	0.54 (0.04)	0.57 (0.04)	0.56 (0.06)	0.58 (0.04)	0.92 (0.02)	0.72 (0.04)	0.93 (0.02)	0.76 (0.03)	**56**
CD deg/nec with no regen	0.64 (0.05)	0.60 (0.07)	0.63 (0.11)	0.52 (0.06)	0.88 (0.03)	0.72 (0.06)	0.85 (0.06)	0.76 (0.06)	19
CD deg/nec with regen	0.51 (0.05)	0.55 (0.05)	0.52 (0.07)	0.61 (0.04)	0.90 (0.02)	0.70 (0.05)	0.92 (0.02)	0.73 (0.04)	37
Medullary tubular regeneration/basophilia	0.59 (0.07)	0.66 (0.07)	0.81 (0.07)	0.51 (0.06)	0.77 (0.05)	0.54 (0.06)	0.84 (0.07)	0.77 (0.04)	14*
Regeneration NOS with no degeneration	0.52 (0.05)	0.58 (0.05)	0.57 (0.06)	0.52 (0.05)	0.52 (0.06)	0.56 (0.05)	0.53 (0.07)	0.56 (0.05)	26
Intratubular casts, granular, cortex	0.62 (0.09)	0.59 (0.08)	0.54 (0.11)	0.71 (0.08)	0.79 (0.07)	0.56 (0.11)	0.51 (0.10)	0.64 (0.09)	12
Intratubular casts, hyaline, cortex	0.79 (0.06)	0.82 (0.05)	0.70 (0.06)	0.78 (0.05)	0.69 (0.07)	0.76 (0.06)	0.71 (0.05)	0.83 (0.05)	32
Inflammation, interstitial, chronic, cortex	0.63 (0.04)	0.64 (0.04)	0.59 (0.04)	0.63 (0.04)	0.62 (0.04)	0.67 (0.04)	0.56 (0.04)	0.61 (0.04)	68

* Animals positive for medullary regeneration/basophilia were observed only in the Han Wistar NPAA study

Figure 2: Reviewer's graph of the AUC_{ROC} values for pooled data by selected diagnosis



Medullary tubular regeneration/basophilia was induced by NPAA only in the Han Wistar strain.

Because Table 7 excluded animals for which a biomarker value was missing, the BQRT requested that the calculations be repeated with all available animals. These results are provided in Table 8 in which the total numbers of positive and negative animals are derived from Table 28. The maximum difference in AUC_{ROC} values between Table 7 and Table 8 was 0.04 for three biomarker/pathology combinations. The AUC_{ROC} values decreased in Table 8 for clusterin in CD regeneration/basophilia and RPA-1 in interstial inflammation, but increased in Table 8 for NAG in CD degeneration/necrosis with regeneration. A side by side version of Tables 7 and 8 by pathology is located in Appendix 6biii. Based on the results in Table 8, HESI concluded that the results were similar to the results obtained with the data set in Table 7 with exclusions. Thus, the statistical analysis can be based on the dataset with exclusions.

Table 8 - AUC_{ROC} estimates (standard error) based on all available animals*

Yellow highlights the principal pathologies claimed. Red text indicates a pathology for which the number of positive animals is <20.

Pathology	BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	Neg.	Pos.
PT degeneration or necrosis	0.63 (0.04)	0.64 (0.04)	0.68 (0.03)	0.72 (0.04)	0.85 (0.03)	0.77 (0.03)	0.60 (0.03)	0.69 (0.03)	340	99
PT deg/nec with no regen	0.52 (0.05)	0.54 (0.04)	0.52 (0.04)	0.53 (0.05)	0.75 (0.04)	0.62 (0.04)	0.56 (0.04)	0.55 (0.04)	382	57
PT deg/nec with regen	0.79 (0.05)	0.82 (0.05)	0.86 (0.02)	0.89 (0.03)	0.87 (0.03)	0.88 (0.03)	0.75 (0.03)	0.94 (0.02)	397	42
PT regeneration/basophilia	0.61 (0.03)	0.63 (0.03)	0.62 (0.04)	0.63 (0.03)	0.52 (0.04)	0.59 (0.04)	0.74 (0.03)	0.79 (0.03)	330	109
DT degeneration or necrosis	0.51 (0.06)	0.66 (0.04)	0.89 (0.03)	0.72 (0.05)	0.94 (0.03)	0.87 (0.04)	0.84 (0.02)	0.63 (0.06)	419	20
CD degeneration or necrosis	0.56 (0.04)	0.59 (0.04)	0.59 (0.06)	0.59 (0.04)	0.90 (0.02)	0.72 (0.04)	0.93 (0.02)	0.74 (0.04)	377	62
CD deg/nec with no PT injury or regen	0.64 (0.05)	0.59 (0.06)	0.63 (0.10)	0.51 (0.06)	0.88 (0.03)	0.72 (0.06)	0.85 (0.06)	0.76 (0.05)	419	20
CD deg/nec and regen with no PT injury	0.51 (0.05)	0.58 (0.05)	0.56 (0.07)	0.62 (0.04)	0.87 (0.03)	0.69 (0.05)	0.93 (0.01)	0.70 (0.04)	397	42
CD regeneration/basophilia	0.61 (0.07)	0.68 (0.06)	0.82 (0.06)	0.53 (0.06)	0.74 (0.05)	0.54 (0.06)	0.86 (0.06)	0.73 (0.05)	422	17
Regeneration NOS with no degeneration	0.53 (0.05)	0.55 (0.05)	0.57 (0.06)	0.50 (0.04)	0.51 (0.05)	0.55 (0.05)	0.56 (0.06)	0.54 (0.05)	404	35
Intratubular casts, granular, cortex	0.61 (0.09)	0.58 (0.08)	0.54 (0.11)	0.71 (0.08)	0.79 (0.07)	0.56 (0.11)	0.51 (0.10)	0.65 (0.09)	427	12
Intratubular casts, hyaline, cortex	0.79 (0.05)	0.82 (0.05)	0.70 (0.05)	0.79 (0.05)	0.70 (0.07)	0.76 (0.06)	0.71 (0.05)	0.84 (0.04)	406	33
Inflammation, interstitial, chronic, cortex	0.64 (0.04)	0.66 (0.04)	0.58 (0.04)	0.62 (0.04)	0.63 (0.04)	0.65 (0.04)	0.53 (0.03)	0.58 (0.04)	356	83

*Except high dose gentamicin Sprague Dawley animals;
Red text indicates a pathology for which the number of positive animals is <20.

AUC_{ROC} values were also calculated for the two rat strains separately as shown in Tables 9 and 10 below. Although the number of positives animals for the three major pathologies (PT, DT, CD) was ≥20 animals for each strain, the number of positives animals for some other pathologies was <20.

Table 9: HESI summary table of AUC_{ROC} estimates (standard error) for Sprague-Dawley animals
Yellow highlights the principal pathologies claimed. Red text indicates a pathology for which the number of positive animals is <20.

Pathology	BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	# pos.
PT degeneration or necrosis	0.78 (0.05)	0.75 (0.06)	0.93 (0.02)	0.86 (0.04)	0.83 (0.04)	0.91 (0.03)	0.80 (0.04)	0.88 (0.04)	**34**
PT deg/nec with no regen	0.67 (0.09)	0.54 (0.12)	0.72 (0.05)	0.70 (0.08)	0.69 (0.11)	0.72 (0.07)	0.60 (0.05)	0.60 (0.08)	9
PT deg/nec with regen	0.79 (0.06)	0.84 (0.06)	0.97 (0.02)	0.88 (0.05)	0.85 (0.04)	0.94 (0.04)	0.84 (0.04)	0.95 (0.03)	25
Cortical tubular regeneration/basophilia	0.63 (0.05)	0.74 (0.04)	0.56 (0.06)	0.59 (0.05)	0.53 (0.06)	0.56 (0.06)	0.92 (0.03)	0.84 (0.04)	**51**
DT degeneration or necrosis	0.53 (0.07)	0.59 (0.05)	0.87 (0.04)	0.74 (0.05)	0.94 (0.03)	0.85 (0.04)	0.93 (0.02)	0.67 (0.06)	20
CD degeneration or necrosis	0.57 (0.06)	0.59 (0.05)	0.91 (0.03)	0.68 (0.06)	0.96 (0.02)	0.89 (0.03)	0.89 (0.03)	0.65 (0.05)	**25**
CD deg/nec with no regen	0.60 (0.09)	0.52 (0.11)	0.94 (0.02)	0.61 (0.14)	0.91 (0.02)	0.90 (0.02)	0.79 (0.09)	0.65 (0.09)	6
CD deg/nec with regen	0.63 (0.06)	0.62 (0.05)	0.87 (0.04)	0.69 (0.06)	0.94 (0.03)	0.86 (0.04)	0.90 (0.03)	0.64 (0.06)	19
Regeneration NOS with no degeneration	0.62 (0.11)	0.53 (0.08)	0.70 (0.11)	0.74 (0.07)	0.65 (0.13)	0.71 (0.11)	0.70 (0.12)	0.63 (0.09)	7
Intratubular casts, granular, cortex	0.93 (0.03)	0.77 (0.14)	0.98 (0.01)	0.89 (0.06)	0.92 (0.03)	0.96 (0.01)	0.86 (0.03)	0.96 (0.02)	5
Intratubular casts, hyaline, cortex	0.69 (0.09)	0.76 (0.08)	0.72 (0.09)	0.69 (0.08)	0.55 (0.10)	0.69 (0.09)	0.81 (0.07)	0.86 (0.05)	16
Inflammation, interstitial, chronic, cortex	0.64 (0.04)	0.62 (0.05)	0.70 (0.04)	0.68 (0.04)	0.65 (0.04)	0.72 (0.04)	0.63 (0.04)	0.65 (0.04)	62

Table 10: HESI Summary table of AUC_{ROC} estimates (standard error) for Wistar animals
Yellow highlights the principal pathologies claimed. Red text indicates a pathology for which the number of positive animals is <20.

Pathology	BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	# pos.
PT degeneration or necrosis	0.53 (0.05)	0.59 (0.05)	0.54 (0.04)	0.64 (0.05)	0.85 (0.03)	0.67 (0.05)	0.54 (0.04)	0.54 (0.05)	**55**
PT deg/nec with no regen	0.58 (0.06)	0.53 (0.05)	0.67 (0.04)	0.52 (0.06)	0.73 (0.05)	0.59 (0.05)	0.62 (0.04)	0.66 (0.04)	39
PT deg/nec with regen	0.76 (0.09)	0.79 (0.08)	0.74 (0.04)	0.92 (0.04)	0.93 (0.03)	0.75 (0.06)	0.65 (0.06)	0.93 (0.02)	16
Cortical tubular regeneration/basophilia	0.61 (0.05)	0.51 (0.06)	0.68 (0.05)	0.66 (0.05)	0.59 (0.05)	0.64 (0.04)	0.63 (0.05)	0.79 (0.04)	**46**
CD degeneration or necrosis	0.65 (0.05)	0.74 (0.05)	0.94 (0.03)	0.53 (0.05)	0.89 (0.03)	0.57 (0.06)	0.96 (0.03)	0.86 (0.03)	**31**
CD deg/nec with no regen	0.68 (0.05)	0.62 (0.09)	0.88 (0.07)	0.51 (0.07)	0.87 (0.04)	0.65 (0.09)	0.87 (0.07)	0.80 (0.07)	13
CD deg/nec with regen	0.60 (0.07)	0.79 (0.06)	0.92 (0.02)	0.55 (0.06)	0.84 (0.04)	0.50 (0.07)	0.95 (0.01)	0.84 (0.03)	18
Medullary tubular regeneration/basophilia	0.63 (0.07)	0.61 (0.08)	0.76 (0.08)	0.54 (0.06)	0.79 (0.05)	0.56 (0.07)	0.82 (0.07)	0.76 (0.05)	14
Regeneration NOS with no degeneration	0.54 (0.06)	0.56 (0.07)	0.58 (0.07)	0.52 (0.05)	0.51 (0.06)	0.50 (0.06)	0.63 (0.08)	0.51 (0.06)	19
Intratubular casts, granular, cortex	0.56 (0.07)	0.54 (0.07)	0.80 (0.06)	0.57 (0.11)	0.69 (0.10)	0.79 (0.06)	0.73 (0.09)	0.60 (0.11)	7
Intratubular casts, hyaline, cortex	0.88 (0.06)	0.88 (0.06)	0.66 (0.06)	0.88 (0.07)	0.84 (0.07)	0.86 (0.06)	0.62 (0.07)	0.81 (0.08)	16
Inflammation, interstitial, chronic, cortex	0.58 (0.18)	0.50 (0.16)	0.62 (0.10)	0.74 (0.14)	0.83 (0.08)	0.51 (0.12)	0.61 (0.14)	0.71 (0.12)	6

Red text indicates the pathologies for which the number of positive animals was <20.

HESI concluded that the similarity of the AUC_{ROC} values in the two rat strains (see Table 11 below) supported pooling the data for further statistical analysis and consequently tests for statistically significant differences in biomarker performance were performed utilizing the pooled data. This comparison was made only for three pathologies.

Table 11 - Sponsor's table comparing AUC_{ROC} estimates for Sprague-Dawley and Han-Wistar animals for selected pathologies and biomarkers

		Strain	
Pathology	**Biomarker**	**Sprague-Dawley**	**Han-Wistar**
PT degeneration or necrosis	α-GST	0.83	0.85
CD degeneration or necrosis	RPA-1	0.89	0.96
Cortical tubular regeneration/basophilia	clusterin	0.84	0.79

A side-by-side comparison of biomarker AUC_{ROC} values for the major pathologies shows differences in the relative (comparison to BUN and sCr) and absolute performance of some biomarkers in the two strains.(see Figure 3 and Table 12) and suggests that analyses of biomarker performance should not be based on pooled results. For PT degeneration/necrosis in the Wistar rat, the AUC_{ROC} value for α-GST is notably greater than the AUC_{ROC} value for all other tested biomarkers; however, for PT degeneration/necrosis in the Sprague Dawley rat, the AUC_{ROC} value for α-GST is similar to the AUC_{ROC} results for the other biomarkers and, in comparison with some of these biomarkers, appears to be lower. For cortical tubular regeneration/basophilia in the Wistar rat, the AUC_{ROC} value for clusterin appears to be greater than the AUC_{ROC} value for all other biomarkers; however, for cortical tubular regeneration/basophilia in the Sprague Dawley rat, the AUC_{ROC} value for RPA-1 is greater than the AUC_{ROC} value for clusterin. For CD degeneration/necrosis in the Wistar rat, RPA-1 appears to outperform the other biomarkers (with possibly the exception of NAG); however, for CD degeneration/necrosis in the Sprague Dawley rat, the AUC_{ROC} values for α-GST appear to be greater than that of RPA-1. Whether such differences represent true differences between strains in the performance of these biomarkers, differences in the type and severity of pathology-induced by the studied nephrotoxicants or simply the inaccuracy of these point estimates is unclear. Nonetheless, these differences raise concern about the pooling of data from different rat strains.

Figure 3: Reviewer's graph – Comparison of AUC_{ROC} values in the Sprague Dawley (SD) and Han Wistar (HW) rat strains for selected diagnoses

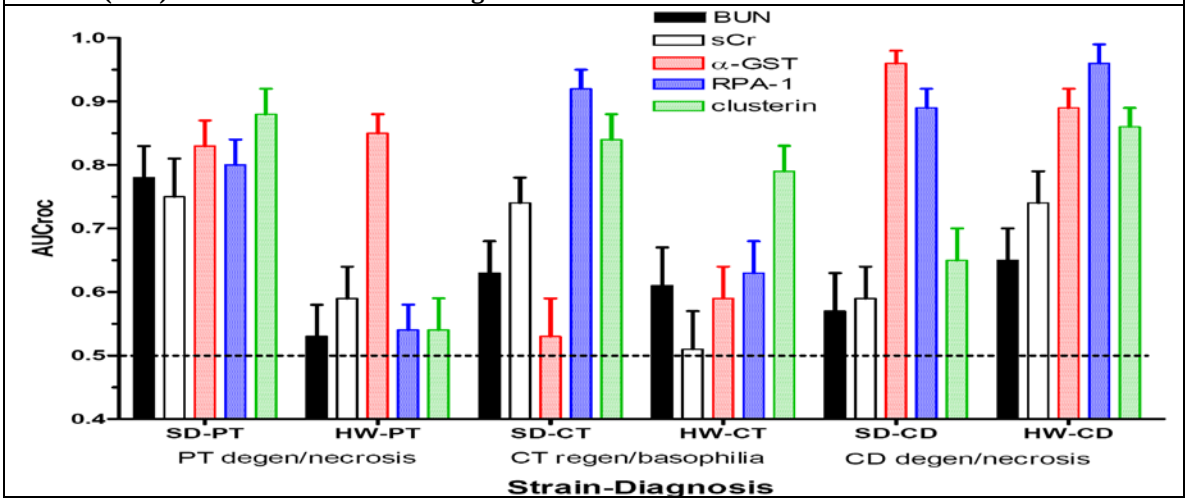


Table 12 - Reviewer's compilation comparing AUC_{ROC} values for the claimed pathologies in the two strains and in the pooled sample (Only the biomarkers shown in Table 11 are presented.)

		BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	Neg	Pos
PT degeneration or necrosis	SD	0.78 (0.05)	0.75 (0.06)	0.93 (0.02)	0.86 (0.04)	0.83 (0.04)	0.91 (0.03)	0.80 (0.04)	0.88 (0.04)	170	34
	HW	0.53 (0.05)	0.59 (0.05)	0.54 (0.04)	0.64 (0.05)	0.85 (0.03)	0.67 (0.05)	0.54 (0.04)	0.54 (0.05)	137	55
	Pool	0.62 (0.04)	0.62 (0.04)	0.69 (0.03)	0.73 (0.04)	0.84 (0.03)	0.77 (0.03)	0.59 (0.03)	0.69 (0.03)	307	89
Cortical tubular regeneration/ basophilia	SD	0.63 (0.05)	0.74 (0.04)	0.56 (0.06)	0.59 (0.05)	0.53 (0.06)	0.56 (0.06)	0.92 (0.03)	0.84 (0.04)	153	51
	HW	0.61 (0.05)	0.51 (0.06)	0.68 (0.05)	0.66 (0.05)	0.59 (0.05)	0.64 (0.04)	0.63 (0.05)	0.79 (0.04)	176	46
	Pool	0.62 (0.04)	0.64 (0.04)	0.63 (0.04)	0.63 (0.03)	0.52 (0.04)	0.59 (0.04)	0.77 (0.03)	0.81 (0.03)	299	97
CD degeneration or necrosis	SD	0.57 (0.06)	0.59 (0.05)	0.91 (0.03)	0.68 (0.06)	0.96 (0.02)	0.89 (0.03)	0.89 (0.03)	0.65 (0.05)	179	25
	HW	0.65 (0.05)	0.74 (0.05)	0.94 (0.03)	0.53 (0.05)	0.89 (0.03)	0.57 (0.06)	0.96 (0.03)	0.86 (0.03)	161	31
	Pool	0.54 (0.04)	0.57 (0.04)	0.56 (0.06)	0.58 (0.04)	0.92 (0.02)	0.72 (0.04)	0.93 (0.02)	0.76 (0.03)	340	56

e. Statistical Analysis

i. Superior diagnostic value

Tables 13 through 18 below summarize the pair-wise statistical analysis performed by HESI using the method of Delong et al (1988) to support the claim that a particular biomarker *outperforms BUN and sCr* and focus on the claimed pathologies. The pairwise statistical analyses for all pathologies are located in Appendix 6biv. These calculations were performed with the pooled data set that used data from both strains and excluded those animals for which a biomarker value was missing. The AUC_{ROC} value for α-GST was significantly greater than those for BUN and sCr for PT degeneration/necrosis and CD degeneration/necrosis, although the direction of the biomarker change was different. Although the AUC_{ROC} value for μ-GST was significantly greater than that BUN and sCr for PT degeneration/necrosis, the AUC_{ROC} value μ-GST was less than that for α-GST. The AUC_{ROC} value for RPA-1 was significantly greater than that for BUN and sCr for CD degeneration/necrosis. The AUC_{ROC} value for clusterin was significantly greater than that for BUN and sCr for cortical tubular regeneration/basophilia. The AUC_{ROC} value for NAG was significantly greater than that for BUN and sCr for distal tubular degeneration/necrosis. Consistent with the PSTC results, AUC_{ROC} value for total urinary protein was not greater than that of BUN and sCr for the three pathologies evaluated here.

Table 13: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for α-GST versus reference biomarkers for the claimed pathologies

	α-GST		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted	**307**	**89**
PT degeneration or necrosis	0.84	+	BUN	0.62	0.22	<.001	*<.001*		
	0.84	+	SCr	0.62	0.22	<.001	*<.001*		
	0.84	+	NAG	0.69	0.15	<.001	*<.001*		
	0.84	+	Protein	0.73	0.11	0.007	0.212		
Cortical tubular regeneration/basophilia	0.52	+	BUN	0.62	-0.10	0.028	0.642	**299**	**97**
	0.52	+	SCr	0.64	-0.12	0.012	0.308		
	0.52	+	NAG	0.63	-0.10	0.009	0.261		
	0.52	+	Protein	0.63	-0.11	<.001	**0.019**		
CD degeneration or necrosis	0.92	-	BUN	0.54	0.38	<.001	*<.001*	**340**	**56**
	0.92	-	SCr	0.57	0.35	<.001	*<.001*		
	0.92	-	NAG	0.56	0.36	<.001	*<.001*		
	0.92	-	Protein	0.58	0.34	<.001	*<.001*		

Table 14: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for RPA-1 versus reference biomarkers for the claimed pathologies

	RPA		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.59	-	BUN	0.62	-0.02	0.560	0.918	307	89
	0.59	-	SCr	0.62	-0.03	0.487	0.918		
	0.59	-	NAG	0.69	-0.09	0.001	*0.039*		
	0.59	-	Protein	0.73	-0.13	0.003	0.092		
Cortical tubular regeneration/basophilia	0.77	+	BUN	0.62	0.14	<.001	*0.031*	299	97
	0.77	+	SCr	0.64	0.12	0.005	0.165		
	0.77	+	NAG	0.63	0.14	0.002	0.057		
	0.77	+	Protein	0.63	0.13	0.004	0.140		
CD degeneration or necrosis	0.93	+	BUN	0.54	0.38	<.001	*<.001*	340	56
	0.93	+	SCr	0.57	0.35	<.001	*<.001*		
	0.93	+	NAG	0.56	0.36	<.001	*<.001*		
	0.93	+	Protein	0.58	0.35	<.001	*<.001*		

Table 15: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for clusterin versus reference biomarkers for the claimed pathologies

	Clusterin		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.69	+	BUN	0.62	0.07	0.063	0.900	307	89
	0.69	+	SCr	0.62	0.07	0.053	0.900		
	0.69	+	NAG	0.69	0.01	0.840	0.900		
	0.69	+	Protein	0.73	-0.03	0.358	0.900		
Cortical tubular regeneration/basophilia	0.81	+	BUN	0.62	0.19	<.001	*<.001*	299	97
	0.81	+	SCr	0.64	0.17	<.001	*<.001*		
	0.81	+	NAG	0.63	0.19	<.001	*<.001*		
	0.81	+	Protein	0.63	0.18	<.001	*<.001*		
CD degeneration or necrosis	0.76	+	BUN	0.54	0.21	<.001	*<.001*	340	56
	0.76	+	SCr	0.57	0.19	0.001	0.053		
	0.76	+	NAG	0.56	0.20	<.001	*0.038*		
	0.76	+	Protein	0.58	0.18	<.001	*<.001*		

Table 16: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for µ-GST versus reference biomarkers for the claimed pathologies

	µ-GST		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.77	+	BUN	0.62	0.15	<.001	*0.002*	307	89
	0.77	+	SCr	0.62	0.15	<.001	*<.001*		
	0.77	+	NAG	0.69	0.08	0.007	0.274		
	0.77	+	Protein	0.73	0.05	0.276	0.937		
Cortical tubular regeneration/basophilia	0.59	+	BUN	0.62	-0.03	0.413	0.937	299	97
	0.59	+	SCr	0.64	-0.05	0.235	0.937		
	0.59	+	NAG	0.63	-0.04	0.274	0.937		
	0.59	+	Protein	0.63	-0.04	0.234	0.937		
CD degeneration or necrosis	0.72	-	BUN	0.54	0.18	<.001	*0.007*	340	56
	0.72	-	SCr	0.57	0.15	0.017	0.642		
	0.72	-	NAG	0.56	0.16	0.003	0.132		
	0.72	-	Protein	0.58	0.14	0.001	0.054		

Table 17: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for NAG versus BUN and SCr for the claimed pathologies

	NAG		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.69	+	BUN	0.62	0.07	0.111	0.942	307	89
	0.69	+	SCr	0.62	0.06	0.119	0.942		
Cortical tubular regeneration/basophilia	0.63	+	BUN	0.62	0.01	0.910	0.942	299	97
	0.63	+	SCr	0.64	-0.01	0.801	0.942		
DT degeneration or necrosis	0.89	-	BUN	0.52	0.37	<.001	*<.001*	376	20
	0.89	-	SCr	0.67	0.22	<.001	*0.006*		
CD degeneration or necrosis	0.56	+	BUN	0.54	0.02	0.799	0.942	340	56
	0.56	+	SCr	0.57	-0.01	0.899	0.942		

Table 18: HESI Statistical analysis - Selected pairwise comparisons of AUC_{ROC} for total protein versus BUN and SCr for the claimed pathologies

	Protein		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted	307	89
PT degeneration or necrosis	0.73	+	BUN	0.62	0.11	0.020	0.479		
	0.73	+	SCr	0.62	0.10	0.016	0.392		
Cortical tubular regeneration/basophilia	0.63	+	BUN	0.62	0.01	0.828	1.000	299	97
	0.63	+	SCr	0.64	-0.01	0.824	1.000		
CD degeneration or necrosis	0.58	-	BUN	0.54	0.04	0.465	1.000	340	56
	0.58	-	SCr	0.57	0.01	0.895	1.000		

ii. Incremental diagnostic value

The incremental value of each novel biomarker individually with two combinations of reference biomarkers was assessed by statistical comparison of the AUC_{ROC} using logistic regression models by pathology. Table 19 below summarizes the AUC_{ROC} results by novel biomarker and pathology. The full tables are located in Appendix 6bv.

For some pathologies, enhanced diagnostic performance was observed when the novel biomarker signal in urine is added to that from the combination with either BUN + SCr in serum or NAG + protein in urine. For PT degeneration or necrosis, α-GST adds diagnostic value to either combination of reference markers (serum and urine), while μ-GST and RPA-1 add diagnostic value only to BUN + SCr in serum. For PT degeneration or necrosis in the absence of regeneration, the added value of α-GST was statistically significant only for BUN + SCr in serum. For cortical tubular regeneration/basophilia the added value for clusterin and RPA-1 was statistically significant for both combinations of reference biomarkers. For DT degeneration or necrosis, RPA-1 adds diagnostic value to either combination of reference markers (serum and urine), while α-GST and μ-GST add diagnostic value only to BUN + SCr in serum. For CD degeneration or necrosis, α-GST, μ-GST, RPA-1 and clusterin had significant added value to either combination of reference BMs. Since the added value for α-GST for CD injury is associated with a consistent decrease of α-GST in urine in response to CD injury, additional studies are needed using compounds that selectively damage the CD as well as compounds that damage more than the CD.

Despite finding that some combinations of the novel markers with traditional markers enhanced diagnostic performance of the traditional markers for a given diagnosis, HESI concluded that the magnitude of the added value was minimal. The HESI conclusion that combination of traditional markers with the novel urinary markers provided minimal or no improvement in diagnostic accuracy relative to that of the novel markers alone was based on comparison between AUC_{ROC} value for the combination of reference markers with novel biomarker compared to the AUC_{ROC} value for the novel marker alone. Contrary to the HESI conclusion, the BQRT concludes that some combinations of reference markers with novel biomarker provided improvement in diagnostic accuracy. In particular, the combination of RPA-1 with BUN + SCr had an AUC_{ROC} value for PT degeneration/necrosis that was greater than the AUC_{ROC} of RPA-1 alone. The combination of RPA-1 with NAG + protein had an AUC_{ROC} value for DT degeneration/necrosis that was greater than the AUC_{ROC} of RPA-1 alone. Also, the

combinations of either α-GST or μ-GST with NAG + protein had an AUC_{ROC} value for CD degeneration/necrosis that was greater than the AUC_{ROC} of α-GST or μ-GST alone. Furthermore, these novel biomarkers have not yet been qualified previously in any context and certainly not by themselves in the absence of reference biomarkers. Therefore, these results demonstrate that α-GST, μ-GST, RPA-1 or clusterin add value either to BUN+sCr or to NAG+protein for a given diagnosis.

Table 19: Reviewer's compilation of HESI Statistical analysis - Summary of incremental analysis from Appendix 21 initial submission

		AUC_{ROC} values					
Pathology	Reference Markers	Reference Alone	Reference + α-GST	Reference + μ-GST	Reference + RPA	Reference + Clusterin	Statistically significant. (AUC for novel BM alone from Table 7)
PT degeneration or necrosis	BUN+SCr	0.63	0.85	0.73	0.66	0.62	α-GST* (0.84), μ-GST† (0.77), **RPA-1† (0.59)**
	NAG+Protein	0.72	0.84	0.77	0.75	0.73	
PT deg/nec with no regen	BUN+SCr	0.58	0.72	0.60	0.62	0.59	α-GST† (0.74)
	NAG+Protein	0.52	0.64	0.55	0.56	0.57	
PT deg/nec with regen	BUN+SCr	0.83	0.85	0.87	0.83	0.84	
	NAG+Protein	0.90	0.91	0.93	0.93	0.91	
Cortical tubular regeneration/basophilia	BUN+SCr	0.65	0.65	0.64	0.75	0.75	RPA-1* (0.77), clusterin* (0.81)
	NAG+Protein	0.66	0.66	0.66	0.75	0.76	
DT degeneration or necrosis	BUN+SCr	0.56	0.95	0.89	0.69	0.58	α-GST† (0.94), μ-GST† (0.87), **RPA-1* (0.85)**
	NAG+Protein	0.89	0.97	0.94	0.96	0.90	
CD degeneration or necrosis	BUN+SCr	0.61	0.93	0.75	0.93	0.71	**α-GST* (0.92)**, RPA-1* (0.93), **μ-GST* (0.72)**, clusterin* (0.76)
	NAG+Protein	0.63	0.96	0.80	0.94	0.77	
CD deg/nec with no regen	BUN+SCr	0.68	0.89	0.73	0.85	0.75	α-GST† (0.88), RPA-1† (0.85)
	NAG+Protein	0.63	0.93	0.79	0.85	0.67	
CD deg/nec with regen	BUN+SCr	0.57	0.90	0.71	0.91	0.67	**α-GST* (0.90),** RPA-1* (0.92), **μ-GST‡ (0.70)**
	NAG+Protein	0.65	0.92	0.80	0.91	0.77	
Medullary tubular regeneration/basophilia	BUN+SCr	0.77	0.82	0.79	0.84	0.77	
	NAG+Protein	0.83	0.86	0.85	0.84	0.82	
Regeneration NOS with no degeneration	BUN+SCr	0.60	0.60	0.63	0.61	0.61	
	NAG+Protein	0.55	0.55	0.56	0.58	0.53	
Intratubular casts, granular, cortex	BUN+SCr	0.57	0.71	0.59	0.52	0.67	
	NAG+Protein	0.59	0.60	0.61	0.73	0.52	
Intratubular casts, hyaline, cortex	BUN+SCr	0.82	0.82	0.83	0.83	0.82	
	NAG+Protein	0.77	0.75	0.80	0.78	0.82	
Inflammation, interstitial, chronic, cortex	BUN+SCr	0.60	0.59	0.68	0.61	0.60	
	NAG+Protein	0.60	0.60	0.69	0.66	0.65	

Statistical significance with both combinations, BUN+SCr and NAG+Protein; † Statistical significance with only BUN+SCr, ‡ Statistical significance with only NAG+Protein,
Bold text indicates biomarkers for which the AUC_{ROC} value for the combination was greater than that for the biomarker alone.

HESI stated that the exploration of combinations of novel biomarkers will be the focus of a future investigation.

f. **Individual Animal Data**

Individual animal data were provided separately in Excel format by HESI. This dataset tabulates histopathology, clinical chemistry, urinalysis, and biomarker data for individual animals by study.

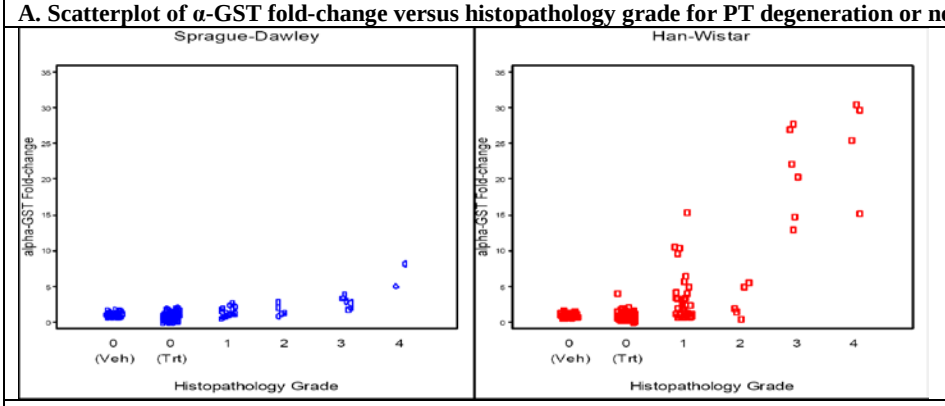
i. Individual results by histopathology grade

HESI provided scatterplots of individual animals for novel biomarkers versus histopathology grade, for selected pathologies. Animals with histopathology grade = 0 were stratified by vehicle (Veh) or toxicant (Trt) dosing. These plots (Figure 4) illustrate the correlation of magnitude of biomarker response and severity of injury as measured by histopathology grade. In general the magnitude of the biomarker response to the same

severity of injury is greater in the Wistar than the Sprague Dawley rat. Box plots of these data were also provided (see Appendix 6bvi).

Figure 4: Sponsor's figures – scatterplots from December submission

A. Scatterplot of α-GST fold-change versus histopathology grade for PT degeneration or necrosis



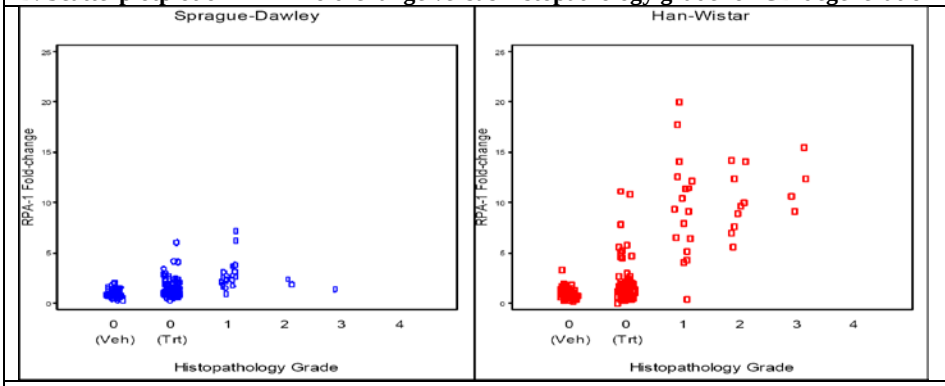
PT degeneration or necrosis

Sprague Dawley
Neg # Pos
170 34

Han Wistar
Neg # Pos
137 55

Total
Neg # Pos
307 89

B. Scatterplotplot of RPA-1 fold-change versus histopathology grade for CD degeneration or necrosis



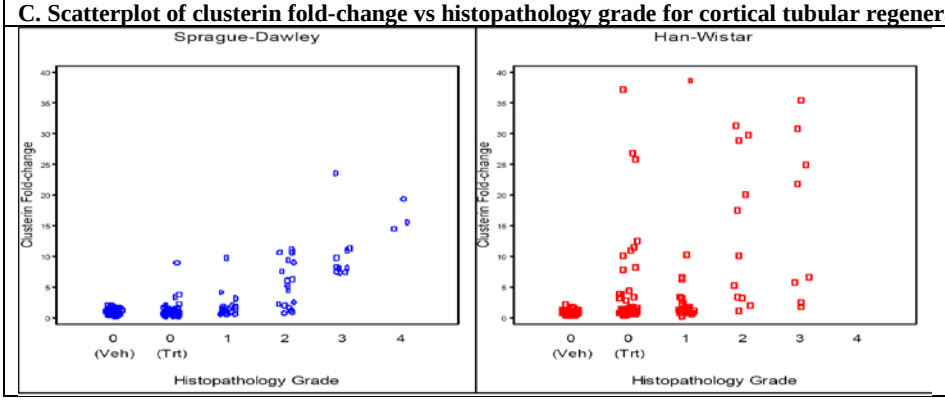
CD degeneration or necrosis

Sprague Dawley
Neg # Pos
179 25

Han Wistar
Neg # Pos
161 31

Total
Neg # Pos
340 56

C. Scatterplot of clusterin fold-change vs histopathology grade for cortical tubular regeneration/basophilia



Cortical tubular regeneration/basophilia

Sprague Dawley
Neg # Pos
153 51

Han Wistar
Neg # Pos
146 46

Total
Neg # Pos
299 97

ii. Individual animal data by study

In individual study reports, biomarker values for individual animals were illustrated as figures. For the major diagnosis in each study, the animals were grouped by study day and within each study day by dose-level. Each animal is represented by its histopathology score and is plotted versus concentration of nephrotoxicant on the x-axis and normalized biomarker values on the y-axis. Each biomarker was plotted separately to show the normalized biomarker values for all the animals in that particular study.

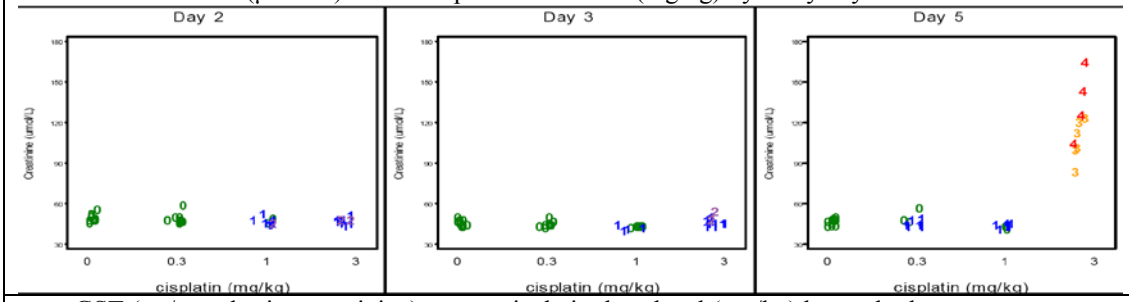
For each of the five studies, the plots of the three proposed novel biomarkers (α-GST, RPA-1 and clusterin) are shown below along with serum creatinine representing a traditional biomarker. Figure 5 is discussed by nephrotoxicant.

In both cisplatin studies (Figure 5A and 5B), animals dosed with 3 mg/kg had high histopathology scores and corresponding high biomarker values on Day 5, the last study day. However, on Day 3, sCr, RPA-1 and clusterin values were negative in animals dosed with 3 mg/kg, while α-GST values were positive. Additionally, on Day 5, α-GST and clusterin values were positive in Wistar rats, while sCr and RPA-1 values were negative. These data indicate that α-GST appears to detect injury earlier than the other biomarkers.

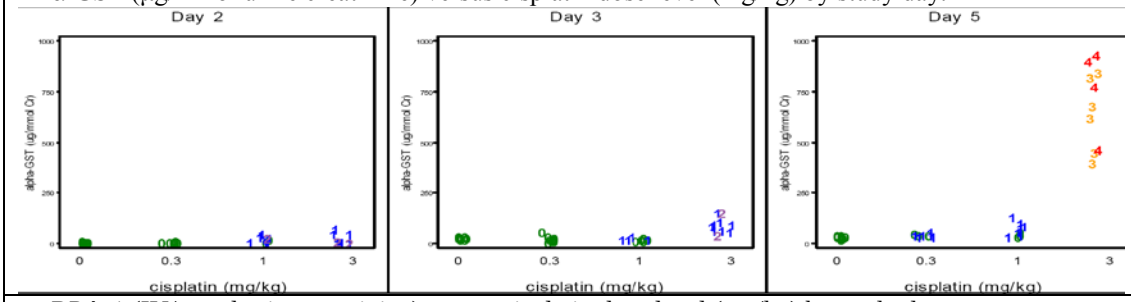
Figure 5: HESI Figures showing biomarker values for individual animals by study

A. Wistar/cisplatin with histopathology grade for tubular degeneration/necrosis of s3 segment

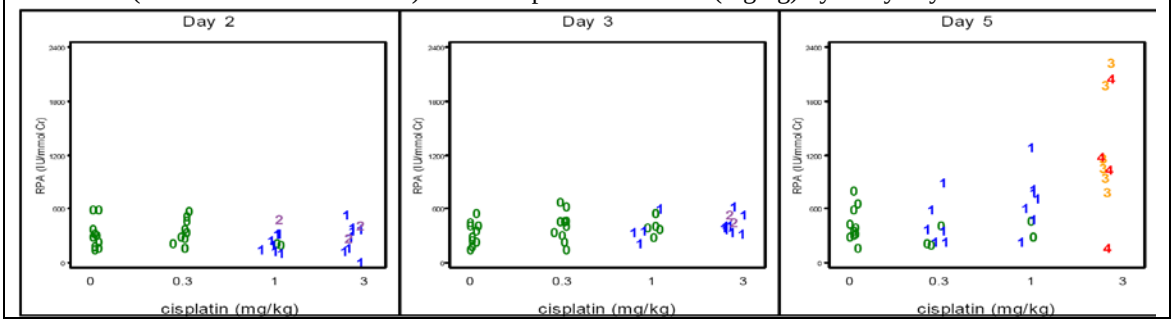
Serum creatinine (µmol/L) versus cisplatin dose level (mg/kg) by study day.



α-GST (µg/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.



RPA-1 (IU/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.



Clusterin (µg/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.

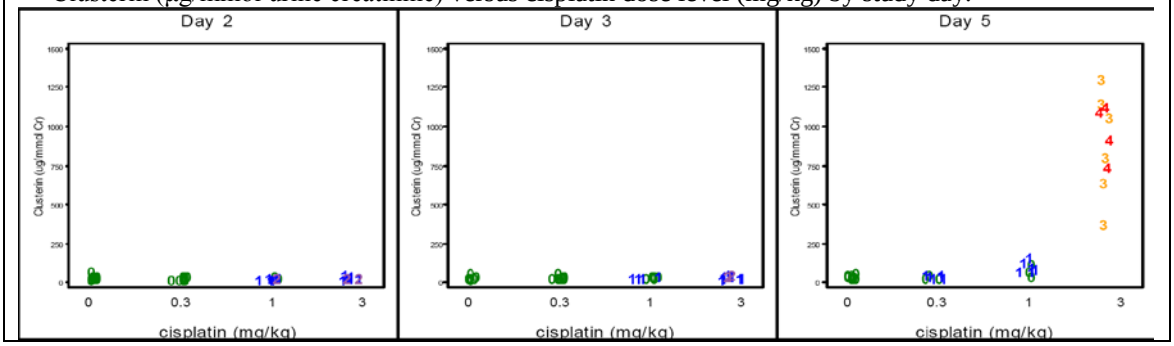
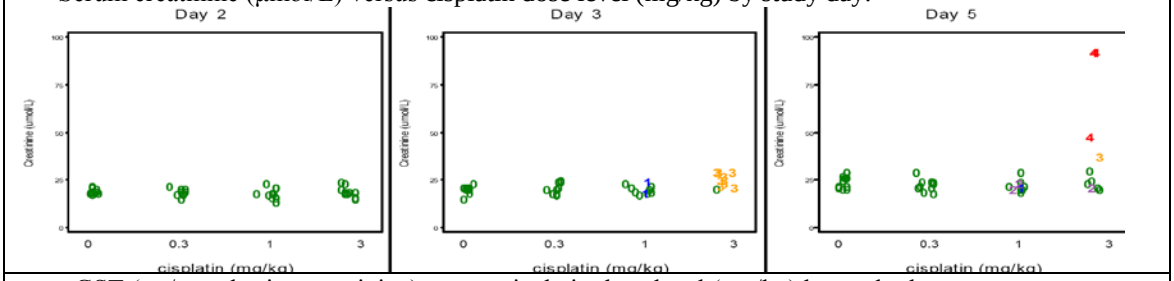


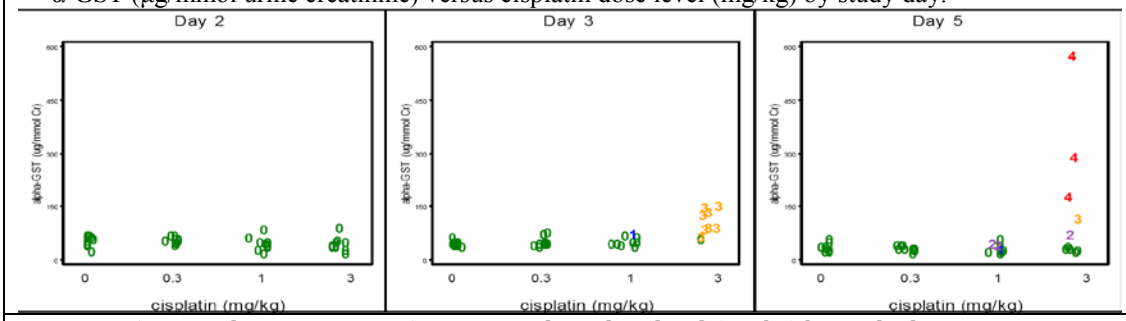
Figure 5 continued: HESI Figures showing biomarker values for individual animals

B. Sprague Dawley/cisplatin with histopathology grade for tubular degeneration/necrosis of s3 segment

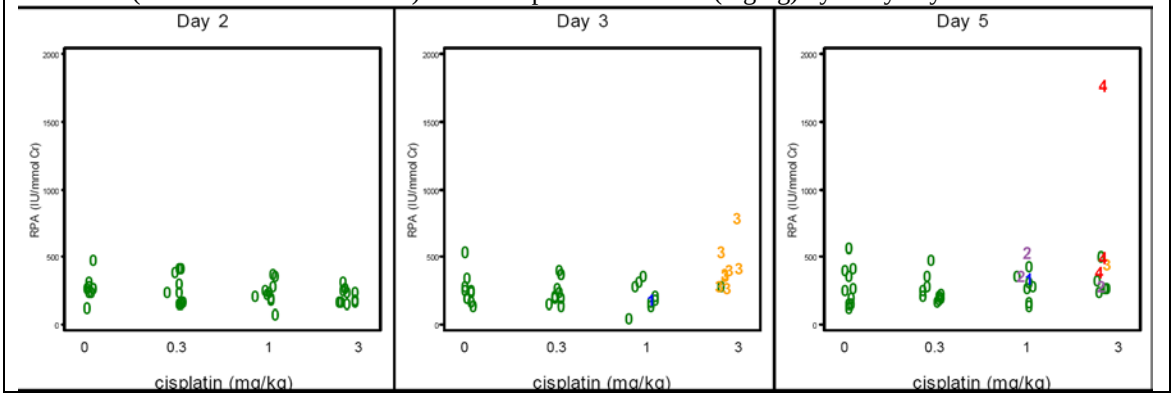
Serum creatinine (µmol/L) versus cisplatin dose level (mg/kg) by study day.



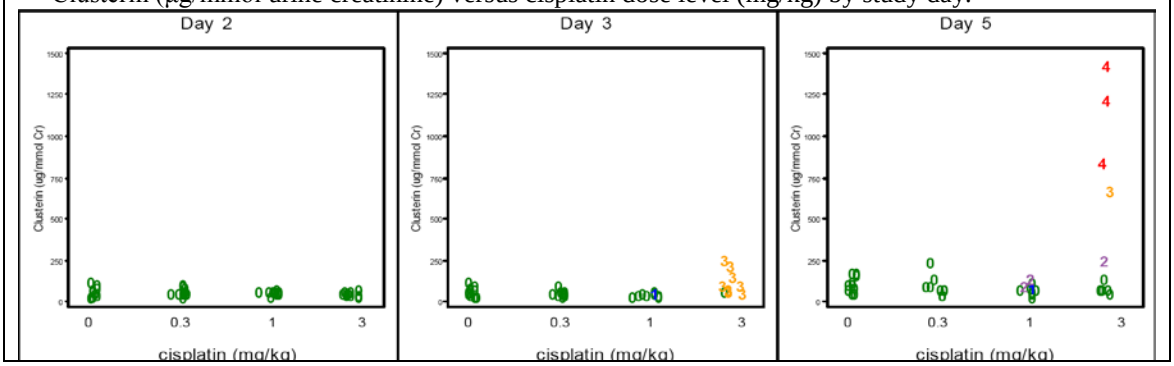
α-GST (µg/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.



RPA-1 (IU/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.



Clusterin (µg/mmol urine creatinine) versus cisplatin dose level (mg/kg) by study day.

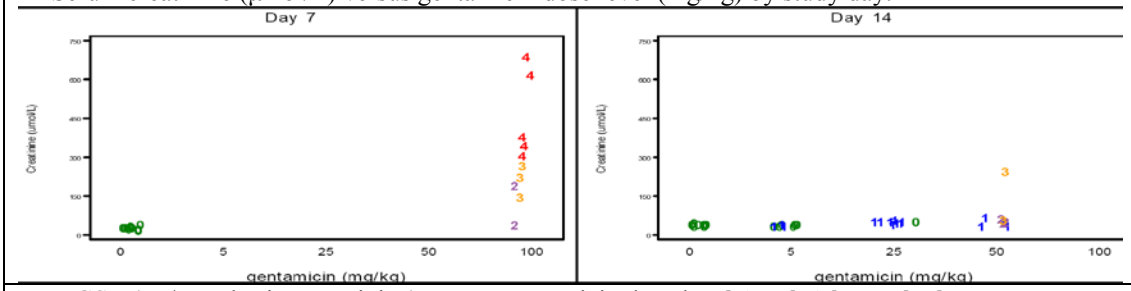


Although histopathology scores and biomarker values were illustrated for all animals in the Sprague Dawley gentamicin study (Figure 5C), the animals dosed at 100 mg/kg were excluded from the ROC analysis. These animals euthanized on Day 7 showed high biomarker values corresponding with high histopathology scores. On day 14, positive clusterin values were observed for animals dosed at 25 and 50 mg/kg, while the values for sCr, α-GST, and RPA-1 were either negative or only slightly positive.

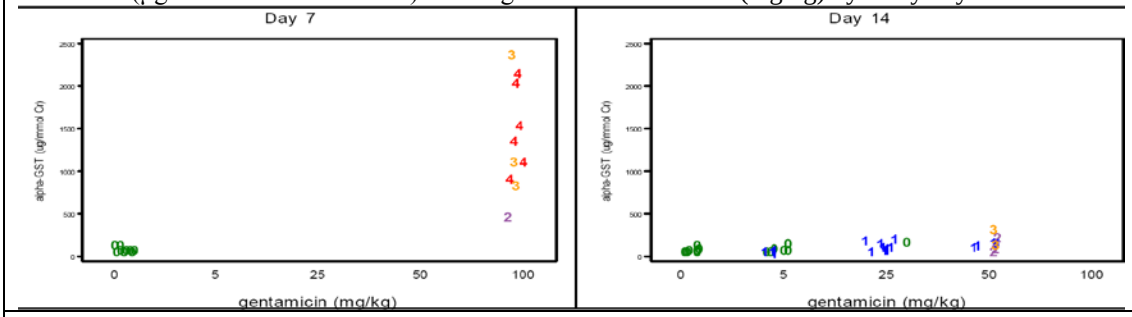
Figure 5 continued: HESI Figures showing biomarker values for individual animals

C. Sprague Dawley/Gentamicin with histopathology grade for proximal tubular degeneration/necrosis

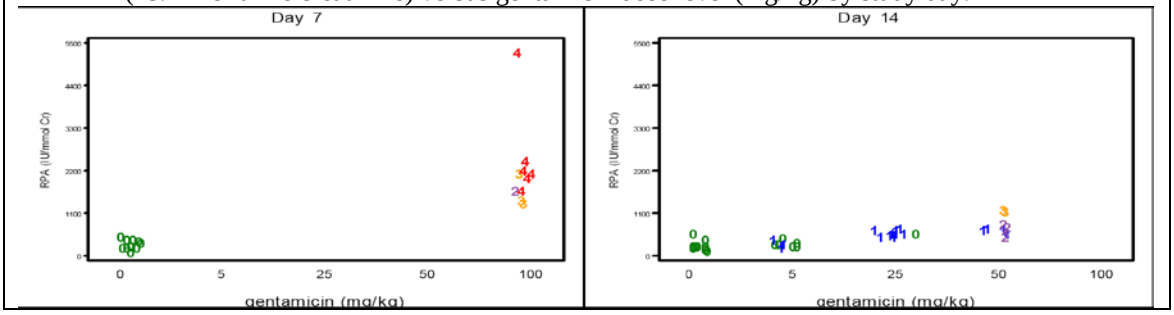
Serum creatinine (µmol/L) versus gentamicin dose level (mg/kg) by study day.



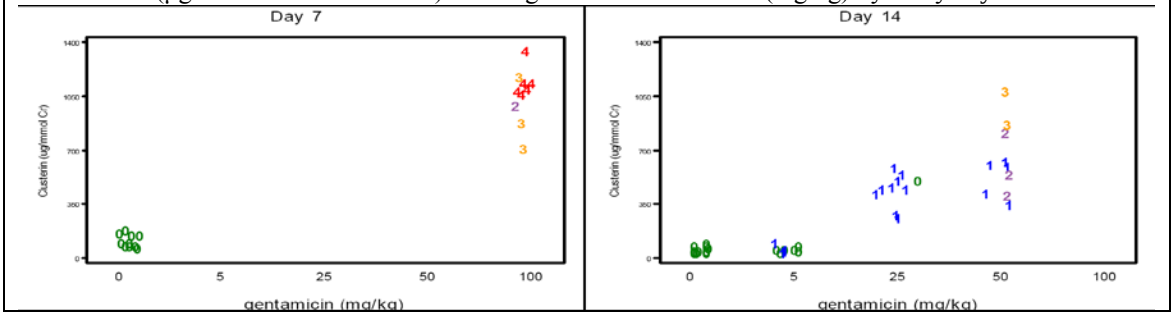
α-GST (µg/mmol urine creatinine) versus gentamicin dose level (mg/kg) by study day.



RPA-1 (IU/mmol urine creatinine) versus gentamicin dose level (mg/kg) by study day.



Clusterin (µg/mmol urine creatinine) versus gentamicin dose level (mg/kg) by study day.



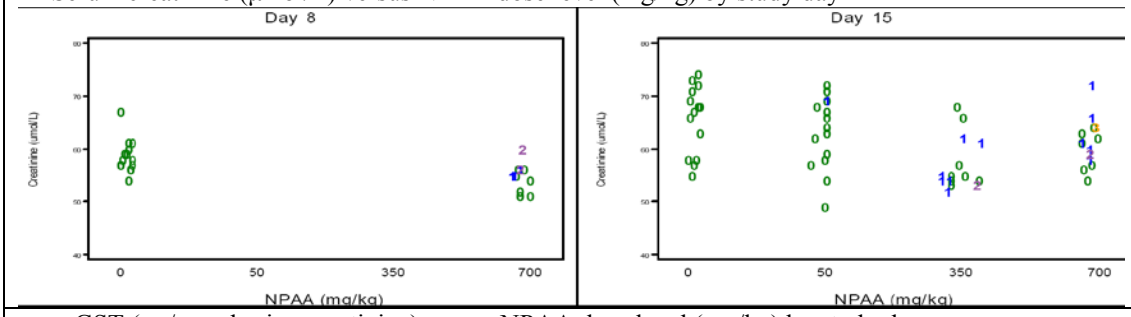
In both NPAA studies (Figure 5D and 5E), the values for sCr are similar to control values, while the values for RPA-1 and clusterin tended to increase with dose of NPAA on Day 14. Importantly, the values for α-GST decreased with increasing dose of NPAA and histopathology score for degeneration/necrosis of papilla collecting duct in the absence of proximal tubular lesions. This decline in α-GST values in the presence of collecting duct injury was unexpected, especially since α-GST is reported to be expressed in the proximal tubules, but not the collecting duct (Sundberg et al 1993). It is unclear how α-GST values respond when a drug induces a combination of tubular and collecting duct injury.

Despite supposedly reporting the maximum histopathology score for degeneration/necrosis of the papilla collecting duct from up to 6 sections, a number of animals at the higher dosages, especially in the Wistar study, have histopathology scores of 0, but positive biomarker values for RPA-1 and clusterin. These cases may represent false positives, the presence of extra-renal organ injury, the presence of focal lesions missed in the tissue sampling or RPA-1 being more sensitive than the histopathology at detecting CD injury.

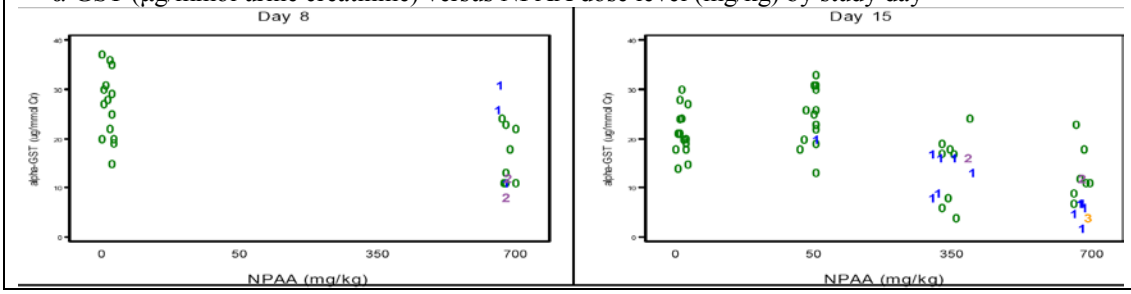
Figure 5 continued: HESI Figures showing biomarker values for individual animals

D. Wistar/NPAA with histopathology grade for degeneration/necrosis of papilla collecting duct.

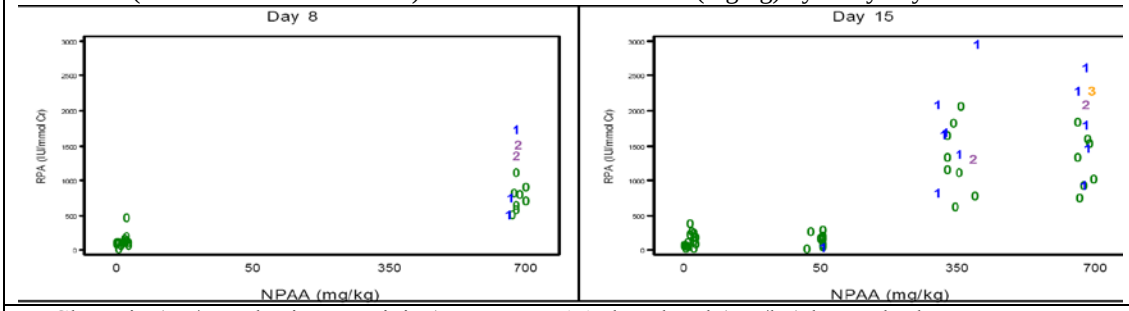
Serum creatinine (µmol/L) versus NPAA dose level (mg/kg) by study day



α-GST (µg/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day



RPA-1 (IU/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day.



Clusterin (µg/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day.

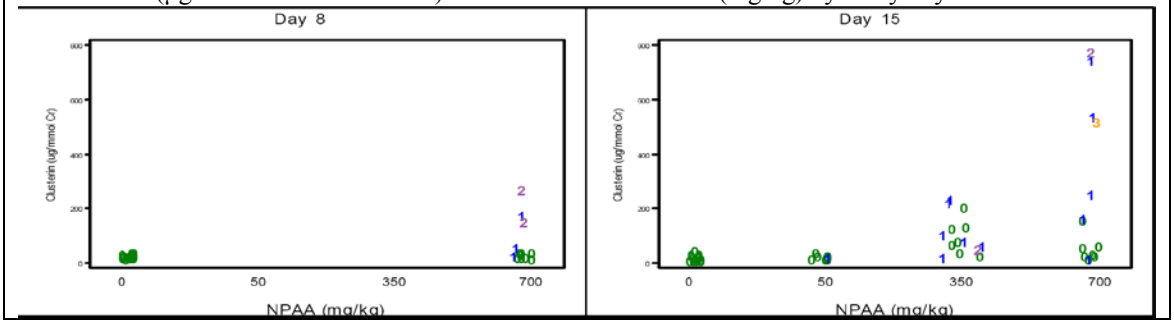
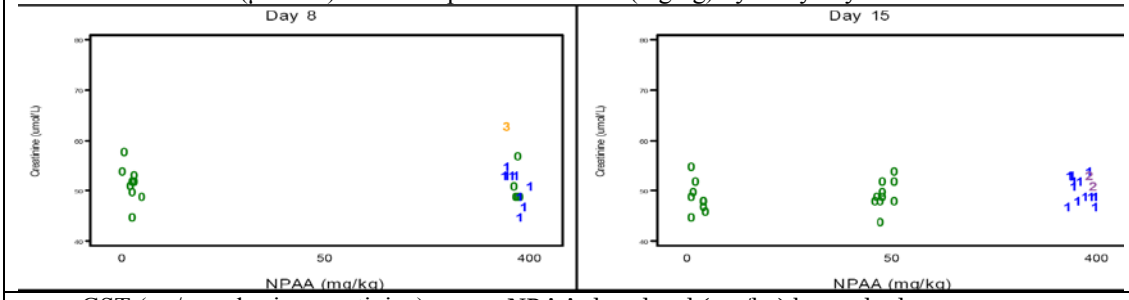


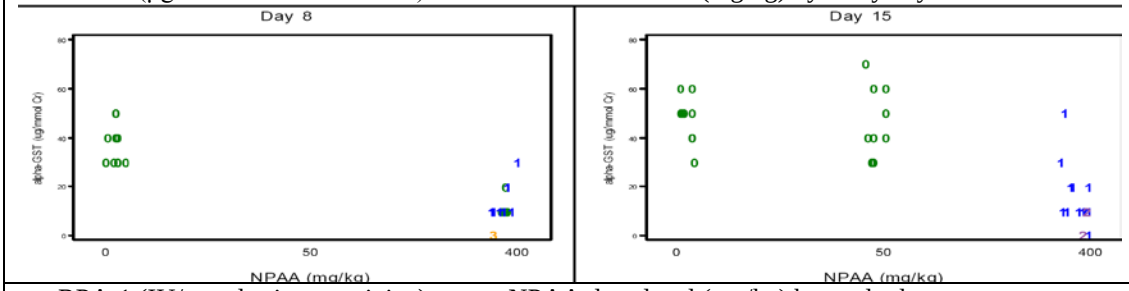
Figure 5 continued : HESI Figures showing biomarker values for individual animals

E. Sprague Dawley/NPAA with histopathology grade for collecting duct degeneration/necrosis.

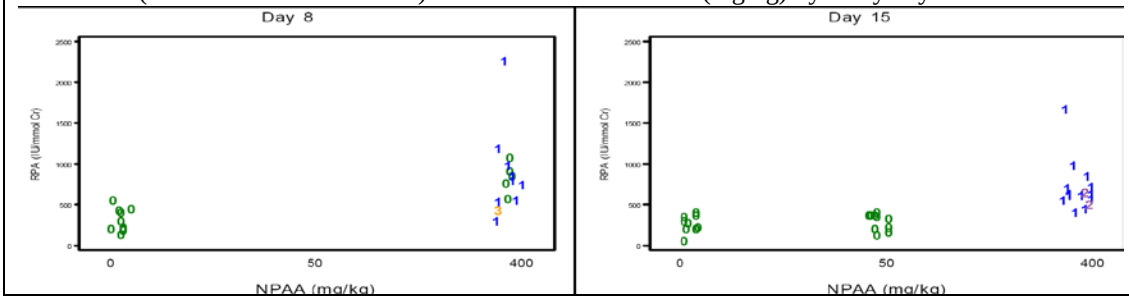
Serum creatinine (µmol/L) versus cisplatin dose level (mg/kg) by study day.



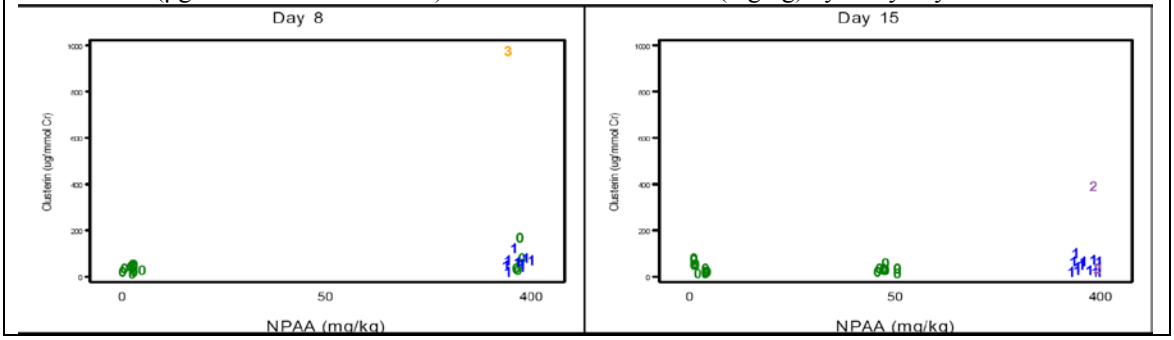
α-GST (µg/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day.



RPA-1 (IU/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day.



Clusterin (µg/mmol urine creatinine) versus NPAA dose level (mg/kg) by study day.



g. Pathology incidence by rat strain

HESI evaluated the biomarker performance in two commonly used rat strains to show that the diagnostic utility of the biomarkers is independent of the strain. Based on Table 20 below, HESI concluded that the incidence of pathologies was broadly similar between strains.

Table 20 Overall incidence of negative and positive animals by pathology and strain

	Sprague-Dawley		Han-Wistar		Total			Total # Excluded	
Pathology	# Neg	# Pos	# Neg	# Pos	# Neg	# Pos	Total # Pos <20	Neg	Pos
PT degeneration or necrosis	170	34	137	55	307	89		33	10
PT deg/nec with no regen	195	9	153	39	348	48		34	9
PT deg/nec with regen	179	25	176	16	355	41		42	1
Cortical tubular regeneration/basophilia	153	51	146	46	299	97		31	12
DT degeneration or necrosis	184	20	192	0	376	20		43	0
CD degeneration or necrosis	179	25	161	31	340	56		37	6
CD deg/nec with no regen	198	6	179	13	377	19	←	42	1
CD deg/nec with regen	185	19	174	18	359	37		38	5
Medullary tubular regeneration/basophilia	204	0	178	14	382	14	←	43	0
Regeneration NOS with no degeneration	197	7	173	19	370	26		34	9
Intratubular casts, granular, cortex	199	5	185	7	384	12	←	43	0
Intratubular casts, hyaline, cortex	188	16	176	16	364	32		42	1
Inflammation, interstitial, chronic, cortex	142	62	186	6	328	68		28	15

Pathologies relating to claims are highlighted in yellow. Those pathologies with less than 20 total positive animals are indicated with an arrow. The incidences of pathologies in the excluded animals are provided.

The sponsor's table above is confounded by inclusion of the gentamicin study results for the Sprague Dawley rat in the overall incidence. A study of gentamicin in Han Wistar rats was not conducted for this submission. The sponsor provided incidence tables for each rat strain by nephrotoxicant (Appendix 6bvii). Since it was difficult to compare the incidence in two strains using the separate tables, the data were combined into one table (see Appendix 6bviii). Table 21 below is a modification of the table in Appendix 6bviii; it omits the sub-pathologies under PT degeneration/necrosis and CD degeneration/necrosis.

The incidence of positive animals in only the cisplatin and NPAA studies given by "Sum C+N" for the pathologies of CT regeneration/basophilia and CD degeneration/necrosis differed by less than 2-fold between the two strains; whereas the incidence of positive animals in only the cisplatin and NPAA studies for PT degeneration/ necrosis differed by 5-fold between the two strains. Thus, the ROC analysis for PT degeneration/ necrosis is based primarily on the data from the study of cisplatin in the Wistar rat.

Table 21 - Reviewer's compilation from sponsor's tables - Pathology incidence by study and strain

		Sprague Dawley		Han Wistar		Total	
Pathology	Study	# Neg.	# Pos	# Neg.	# Pos	# Neg.	# Pos
PT degeneration or necrosis	Cisplatin (C)	83	11	64	55	147	66
	Gentamicin (G)	27	23	0	0	27	23
	NPAA (N)	60	0	73	0	133	0
	All (C +G + N)	170	34	137	55	307	89
	Sum C + N	143	**11**	137	**55**	280	**66**
CT regeneration/basophilia	Cisplatin (C)	89	5	99	20	188	25
	Gentamicin (G)	27	23	0	0	27	23
	NPAA (N)	37	23	47	26	84	49
	All (C +G + N)	153	51	146	46	299	97
	Sum C + N	126	**28**	146	**46**	272	**74**
DT degeneration or necrosis	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	40	20	73	0	113	20
	All (C +G + N)	184	20	192	0	376	20
	Sum C + N	134	**20**	192	**0**	326	**20**
CD degeneration or necrosis	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	35	25	42	31	77	56
	All (C +G + N)	179	25	161	31	340	56
	Sum C + N	129	**25**	161	**31**	290	**56**
CD regeneration or basophilia	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	60	0	59	14	119	14
	All (C +G + N)	204	0	178	14	382	14
	Sum C + N	154	**0**	178	**14**	332	**14**
Regeneration NOS with no degeneration	Cisplatin (C)	94	0	115	4	209	4
	Gentamicin (G)	47	3	0	0	47	3
	NPAA (N)	56	4	58	15	114	19
	All (C +G + N)	197	7	173	19	370	26
	Sum C + N	150	**4**	173	**19**	323	23
Intratubular casts, granular, cortex	Cisplatin (C)	94	0	112	7	206	7
	Gentamicin (G)	45	5	0	0	45	5
	NPAA (N)	60	0	73	0	133	0
	All (C +G + N)	199	5	185	7	384	12
	Sum C + N	154	**0**	185	**7**	339	**7**
Intratubular casts, hyaline, cortex	Cisplatin (C)	90	4	103	16	193	20
	Gentamicin (G)	42	8	0	0	42	8
	NPAA (N)	56	4	73	0	129	4
	All (C +G + N)	188	16	176	16	364	32
	Sum C + N	146	**8**	176	**16**	322	**24**
Inflammation, interstitial, chronic, cortex	Cisplatin (C)	67	27	113	6	180	33
	Gentamicin (G)	20	30	0	0	20	30
	NPAA (N)	55	5	73	0	128	5
	All (C +G + N)	142	62	186	6	328	68
	Sum C + N	122	**32**	186	**6**	308	**38**

Yellow color highlights the principal pathologies claimed.
Blue color highlights the sum of the animals from the cisplatin and NPAA studies; the positive animals are in bold text.
Red text indicates a pathology for which the number of positive animals is >2-fold between the two strains.

h. Recovery Studies

The recovery or reversal of the biomarkers was not evaluated in the studies submitted for the HESI qualification. Prior to use in the clinic, it will be important to characterize the abilities of the biomarkers to monitor not only injury but also recovery from injury.

4. Reviewer Discussion of Qualification Data

a. The Qualification Process

Data submitted by the HESI for biomarker qualification were originally received by the BQRT in May 2008. Additional data requested by the BQRT to bridge information gaps were submitted on December 8, 2008 and April 30, 2009. These submissions and meetings with the BQRT are summarized in Table 22 below. The December 2008 and April 2009 responses to the EMEA/FDA questions were not integrated into the original submission.

Table 22: Summary of HESI submissions and meetings with the BQRT

Date	Description
05_01_08	Initial submission containing data and primary literature references used to support key claims
07_08_08	HESI Meeting minutes of VXDS meeting with FDA/EMEA/PMDA along with FDA/EMEA Preliminary review comments and questions HESI presentations from July 12 meeting
12_08_08	Responses to EMEA/FDA questions.
04_30_09	Responses to EMEA/FDA questions:

b. Analytical Validation

According to the Bioanalytical Method Validation Guidance (2001), the key parameters for bioanalytical method validation are: accuracy, precision, selectivity, sensitivity, reproducibility and stability. Measurements of the biomarkers in the biological matrices should be validated and the stability of the biomarkers in spiked samples determined. The chemical identity and purity of the reference standard used to spike samples and to generate quality control samples is critical since validation data can be affected. With respect to the accuracy of the assay, the Bioanalytical Method Validation Guidance recommends that the mean value of replicate analyses of samples should be within 15% of the actual value except at the lower limit of quantification, where it should not deviate by more than 20%. The precision at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the lower limit of quantification, where it should not exceed 20% of the CV.

In the HESI submission, analytical validation data included measuring range, limit of detection, linearity, recovery, intra-assay reproducibility, inter-assay reproducibility, recovery and inter-laboratory variability for each novel biomarker assay and the evaluation of some potential interfering substances in some of the assays. HESI provided an integrated discussion concerning the assay validation of the four novel biomarkers in the December 2008 submission along with summary tables (see Appendix 6bix). The following more detailed discussion focuses on the α-GST, clusterin, and RPA-1 assays for which claims are made.

i. Immunoassays

Levels of the three claimed biomarkers in urine were measured in enzyme immunoassays that are summarized in Table 23 below. The kits were provided by Biotrin.

Table 23 - Reviewer's summary of biomarker immunoassays

	α-GST	**Clusterin**	**RPA-1**
Antibody on plate	Polyclonal rabbit anti-rat GST Ya (Ya-1 Swiss protein P00502) Polyclonal rabbit anti-rat GST Yc (Yc-1 Swiss protein P04904)IgG	Polyclonal rabbit anti-recombinant Rat Clusterin (AA 146-360)	Monoclonal mouse anti rat renal papillary antigen antibody of the subclass IgG1 (PapX5C10)
Detection reagent	Anti-rat α-GST IgG conjugated to HRP	Anti-rabbit IgG conjugated to HRP	Anti-rat RPA-1 IgG conjugated to HRP
Calibrator	Purified rat α-GST (YaYc isoform)	Purified recombinant rat clusterin	Partially purified rat RPA-1
Substrate	TMB	TMB	TMB
Absorbance at	450 nm	450 nm	450 nm

HRP = horse radish peroxidase; TMB = Tetramethylbenzidine

ii. Standards/Calibrators and Alternative Methodology

1. The standards/calibrators were provided with the Biotrin kit. These consisted of the purified analyte for α-GST and clusterin and a partially purified analyte for RPA-1 in stabilizer buffer. A positive control consisted of a rat urine sample with a defined level of the particular analyte.

2. No methodology was indicated as used to establish equivalence of methodology or to cross check the accuracy of the assays. Ideally, an alternate method should have been described or proposed for establishing accuracy of the assays. For instance, a liquid chromatography/tandem mass spectrometry method has been developed for the analysis of signature peptides of α-GST and used for the quantification of α-GST in human liver tissue (Zhang et al 2004)

Recovery/accuracy studies summarized in Table 24 below were conducted at each site using the positive controls for each assay provided by Biotrin. HESI concluded that recovery at all sites for all assays was within the range stated by Biotrin. However, the recovery at one site each for α-GST and μ-GST was >15% and the recovery at three sites for clusterin was >15%.

Table 24 - Reviewer's summary of HESI recovery data

		HESI results by site (number of sites)		
Biomarker	Biotrin control range	Mean % recovery	Range % Recovery	# Sites > 15%
α–GST	≤ +/- 40%	109.6% (5)	90 – 133%	1
μ–GST	≤ +/- 30%	102% (5)	90 – 119%	1
RPA-1	≤ +/- 25%	102.5% (4)	93 – 114%	0
Clusterin	≤ +/- 35%	84.5% (5)	76 - 105%	3

iii. Cross Reactants/Assay specificity

The RPA-1 assay used a monoclonal antibody (PapX5C10). However, the epitope on RPA-1 has not been identified, since RPA-1 protein has not been fully identified and characterized. According to HESI, the monoclonal mouse anti RPA-1 antibody only stained the collecting ducts from the cortex, medulla and papilla of the kidney, the urothelium of the renal pelvis and ureter plus some epididymal granular epithelial cells in the testis when used against full tissue microarrays. Western blotting of urine and kidney

homogenates under a variety of conditions and with deglycosylation and protease treatments indicates the epitope is likely a three-dimensional structure of a very high molecular weight protein. This antigen is released into urine upon exposure to renal toxins, e.g., bromoethanamine, propyleneamine, ipsapirone and indomethacin (Hildebrand et al 1999). The specificity of the RPA-1 immunoassay was determined solely through immunohistochemistry of frozen and fixed sections using the PapX5C10 antibody. Results of assays with potential cross-reactants were not reported.

Since the clusterin assay used a polyclonal antibody, no epitope was identified. The specificity of the clusterin immunoassay was determined solely through immunohistochemistry of kidney sections. The anti-rat clusterin antibody localized to tubular basement membrane and glomerular mesangium in cortex. Results of assays with potential cross-reactants were not reported.

Since the α-GST assay used a polyclonal antibody, no epitope was identified. The specificity of the α-GST immunoassay was determined through immunohistochemistry of kidney sections showing staining of the α-GST polyclonal antibodies to the proximal tubule of rat kidney. In addition, binding of the α-GST polyclonal antibodies to a dot blot of dilution series of recombinant GST isotypes Ya, Yc, Yb1, purified native rat α– and μ–GST purified from rat liver showed binding only to rat YaYc isotypes and no significant binding to Yp or Yb1 isoforms. The α-GST polyclonal antibodies did not detect human, canine, and porcine α-GST. The results of biomarker immunoassays with potential cross-reactants were not reported.

iv. Matrix Interference

For the α-GST, μ-GST and RPA-1 assays, matrix interference was examined by spiking hemoglobin (up to 5000 mg/dL), conjugated bilirubin (up to 5 mg/dL), albumin (up to 1000 mg/dL), and sodium chloride (up to 10 gm/dL) into a mid-level control and determining the percent recovery (observed/expected). The results indicated hemoglobin interference at 100-500 mg/dL in the rat μ-GST assay and albumin interference at 20 and 50 μg/mL in the clusterin assay. Only the potential interference of albumin and rat IgG was evaluated in the clusterin assay. Statements based on the literature and product inserts were made concerning the interference of hemoglobin, bilirubin, urea and aminoglucoside-like antibiotics in the NAG assay and hemoglobin and albumin in the total protein assay. The potential interference of heavy metals (mercury, cadmium, lead, lithium, gadolinium) was not evaluated in any assay.

v. Other matrix interference issues

Linearity of the assays was shown by evaluation of a dilution series of rat urine samples, the positive control or a high calibrator. No uniform procedure for linearity was used across laboratories. Correlation coefficients of 0.98 to 1.0 were reported for each assay.

vi. Stability

The stability of the biomarkers in urine was minimally addressed by statements in Table 25 below in the Biotrin product inserts. No data was provided to support these statements.

Table 25 – Reviewer's summary of statements in product inserts	
Biomarker	**Statement in Biotrin product insert**
α–GST	Addition of a stabilizing buffer required (100 µL to 400 µL sample. Samples can then be stored 2-8 C for 48 hr or -20 C for a month
Clusterin	Addition of a stabilizing buffer required (100 µL to 400 µL sample. Samples can then be stored 2-8 C for 48 hr or -20 C for 2 years
µ–GST	Addition of a stabilizing buffer required (100 µL to 400 µL sample. Samples can then be stored 2-8 C for 14 days or -20 C for a month
RPA-1	Addition of a stabilizing buffer required (100 µL to 400 µL sample. Samples can then be stored 2-8 C for 48 hr or -20 C for 1 year

vii. Intra-assay reproducibility

Three of the HESI sites evaluated intra-assay precision by testing of replicate calibrator/positive control samples or native specimen within one assay. The number of aliquots used in these assays varied from 12-24 replicates at the HESI sites and from 10-24 replicates at Biotrin. Table 26 shows the maximum intra-assay % CV was 10.4% for µ–GST.

Table 26 - Reviewer's summary of HESI Intra-assay reproducibility			
		HESI results by site (number of sites)	
Biomarker	Biotrin % CV	Mean %CV	Range % CV by site
α–GST	6.0	6.6 (3)	5.2 – 7.4
µ–GST	7.1	8.0 (3)	4.0 – 10.4
RPA-1	6	4.9 (3)	4.0 – 5.4
Clusterin	7.0	7.6 (3)	7.3 – 7.8

viii. Inter-assay reproducibility

Each HESI site evaluated inter-assay precision by testing of calibrator/positive control samples or native specimen in assays conducted on different days. The number of different assays varied from 4-30 at the HESI sites and from 10-20 at Biotrin. As shown in Table 27 below, two sites had inter-assay % CVs of greater than 15% for the α–GST assay and all five sites had inter-assay % CVs of greater than 15% for the clusterin assay.

Table 27 - Reviewer's summary of HESI Inter-assay reproducibility				
		HESI results by site (number of sites)		
Biomarker	Biotrin % CV	Mean % CV	Range % CV	# Sites > 15%
α–GST	7.2	12.9 (5)	7.9 – 17.0	2
µ–GST	9.4	10.9 (5)	8.8 – 12.9	0
RPA-1	11	7.8 (4)	1.8 – 13.5	0
Clusterin	24.7	22.4 (5)	16 - 30	5

ix. Inter-site reproducibility

Inter-laboratory reproducibility of a-GST, µ-GST, clusterin, and RPA-1 immunoassays was assessed through the analysis at each of six participating laboratories of three blinded urine samples (low, mid, and high concentrations) that had been prepared at a single reference laboratory and then shipped to the other participating laboratories for analysis. Table 28 below summarizes the range of percent differences from the overall mean for each blinded sample for each assay. The sponsor summarized the results in Figure 6. The sponsor's summary tables are in Appendix 6bx.

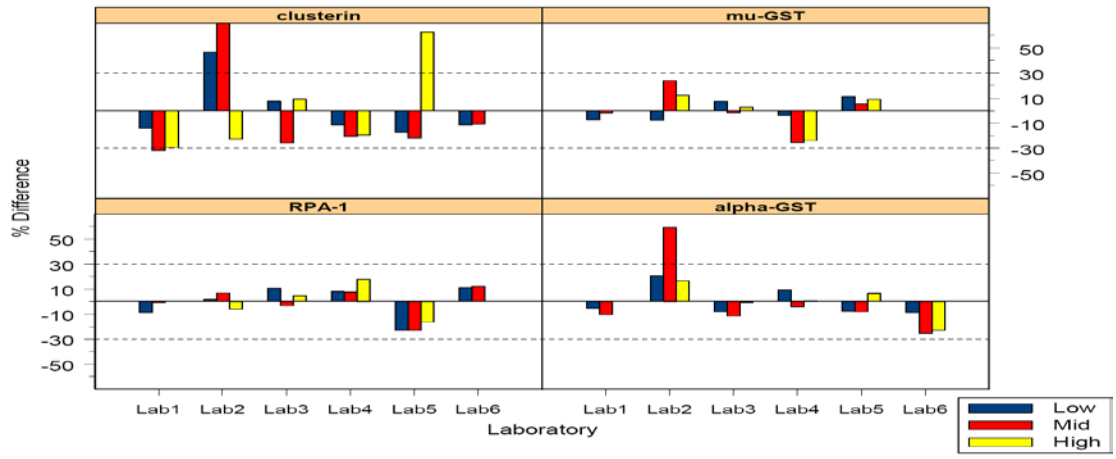
All sites had values within 25% of the overall mean for the RPA-1 and μ-GST samples. All sites, but one (59%), had values ≤25% of the overall mean for the α-GST samples. The results for the clusterin samples were more variable than the other assays with one apparent outlier laboratory for each concentration. Only the outlier for the mid-concentration clusterin sample could be excluded based on a Q-test.

Table 28 - Reviewer's summary of HESI inter-site reproducibility data

	Range of % difference from overall mean (# > 15%/#sites)		
Biomarker	Low	Mid	High
α–GST	-8.8 to +20.6 (1/6)	-25.4 to +59.4 (2/6)	-23.0 to +16.5 (2/5)
μ–GST	-7.6 to +11.3 (0/5)	-25.5 to +23.9 (2/5)	-24.0 to +12.5 (1/4)
RPA-1	-22.9 to +11.0 (1/6)	-22.8 to 12.3 (1/6)	-16.5 to +17.9 (2/4)
Clusterin	-17.5 to +46.6 (2/6)	-31.7 to 111 (5/6) *-12.3 to 14.9 (1 at 171*)*	-29.3 to 62.7 (4/5) *-16.2 to 29.4 (1 at 93)*

Clusterin values in italics indicate % difference from mean calculated excluding outlier value
* Result could be omitted based on Q-test.

Figure 6 - Sponsor's figure - Reproducibility (Inter-lab variability) shown as percent difference of lab mean from global mean for blinded samples



HESI cited the 2003 American Association of Pharmaceutical Scientists Biomarkers Workshop conference report (Lee et al 2005) which suggested that validation demonstrate that a method is fit for its intended purpose and acceptance criteria of 20-30% coefficient of variation (%CV) was reasonable for intermediate precision, especially for immunoassays. Using these criteria, the RPA-1 and μ-GST assays had acceptable precision on all five laboratories, while the α-GST assay had acceptable precision in four of the five laboratories. The precision for the clusterin assay was higher than 30% at two laboratories.

c. **Correlation between Histopathology and Biomarker Data**

i. *Blinding of histopathology analysis*

The initial kidney histopathology read by each study pathologist was conducted blinded to biomarker datasets but with knowledge of dose group, necropsy, organ weight and standard clinical pathology datasets. Subsequently, the April 2009 submission indicated that only the pathologist in the Sprague Dawley/cisplatin study was aware of group mean

BUN and sCr values, but not values for individual animals. The pathologists on the other studies were indicated as having no knowledge of the clinical pathology data at the time of the initial slide evaluation. A full PWG review evaluated all slides unblinded to treatment group, but blinded to novel biomarker values. Since this limited blinding can bias the results either in favor or against the sensitivity and specificity of the biomarkers, the results would be more reliable if the histopathology was conducted fully blinded to all other data.

ii. *Basis for histopathology evaluation*

In the cisplatin and gentamicin studies, both kidneys of each animal were evaluated on the basis of one section per kidney. In the NPAA studies, both kidneys of each animal were evaluated on the basis of up to six sections per kidney, although only one section, stated as being the one with the most severe histopathology, was scored. Furthermore, the orientation of the section varied among the studies (see Table 6). No information was provided concerning potential differences in histopathology score between kidneys in any study or among the multiple sections in the NPAA studies.

In the absence of data to demonstrate that all lesions, even those at low doses and early after dosing, are detected uniformly throughout the kidney, the assessment of the histopathology reference standard would be more reliable if multiple sections from each kidney were examined.

iii. *Exclusion of animals from analysis*

Animals were excluded from the ROC analyses based on three conditions.

First, animals from the high dose 100 mg/kg gentamicin group were excluded because of the unscheduled deaths in this group. Although no animals in this group survived to Day 14, histopathology and biomarker evaluations of these animals were conducted at earlier timepoints. These results could have been included in the analyses, since biomarker values and histopathology scores are available for these animals.

Second, a total of 43 animals were excluded because at least one biomarker value was missing. HESI's listing of excluded animals is in Appendix xi. HESI decided to analyze only those animals with all biomarker values. As shown in Table 29, almost all of the excluded animals were from the cisplatin study in Sprague-Dawley rats and the NPAA study in Han-Wistar rats. Missing data occurred in control group animals with similar frequency as that in toxicant-treated animals and were not related to treatment and/or toxicity. However, 19 of the animals were excluded solely because a value for NAG was missing.

Table 29 - Reviewer's summary of excluded animals

Study		Number Excluded			Exclusion solely because of lack of				
Drug	Strain	Total	Control	Treated	BUN/SCr	NAG	N+P	Novel	Multiple
Cisplatin	SD	26	6	20	0	17	3	6	0
Cisplatin	HW	2	0	2	0	0	0	2	0
NPAA	HW	15	6	9	2	2	0	9	2
Total		43	12	31	2	19	3	17	2

N+P = NAG and protein

HESI argued that the incidence of specific pathologies was similar in the excluded animals and the included animals based on the frequency distribution in Table 30. Although this statement is true for the three major pathologies considered by HESI, it is not true for some of the other pathologies (e.g. distal tubule degeneration or necrosis).

Table 30 - From sponsor's tables - Frequency distribution of animals by pathology

	Excluded animals						Included	
	Sprague-Dawley		Han-Wistar		Total		Total	
Pathology	# Neg	# Pos	# Neg	# Pos	# Neg	# Pos	# Neg	# Pos
PT degeneration or necrosis	18	8	15	2	33	10	307	89
PT deg/nec with no regen	19	7	15	2	34	9	348	48
PT deg/nec with regen	25	1	17	0	42	1	355	41
PT regeneration/basophilia	21	5	10	7	31	12	299	97
DT degeneration or necrosis	26	0	17	0	43	0	376	20
CD degeneration or necrosis	26	0	11	6	37	6	340	56
CD deg/nec with no PT injury or regen	26	0	16	1	42	1	377	19
CD deg/nec and regen with no PT injury	26	0	12	5	38	5	359	37
CD regeneration/basophilia	26	0	14	3	40	3	382	14
Regeneration NOS with no degeneration	22	4	12	5	34	9	370	26
Intratubular casts, granular, cortex	26	0	17	0	43	0	384	12
Intratubular casts, hyaline, cortex	25	1	17	0	42	1	364	32
Inflammation, interstitial, chronic, cortex	12	14	16	1	28	15	328	68

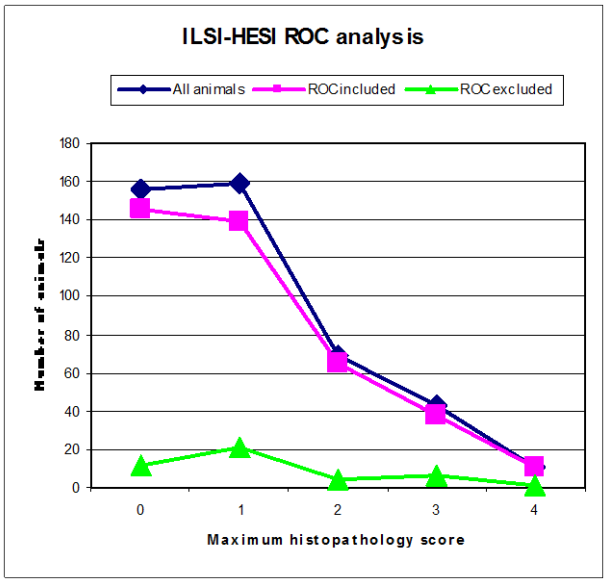
In addition, the distribution of histopathology scores for the excluded set of animals should be similar to that for the included set of animals. The distribution of histopathology scores in section iv below indicates a grossly similar distribution of excluded and included animals. However, in contrast to the included set with a similar number of animals with scores of 0 and 1, the excluded set has almost 2-fold more excluded animals with a score of 1 than excluded animals with a score of 0.

Third, HESI calculated for each major pathology a threshold for each biomarker in two ways. One calculation used all available animals for a specific pathology, while the other excluded toxicant-dosed animals with no observable pathology. See Section 3d.

iv. Generation of ROC curves

The distribution of histopathology scores for total, included and excluded animals is presented in Figure 7 below. Of the total number of included animals, 63% were considered positive by histopathology However, most of these included positive animals had a histopathology score of 1. The percentage of included animals with a score of 1 (35%) is similar to the percentage of included animals that were considered negative with a score of 0 (36.5%). This means that the ROC analysis, a binary evaluation, was based primarily on the distinction between no histopathology and the lowest histopathology grade. This determination is more subject to unconscious bias and improper diagnosis when there is inadequate sampling of the tissue.

Figure 7 – Reviewer's analysis of the distribution of histopathology scores in HESI submission



		Maximum Histo score					
	Lab	0	1	2	3	4	Total
All	1	18	48	15	6	0	87
	2	17	14	7	9	3	50
	3	27	24	8	1	0	60
	4	24	40	35	17	4	120
	5	70	33	4	10	3	120
	Total	156	153	69	43	10	**437**
Included	1	16	39	12	6	0	73
	2	17	14	7	9	3	50
	3	27	24	8	1	0	60
	4	24	40	34	16	4	118
	5	61	22	4	6	3	96
	Total	145	139	65	38	10	**397**
Excluded	1	2	10	3	0	0	15
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	1	1	0	2
	5	9	11	0	5	1	26
	Total	11	21	4	6	1	**43**

d. **Performance of Proposed Biomarkers Compared with Accessible Biomarkers in Current Use.**

i. Collection of samples

Urines were collected over ice for a timed period as a 16 hour overnight fasted urine collection in four laboratories and as a 16 hour overnight fed urine collection in one laboratory (due to specific requirements of the local animal care legislation). In the NPAA study in Wistar rats, data from a comparison of the urinary biomarker data collected under fasted collections over 6/7 hours with data from the same animals collected over 16/17 hours under fed conditions was used to support inclusion on data from overnight (fed) urine samples alongside the data from the overnight (fasted) urine sample in the biomarker analyses. As shown in Table 31 below, most of the mean values of the 17 hour (fed) samples were within 2-fold of the mean values for the 7 hour (fasted) samples. However, the mean fold values for α-GST and NAG are 2 to 2.6-fold higher for the 17 hour (fed) samples and the clusterin control value is 2.3 fold higher for the 17 hour (fed) samples than for the 7 hour (fasted) samples.

Table 31 - Reviewer's compilation from sponsor's tables – NPAA study in Wistar rats

Group	7 hr collection						17 hr collection					
N = 13-15	αGST - µg/mmol Crt	µGST - µg/mmol Crt	RPA1 -U/mmol/Crt	Clusterin - µg/mmol Crt	Total protein - mg/mmol Crt	NAG -IU/mmol Crt	αGST - µg/mmol Crt	µGST - µg/mmol Crt	RPA1 -U/mmol/Crt	Clusterin - µg/mmol Crt	Total protein - mg/mmol Crt	NAG -IU/mmol Crt
Day 7/8												
Control	36.09	4.13	223.80	16.09	146.05	1.65	27.11	5.33	116.23	22.73	211.75	1.29
750/350*	0.22	0.70	11.71	5.78	1.06	1.86	0.38	0.92	9.97	6.25	0.85	3.64
Day 14/15												
Control	28.46	3.52	234.02	9.07	126.51	0.78	21.11	5.13	147.94	19.99	178.90	0.67
50*	0.57	1.15	1.59	0.90	0.78	1.37	1.14	1.12	0.89	1.04	1.06	2.09
350*	0.26	0.72	7.75	7.19	1.27	2.02	0.66	0.73	10.18	5.03	1.08	4.51
700/500*	0.18	1.82	10.58	21.50	2.10	2.67	0.46	1.52	10.93	12.07	1.85	6.02

* Fold change

ii. Blinding of biomarker assays

Each technician who measured the novel biomarkers (α-GST, µ-GST, clusterin or RPA-1) in the urines from all individual animals was blinded regarding the pathology results. However, they had knowledge of the treatment groups and study design.

iii. Urinary creatinine normalization

HESI stated that preliminary analyses produced similar AUC_{ROC} values when biomarker data was normalized to urinary creatinine or as total excretion, despite up to a 6-fold variation in urine volume in control animals. To support the use of urinary creatinine to adjust for variation in urine volume, HESI provided graphs showing a similar mean urine creatinine excretion across dose groups. The mean urine creatinine excretion in the Sprague Dawley NPAA, the Sprague Dawley gentamicin, and Han Wistar NPAA studies were 75-90, 55-65, and 75-85 µmoles per collection, respectively. In contrast the mean urine creatinine excretion in the Han Wistar cisplatin study was 14-20 µmoles per collection. No explanation was provided for this difference. Although urine creatinine levels vary with change in kidney function, it is reasonable to adjust urinary biomarkers by it during the acute phase of injury because it normalizes for differences in urine volume.

iv. Background (control range) biomarker levels

A comparison of control ranges for the novel biomarkers (α-GST, clusterin, µ-GST, RPA-1) in each strain are provided below in Tables 32 and 33 for fold change and urine creatinine-normalized values, respectively. In general, the variation in 95th percentile values across individual sites is less for the fold-change values (mean = 1.2) than for the urine creatinine-normalized values (mean = 1.8). The 95th percentile fold change values are generally similar between the two strains, except for µ-GST and NAG. Despite the variability in the 95th percentile urine creatinine-normalized values between sites, the values for α-GST and clusterin are significantly different between the two strains.

Table 32 - Reviewer's summary - Fold-change ranges for control animals across all studies by rat strain								
	Sprague Dawley percentile				Han Wistar percentile			
	Across sites				Across sites			
Assay	50th	5th	95th	95th by site	50th	5th	95th	95th by site
α-GST	0.99	0.62	1.55	1.36, 1.39, 1.59	0.98	0.62	1.44	1.44, 1.46
μ-GST	0.90	0.14	2.11	1.86, 2.06, 2.43	0.94	0.46	1.74	1.72, 2.02
clusterin	0.92	0.49	1.87	1.77, 1.99, 1.81	0.92	0.50	1.69	1.54, 1.90
RPA-1	0.91	0.43	1.77	1.69, 1.77, 1.97	0.93	0.38	1.85	1.84, 2.22
BUN	0.98	0.77	1.30	1.22, 1.29, 1.32	1.00	0.81	1.16	1.15, 1.22
sCr	1.00	0.84	1.15	1.13, 1.16, 1.16	1.01	0.88	1.11	1.09, 1.11
NAG	0.97	0.71	1.39	1.24, 1.39, 1.42	0.96	0.25	1.77	1.51, 2.15
Protein	0.98	0.53	1.50	1.39, 1.57, 1.63	1.05	0.00	1.48	1.24, 1.67
Yellow highlight indicates 5-95 percentile ranges that are similar between the two strains.								

Table 33 - Reviewer's summary of biomarker urine creatinine-normalized values				
	Sprague Dawley – by site		Han Wistar – by site	
Biomarker	50th percentile	95th percentile	50th percentile	95th percentile
α-GST, μg	0.080, 0.050, 0.042	0.140, 0.080, 0.067	0.027, 0.023	0.050, 0.037
μ-GST, μg	0.004, 0.006, 0.003	0.014, 0.013, 0.008	0.006, 0.002	0.011, 0.006
Clusterin, μg	0.070, 0.042, 0.073	0.163, 0.084, 0.166	0.021, 0.029	0.039, 0.062
RPA-1, IU	0.280, 0.290, 0.263	0.545, 0.610, 0.535	0.110, 0.329	0.283, 0.664
BUN, mM	4.10, 6.00, 4.57	6.1, 8.0, 5.63	6.00, 7.32	7.1, 9.28
sCr, μM	34.0, 50.0, 20.5	44, 56, 27	57.0, 47.74	72, 52.2
NAG, IU	0.00190, 0.00300, 0.00350	0.00295, 0.00450, 0.00490	0.00130, 0.00180	0.00230, 0.00274
Protein, gm	0.00010, 0.00013, 0.00009	0.00017, 0.00019, 0.00016	0.00010, 0.00010	0.00029, 0.00013
BMS = Bristol-Myers Squibb; GSK = GlaxoSmithKline; SA = sanofi Aventis; AZ = AstraZeneca; Sc = Schering Plough				

v. Control animal variability

HESI made repeated measurements from control rats of both strains in the NPAA studies to estimate of the likely variability among controls in the study as a whole. All biomarker values were normalized to urinary creatinine prior to calculation of variability.

Table 34 - Sponsor's table - Intra-animal and inter-animal %CV estimates for Sprague-Dawley and Han-Wistar control animals in the NPAA studies

	Intra-animal %CV		Inter-animal %CV	
Marker	S-D[1]	Wistar[2]	S-D[1]	Wistar[2]
α-GST	24.3	31.0	19.0	22.5
μ-GST	29.1	39.1	46.9	0.0
RPA-1	35.1	65.5	34.3	9.8
clusterin	22.0	26.4	38.8	25.9

[1]Estimates obtained from 20 control animals with 3-4 repeated measurements per animal
[2]Estimates obtained from 30 control animals with 2 repeated measurements per animal (16-hour overnight collection samples only)

Variability in the above control animal data (Table 34) was attributed to the biologic variation inherent in urinary markers and analytical variability of the assays based on reproducibility of the data in the analytical validation studies. However, the most important source of variability may have been the differences in urine collection methods/handling. The 16-hour urine samples for the Wistar animals were collected under fed conditions, whereas the 16-hour urine samples for the Sprague Dawley animals were collected under fasted conditions. The sponsor also cited diurnal or circadian

alterations in feeding and drinking behaviour leading to fluctuations in both fluid balance and urinary excretion as an additional source of variability in the control rat data presented above. However, if all samples were derived from 16-hour overnight collections, any diurnal variation should have been minimized. A more obvious reason for the difference is the collection under fasted versus fed conditions. The effect of dropped food on biomarker values was not examined. In addition, the values in Wistar rats were derived from only two repeated measurements per animal.

.

vi. Threshold definition

Although definitive thresholds require a much larger and more extensive set of data than those currently available, HESI provided the following tables of threshold values based on the current data. For each novel biomarker and pathology, threshold values corresponding to an estimated 95% specificity were calculated based on study data for the compounds which induced the pathology of interest. For each threshold, estimates and 95% confidence intervals for the specificity and sensitivity are given in Table 35 below. For comparative purposes, thresholds for each traditional marker were calculated in an identical manner and are also given. At 95% specificity, α-GST, RPA-1 and clusterin had higher sensitivity than the comparator biomarkers. The diagnostic likelihood ratio (DLR) for a positive test result is defined as LR(+)= sensitivity/(1 - specificity), and the DLR for a negative test result is defined as LR(-)= (1-sensitivity)/specificity. Better biomarker performance is indicated by a larger positive DLR and a negative DLR close to 0. As indicated below in Table 35, α-GST, RPA-1, and clusterin exhibit the best biomarker performance for the given pathology.

Table 35 – Sponsor's thresholds corresponding to estimated 95% specificity for indicated pathology and diagnostic likelihood ratios

Pathology	Provided in December 2008 submission -								April 2009	
PT degeneration or necrosis (cisplatin and gentamicin studies only)		#	#	Fold-change	Specificity		Sensitivity		Diagnostic LR	
	Marker	Neg	Pos	Threshold	Estimate	95% CI	Estimate	95% CI	Positive	Negative
	α-GST	191	95	1.69	0.958	(0.920, 0.979)	0.589	(0.489, 0.683)	14.07	0.43
	SCr	192	98	1.21	0.953	(0.913, 0.975)	0.347	(0.260, 0.445)	7.40	0.69
	BUN	192	98	1.31	0.958	(0.920, 0.979)	0.347	(0.260, 0.445)	8.33	0.68
	NAG	180	90	1.47	0.950	(0.908, 0.973)	0.467	(0.367, 0.569)	9.33	0.56
	Protein	191	96	1.74	0.953	(0.913, 0.975)	0.438	(0.343, 0.537)	9.28	0.59
CD degeneration or necrosis (NPAA studies only)		#	#	Fold-change	Specificity		Sensitivity		Diagnostic LR	
	Marker	Neg	Pos	Threshold	Estimate	95% CI	Estimate	95% CI	Positive	Negative
	RPA-1	84	61	5.62	0.952	(0.884, 0.981)	0.492	(0.371, 0.614)	10.33	0.53
	SCr	84	61	0.82	0.952	(0.884, 0.981)	0.098	(0.046, 0.198)	2.07	0.95
	BUN	84	61	1.30	0.952	(0.884, 0.981)	0.131	(0.068, 0.238)	2.75	0.91
	NAG	84	61	3.33	0.964	(0.900, 0.988)	0.344	(0.237, 0.470)	9.64	0.68
	Protein	86	61	1.34	0.965	(0.902, 0.988)	0.180	(0.104, 0.295)	5.17	0.85

cortical tubular regeneration / basophilia (all studies)	Marker	# Neg	# Pos	Fold-change Threshold	Specificity Estimate	Specificity 95% CI	Sensitivity Estimate	Sensitivity 95% CI	Diagnostic LR Positive	Diagnostic LR Negative
	Clusterin	324	108	3.24	0.951	(0.921, 0.969)	0.426	(0.337, 0.520)	8.63	0.60
	SCr	327	108	1.21	0.954	(0.926, 0.972)	0.269	(0.194, 0.359)	5.85	0.77
	BUN	327	108	1.38	0.951	(0.922, 0.970)	0.204	(0.139, 0.289)	4.16	0.84
	NAG	310	105	3.71	0.952	(0.922, 0.970)	0.076	(0.039, 0.143)	1.57	0.97
	Protein	325	109	1.98	0.951	(0.922, 0.969)	0.239	(0.168, 0.327)	4.85	0.80

Additionally, the thresholds were re-calculated after exclusion of toxicant-dosed animals which exhibited no observable pathology. After exclusion of treated animals that had no pathology, the thresholds decreased for RPA-1 and NAG in CD degeneration or necrosis and for clusterin and NAG in cortical tubular regeneration/basophilia.

vii. Secondary organ effects

The liver was the only organ examined microscopically other than the kidney. Liver histopathology and/or hepatobiliary markers were evaluated in the studies as indicated in Table 36 below. Both liver histopathology and hepatobiliary marker values were assessed only in the cisplatin study in Sprague Dawley rats. However, HESI indicated that liver histopathology was evaluated in dose range finding studies at comparable or higher doses of NPAA (SD) and gentamicin (SD). Therefore, HESI concluded that no manifestations of hepatotoxicity were identified for any of the three nephrotoxicants at the highest doses tested in dose range and/or definitive studies in at least one strain.

Table 36 – Sponsor's overview of assessment of hepatotoxicity in dose-range-finding or definitive studies by compound and strain – From December submission

Laboratory	Study	Doses	Test Article	Strain	Liver Sections examined	Liver findings	Blinded initial	Blinded targeted*	Peer Review
AZ	Definitive	1 week oral 50 and 350 (700) mg/kg/day	NPAA	Wistar	Sampled but not processed	ALT, AST, ALP Tbili and liver weights all within normal limits	N/A	N/A	N/A
Pfizer	Dose range	2 week oral 50, 350 and 700 mg/kg/day	NPAA	SD	Left lateral and right medial	No microscopic change	No	Yes	No
BMS	Definitive	2 week IP 5, 25, 50 or 100 mg/kg/day	Gentamicin	SD	Sampled but not processed	ALT, AST, ALP and Tbili all within normal limits	N/A	N/A	N/A
Pfizer	Dose range	2 week SC 5, 50 and 100 mg/kg/day	Gentamicin	SD	Left lateral and right medial	No microscopic change	No	Yes	No
Sanofi-aventis	Definitive	Up to 5 day IP, 0.3, 1 or 3 mg/kg/day	Cisplatin	SD	Left lateral, right medial, caudate	No microscopic change or change in liver weight, ALT, AST, Tbili, or ALP	No	Yes	Yes
Bayer	Definitive	Up to 5 day IP, 0.3, 1 or 3 mg/kg/day	Cisplatin	Wistar	Single section , not otherwise specified	No microscopic finding or liver weight change in DRF or definitive study, serum chemistry not performed	No	Yes	Yes

*Blinded targeted evaluation refers to re-evaluation of selected slides to further resolve identification of specific findings. The slides are randomized so that the pathologist is blinded to treatment groups and the presence (with severity grading) or absence of a particular finding is specified for each study animal, independent of animal number or dose group designation.

Hepatotoxicity data provided in the studies listed in Table 34 are not adequate to assess hepatoxicity, because all the studies did not examine histopathology and hepatobiliary

markers simultaneously. The sponsors provided a single evaluation, either histopathology or hepatobiliary markers in all studies, except one study.

The hepatotoxicity of cisplatin was evaluated in one definitive study via liver histopathology and hepatobiliary makers. However, the duration of the treatment was only up to 5 days. For short duration of treatment this data may be acceptable. But the 5-day treatment period may not be sufficient to show any liver toxicity. Therefore, it only can conclude that cisplatin at a dose up to 3 mg/kg/day for 5 days did not cause hepatotoxicity in Sprague Dawley rats.

It remains inconclusive whether the NPAA causes hepatotoxicity at tested doses because two different strains, and different treatment durations were used in these two studies that evaluated either histopathology or hepatobiliary markers.

The hepatotoxicity of gentamicin was evaluated in two 2-week studies in Sprague Dawley rats via intraperitoneal or subcutaneous administrations of the same doses. The histopathology and hepatobiliary markers were not evaluated simultaneously in these studies. The sponsor did not submit the report of the 2-week study via intraperitoneal route. It is unknown whether the animals had similar systemic exposures to gentamicin when administered via the two different routes to administration. Therefore, the combined data from these two studies cannot conclude that gentamicin has no hepatoxicity.

viii. Specificity

No studies were conducted with non-nephrotoxicant drugs. Therefore, a full assessment of the specificity of the biomarkers for detecting acute kidney injury can not be made.

Considering that serum α-GST is a very sensitive, if not the most sensitive hepatotoxicity biomarker, the specificity of urinary α-GST is a concern. HESI maintains that urinary α-GST is expected to reflect renal rather than liver injury based on the following rationale. Although circulating α-GST would be expected to cross the glomerulus based on molecular weight of approximately 50 kD, with normal proximal tubular function > 90% of the protein content of the tubular filtrate is resorbed, such that an increase in urinary α-GST would reflect proximal tubular rather than hepatic injury. Further, serum α-GST has a short circulating half life (~90 minutes in man, Kilty et al 1998) and would be associated with transaminase increases (half-life ~50 hours, Burtis and Ashwood 1986), such that α-GST increases would be expected to be associated with increases in other hepatobiliary markers. Although HESI's rationale seems plausible, a number of citations indicate that increases in serum α-GST can occur in the absence of changes in hepatobiliary markers at least in humans (Giannini et al 2000; Helaly and Mahmoud 2003; Ozturk et akl 2009)

The epitopes recognized in each of the antibodies in the biomarker assays have not been identified to date. The rat alpha GST and rat clusterin enzyme immunoassays utilize polyclonal antibodies against the full length protein, which can be expected to recognize a multitude of epitopes. Although the specificity of the RPA-1 monoclonal antibody had been defined through extensive immunohistochemical analysis, the corresponding protein has not been successfully neither identified nor isolated and, therefore, the binding epitope is also unknown.

Immunohistochemistry (IHC) was used as an adjunct (secondary) to histopathology and topography to confirm localization of nephrotoxic injury and anchor segment-specific biomarker changes. For immunohistochemistry (IHC), negative immunoglobulin isotype controls were run at matched concentrations on a known control tissue for each antibody per standard operating procedure. For α-GST, IHC of normal kidney confirmed specificity of α-GST localization to both S1/S2 and S3 segments of the proximal tubule throughout the cortex based on morphologic features such as presence of a brush border and origin from the urinary pole of the glomerulus. In normal kidney, RPA-1 produced diffuse cytoplasmic staining along the length of the collecting duct, while clusterin was localized to tubular basement membranes throughout the cortex and glomerular mesangium, with greatest immunofluorescene along the corticomedullary junction and renal papilla. In the case of RPA-1, morphologic features and co-localization with aquaporin-2 by IHC confirmed specificity of this marker for collecting duct, however RPA-1 expression is restricted to medullary collecting duct in normal kidney. HESI stated that no inconsistencies between histopathology and IHC were identified. IHC confirmed that α-GST and RPA-1 provide information on injury to specific segments of the tubule, while clusterin lacking a specific localization in the nephron is likely to report injury to the tubule without such specificity.

HESI indicated that tissues other than kidney were also evaluated for immunoreactivity for α-GST, clusterin, and RPA-1. Although HESI indicated that α-GST was present in liver as well as kidney (cytoplasmic and nuclear staining), a listing of any other tissues examined for α-GST was not provided. Although clusterin was not detectable in normal kidney, specific clusterin immunoreactivity was observed in the cytoplasm of interstitial macrophages within stomach, uterus, skeletal muscle, heart, tongue, as well as macrophages within the medulla of thymus and lymph node of an untreated control rat. However, clusterin is synthesized in many tissues and found in plasma and cerebrospinal fluid (CSF).

RPA-1 antibody cross-reactivity studies were performed with formalin fixed tissue microarrays from normal Han Wistar rats. Positive immunostaining due to specific RPA-1 antibody binding was found in the urothelium of the renal pelvis and ureter and collecting ducts from the cortex, medulla and papilla plus some epididymal granular epithelial cell staining in the testis (Betton, et al 2007). Organs showing no specific immunostaining included; brain (cerebrum, cerebellum, medulla), eye (retina, lens), stomach, duodenum, jejunum, ileum, colon, liver, pancreas, salivary glands (mandibular, parotid), testis, prostate, seminal vesicle, ovary, uterus, cervix, vagina, skeletal muscle, heart, aorta, spleen and lymph node.

vii. Unusual findings:

Consistent with the immunohistochemistry localization of α-GST to the proximal tubule, increases in urinary α-GST were seen with PT injury in the absence of CD injury. However, when isolated CD injury was induced by NPAA, α-GST values were consistently decreased in urine of both strains and α-GST was superior to all of the reference biomarkers for the diagnosis of CD injury in the absence of PT injury. None of the studied nephrotoxicants induced both proximal tubule and collecting duct injury and hence the performance of α-GST in this setting remains unclear.

The magnitude of the rise in α-GST levels seen with PT injury was much greater than the fall observed with CD injury induced by the studied nephrotoxicants. To explore whether these opposing effects on α-GST levels might impact the biomarker's performance in the setting of concomitant PT and CD injury, the mean decrease in α-GST levels observed with NPAA-induced CD injury was subtracted from the values obtained for individual animals with drug-induced PT injury. The impact of this correction on the fold-change in α-GST levels was determined and biomarker results were categorized as being "positive" or "negative" based on whether the corrected fold-change was above or below the 1.7 threshold (the threshold with 95% specificity for detecting injury). The results of this analysis are shown in Table 37 below. Using this approach, there were 24 animals (out of a total 116 positive animals) for which the α-GST result changed from "positive" to "negative" in the gentamicin and cisplatin studies conducted in the two rat strains. Of these 24 animals, 18 had α-GST values that were no longer consistent with the histopathology score for PT degeneration/necrosis; many of these animals had minimal injury (very low histopathology score). These results raise concern that α-GST levels may not be useful in detecting minimal proximal tubule injury in the setting of concomitant collecting duct injury.

Table 37 - Reviewer's examination of α-GST values relative to histopathology scores

			Histoathology			α-GST, uCr-normalized, (µg/µM)			
	Day	Dose	#N	#P	Score	Mean	Maximum	Minimum	#P → N
Wistar									
NPAA	8	0	15	0	0	0.027	0.037	0.015	
NPAA	8	700	3	10	1,2,3	0.017	0.031	0.008	
NPAA	15	0	15	0	0	0.021	0.030	0.014	
NPAA	15	50	14	1	1	0.024	0.033	0.013	
NPAA	15	350	3	12	1.2	0.014	0.024	0.004	
NPAA	15	700	1	14	1,2,3	0.010	0.023	0.002	
Cisplatin	2	0	10	0	0	0.005	0.007	0.003	
Cisplatin	2	0.3	10	0	0	0.005	0.008	0.002	
Cisplatin	2	1	2	8	1,2	0.017	0.045	0.000	2 (2*)
Cisplatin	2	3	0	10	1,2	0.021	0.073	0.002	1
Cisplatin	3	0	10	0	0	0.027	0.036	0.016	
Cisplatin	3	0.3	10	0	0	0.023	0.055	0.007	1
Cisplatin	3	1	6	4	1	0.019	0.030	0.008	
Cisplatin	3	3	0	10	1,2	0.089	0.153	0.038	1 (1*)
Cisplatin	5	0	10	0	0	0.030	0.038	0.020	
Cisplatin	5	0.3	3	7	1	0.042	0.058	0.033	1
Cisplatin	5	1	3	7	1	0.064	0.129	0.029	2
Cisplatin	5	3	0	10	3,4	0.685	0.926	0.395	
Sprague Dawley									
NPAA	8	0	10	0	0	0.037	0.050	0.030	
NPAA	8	400	5	10	1,3	0.013	0.030	0.000	
NPAA	15	0	10	0	0	0.048	0.060	0.030	
NPAA	15	50	10	0	0	0.045	0.070	0.030	
NPAA	15	400	0	15	1,2	0.015	0.050	0.000	
Cisplatin	2	0	10	0	0	0.052	0.068	0.024	
Cisplatin	2	0.3	10	0	0	0.056	0.068	0.041	
Cisplatin	2	1	10	0	0	0.048	0.087	0.020	
Cisplatin	2	3	10	0	0	0.046	0.092	0.020	1
Cisplatin	3	0	10	0	0	0.046	0.063	0.036	
Cisplatin	3	0.3	10	0	0	0.054	0.077	0.038	
Cisplatin	3	1	8	2	1	0.053	0.073	0.040	
Cisplatin	3	3	1	9	3	0.106	0.154	0.060	3
Cisplatin	5	0	10	0	0	0.035	0.058	0.024	
Cisplatin	5	0.3	10	0	0	0.033	0.041	0.019	
Cisplatin	5	1	7	3	1,2	0.035	0.058	0.021	
Cisplatin	5	3	5	5	2,3,4	0.139	0.576	0.026	1
Gentamicin	7	0	10	0	0	0.085	0.140	0.060	
Gentamicin	7	100	0	11	2,3,4	1.397	2.380	0.470	
Gentamicin	8	100	0	4	3	0.310	0.470	0.210	
Gentamicin	10	100	1	3	3	0.190	0.220	0.150	1
Gentamicin	14	0	10	0	0	0.081	0.130	0.060	
Gentamicin	14	5	6	4	1	0.081	0.150	0.040	1
Gentamicin	14	25	1	9	1	0.128	0.220	0.060	2
Gentamicin	14	50	0	10	1,2,3	0.163	0.320	0.070	4

#P → N = number of animals for which α-GST value might decrease below the threshold of 1.7 fold; * Borderline change

HESI did not offer an explanation for the decrease in α-GST values in response to CD injury. Although α-GST is not considered to be expressed in the collecting duct, the possibility exists that some other protein released from the collecting duct during injury interferes in the α-GST assay utilizing polyclonal antibodies against rat GST Ya and rat GST Yc. An alternative explanation is that although NPAA did not apparently affect

proximal tubule pathology, it may have altered the synthesis of α-GST in the proximal tubule or the background release of α-GST into the urine.

f. **Format issues**

i. Initial Submission

Although the summary report in initial submission was consecutively paginated, the appendices were separately paginated, making the review difficult. Also, a list of appendices was not provided.

ii. Subsequent submissions

The two subsequent submissions were not integrated into the initial submission. Since the subsequent responses were labeled only by the number of the EMEA/FDA question, specific information in the subsequent submissions was difficult to locate.

5. Qualification Conclusions

a. BQRT Conclusions:

The HESI submission is an example of an initial, context-dependent qualification proposal, suitable for evaluation by the pilot FDA qualification process. This submission pooled the results of nephrotoxicant studies performed in different rat strains to determine the sensitivity and specificity of several urinary biomarkers for acute drug-induced renal tubular alterations in male rats. The performance of each biomarker was compared to that of sCr and BUN against the reference standard of histopathology using Receiver Operating Characteristic (ROC) analysis. Comparisons of the area under the ROC curve (AUCroc) showed the performance of clusterin, RPA-1 and α-GST was statistically superior to sCr and BUN in these studies for the diagnosis of specific kidney pathologies.

The BQRT recommends the qualification of RPA-1 and clusterin, but not the qualification of α-GST. In making recommendations for qualification of these biomarkers, the BQRT took into consideration the results discussed below as well as limitations of the data. Consequently, the BQRT recommends that the qualification context of RPA-1 and clusterin be limited.

Clusterin was previously qualified by the FDA in 2008 based on data reported in a PSTC submission. The HESI data support the qualification of urinary clusterin as a more sensitive biomarker of drug-induced nephrotoxicity as evidenced by an AUCroc value for clusterin that was significantly greater ($p<0.001$) than the AUCroc values for sCr and BUN for the diagnosis of tubular toxicity (nonspecific with respect to location). The HESI submission provided data not only for the male Han Wistar rat (provided in the PSTC submission), but also for the male Sprague Dawley rat. The HESI submission also provided inter-laboratory validation data on the clusterin assay to support the decision to pool data from different laboratories (data not contained in the PSTC submission). The HESI submission provides additional support for the use of urinary clusterin in

nonclinical toxicity studies in the male rat when drug related tubular pathology changes, particularly in the presence of tubular regeneration, are observed.

RPA-1 is a novel biomarker not previously qualified by the FDA. The HESI data show that the AUCroc value for RPA-1 diagnosis of collecting duct injury was significantly greater ($p<0.001$) than AUCroc values for sCr and BUN. In addition, the curves did not cross each other at different levels of specificity. The significant increase in AUCroc values without crossing of the curves indicate that RPA-1 is a more sensitive biomarker of collecting duct injury at all levels of specificity. Furthermore, the AUCroc value for RPA-1 remained high whereas the AUCroc values for clusterin and α-GST decreased when distinguishing between histopathology scores of zero (no pathology) and one (minimal pathology).

HESI also proposed α-GST as a novel biomarker proposed for use in drug-induced kidney toxicity. The HESI data show that the AUCroc value for α-GST was significantly greater ($p<0.001$) than AUCroc values for sCr and BUN for the detection of proximal tubule and collecting duct injury. However, increases in urinary α-GST showed greater sensitivity than sCr and BUN for the detection of proximal tubule injury and decreases in urinary α-GST showed greater sensitivity than BUN and sCr for the detection of collecting duct injury. The opposing effects of proximal tubule and collecting duct injury on α-GST levels may confound the interpretation of urinary α-GST measurements, particularly for compounds for which there is limited mechanistic information. Therefore, the BQRT does not recommend the qualification of α-GST at this time.

The BQRT does recommend the qualification of RPA-1 and clusterin. However, based on the limitations of the data the BQRT recommends qualification context of RPA-1 and clusterin be limited.

b. Limitations of Submitted Data

In reaching a conclusion about the qualification of these biomarkers and their application context, the BQRT considered the following aspects of the data:

1. The amount of data used to construct the ROC curves is limited by three main concerns:
    a. No non-nephrotoxins and only three nephrotoxins, two of which induce similar proximal tubule injury were used. It is unclear how well clusterin and RPA-1 will perform in rats for the evaluation of new compounds without nephrotoxicity and new compounds that have mechanisms of toxicity different than the compounds studied by HESI. Therefore, the BQRT recommends that traditional clinical chemistry markers and histopathology assessments should also be made when clusterin and RPA-1 are used in a preclinical development program.
    b. Only male rats were used. It is unclear how well clusterin and RPA-1 will perform in female rats. Although the mechanisms of toxicity should be similar in both genders, differences in basal biomarker levels and the extent and timing of response to injury may differ in males and females.

Therefore, the BQRT recommends that the nonclinical qualification of urinary clusterin and RPA-1 should be limited to use in male rats.

c. The temporal relationship between changes in histopathology and changes in urinary clusterin and RPA-1 levels was minimally examined with two or three timepoints defining the evolution of injury and no timepoints examining reversibility of the drug-induced renal injury. Therefore, uncertainty exists as to how well clusterin and RPA-1 will perform at different time points post injury, particularly early time points, and whether repair of injury will be reflected by changes in clusterin or RPA-1 levels. Although this information is needed for a qualification with a context of use that excludes the need for accompanying histopathology, this information is not essential for a qualification with a context of use that requires accompanying histopathology.

2. While data pooled across rat strains were used to support the qualification of these biomarkers, there were differences between rat strains in the performance of individual biomarkers. These differences raise concern about the appropriateness of pooling data across strains. Confidence in a biomarker's performance is increased when both rat strains show higher sensitivity and specificity than sCr and BUN as was observed for clusterin for cortical tubular regeneration/basophilia and RPA-1 for collecting duct degeneration/necrosis, For this reason, the BQRT feels that it is important to limit the qualification of clusterin for the detection of cortical tubular regeneration/basophilia and the qualification of RPA-1 for the detection of collecting duct degeneration/necrosis.

3. Since knowledge of the treatment group may have introduced bias into the study results, the BQRT would be more confident of the results if the pathologists had been fully blinded to all information. The initial pathologist, a peer-review pathologist, and a subsequent HESI Pathology Working Group (PWG) were unblinded to treatment group, but were blinded to novel biomarker results. Although the PWG harmonized terminology and severity grading and arrived at a consensus opinion, the BQRT believes that fully blinded readings of histopathology are needed in future qualification studies.

4. A few of animals had positive urinary clusterin and RPA-1 values in the absence of positive histopathology. Whether this finding reflects the ability of these biomarkers to detect injury prior to histopathology changes, a non-specific change in biomarker levels, or inadequate tissue sampling (and possible underdetection of the underlying histopathology findings) cannot be determined. In the submitted studies, only a single section per kidney per animal was examined microscopically. The minimum number of tissue samples needed in biomarker qualification studies to adequately characterize renal injury, particularly low levels of injury, remains unknown and should be better characterized. At this time, we do not have sufficient information to conclude that positive urinary clusterin and RPA-1 values in the absence of histopathology changes are prodromal signs of injury.

Together the above limitations indicate that application of clusterin and RPA-1 to monitor renal toxicity has not yet been sufficiently demonstrated to stand on its own without histopathology and traditional clinical chemistry as measures of renal toxicity.

d. BQRT Recommendations for Qualification

Despite the aforementioned limitations, the BQRT concludes that the data contained in the HESI submission support the qualification of

- urinary clusterin for voluntary use in rat safety assessment studies for the detection of acute drug-induced tubular injury and tubular regeneration/basophilia.
- urinary RPA-1 for voluntary use in rat safety assessment studies for the detection acute drug-induced collecting duct injury.

We recommend that urinary clusterin and RPA-1 should be used along with traditional clinical chemistry markers and histopathology for the detection of acute drug-induced nephrotoxicity in toxicology studies. Sponsors may use these biomarkers in GLP toxicology studies in the development of drugs for which evidence of drug induced nephrotoxicity already exists or is likely based on prior experience with the pharmacologic class of the drug being developed. Specifically, sponsors may use these biomarkers to determine more conservative NOAELs for estimating starting doses in the initial human clinical trial of a drug that displays preclinical nephrotoxicity as determined by histopathology.

The HESI data support clusterin and RPA-1 as qualified for the following contexts of use:

> **Urinary Clusterin** is a qualified biomarker for voluntary use in the detection of acute drug-induced renal tubule alterations, particularly when regeneration is present, in male rats when used in conjunction with traditional clinical chemistry markers and histopathology in GLP toxicology studies for drugs for which there is previous preclinical evidence of drug induced nephrotoxicity or where it is likely given the experience with other members of the pharmacologic class.
>
> **Urinary RPA-1** is a qualified biomarker for voluntary use in detecting acute drug-induced renal tubule alterations, particularly in the collecting duct, in male rats when used in conjunction with traditional clinical chemistry markers and histopathology in GLP toxicology studies for drugs for which there is previous preclinical evidence of drug induced nephrotoxicity or where it is likely given the experience with other members of the pharmacologic class.

e. Recommendations for Future Research to Address Gaps in Understanding of the Performance of These Urinary Biomarkers

The BQRT has concluded that the data contained in the HESI submission support the qualification of urinary clusterin and RPA-1 as acceptable biomarkers for voluntary use in male rats along with traditional clinical chemistry markers and histopathology for the detection of acute drug-induced nephrotoxicity in safety assessment studies. However, further studies are needed to improve our understanding of how these markers respond in

different animal models and with different drugs, and how best to interpret different biomarker levels. In order to gain useful information about the biomarker performance in different contexts, including the clinical setting, the BQRT recommends the following gaps be addressed.

1. The BQRT recommends that the urinary clusterin and RPA-1 be qualified as acceptable biomarkers for voluntary use along with traditional clinical chemistry markers and histopathology for the detection of acute drug-induced nephrotoxicity in GLP toxicology studies in *male* rats, but not in *female* rats. Testing of these biomarkers should be done in the female rat and should be extended to other animal species when appropriate assays become available.

2. To support conclusions based on the association with specific histopathologic lesions, future submissions should include:

   a. Data on behavior of the novel biomarkers using multiple nephrotoxic and nonnephrotoxic compounds from different mechanistic classes and in altered physiologic conditions to broaden our understanding of the generalizability of conclusions about the ability of the biomarkers to detect localizable lesions

   b. Use of appropriate doses of nephrotoxic compounds and study design so that histopathology specimens can be gathered when the injury is more localized and/or is milder and also when the injury covers a broad range of severity.

3. The submitted studies examined the evolution of the drug-induced renal injury using two or three timepoints, but did not address reversibility or recovery from injury. The BQRT recommends that nonclinical studies be conducted to better characterize the evolution of drug-induced injury and demonstrate reversibility of injury by histopathology and biomarker levels when drug administration is stopped based on elevation of biomarker levels.

4. The characterization of an endogenous substance in blood or urine requires a different testing paradigm than characterizing the effects of a xenobiotic. The data that was used in this submission were collected from studies that were designed for the characterization of a xenobiotic. Future studies will be more informative if designed specifically for the purpose of assessing the putative biomarker. These studies should address the issues of adequacy of tissue sampling, background lesions, and blinding of histopathology.

   a. In the absence of data establishing the ability of a single section to accurately characterize the presence, extent, severity, and location(s) of minimal injury, the BQRT recommends that multiple histopathology sections be taken and evaluated in biomarker qualification studies. Studies using multiple histopathology sections will be needed to support any claims concerning the ability of these biomarkers to detect injury prior to histopathology changes.

   b. Variation in biomarker levels in control and treated animals may be influenced by so-called “background lesions” and morphologic variations. Future biomarker qualification studies should assess the impact of “background” lesions and morphologic variations on biomarker performance and include a list by

animal of all the variations (common as well as uncommon lesions) in the target tissue.

c. In future biomarker qualification studies, pathologists need to be blinded to the results of biomarker analyses (including novel and traditional biomarkers such as BUN or sCr) at a minimum. To avoid bias, the BQRT strongly recommends that the evaluation of histopathology and biomarker results in future biomarker studies be conducted in a fully blinded manner such that the pathologist is blinded to any aspect of study design or results that could potentially unblind the pathologist to treatment assignment or biomarker level.

5. Prospectively designed, hypothesis driven preclinical studies are needed to address the correlation between biomarker levels and evolution of lesions with secondary confirmation using appropriate techniques, such as immunohistochemistry, in-situ hybridization and/or electron microscopy, when appropriate relative to the biology of the biomarker and any claims concerning localization of injury. Immunohistochemistry or other appropriate techniques should be used to define the temporal relationship between changes in histopathology, changes in tissue levels of the biomarkers and changes in urinary biomarker levels.

6. The opposing behavior of urinary α-GST levels in response to proximal tubule and collecting duct injury raise uncertainty about the usefulness of α-GST for the detection of early and/or mild renal injury; hence the BQRT does not currently recommend the qualification of urinary α-GST. Given the limited amount of data on the specificity of the α-GST biomarker assay, future studies should address the effect of potential interfering substances as well as dilutional effects and the cross-reactivity of other GST isoforms as possible explanations for the decrease in urinary α-GST observed with collecting duct injury. Studies utilizing immunohistochemistry to localize the expression of various GST isoforms before and after collecting duct injury should be conducted to clarify the response of α-GST to different areas of renal injury and provide a better understanding of the mechanistic basis for the observed decreases following collecting duct injury. Additional nephrotoxicants should also be studied to explore the effect of isolated collecting duct injury as well as the effect of concomitant proximal tubule and collecting duct injury on α-GST levels.

7. An efficient and accurate review of biomarker submissions requires that information provided in subsequent submissions be integrated into the initial submission, which is consecutively paginated and the numbers shown. Future submissions should include a detailed integrated section on the methods and results of analytical validation of assays, including assay interferences, specificity, biomarker stability and sample handling.

8. Although the data to support the analytical validation of the biomarker assays were generally acceptable for the intended analytical application (see criteria in the Bioanalytical Method Validation Guidance (2001), some potentially interfering substances and cross-reactants were not evaluated in the biomarker immunoassays. In particular, the specificity of antibodies used in the biomarker immunoassays and the specificity of the biomarker assays were not fully characterized. A better

understanding of their specificity is needed to ensure meaningful interpretation of changes in biomarker levels in drug development studies.

9. Preclinical studies to support a specific drug development program should demonstrate that the novel biomarkers can detect early drug induced renal injury and reversibility of injury after drug cessation before proceeding to clinical studies.

10. The BQRT recognizes the need for biomarkers that can reliably predict injury in both the preclinical and clinical setting. With respect to the clinical use of urinary clusterin and RPA-1, the BQRT recommends the exploration of these novel renal biomarkers in humans when and if sufficiently validated assays become available. While these novel renal biomarkers should be tested in humans, they are not currently qualified to be used as primary renal injury monitoring tests or dose-stopping criteria. For the time being, the sponsor and regulatory division will decide on a case by case basis how best to implement these biomarkers in the clinical development program. Demonstration that a biomarker or a panel of biomarkers consistently detects toxicity at an early stage in animal models may justify incorporating them into clinical studies as sentinels for toxicity. Using novel renal biomarkers in early clinical trials for renal toxicity monitoring may represent a reasonable risk for the development of promising therapies which would otherwise be abandoned. Use of a particular biomarker in a clinical trial will be dependent on demonstration of reversibility of both biomarker levels and histopathology and establishment of a pre-specified cut-off value of abnormality.

6. Appendices

This section includes detailed information referenced in the main text of this review, including additional background information, as well as data submitted by the HESI to support qualification of the proposed biomarkers of nephrotoxicity.

a. Background information about the proposed biomarkers submitted by HESI

i. GST isoforms

The GSTs are phase II detoxifying enzymes that exist in the kidney in various isoforms (Beckett and Hayes 1993). Immunohistochemical studies reveal that the distribution of the different isoforms varies along the nephron and between species (Campbell et al 1991; Harrison et al 1989; Rozell et al 1993; Sundberg et al 1993; Sundberg et al 1994). The isoform found in the proximal tubule in both rat and human is α-GST whereas, in the distal tubule, μ-GST (GSTYb1) is the isoform found in rats and π-GST is the human isoform. While the expression of these isoforms may be up-regulated after exposure to some xenobiotics and renal toxins (Derbel et al 1993; Daggett et al 1997), α-GST is known to exist constitutively in high concentration (approximately 2% of soluble protein) in the cells of the proximal tubule (Beckett and Hayes 1993). The increased presence of GSTs in the urine after nephrotoxic injury to rats has been known for about 30 years (Bass et al 1979) and is attributed to leakage from the cells into the lumen of the tubule secondary to epithelial cell damage (Harrison et al 1989). In a study of the effects of volatile anesthetics on the kidney in rats, Kharasch et al (1997) reported that, of the biomarkers they examined, urinary excretion of α-GST was the most sensitive biomarker of mild proximal tubular cell necrosis. Measurement of the GST isoforms in urine also was more sensitive than either BUN or creatinine for detection of tubular injury in a study in human volunteers administered volatile anesthetics (Eger et al 1997). Although there has been no systematic study of the potential of measurement of urinary GSTs as biomarkers of renal tubular injury, urinary levels of specific isoforms of GST have been proposed not only as markers of renal tubular damage in general but also as indicators of the location of the injury along the nephron (Eger et al 1997). Thus, the quantitative measurement of the GST isoforms in urine has potential in monitoring drug-induced proximal and distal tubular damage in animals and humans as well as monitoring the progression of renal diseases in humans (Dvergsten et al 1994; Kilty et al 2007).

ii. Clusterin

Clusterin, also known as sulfated glycoprotein-2 (SGP-2), is a ubiquitously expressed dimeric glycoprotein. It is highly expressed during early stages of renal development and is up-regulated in a variety of renal diseases and in response to renal tubular injury (Rosenberg and Silkensen 1995b). Secreted clusterin has been variously suggested to play an anti-apoptotic role or to be involved in cell protection, cell aggregation and cell attachment (Rosenberg and Silkensen 1995b). The exact role that clusterin plays in renal injury is not well understood but it is thought to be involved in tissue remodeling and repair. Girton et al (2002) provided some evidence to support the hypothesis that

induction of clusterin due to tissue injury might provide a protective mechanism by eliminating excess lipid or scavenging toxic lipid by-products. The clusterin gene is up-regulated in different parts of the nephron andfollowing various types of kidney injury e.g. in rats following nephrectomy (Correa-Rotter et al 1992), unilateral ureteral obstruction (Ishii et al 2007), renal ischemia-reperfusion (Yoshida et al 2002) or nephrotoxicity (Kharasch et al 2006; 61) as well as in dogs with renal papillary necrosis induced by nefiracetam (Tsuchiya et al 2005). Increased levels of clusterin protein have been detected in the urine of rats or dogs following ischemic or chemically-induced injury (Aulitzky et al 1992; Eti et al 1997; Hidaka et al 2002; Tsuchiya et al 2005). While increased expression of clusterin is seen in humans in a variety of renal disorders (Rosenberg and Silkensen 1995b), to date there has been no clinical study demonstrating the use of clusterin as a diagnostic marker of renal injury.

Clusterin is a highly glycosylated and sulfated secreted glycoprotein first isolated from ram rete testes fluid in 1983 (Blashuck et al 1983). It was named clusterin because of its ability to cause clustering of Sertoli cells *in vitro* (Fritz et al 1983. Clusterin is primarily found in the epithelial cells of most organs. Tissues with the highest levels of clusterin include: testis, epididymis, liver, stomach and brain. Metabolic and cell specific functions assigned to clusterin include: sperm maturation, cell transformation, complement regulation, lipid transport, secretion, apoptosis, and metastasis (Rosenberg and Silkensen 1995b).

Table 1. Summary of the isolation and/or cloning of clusterin from different tissues

Tissue	Species	Name	Association
Rete testes fluid	Ram	Clusterin	Reproduction-sperm maturation
Adrenal medulla	Bovine	GP III	Chromaffin granules-secretion
Sertoli cells	Rat	SGP-2 (DAG)	Reproduction
Prostate	Rat	TRPM-2	Apoptosis
Testes	Rat	Clusterin	Reproduction
Prostate	Rat	SGP-2	Reproduction
Neuroretinal cells	Quail	T64	Cell transformation
Serum (liver)	Human	SP-40, 40	Complement regulation
Serum (liver)	Human	CL1	Complement regulation
Brain	Hamster	SGP-2	scrapie
Adrenal medulla	Bovine	Glycoprotein III	Chromaffin granules
Blood	Human	apo J	Lipid transport
Brain	Human	pADHC-9	Alzheimer's disease
Renal cells	Canine	gp 80	Vectorial secretion
Blood	Human	NA1/NA2	Lipid transport
Brain	Human	pTB16	Gliomas and epileptic foci
Retina	Human	K611	Retinitis pigmentosa
Vasc smooth muscle	Porcine	pc38K	Nodule formation in vitro

from Mark E Rosenberg and John Sikensen[3], 1995.

Clusterin is also known by a number of synonyms as a consequence of having been identified simultaneously in many parallel lines of inquiry. Names include: glycoprotein III (GPIII), sulfated glycoprotein-2 (SG-2), apolipoprotein J (apo J), testosterone-repressed message-2 (TRPM-2), complement associated protein SP-40, 40 and complement cytolysis inhibitor protein (see Table 1). Clusterin has been cloned from a number of species including the rat (Collard et al 1987). The human homologue is 449 amino acids in length, coding for a protein with a molecular weight of 52,495 Daltons (Kirszbaum et al 1992). However, due to extensive post translational modification the protein migrates to an apparent molecular weight of 70-80 kDa following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Amino acid identity between species is moderate. Human clusterin shares 70.3%, 76.6%, 71.7% and 77% with the bovine, mouse, pig and rat homologues, respectively (www.expasy.org). Clusterin is a heterodimer comprised of an α and β subunit, each having an apparent mass of 40 kDa by SDS-PAGE. The subunits result from the proteolytic cleavage of the translated polypeptide at amino acid positions 23 and 277. This eliminates the leader sequence and produces the mature 205 amino acid β subunit and the remaining 221 amino acid α subunit. The α and β subunits are held together by 5 sulfhydryl bonds afforded by cysteine residues clustered within each of the subunits6. In addition, each subunit has three N-linked carbohydrates that are also heavily sulfated giving rise to the observed higher apparent molecular weight following SDS-PAGE.

Considerable evidence exists which suggest that clusterin plays an important role in development. For example, clusterin mRNA expression is present at 12.5 days post gestation in mice where it is present in all germ cell layers (French et al 1993). Furthermore, stage-specific variations of the transcript have been identified as have changes in specific localization during development. Similarly, changes in the developmental expression of clusterin in kidney, lung and nervous system (O'Bryan et al 1993) have also been reported. These observations suggest that clusterin might play a role in tissue remodeling.

In the developing murine kidney, clusterin is expressed in the tubular epithelium and later in development expression is diminished as tubular maturation progresses9. Interestingly, clusterin is observed in newly formed tubules but appears to be absent in glomeruli. In mature kidney, basal expression of clusterin is low, with localization in tubular basement membranes and glomerular mesangium(Yamada et al 2003). Clusterin upregulation is observed following induction of a variety of kidney diseases and compound-induced renal injury. Clusterin induction has been observed following ureteral obstruction (Pearse et al 1992) and ischemia reperfusion injury (Witzgall et al 1994). Elevations in the levels of clusterin have also been observed following subtotal nephrectomy (Correa-Rotter et al 1992) and in animal models of hereditary polycystic kidney disease (Cowley et al 1995). Marked increases of clusterin released in urine have also been recorded in animal models of aminoglycoside-induced nephrotoxicity (Aulitzky 1992; Eti et al 1992; (Rosenberg and Silkensen 1995a). Authors have proposed that clusterin functions in either a protective role by scavenging cell debris or may play a role in the process of tissue remodeling following cellular injury based on these data. Collectively, the body of work linking elevated levels of urinary clusterin to kidney damage suggest that measurement of urinary clusterin may be useful as a marker of renal tubular injury. Indeed, an early study comparing urinary levels of clusterin against N-acetylglucosamine (NAG) following

chronic administration of gentamicin over a two month period demonstrated that while the excretion rate of both proteins rose rapidly, peaked and then declined, clusterin levels remained significantly higher than control values over the duration of the experiment while NAG levels dropped to within control values within 10 days of treatment despite evidence of persistent tubulointerstitial disease (Eti et al 1993). More recent work examining the levels of urinary clusterin in the autosomal-dominant polycystic kidney disease (cy/+) rat model compared to the FHH rat model of focal segmental glomerulosclerosis following bilateral renal ischemia demonstrated that clusterin levels correlated with the severity of tubular damage and suggested use as a marker for differentiating between tubular and glomerular damage (Hidaka et al 2002). It is within the scope of the ILSI-HESI work to determine if this hypothesis is valid for site-specific, compound induced nephrotoxicity.

iii. RPA-1

RPN can be induced experimentally in rats by compounds such as 2-bromethanamine or NSAIDs and the differential release of segment specific proteins has been demonstrated in rats treated with such compounds. Monoclonal antibodies were raised against the proteins released in urine from these studies (as potential BMs) and then used, through a process of immunohistochemical screening to confirm the nephronal origin of the released proteins (Falkenberg et al 1996; Hildebrand et al 1999). By this process, a collecting duct antigen named renal papillary antigen-1 (RPA-1) was selected for evaluation as a potential urinary BM of collecting duct injury.

Although there are a large number of renal proximal and some distal tubule biomarkers reported, there is a paucity of biomarkers specific to the collecting duct. RPA-1 has been shown experimentally to be a highly specific marker for the rat collecting duct and is an early predictive and sensitive urinary biomarker for renal papillary necrosis, including effects of NPAA and other toxicants such as 2-bromethanamine and propyleneimine. The absence of expression in organs outside the urinary tract confers additional specificity. Because of the empirical way in which the antibody was generated and selected, it has been necessary to employ a range of molecular techniques to characterise this marker. Because of the very high molecular weight and variety of post-translational modifications involved, it has not been possible to assign a molecular identity to the biomarker epitope or epitopes recognised. Sensitivity to both proteases and deglycosylation enzyme treatments implies a glycoprotein epitope. Since the same monoclonal is effective in both capture and detection in the Biotrin ELISA, the epitope may be repeated along the molecule. The membrane localisation along the collecting duct is also likely to be a result of hydrophobic domains. Until the identity of the RPA-1 epitope is defined, its biological role remains unknown but as a functional leakage marker of collecting duct injury in the rat, it is the only currently proven urinary biomarker for the detection of renal papillary necrosis in the rat (Betton et al 2007)

b. Additional data supporting qualification of proposed biomarkers submitted by HESI

i. Summary of individual studies

HESI detailed summary of studies

Sponsor's Table - Dose groups, compound administration and numbers of animals in gentamicin study (Sprague-Dawley rats)

Group	Dose of gentamicin (mg/kg/day)[a]	Dose volume (mL/kg)	Total No. of Animals	No. of animals per necropsy (Day of necropsy)
1	0 (vehicle [b])	1.0	20	10 (Days 8 or15)
2	5	1.0	10	10 (Day 15)
3	25	1.0	10	10 (Day 15)
4	50	1.0	10	10 (Day 15)
5	100	1.0	20	10 (Day 8 or 15[c])

[a] Doses are expressed in terms of gentamicin base and dose volumes were based on the most recent body weight.
[b] 0.9% saline
[c] No high dose animals survived to study day 15

O/N Urine collected Day 3/4, Day 7/8 and 14/15 in all doses groups

Dose groups, compound administration and numbers of animals in cisplatin studies (Han-Wistar and Sprague-Dawley rats)

Group Number	Dose of Cisplatin (mg/kg)[a]	Dose volume (mL/kg)	Total No. of Animals	No. of animals per necropsy (Day of necropsy)
1	0(vehicle[b])	5	30	10 (Days 2, 3 or 5)
2	0.3	5	30	10 (Days 2, 3 or 5)
3	1	5	30	10 (Days 2, 3 or 5)
4	3	5	30	10 (Days 2, 3 or 5)

[a] All doses are expressed in terms of the pure parent compound, and dose volume was based on the most recent body weight.
[b] 0.9% saline

O/N urine was collected for biomarker analysis on Day 1/2; Day 2/3 and Day 4/5

Dose groups, compound administration and numbers of animals in NPAA studies (Han-Wistar and Sprague-Dawley rats)

Group	Strain of rat	Dose of NPAA(mg/kg/day)[a]	Dose volume (mL/kg)	Total No. of Animals	No. of animals per necropsy (Day of necropsy)
1	Han-Wistar	0 (vehicle[b])	10	30	15 (Days 8 or 15)
	Sprague-Dawley			20	10 (Days 8 or 15)
2	Han-Wistar	50	10	15	15 (Day 15)
	Sprague-Dawley			10	10 (Day 15)
3	Han-Wistar	350	10	15	15 (Day 15)
	Sprague-Dawley	400		30	15 (Day 8 or 15)
4	Han-Wistar	700[c]	10	30	15 (Day 8 or 15)

[a] Doses are expressed in terms of pure parent compound and dose volumes were based on the most recent body weight.
[b] Vehicle was 1.25% carboxymethylcellulose in both strains
[c] Dose reduced from Day 4 to 350 mg/kg for animals sacrificed on Day 8 and to 500 mg/kg for animals sacrificed on Day 14

O/N urine was collected for biomarker analysis on Day 3/4 (both strains, but only Control and high dose in Wistar); Day 7/8 and Day 14/15

ii. HESI Standardized kidney histopathology lexicon

HESI Standardized kidney histopathology lexicon

Primary Histopathology Process	Secondary Histopathology Lesion	Structural element / Segment
Tubular Cell Degeneration/Necrosis/Apoptosis	Necrosis	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Apoptosis	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
Necrosis/Infarction		Cortex Medulla Papilla
Tubular Cell Regeneration	Basophilia	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Mitosis increase	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule
Tubular Cell Alterations	Hyaline droplet formation	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Hypertrophy/Enlargement	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Nuclear change	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Cellular sloughing	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Pigmentation accumulation	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Thick ascending tubule Distal convoluted tubule Collecting duct
	Vacuolation	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
Tubular Dilatation	Tubular Dilatation	Cortex Medulla Papilla
	Tubular Cystic Dilatation / Tubular Cyst(s)	Cortex Medulla Papilla
Pelvis Dilatation		Pelvis

Primary Histopathology Process	Secondary Histopathology Lesion	Structural element / Segment
Intratubular Casts	Crystalline	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Granular	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Hyaline (proteinaceous, pigmented)	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Leukocytic	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
	Mineralization	No precise localization possible Prox. convoluted tubule (PCT, s1-s2) Thick descending tubule (s3) Loop of Henle Thick ascending tubule Distal convoluted tubule Collecting duct
Inflammation	Interstitial,acute	Cortex Medulla Papilla Pelvis
	Interstitial,chronic	Cortex Medulla Papilla Pelvis
	Acute	Glomerulus
	Chronic	Glomerulus
Fibrosis	Perivascular	Cortex Medulla Papilla
	Interstitial	Cortex Medulla Papilla
	Interstitial Bowman's capsule	Glomerulus
	Fibrosis of glomerulus / Glomerulosclerosis	Glomerulus
Glomerular Alteration	Enlargement of Bowman's space	Glomerulus
	Decrease of Bowman's space	Glomerulus
	Mesangial proliferation/expansion	Glomerulus
	Glomerular Vacuolation	Glomerulus
Edema		Diffuse Cortex Medulla Papilla
Vascular alteration	Vasculitis	Diffuse Cortex Medulla Hilum
	Medial hypertrophy	Diffuse Cortex Medulla
	Necrosis	Diffuse Cortex Medulla Hilum
	Thrombosis-thrombus	Diffuse Cortex Medulla Hilum
Mineralisation-parenchymal		Cortex Cortico-medullary junction Medulla Papilla
Urothelial hypertrophy-hyperplasia		Papilla Pelvis
Juxtaglomerular Apparatus Hypertrophy		Juxtaglomerular
Concentric Lamellar Bodies		Cortex Cortico-medullary junction Medulla Papilla

iii. Comparison of AUC_{ROC} values for all pathologies in the Excluded (Table 7) and Included (Table 8) datasets

Reviewer's compilation comparing AUC_{ROC} values for all pathologies in the Excluded (Table 7) and Included (Table 8) datasets (Yellow highlights the principal pathologies claimed.)

	Dataset	BUN	SCr	NAG	Protein	α-GST	μ-GST	RPA	Clust	Neg	Pos
PT degeneration or necrosis	Excluded	0.63 (0.04)	0.63 (0.04)	0.69 (0.03)	0.73 (0.04)	0.84 (0.03)	0.78 (0.03)	0.60 (0.03)	0.70 (0.03)	307	89
	Included	0.63 (0.04)	0.64 (0.04)	0.68 (0.03)	0.72 (0.04)	0.85 (0.03)	0.77 (0.03)	0.60 (0.03)	0.69 (0.03)	340	99
	Difference	**0**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0**	**0.01**		10
PT degeneration or necrosis with no regeneration	Excluded	0.55 (0.05)	0.57 (0.04)	0.51 (0.04)	0.54 (0.06)	0.73 (0.04)	0.63 (0.04)	0.56 (0.04)	0.56 (0.04)	348	48
	Included	0.52 (0.05)	0.54 (0.04)	0.52 (0.04)	0.53 (0.05)	0.75 (0.04)	0.62 (0.04)	0.56 (0.04)	0.55 (0.04)	382	57
	Difference	**0.03**	**0.03**	**0.01**	**0.01**	**0.02**	**0.01**	**0**	**0.01**		9
PT degeneration or necrosis with regeneration	Excluded	0.79 (0.05)	0.82 (0.05)	0.87 (0.02)	0.89 (0.03)	0.87 (0.03)	0.87 (0.03)	0.76 (0.04)	0.94 (0.02)	355	41
	Included	0.79 (0.05)	0.82 (0.05)	0.86 (0.02)	0.89 (0.03)	0.87 (0.03)	0.88 (0.03)	0.75 (0.03)	0.94 (0.02)	397	42
	Difference	**0**	**0**	**0.01**	**0.01**	**0**	**0.01**	**0.01**	**0**		1
PT regeneration/ basophilia	Excluded	0.62 (0.04)	0.64 (0.04)	0.63 (0.04)	0.63 (0.03)	0.53 (0.04)	0.59 (0.04)	0.77 (0.03)	0.81 (0.03)	299	97
	Included	0.61 (0.03)	0.63 (0.03)	0.62 (0.04)	0.63 (0.03)	0.52 (0.04)	0.59 (0.04)	0.74 (0.03)	0.79 (0.03)	330	109
	Difference	**0.01**	**0.01**	**0.01**	**0**	**0.01**	**0**	**0.03**	**0.02**		12
DT degeneration or necrosis	Excluded	0.51 (0.06)	0.67 (0.04)	0.89 (0.03)	0.73 (0.05)	0.94 (0.03)	0.87 (0.04)	0.85 (0.02)	0.63 (0.06)	376	20
	Included	0.51 (0.06)	0.66 (0.04)	0.89 (0.03)	0.72 (0.05)	0.94 (0.03)	0.87 (0.04)	0.84 (0.02)	0.63 (0.06)	419	20
	Difference	**0**	**0.01**	**0**	**0.01**	**0**	**0**	**0.01**	**0**		0
CD degeneration or necrosis	Excluded	0.54 (0.04)	0.57 (0.04)	0.56 (0.06)	0.58 (0.04)	0.92 (0.02)	0.72 (0.04)	0.93 (0.02)	0.76 (0.03)	340	56
	Included	0.56 (0.04)	0.59 (0.04)	0.59 (0.06)	0.59 (0.04)	0.90 (0.02)	0.72 (0.04)	0.93 (0.02)	0.74 (0.04)	377	62
	Difference	**0.02**	**0.02**	**0.03**	**0.01**	**0.02**	**0**	**0**	**0.02**		6
CD degen./necrosis + regenera-tion with no PT injury	Excluded	0.64 (0.05)	0.61 (0.07)	0.63 (0.11)	0.52 (0.06)	0.88 (0.03)	0.72 (0.06)	0.85 (0.06)	0.76 (0.06)	377	19
	Included	0.64 (0.05)	0.59 (0.06)	0.63 (0.10)	0.51 (0.06)	0.88 (0.03)	0.72 (0.06)	0.85 (0.06)	0.76 (0.05)	419	20
	Difference	**0**	**0.02**	**0**	**0.01**	**0**	**0**	**0**	**0**		1
CD degen./ necrosis with no PT injury or regeneration	Excluded	0.51 (0.05)	0.55 (0.05)	0.52 (0.07)	0.61 (0.04)	0.90 (0.02)	0.70 (0.05)	0.92 (0.02)	0.73 (0.04)	359	37
	Included	0.51 (0.05)	0.58 (0.05)	0.56 (0.07)	0.62 (0.04)	0.87 (0.03)	0.69 (0.05)	0.93 (0.01)	0.70 (0.04)	397	42
	Difference	**0**	**0.03**	**0.04**	**0.01**	**0.03**	**0.01**	**0.01**	**0.03**		5
CD regeneration or basophilia	Excluded	0.58 (0.07)	0.66 (0.07)	0.81 (0.07)	0.51 (0.06)	0.77 (0.05)	0.54 (0.06)	0.84 (0.07)	0.77 (0.04)	382	14
	Included	0.61 (0.07)	0.68 (0.06)	0.82 (0.06)	0.53 (0.06)	0.74 (0.05)	0.54 (0.06)	0.86 (0.06)	0.73 (0.05)	422	17
	Difference	**0.03**	**0.02**	**0.01**	**0.02**	**0.03**	**0**	**0.02**	**0.04**		3
Regeneration NOS with no degeneration	Excluded	0.52 (0.05)	0.58 (0.05)	0.57 (0.06)	0.52 (0.05)	0.52 (0.06)	0.56 (0.05)	0.54 (0.07)	0.56 (0.05)	370	26
	Included	0.53 (0.05)	0.55 (0.05)	0.57 (0.06)	0.50 (0.04)	0.51 (0.05)	0.55 (0.05)	0.56 (0.06)	0.54 (0.05)	404	35
	Difference	**0.01**	**0.03**	**0**	**0.02**	**0.01**	**0.01**	**0.02**	**0.02**		9
Intratubular casts, granular, cortex	Excluded	0.62 (0.09)	0.59 (0.08)	0.54 (0.11)	0.71 (0.08)	0.79 (0.07)	0.56 (0.11)	0.51 (0.10)	0.64 (0.09)	384	12
	Included	0.61 (0.09)	0.58 (0.08)	0.54 (0.11)	0.71 (0.08)	0.79 (0.07)	0.56 (0.11)	0.51 (0.10)	0.65 (0.09)	427	12
	Difference	**0.01**	**0.01**	**0**	**0**	**0**	**0.02**	**0**	**0.01**		0
Intratubular casts, hyaline, cortex	Excluded	0.79 (0.06)	0.82 (0.05)	0.70 (0.06)	0.78 (0.06)	0.69 (0.07)	0.76 (0.06)	0.71 (0.05)	0.83 (0.05)	364	32
	Included	0.79 (0.05)	0.82 (0.05)	0.70 (0.05)	0.79 (0.05)	0.70 (0.07)	0.76 (0.06)	0.71 (0.05)	0.84 (0.04)	406	33
	Difference	**0**	**0**	**0**	**0.01**	**0.01**	**0**	**0**	**0.01**		1
Inflammation, interstitial, chronic, cortex	Excluded	0.64 (0.04)	0.64 (0.04)	0.59 (0.04)	0.64 (0.04)	0.62 (0.04)	0.68 (0.04)	0.57 (0.04)	0.61 (0.04)	328	68
	Included	0.64 (0.04)	0.66 (0.04)	0.58 (0.04)	0.62 (0.04)	0.63 (0.04)	0.65 (0.04)	0.53 (0.03)	0.58 (0.04)	356	83
	Difference	**0**	**0.02**	**0.01**	**0.02**	**0.01**	**0.03**	**0.04**	**0.03**		15

iv. Pairwise statistical analysis

Pairwise comparisons of AUC_{ROC} for α-GST vs. reference biomarkers

	α-GST		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.84	+	BUN	0.62	0.22	<.001	*<.001*	**307**	**89**
	0.84	+	SCr	0.62	0.22	<.001	*<.001*		
	0.84	+	NAG	0.69	0.15	<.001	*<.001*		
	0.84	+	Protein	0.73	0.11	0.007	0.212		
PT deg/nec with no regen	0.74	+	BUN	0.56	0.18	0.002	0.055	348	48
	0.74	+	SCr	0.58	0.16	0.001	*0.039*		
	0.74	+	NAG	0.52	0.22	<.001	*<.001*		
	0.74	+	Protein	0.53	0.20	0.002	0.060		
PT deg/nec with regen	0.87	+	BUN	0.79	0.08	0.068	0.993	355	41
	0.87	+	SCr	0.82	0.05	0.371	0.993		
	0.87	+	NAG	0.87	0.00	0.982	0.993		
	0.87	+	Protein	0.89	-0.02	0.645	0.993		
Cortical tubular regeneration/basophilia	0.52	+	BUN	0.62	-0.10	0.028	0.642	**299**	**97**
	0.52	+	SCr	0.64	-0.12	0.012	0.308		
	0.52	+	NAG	0.63	-0.10	0.009	0.261		
	0.52	+	Protein	0.63	-0.11	<.001	**0.019**		
DT degeneration or necrosis	0.94	-	BUN	0.52	0.42	<.001	*<.001*	376	20
	0.94	-	SCr	0.67	0.27	<.001	*<.001*		
	0.94	-	NAG	0.89	0.05	0.204	0.993		
	0.94	-	Protein	0.73	0.21	<.001	*<.001*		
CD degeneration or necrosis	0.92	-	BUN	0.54	0.38	<.001	*<.001*	**340**	**56**
	0.92	-	SCr	0.57	0.35	<.001	*<.001*		
	0.92	-	NAG	0.56	0.36	<.001	*<.001*		
	0.92	-	Protein	0.58	0.34	<.001	*<.001*		
CD deg/nec with no regen	0.88	-	BUN	0.64	0.24	<.001	*0.004*	377	19
	0.88	-	SCr	0.60	0.28	<.001	*0.002*		
	0.88	-	NAG	0.63	0.26	0.011	0.289		
	0.88	-	Protein	0.52	0.36	<.001	*<.001*		
CD deg/nec with regen	0.90	-	BUN	0.51	0.38	<.001	*<.001*	359	37
	0.90	-	SCr	0.55	0.35	<.001	*<.001*		
	0.90	-	NAG	0.52	0.38	<.001	*<.001*		
	0.90	-	Protein	0.61	0.29	<.001	*<.001*		
Medullary tubular regeneration/basophilia	0.77	-	BUN	0.59	0.18	0.072	0.993	382	14
	0.77	-	SCr	0.66	0.11	0.197	0.993		
	0.77	-	NAG	0.81	-0.04	0.675	0.993		
	0.77	-	Protein	0.51	0.26	<.001	*0.002*		
Regeneration NOS with no degeneration	0.52	-	BUN	0.52	0.00	0.993	0.993	370	26
	0.52	-	SCr	0.58	-0.06	0.496	0.993		
	0.52	-	NAG	0.57	-0.05	0.541	0.993		
	0.52	-	Protein	0.52	0.01	0.918	0.993		
Intratubular casts, granular, cortex	0.79	+	BUN	0.62	0.17	0.037	0.777	384	12
	0.79	+	SCr	0.59	0.20	0.026	0.614		
	0.79	+	NAG	0.54	0.25	0.032	0.697		
	0.79	+	Protein	0.71	0.08	0.514	0.993		
Intratubular casts, hyaline, cortex	0.69	+	BUN	0.79	-0.11	0.055	0.993	364	32
	0.69	+	SCr	0.82	-0.14	0.013	0.329		
	0.69	+	NAG	0.70	-0.01	0.735	0.993		
	0.69	+	Protein	0.78	-0.09	0.066	0.993		
Inflammation, interstitial, chronic, cortex	0.62	+	BUN	0.63	-0.01	0.909	0.993	328	68
	0.62	+	SCr	0.64	-0.01	0.811	0.993		
	0.62	+	NAG	0.59	0.04	0.364	0.993		
	0.62	+	Protein	0.63	-0.01	0.893	0.993		

Pairwise comparisons of AUC_{ROC} for µ-GST vs. reference biomarkers

	µ-GST		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.77	+	BUN	0.62	0.15	<.001	*0.002*	307	89
	0.77	+	SCr	0.62	0.15	<.001	*<.001*		
	0.77	+	NAG	0.69	0.08	0.007	0.274		
	0.77	+	Protein	0.73	0.05	0.276	0.937		
PT deg/nec with no regen	0.62	-	BUN	0.56	0.06	0.236	0.937	348	48
	0.62	-	SCr	0.58	0.04	0.427	0.937		
	0.62	-	NAG	0.52	0.10	0.009	0.351		
	0.62	-	Protein	0.53	0.08	0.173	0.937		
PT deg/nec with regen	0.87	+	BUN	0.79	0.09	0.027	0.937	355	41
	0.87	+	SCr	0.82	0.05	0.079	0.937		
	0.87	+	NAG	0.87	0.01	0.864	0.937		
	0.87	+	Protein	0.89	-0.01	0.789	0.937		
Cortical tubular regeneration/basophilia	0.59	+	BUN	0.62	-0.03	0.413	0.937	299	97
	0.59	+	SCr	0.64	-0.05	0.235	0.937		
	0.59	+	NAG	0.63	-0.04	0.274	0.937		
	0.59	+	Protein	0.63	-0.04	0.234	0.937		
DT degeneration or necrosis	0.87	-	BUN	0.52	0.36	<.001	*<.001*	376	20
	0.87	-	SCr	0.67	0.20	<.001	*0.001*		
	0.87	-	NAG	0.89	-0.01	0.756	0.937		
	0.87	-	Protein	0.73	0.15	0.013	0.497		
CD degeneration or necrosis	0.72	-	BUN	0.54	0.18	<.001	*0.007*	340	56
	0.72	-	SCr	0.57	0.15	0.017	0.642		
	0.72	-	NAG	0.56	0.16	0.003	0.132		
	0.72	-	Protein	0.58	0.14	0.001	0.054		
CD deg/nec with no regen	0.72	-	BUN	0.64	0.08	0.249	0.937	377	19
	0.72	-	SCr	0.60	0.12	0.151	0.937		
	0.72	-	NAG	0.63	0.10	0.380	0.937		
	0.72	-	Protein	0.52	0.20	<.001	*0.035*		
CD deg/nec with regen	0.70	-	BUN	0.51	0.18	<.001	*0.043*	359	37
	0.70	-	SCr	0.55	0.15	0.073	0.937		
	0.70	-	NAG	0.52	0.18	0.001	0.053		
	0.70	-	Protein	0.61	0.09	0.096	0.937		
Medullary tubular regeneration/basophilia	0.54	-	BUN	0.59	-0.04	0.614	0.937	382	14
	0.54	-	SCr	0.66	-0.12	0.168	0.937		
	0.54	-	NAG	0.81	-0.27	0.007	0.292		
	0.54	-	Protein	0.51	0.03	0.719	0.937		
Regeneration NOS with no degeneration	0.56	-	BUN	0.52	0.04	0.597	0.937	370	26
	0.56	-	SCr	0.58	-0.01	0.837	0.937		
	0.56	-	NAG	0.57	-0.01	0.937	0.937		
	0.56	-	Protein	0.52	0.05	0.432	0.937		
Intratubular casts, granular, cortex	0.56	+	BUN	0.62	-0.06	0.226	0.937	384	12
	0.56	+	SCr	0.59	-0.03	0.739	0.937		
	0.56	+	NAG	0.54	0.02	0.710	0.937		
	0.56	+	Protein	0.71	-0.15	0.179	0.937		
Intratubular casts, hyaline, cortex	0.76	+	BUN	0.79	-0.04	0.494	0.937	364	32
	0.76	+	SCr	0.82	-0.07	0.141	0.937		
	0.76	+	NAG	0.70	0.05	0.114	0.937		
	0.76	+	Protein	0.78	-0.02	0.539	0.937		
Inflammation, interstitial, chronic, cortex	0.67	+	BUN	0.63	0.04	0.366	0.937	328	68
	0.67	+	SCr	0.64	0.04	0.472	0.937		
	0.67	+	NAG	0.59	0.08	0.027	0.937		
	0.67	+	Protein	0.63	0.04	0.269	0.937		

Pairwise comparisons of AUC_{ROC} for RPA-1 vs. reference biomarkers

	RPA		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.59	-	BUN	0.62	-0.02	0.560	0.918	307	89
	0.59	-	SCr	0.62	-0.03	0.487	0.918		
	0.59	-	NAG	0.69	-0.09	0.001	*0.039*		
	0.59	-	Protein	0.73	-0.13	0.003	0.092		
PT deg/nec with no regen	0.57	-	BUN	0.56	0.01	0.826	0.918	348	48
	0.57	-	SCr	0.58	-0.01	0.828	0.918		
	0.57	-	NAG	0.52	0.05	0.141	0.918		
	0.57	-	Protein	0.53	0.03	0.572	0.918		
PT deg/nec with regen	0.76	+	BUN	0.79	-0.03	0.502	0.918	355	41
	0.76	+	SCr	0.82	-0.07	0.166	0.918		
	0.76	+	NAG	0.87	-0.11	<.001	*0.020*		
	0.76	+	Protein	0.89	-0.13	0.005	0.165		
Cortical tubular regeneration/basophilia	0.77	+	BUN	0.62	0.14	<.001	*0.031*	299	97
	0.77	+	SCr	0.64	0.12	0.005	0.165		
	0.77	+	NAG	0.63	0.14	0.002	0.057		
	0.77	+	Protein	0.63	0.13	0.004	0.140		
DT degeneration or necrosis	0.85	+	BUN	0.52	0.33	<.001	*<.001*	376	20
	0.85	+	SCr	0.67	0.18	<.001	*0.019*		
	0.85	+	NAG	0.89	-0.04	0.273	0.918		
	0.85	+	Protein	0.73	0.12	0.015	0.437		
CD degeneration or necrosis	0.93	+	BUN	0.54	0.38	<.001	*<.001*	340	56
	0.93	+	SCr	0.57	0.35	<.001	*<.001*		
	0.93	+	NAG	0.56	0.36	<.001	*<.001*		
	0.93	+	Protein	0.58	0.35	<.001	*<.001*		
CD deg/nec with no regen	0.85	+	BUN	0.64	0.21	0.002	0.060	377	19
	0.85	+	SCr	0.60	0.25	0.018	0.514		
	0.85	+	NAG	0.63	0.23	0.033	0.866		
	0.85	+	Protein	0.52	0.34	<.001	*0.003*		
CD deg/nec with regen	0.92	+	BUN	0.51	0.41	<.001	*<.001*	359	37
	0.92	+	SCr	0.55	0.37	<.001	*<.001*		
	0.92	+	NAG	0.52	0.40	<.001	*<.001*		
	0.92	+	Protein	0.61	0.31	<.001	*<.001*		
Medullary tubular regeneration/basophilia	0.84	+	BUN	0.59	0.26	0.004	0.140	382	14
	0.84	+	SCr	0.66	0.18	0.118	0.918		
	0.84	+	NAG	0.81	0.03	0.572	0.918		
	0.84	+	Protein	0.51	0.33	<.001	*0.002*		
Regeneration NOS with no degeneration	0.53	+	BUN	0.52	0.01	0.867	0.918	370	26
	0.53	+	SCr	0.58	-0.04	0.645	0.918		
	0.53	+	NAG	0.57	-0.03	0.669	0.918		
	0.53	+	Protein	0.52	0.02	0.840	0.918		
Intratubular casts, granular, cortex	0.51	-	BUN	0.62	-0.11	0.205	0.918	384	12
	0.51	-	SCr	0.59	-0.08	0.371	0.918		
	0.51	-	NAG	0.54	-0.04	0.539	0.918		
	0.51	-	Protein	0.71	-0.20	0.006	0.165		
Intratubular casts, hyaline, cortex	0.71	+	BUN	0.79	-0.09	0.218	0.918	364	32
	0.71	+	SCr	0.82	-0.12	0.028	0.744		
	0.71	+	NAG	0.70	0.01	0.918	0.918		
	0.71	+	Protein	0.78	-0.07	0.289	0.918		
Inflammation, interstitial, chronic, cortex	0.56	-	BUN	0.63	-0.07	0.167	0.918	328	68
	0.56	-	SCr	0.64	-0.07	0.141	0.918		
	0.56	-	NAG	0.59	-0.02	0.549	0.918		
	0.56	-	Protein	0.63	-0.07	0.124	0.918		

Pairwise comparisons of AUC_{ROC} for clusterin vs. reference biomarkers

	Clusterin		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.69	+	BUN	0.62	0.07	0.063	0.900	307	89
	0.69	+	SCr	0.62	0.07	0.053	0.900		
	0.69	+	NAG	0.69	0.01	0.840	0.900		
	0.69	+	Protein	0.73	-0.03	0.358	0.900		
PT deg/nec with no regen	0.57	-	BUN	0.56	0.01	0.839	0.900	348	48
	0.57	-	SCr	0.58	-0.01	0.774	0.900		
	0.57	-	NAG	0.52	0.05	0.162	0.900		
	0.57	-	Protein	0.53	0.03	0.562	0.900		
PT deg/nec with regen	0.94	+	BUN	0.79	0.15	0.001	0.057	355	41
	0.94	+	SCr	0.82	0.11	0.008	0.272		
	0.94	+	NAG	0.87	0.07	<.001	*0.001*		
	0.94	+	Protein	0.89	0.05	0.116	0.900		
Cortical tubular regeneration/basophilia	0.81	+	BUN	0.62	0.19	<.001	*<.001*	299	97
	0.81	+	SCr	0.64	0.17	<.001	*<.001*		
	0.81	+	NAG	0.63	0.19	<.001	*<.001*		
	0.81	+	Protein	0.63	0.18	<.001	*<.001*		
DT degeneration or necrosis	0.63	+	BUN	0.52	0.11	0.138	0.900	376	20
	0.63	+	SCr	0.67	-0.04	0.451	0.900		
	0.63	+	NAG	0.89	-0.26	0.002	0.073		
	0.63	+	Protein	0.73	-0.10	0.128	0.900		
CD degeneration or necrosis	0.76	+	BUN	0.54	0.21	<.001	*<.001*	340	56
	0.76	+	SCr	0.57	0.19	0.001	0.053		
	0.76	+	NAG	0.56	0.20	<.001	*0.038*		
	0.76	+	Protein	0.58	0.18	<.001	*<.001*		
CD deg/nec with no regen	0.76	+	BUN	0.64	0.12	0.100	0.900	377	19
	0.76	+	SCr	0.60	0.15	0.101	0.900		
	0.76	+	NAG	0.63	0.13	0.220	0.900		
	0.76	+	Protein	0.52	0.24	<.001	*0.033*		
CD deg/nec with regen	0.73	+	BUN	0.51	0.22	<.001	*0.002*	359	37
	0.73	+	SCr	0.55	0.18	0.011	0.350		
	0.73	+	NAG	0.52	0.21	0.002	0.065		
	0.73	+	Protein	0.61	0.13	0.007	0.255		
Medullary tubular regeneration/basophilia	0.77	+	BUN	0.59	0.19	0.006	0.227	382	14
	0.77	+	SCr	0.66	0.11	0.241	0.900		
	0.77	+	NAG	0.81	-0.04	0.543	0.900		
	0.77	+	Protein	0.51	0.26	<.001	*0.019*		
Regeneration NOS with no degeneration	0.56	-	BUN	0.52	0.04	0.568	0.900	370	26
	0.56	-	SCr	0.58	-0.02	0.799	0.900		
	0.56	-	NAG	0.57	-0.01	0.900	0.900		
	0.56	-	Protein	0.52	0.04	0.464	0.900		
Intratubular casts, granular, cortex	0.64	+	BUN	0.62	0.02	0.758	0.900	384	12
	0.64	+	SCr	0.59	0.05	0.589	0.900		
	0.64	+	NAG	0.54	0.10	0.102	0.900		
	0.64	+	Protein	0.71	-0.07	0.368	0.900		
Intratubular casts, hyaline, cortex	0.83	+	BUN	0.79	0.04	0.465	0.900	364	32
	0.83	+	SCr	0.82	0.01	0.811	0.900		
	0.83	+	NAG	0.70	0.13	0.002	0.059		
	0.83	+	Protein	0.78	0.06	0.260	0.900		
Inflammation, interstitial, chronic, cortex	0.61	+	BUN	0.63	-0.02	0.646	0.900	328	68
	0.61	+	SCr	0.64	-0.03	0.556	0.900		
	0.61	+	NAG	0.59	0.02	0.616	0.900		
	0.61	+	Protein	0.63	-0.02	0.590	0.900		

Pairwise comparisons of AUC_{ROC} for NAG versus BUN and serum creatinine

	NAG		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.69	+	BUN	0.62	0.07	0.111	0.942	307	89
	0.69	+	SCr	0.62	0.06	0.119	0.942		
PT deg/nec with no regen	0.52	-	BUN	0.56	-0.04	0.482	0.942	348	48
	0.52	-	SCr	0.58	-0.06	0.222	0.942		
PT deg/nec with regen	0.87	+	BUN	0.79	0.08	0.097	0.942	355	41
	0.87	+	SCr	0.82	0.05	0.297	0.942		
Cortical tubular regeneration/basophilia	0.63	+	BUN	0.62	0.01	0.910	0.942	299	97
	0.63	+	SCr	0.64	-0.01	0.801	0.942		
DT degeneration or necrosis	0.89	-	BUN	0.52	0.37	<.001	*<.001*	**376**	**20**
	0.89	-	SCr	0.67	0.22	<.001	*0.006*		
CD degeneration or necrosis	0.56	+	BUN	0.54	0.02	0.799	0.942	340	56
	0.56	+	SCr	0.57	-0.01	0.899	0.942		
CD deg/nec with no regen	0.63	+	BUN	0.64	-0.02	0.902	0.942	377	19
	0.63	+	SCr	0.60	0.02	0.881	0.942		
CD deg/nec with regen	0.52	+	BUN	0.51	0.01	0.932	0.942	359	37
	0.52	+	SCr	0.55	-0.03	0.805	0.942		
Medullary tubular regeneration/basophilia	0.81	+	BUN	0.59	0.22	0.005	0.127	382	14
	0.81	+	SCr	0.66	0.15	0.251	0.942		
Regeneration NOS with no degeneration	0.57	-	BUN	0.52	0.05	0.636	0.942	370	26
	0.57	-	SCr	0.58	-0.01	0.942	0.942		
Intratubular casts, granular, cortex	0.54	+	BUN	0.62	-0.08	0.298	0.942	384	12
	0.54	+	SCr	0.59	-0.05	0.619	0.942		
Intratubular casts, hyaline, cortex	0.70	+	BUN	0.79	-0.09	0.146	0.942	364	32
	0.70	+	SCr	0.82	-0.12	0.036	0.819		
Inflammation, interstitial, chronic, cortex	0.59	+	BUN	0.63	-0.04	0.385	0.942	328	68
	0.59	+	SCr	0.64	-0.05	0.339	0.942		

Pairwise comparisons of AUC_{ROC} for protein versus BUN and serum creatinine

	Protein		Reference	Reference	AUC	P-value		Neg	Pos
Pathology	AUC	Direction	Marker	AUC	Difference	Raw	Adjusted		
PT degeneration or necrosis	0.73	+	BUN	0.62	0.11	0.020	0.479	307	89
	0.73	+	SCr	0.62	0.10	0.016	0.392		
PT deg/nec with no regen	0.53	-	BUN	0.56	-0.02	0.728	1.000	348	48
	0.53	-	SCr	0.58	-0.05	0.446	1.000		
PT deg/nec with regen	0.89	+	BUN	0.79	0.10	0.085	1.000	355	41
	0.89	+	SCr	0.82	0.06	0.192	1.000		
Cortical tubular regeneration/basophilia	0.63	+	BUN	0.62	0.01	0.828	1.000	299	97
	0.63	+	SCr	0.64	-0.01	0.824	1.000		
DT degeneration or necrosis	0.73	-	BUN	0.52	0.21	0.015	0.391	376	20
	0.73	-	SCr	0.67	0.06	0.303	1.000		
CD degeneration or necrosis	0.58	-	BUN	0.54	0.04	0.465	1.000	340	56
	0.58	-	SCr	0.57	0.01	0.895	1.000		
CD deg/nec with no regen	0.52	+	BUN	0.64	-0.12	0.077	1.000	377	19
	0.52	+	SCr	0.60	-0.09	0.307	1.000		
CD deg/nec with regen	0.61	-	BUN	0.51	0.09	0.148	1.000	359	37
	0.61	-	SCr	0.55	0.06	0.441	1.000		
Medullary tubular regeneration/basophilia	0.51	-	BUN	0.59	-0.08	0.447	1.000	382	14
	0.51	-	SCr	0.66	-0.15	0.125	1.000		
Regeneration NOS with no degeneration	0.52	-	BUN	0.52	-0.00	0.956	1.000	370	26
	0.52	-	SCr	0.58	-0.06	0.433	1.000		
Intratubular casts, granular, cortex	0.71	+	BUN	0.62	0.09	0.410	1.000	384	12
	0.71	+	SCr	0.59	0.12	0.292	1.000		
Intratubular casts, hyaline, cortex	0.78	+	BUN	0.79	-0.02	0.728	1.000	364	32
	0.78	+	SCr	0.82	-0.05	0.300	1.000		
Inflammation, interstitial, chronic, cortex	0.63	+	BUN	0.63	0.00	1.000	1.000	328	68
	0.63	+	SCr	0.64	-0.01	0.891	1.000		

v. Incremental statistical analysis

Incremental analyses

AUC_{ROC} for reference biomarker sets alone and in conjunction with α-GST

Pathology	Reference Markers	AUC: Reference Alone	AUC: Reference + α-GST	P-value: Raw	P-value: Adjusted
PT degeneration or necrosis	BUN+SCr	0.63	0.85	<.001	***<.001***
	NAG+Protein	0.72	0.84	<.001	***0.010***
PT deg/nec with no regen	BUN+SCr	0.58	0.72	<.001	***<.001***
	NAG+Protein	0.52	0.64	0.004	0.066
PT deg/nec with regen	BUN+SCr	0.83	0.85	0.034	0.373
	NAG+Protein	0.90	0.91	0.139	0.833
Cortical tubular regeneration/basophilia	BUN+SCr	0.65	0.65	0.092	0.735
	NAG+Protein	0.66	0.66	0.060	0.564
DT degeneration or necrosis	BUN+SCr	0.56	0.95	<.001	***<.001***
	NAG+Protein	0.89	0.97	0.008	0.124
CD degeneration or necrosis	BUN+SCr	0.61	0.93	<.001	***<.001***
	NAG+Protein	0.63	0.96	<.001	***<.001***
CD deg/nec with no regen	BUN+SCr	0.68	0.89	<.001	***0.009***
	NAG+Protein	0.63	0.93	0.003	0.053
CD deg/nec with regen	BUN+SCr	0.57	0.90	<.001	***<.001***
	NAG+Protein	0.65	0.92	<.001	***<.001***
Medullary tubular regeneration/basophilia	BUN+SCr	0.77	0.82	0.031	0.373
	NAG+Protein	0.83	0.86	0.136	0.833
Regeneration NOS with no degeneration	BUN+SCr	0.60	0.60	0.875	0.975
	NAG+Protein	0.55	0.55	0.975	0.975
Intratubular casts, granular, cortex	BUN+SCr	0.57	0.71	0.034	0.373
	NAG+Protein	0.59	0.60	0.943	0.975
Intratubular casts, hyaline, cortex	BUN+SCr	0.82	0.82	0.063	0.564
	NAG+Protein	0.77	0.75	0.026	0.369
Inflammation, interstitial, chronic, cortex	BUN+SCr	0.60	0.59	0.761	0.975
	NAG+Protein	0.60	0.60	0.618	0.975

AUC_{ROC} for reference biomarker sets alone and in conjunction with μ-GST

Pathology	Reference Markers	AUC: Reference Alone	AUC: Reference + μ-GST	P-value: Raw	P-value: Adjusted
PT degeneration or necrosis	BUN+SCr	0.63	0.73	<.001	***<.001***
	NAG+Protein	0.72	0.77	0.006	0.120
PT deg/nec with no regen	BUN+SCr	0.58	0.60	0.249	0.898
	NAG+Protein	0.52	0.55	0.127	0.898
PT deg/nec with regen	BUN+SCr	0.83	0.87	0.043	0.677
	NAG+Protein	0.90	0.93	0.052	0.729
Cortical tubular regeneration/basophilia	BUN+SCr	0.65	0.64	0.436	0.898
	NAG+Protein	0.66	0.66	0.898	0.898
DT degeneration or necrosis	BUN+SCr	0.56	0.89	<.001	***<.001***
	NAG+Protein	0.89	0.94	0.066	0.851
CD degeneration or necrosis	BUN+SCr	0.61	0.75	0.002	***0.041***
	NAG+Protein	0.63	0.80	<.001	***<.001***
CD deg/nec with no regen	BUN+SCr	0.68	0.73	0.338	0.898
	NAG+Protein	0.63	0.79	0.003	0.071
CD deg/nec with regen	BUN+SCr	0.57	0.71	0.021	0.383
	NAG+Protein	0.65	0.80	<.001	***0.014***
Medullary tubular regeneration/basophilia	BUN+SCr	0.77	0.79	0.088	0.898
	NAG+Protein	0.83	0.85	0.071	0.851
Regeneration NOS with no degeneration	BUN+SCr	0.60	0.63	0.510	0.898
	NAG+Protein	0.55	0.56	0.835	0.898
Intratubular casts, granular, cortex	BUN+SCr	0.57	0.59	0.849	0.898
	NAG+Protein	0.59	0.61	0.887	0.898
Intratubular casts, hyaline, cortex	BUN+SCr	0.82	0.83	0.683	0.898
	NAG+Protein	0.77	0.80	0.005	0.105
Inflammation, interstitial, chronic, cortex	BUN+SCr	0.60	0.68	0.041	0.677
	NAG+Protein	0.60	0.69	0.045	0.677

AUC_{ROC} for reference biomarker sets alone and in conjunction with RPA-1

Pathology	Reference Markers	AUC: Reference Alone	AUC: Reference + RPA	P-value: Raw	P-value: Adjusted
PT degeneration or necrosis	BUN+SCr	0.63	0.66	<.001	***0.013***
	NAG+Protein	0.72	0.75	0.056	0.723
PT deg/nec with no regen	BUN+SCr	0.58	0.62	0.292	0.723
	NAG+Protein	0.52	0.56	0.071	0.723
PT deg/nec with regen	BUN+SCr	0.83	0.83	0.062	0.723
	NAG+Protein	0.90	0.93	0.009	0.137
Cortical tubular regeneration/basophilia	BUN+SCr	0.65	0.75	<.001	***0.006***
	NAG+Protein	0.66	0.75	<.001	***<.001***
DT degeneration or necrosis	BUN+SCr	0.56	0.69	<.001	***0.004***
	NAG+Protein	0.89	0.96	<.001	***0.008***
CD degeneration or necrosis	BUN+SCr	0.61	0.93	<.001	***<.001***
	NAG+Protein	0.63	0.94	<.001	***<.001***
CD deg/nec with no regen	BUN+SCr	0.68	0.85	0.002	***0.037***
	NAG+Protein	0.63	0.85	0.008	0.127
CD deg/nec with regen	BUN+SCr	0.57	0.91	<.001	***<.001***
	NAG+Protein	0.65	0.91	<.001	***0.004***
Medullary tubular regeneration/basophilia	BUN+SCr	0.77	0.84	0.105	0.723
	NAG+Protein	0.83	0.84	0.472	0.723
Regeneration NOS with no degeneration	BUN+SCr	0.60	0.61	0.723	0.723
	NAG+Protein	0.55	0.58	0.514	0.723
Intratubular casts, granular, cortex	BUN+SCr	0.57	0.52	0.700	0.723
	NAG+Protein	0.59	0.73	0.083	0.723
Intratubular casts, hyaline, cortex	BUN+SCr	0.82	0.83	0.591	0.723
	NAG+Protein	0.77	0.78	0.294	0.723
Inflammation, interstitial, chronic, cortex	BUN+SCr	0.60	0.61	0.547	0.723
	NAG+Protein	0.60	0.66	0.046	0.648

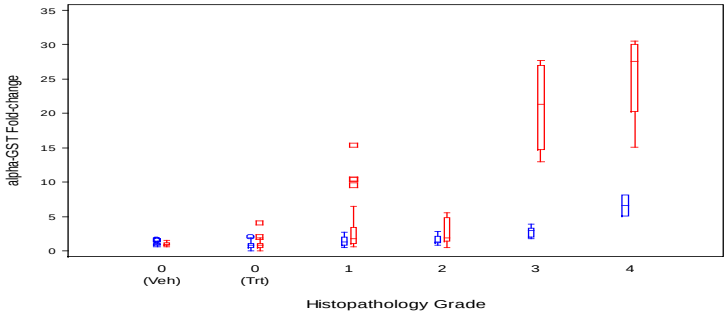
AUC_{ROC} for reference biomarker sets alone and in conjunction with clusterin

Pathology	Reference Markers	AUC: Reference Alone	AUC: Reference + Clusterin	P-value: Raw	P-value: Adjusted
PT degeneration or necrosis	BUN+SCr	0.63	0.62	0.490	0.990
	NAG+Protein	0.72	0.73	0.061	0.968
PT deg/nec with no regen	BUN+SCr	0.58	0.59	0.729	0.990
	NAG+Protein	0.52	0.57	0.398	0.990
PT deg/nec with regen	BUN+SCr	0.83	0.84	0.111	0.990
	NAG+Protein	0.90	0.91	0.007	0.138
Cortical tubular regeneration/basophilia	BUN+SCr	0.65	0.75	<.001	***0.001***
	NAG+Protein	0.66	0.76	<.001	***<.001***
DT degeneration or necrosis	BUN+SCr	0.56	0.58	0.822	0.990
	NAG+Protein	0.89	0.90	0.227	0.990
CD degeneration or necrosis	BUN+SCr	0.61	0.71	0.002	***0.043***
	NAG+Protein	0.63	0.77	<.001	***<.001***
CD deg/nec with no regen	BUN+SCr	0.68	0.75	0.026	0.492
	NAG+Protein	0.63	0.67	0.045	0.804
CD deg/nec with regen	BUN+SCr	0.57	0.67	0.013	0.262
	NAG+Protein	0.65	0.77	0.004	0.094
Medullary tubular regeneration/basophilia	BUN+SCr	0.77	0.77	0.409	0.990
	NAG+Protein	0.83	0.82	0.418	0.990
Regeneration NOS with no degeneration	BUN+SCr	0.60	0.61	0.882	0.990
	NAG+Protein	0.55	0.53	0.211	0.990
Intratubular casts, granular, cortex	BUN+SCr	0.57	0.67	0.311	0.990
	NAG+Protein	0.59	0.52	0.228	0.990
Intratubular casts, hyaline, cortex	BUN+SCr	0.82	0.82	0.622	0.990
	NAG+Protein	0.77	0.82	0.085	0.990
Inflammation, interstitial, chronic, cortex	BUN+SCr	0.60	0.60	0.990	0.990
	NAG+Protein	0.60	0.65	0.056	0.960

vi. Box-plots – biomarker versus histopathology grade

Figure 6: Sponsor's figures – box-plots from initial submission

Box-plot of α-GST fold-change versus histopathology grade for PT degeneration or necrosis



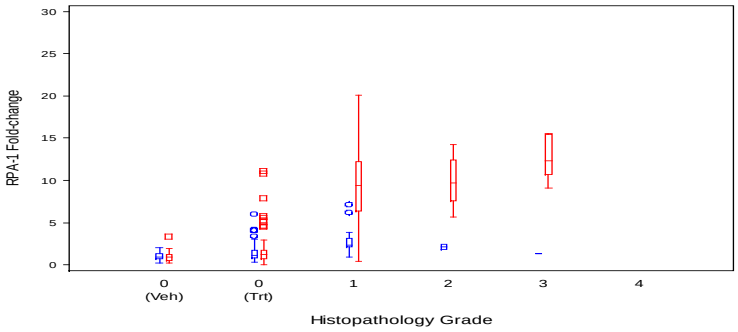
PT degeneration or necrosis

Sprague Dawley
Neg # Pos
170 34

Han Wistar
Neg # Pos
137 55

Total
Neg # Pos
307 89

Box-plot of RPA-1 fold-change versus histopathology grade for CD degeneration or necrosis



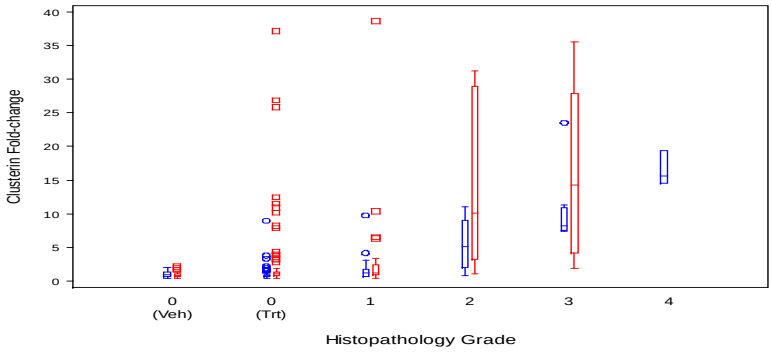
CD degeneration or necrosis

Sprague Dawley
Neg # Pos
179 25

Han Wistar
Neg # Pos
161 31

Total
Neg # Pos
340 56

Box-plot of clusterin fold-change vs histopathology grade for cortical tubular regeneration/basophilia



Cortical tubular regeneration/basophilia

Sprague Dawley
Neg # Pos
153 51

Han Wistar
Neg # Pos
146 46

Total
Neg # Pos
299 97

vii. Pathology incidence by strain and nephrotoxicant

Cisplatin incidence of observed pathology by strain

	Sprague-Dawley		Han-Wistar		Total		SD >20 pos	HW >20 pos	Tot ≥20 Pos
Pathology	# Neg	# Pos	# Neg	# Pos	# Neg	# Pos			
PT degeneration or necrosis	83	11	64	55	147	66		+	+
PT deg/nec with no regen	88	6	80	39	168	45		+	+
PT deg/nec with regen	89	5	103	16	192	21			+
Cortical tubular regeneration/basophilia	89	5	99	20	188	25		+	+
Regeneration NOS with no degeneration	94	0	115	4	209	4			
Intratubular casts, granular, cortex	94	0	112	7	206	7			
Intratubular casts, hyaline, cortex	90	4	103	16	193	20			+
Inflammation, interstitial, chronic, cortex	67	27	113	6	180	33	+		+

Pathologies relating to claims are highlighted in yellow. Those pathologies with more than 20 total positive animals are indicated with a +.

NPAA incidence of observed pathology by strain

	Sprague-Dawley		Han-Wistar		Total		SD >20 pos	HW >20 pos	Tot ≥20 Pos
Pathology	# Neg	# Pos	# Neg	# Pos	# Neg	# Pos			
Cortical tubular regeneration/basophilia	37	23	47	26	84	49	+	+	+
DT degeneration or necrosis	40	20	73	0	113	20	+		+
CD degeneration or necrosis	35	25	42	31	77	56	+	+	+
CD deg/nec with no regen	54	6	60	13	114	19			
CD deg/nec with regen	41	19	55	18	96	37			+
Medullary tubular regeneration/basophilia	60	0	59	14	119	14			
Regeneration NOS with no degeneration	56	4	58	15	114	19			
Intratubular casts, hyaline, cortex	56	4	73	0	129	4			
Inflammation, interstitial, chronic, cortex	55	5	73	0	128	5			

Gentamicin incidence of observed pathology in Sprague Dawley rats

	Sprague-Dawley		Total		Tot ≥20 Pos
Pathology	# Neg	# Pos	# Neg	# Pos	
PT degeneration or necrosis	27	23	27	23	+
PT deg/nec with no regen	47	3	47	3	
PT deg/nec with regen	30	20	30	20	+
Cortical tubular regeneration/basophilia	27	23	27	23	+
Regeneration NOS with no degeneration	47	3	47	3	
Intratubular casts, granular, cortex	45	5	45	5	
Intratubular casts, hyaline, cortex	42	8	42	8	
Inflammation, interstitial, chronic, cortex	20	30	20	30	+

viii. Reviewer's compilation- Pathology incidence by study and strain

Reviewer's compilation from sponsor's tables - Pathology incidence by study and strain							
		Sprague Dawley		Han Wistar		Total	
Pathology	Study	# Neg.	# Pos	# Neg.	# Pos	# Neg.	# Pos
PT degeneration or necrosis	Cisplatin (C)	83	11	64	55	147	66
	Gentamicin (G)	27	23	0	0	27	23
	NPAA (N)	60	0	73	0	133	0
	Sum C +G + N	170	34	137	55	307	89
	Sum C + N	143	**11**	137	**55**	280	**66**
PT degeneration or necrosis with no regeneration	Cisplatin (C)	88	6	80	39	168	45
	Gentamicin (G)	47	3	0	0	47	3
	NPAA (N)	60	0	73	0	133	0
	Sum C +G + N	195	9	153	39	348	48
	Sum C + N	148	**6**	153	**39**	301	45
PT degeneration or necrosis with regeneration	Cisplatin (C)	89	5	103	16	192	21
	Gentamicin (G)	30	20	0	0	30	20
	NPAA (N)	60	0	73	0	133	0
	Sum C +G + N	179	25	176	16	355	41
	Sum C + N	149	**5**	176	**16**	325	21
CT regeneration/basophilia	Cisplatin (C)	89	5	99	20	188	25
	Gentamicin (G)	27	23	0	0	27	23
	NPAA (N)	37	23	47	26	84	49
	Sum C +G + N	153	51	146	46	299	97
	Sum C + N	126	**28**	146	**46**	272	**74**
DT degeneration or necrosis	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	40	20	73	0	113	20
	Sum C +G + N	184	20	192	0	376	20
	Sum C + N	134	**20**	192	**0**	326	**20**
CD degeneration or necrosis	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	35	25	42	31	77	56
	Sum C +G + N	179	25	161	31	340	56
	Sum C + N	129	**25**	161	**31**	290	**56**
CD degen./necrosis + regenera-tion with no PT injury	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	54	6	60	13	114	19
	Sum C +G + N	198	6	179	13	377	19
	Sum C + N	148	6	179	13	327	19
CD degen./ necrosis with no PT injury or regeneration	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	41	19	55	18	96	37
	Sum C +G + N	185	19	174	18	359	37
	Sum C + N	135	19	174	18	309	37
CD regeneration or basophilia	Cisplatin (C)	94	0	119	0	213	0
	Gentamicin (G)	50	0	0	0	50	0
	NPAA (N)	60	0	59	14	119	14
	Sum C +G + N	204	0	178	14	382	14
	Sum C + N	154	**0**	178	**14**	332	**14**
Regeneration NOS with no degeneration	Cisplatin (C)	94	0	115	4	209	4
	Gentamicin (G)	47	3	0	0	47	3
	NPAA (N)	56	4	58	15	114	19
	Sum C +G + N	197	7	173	19	370	26
	Sum C + N	150	**4**	173	**19**	323	23

Intratubular casts, granular, cortex	Cisplatin (C)	94	0	112	7	206	7
	Gentamicin (G)	45	5	0	0	45	5
	NPAA (N)	60	0	73	0	133	0
	Sum C +G + N	199	5	185	7	384	12
	Sum C + N	154	**0**	185	**7**	339	**7**
Intratubular casts, hyaline, cortex	Cisplatin (C)	90	4	103	16	193	20
	Gentamicin (G)	42	8	0	0	42	8
	NPAA (N)	56	4	73	0	129	4
	Sum C +G + N	188	16	176	16	364	32
	Sum C + N	146	**8**	176	**16**	322	**24**
Inflammation, interstitial, chronic, cortex	Cisplatin (C)	67	27	113	6	180	33
	Gentamicin (G)	20	30	0	0	20	30
	NPAA (N)	55	5	73	0	128	5
	Sum C +G + N	142	62	186	6	328	68
	Sum C + N	122	**32**	186	**6**	308	**38**

Yellow color highlights the principal pathologies claimed.
Blue color highlights the sum of the animals from the cisplatin and NPAA studies; the positive animals are in bold text.
Red text indicates a pathology for which the number of positive animals is >2-fold between the two strains.

ix. Summary of analytical validation

Sponsor's tables provided in December 2008 concerning assay validation

Measuring range

Parameter	Rat α–GST	Rat μ–GST	Rat RPA-1	Rat Clusterin
Calibration curve range	1.56 - 100 µg/L	1.56 - 100 µg/L	3.12 - 100 U/L	0.075 - 4.8 ug/L
Recommended Dilution Factor for Urine	5	10	25	500
Covered urine conc. range	9.75 - 625 µg/L	19.5 – 1250 µg/L	97.5 - 3125 U/L	46.9 - 3000 µg/L

Limit of detection

Parameter	Rat α–GST	Rat μ–GST	Rat RPA-1	Rat Clusterin
Limit of Detection in Assay	0.2 ug/L	0.2 ug/L	3.12 U/L	0.017 ug/L
Limit of Detection in Urine	1.25 ug/L	2.5 ug/L	97.5 U/L	10.6 ug/L

Interference

	rat alpha GST	rat mu GST	rat RPA-1	rat Clusterin	NAG	Pyrogallol Red
Hemoglobin	no	yes	no	-	yes	yes
Conjugated Billirubin	no	no	no	-	yes*	-
Albumin	no	no	no	yes	-	N/A
Sodium Cloride	no	no	no	-	-	-
Metal	-	-	-	-	-	-
Others						
rat IgG	-	-	-	no	-	-
Urea	-	-	-	-	yes	-
aminoglucoside-like antibiotics	-	-	-	-	yes	-

Remarks:

* not specified what type of billirubin

- not tested

Hemoglobin at 100-500 mg/dL interfered in the rat µGST assay
Albumin at 20 and 50 µg/mL interfered with mid and high controls in the clusterin assay

Linearity

Biomarker	Biotrin (2)	R (3) BMS (5)	R (3) SA (4)
Rat α–GST	shown	0.9952	0.98
Rat μ–GST	N/A	0.9933	0.99
Rat RPA-1	shown	1.000	0.99
Rat Clusterin	shown	0.9898	0.99

No uniform procedure for linearity was used across laboratories:
- Biotrin used stabilised native urine samples and/or spiked urine samples,
- SA diluted PC's for alpha and mu GST, calibrator for Clusterin and urine samples for RPA-1
- BMS used pooled native urine samples

Intra-assay reproduce-bility

Biomarker	≤%CV (N) Biotrin (2)	≤%CV (N) BMS (5)	≤%CV (N) SA (4)	≤%CV (N) AZ (6)
Rat α–GST	6.0 (20)	5.2 (12)	7.4 (20)	7.1 (20)
Rat μ–GST	7.1 (10)	4.0 (12)	9.7 (20)	10.4 (20)
Rat RPA-1	6 (24)	5.4 (12)	4.9 (24)	4.0 (20)
Rat Clusterin	7.0 (10)	7.8 (12)	7.6 (24)	7.3 (20)

Inter-assay reproduce-bility

Biomarker	≤%CV (N) Biotrin (2)	≤%CV (N) BMS (5, 6)	≤%CV (N) SA (4, 6)	≤%CV (N) AZ (6, 7)	≤%CV (N) Bayer (7)	≤%CV (N) GSK (7)
Rat α–GST	7.2 (10)	9.7 (24)	12.8 (20)	17.0 (12)	16.4 (4)	7.9 (6)
Rat μ–GST	9.4 (10)	12.9 (16)	12.2 (20)	10.2 (20)	8.8 (4)	10.5 (9)
Rat RPA-1	11 (20)	13.5 (15)	8.6 (100)	7.1 (8)	N/A	1.8 (3)
Rat Clusterin	24.7 (10)	16 (7)	30 (9)	21.0 (30)	26.6 (4)	18.4 (7)

Recovery/ Accuracy

Biomarker	Control Range Mean Biotrin (3)	%Recovery BMS (5, 6)	%Recovery SA (4)	%Recovery AZ (6, 7)	%Recovery Bayer (7)	%Recovery GSK (7)
Rat α–GST	≤ +/- 40%	133%	90%	100%	115%	110%
Rat μ–GST	≤ +/- 30%	119%	90%	94%	96%	111%
Rat RPA-1	≤ +/- 25%	114%	93%	98%	N/A	105 %
Rat Clusterin	≤ +/- 35%	76%	105%	87%	75%	81%

In the above tables, the numbers in parentheses next to a laboratory's name in the header row are references generally referring to specific appendices in the initial submission.

x. Inter-laboratory data by site

Sponsor's tables summarizing inter-laboratory data by site for blinded samples

		Laboratory					
	Level	**AZ**	**BMS**	**Biotrin**	**GSK**	**S-A**	**Schering**
α–GST	Low	281.0 (10.1) n=2	357.5 (15.2) n=2	273.0 (4.7) n=2	324.0 (2.2) n=2	273.2 (11.1) n=9	270.5 (2.4) n=2
	Mid	1156.0 (9.8) n=2	***2056.5*** (6.0) n=2	1142.5 (3.4) n=2	1237.5 (2.6) n=2	1186.1 (4.5) n=8	963.0 (3.4) n=2
	High	n/a	5575.0 (9.0) n=2	4753.5 (15.9) n=2	4810.0 (13.1) n=2	5110.6 (9.6) n=5	3688.5 (2.4) n=2
μ–GST	Low	301.0 (14.6) n=2	***436.5*** (17.0) n=2	287.5 (6.6) n=2	294.0 (15.4) n=2	301.6 (9.9) n=7	317.5 (5.6) n=2
	Mid	1002.5 (16.3) n=2	1258.0 (7.0) n=2	978.5 (16.1) n=2	1000.0 (13.9) n=2	1014.5 (12.5) n=8	788.5 (3.9) n=2
	High	***2372.0*** (11.4) n=2	1625.0 (6.0) n=2	1494.0 (22.6) n=2	2072.5 (9.7) n=2	2107.6 (5.6) n=7	***1178.0*** (8.3) n=2
RPA-1	Low	411.0 (18.9) n=2	458.0 (12.0) n=2	498.0 (8.8) n=2	486.0 (2.6) n=2	346.8 (19.9) n=4	499.5 (7.8) n=2
	Mid	1375.5 (14.1) n=2	1483.0 (6.7) n=2	1342.0 (5.0) n=2	1493.5 (0.3) n=2	1070.8 (21.9) n=8	1557.5 (8.6) n=2
	High	n/a	6500.3 (22.8) n=4	7240.5 (1.8) n=2	8158.5 (0.6) n=2	5779.2 (8.3) n=6	n/a
Clusterin	Low	128.5 (17.1) n=2	***219.0*** (n/a) n=1	160.5 (19.8) n=2	132.5 (36.8) n=2	123.3 (31.4) n=3	132.5 (3.7) n=2
	Mid	***344.0*** (10.3) n=2	***1063.0*** (n/a) n=1	373.5 (12.3) n=2	399.5 (31.3) n=2	393.0 (2.9) n=3	450.5 (1.7) n=2
	High	3154.3 (28.3) n=3	3444.0 (n/a) n=1	4868.5 (10.9) n=2	3581.5 (1.2) n=2	***7259.0*** (49.0) n=5	n/a

xi. HESI's listing of excluded animals

Listing of animals with missing data for one or more biomarkers

Compound	Strain	Dose (mg/kg)	Animal ID	BUN	SCr	NAG	Prot	α-GST	μ-GST	RPA-1	Clust
Cisplatin	Sprague-Dawley	0	50094105			x	x				
Cisplatin	Sprague-Dawley	0	50094110						x		
Cisplatin	Sprague-Dawley	0	50094116			x					
Cisplatin	Sprague-Dawley	0	50094122			x					
Cisplatin	Sprague-Dawley	0	50094125						x		
Cisplatin	Sprague-Dawley	0	50094129			x					
Cisplatin	Sprague-Dawley	0.3	50094213			x					
Cisplatin	Sprague-Dawley	0.3	50094216			x					
Cisplatin	Sprague-Dawley	0.3	50094219			x					
Cisplatin	Sprague-Dawley	0.3	50094223			x					
Cisplatin	Sprague-Dawley	0.3	50094227						x		
Cisplatin	Sprague-Dawley	1	50094311			x					
Cisplatin	Sprague-Dawley	1	50094313			x					
Cisplatin	Sprague-Dawley	1	50094316						x		
Cisplatin	Sprague-Dawley	1	50094318			x	x				
Cisplatin	Sprague-Dawley	1	50094319			x					

Compound	Strain	Dose (mg/kg)	Animal ID	BUN	SCr	NAG	Prot	α-GST	μ-GST	RPA-1	Clust
Cisplatin	Sprague-Dawley	1	50094322			x					
Cisplatin	Sprague-Dawley	1	50094325			x					
Cisplatin	Sprague-Dawley	1	50094326						x		
Cisplatin	Sprague-Dawley	3	50094407						x		
Cisplatin	Sprague-Dawley	3	50094411			x					
Cisplatin	Sprague-Dawley	3	50094413			x					
Cisplatin	Sprague-Dawley	3	50094414			x	x				
Cisplatin	Sprague-Dawley	3	50094415			x					
Cisplatin	Sprague-Dawley	3	50094417			x					
Cisplatin	Sprague-Dawley	3	50094423			x					
Cisplatin	Wistar	0.3	58					x	x	x	x
Cisplatin	Wistar	3	100							x	
NPAA	Wistar	0	01-003						x		
NPAA	Wistar	0	01-007					x			
NPAA	Wistar	0	01-013	x	x				x		
NPAA	Wistar	0	01-018	x	x						
NPAA	Wistar	0	01-021			x					
NPAA	Wistar	0	01-022			x					

Compound	Strain	Dose (mg/kg)	Animal ID	BUN	SCr	NAG	Prot	α-GST	μ-GST	RPA-1	Clust
NPAA	Wistar	50	02-031							x	
NPAA	Wistar	50	02-032							x	
NPAA	Wistar	50	02-038								x
NPAA	Wistar	700/500	04-061						x		
NPAA	Wistar	700/500	04-064						x		
NPAA	Wistar	700/500	04-068						x		
NPAA	Wistar	700/500	04-069						x		
NPAA	Wistar	700/500	04-076	x	x						
NPAA	Wistar	700/500	04-080			x	x	x	x	x	x

7. References

Aulitzky W.K., Schlegel P.N., Wu D., Cheng C.Y., Chen C.L.C., Li P.S., Goldstein M., Reidenberg M. and Bardin C.W. (1992) Measurement of urinary clusterin as an index of nephrotoxicity. Proc. Soc. Exp. Biol. Med. 199, 93-96.

Bass NM, Kirsch RE, Tuff SA et al (1979) Radioimmunoassay measurement of urinary ligandin excretion in nephrotoxin-treated rats. Clinical Science 56: 419-426

Beckett GJ and Hayes JD (1993) Glutathione S-transferases: biomedical applications. Adv Clin Chem 30: 281-380

Betton, G.R., Falkenberg, F.W., Price, S., Somers, R.L. and Roche, A. (2007) Immunohistochemical protein expression studies of novel monoclonal kidney biomarkers in the Han Wistar rat. Toxicol Pathol 35, 187

Blashuck O., Burdzy K. and Fritz I.B. (1983) Purification and characterization of cell-aggregating factor (clusterin), the major glycoproteoin in ram rete testis fluid. J. Biol. Chem. 12, 7714-7720.

Branten AJ, Mulder TP, Peters WH et al (2000) Urinary excretion of glutathione S transferases alpha and pi in patients with proteinuria: reflection of the site of tubular injury.Nephron 85: 120-126

Burtis, CA and ER Ashwood, eds. (1986). Tietz Textbook of Clinical Chemistry, 2nd ed. Philadelphia, WB Saunder & Co.

Campbell JAH, Corrigall AV, Guy A et al (1991) Immunohistologic localization of alpha, mu and pi class glutathione-s-transferases in human tissues. Cancer 67: 1608-13

Coca SG, Yalavarthy R, Concato J et al (2007) Biomarkers for the diagnosis and risk stratification of acute kidney injury: A systematic review. Kid Int 73: 1008-1016

Collard M.W. and Griswold M.D. (1987) Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. Biochemistry 26, 3297-3303.

Correa-Rotter R., Hostetter T.M., Manivel J.C., Eddy A.A., and Rosenberg M.E. (1992) Intrarenal distribution of clusterin following reduction of renal mass. Kidney Int. 41, 938-950.

Cowley B.D. Jr. and Rupp J.C. (1995) Abnormal expression of epidermal growth factor and sulfated glycoprotein SGP-2 messenger RNA in a rat model of autosomal dominant polycystic kidney disease. J. Am. Soc. Nephrol. 6, 1679-1681.

Daggett DA, Nuwaysir EF, Nelson SA et al (1997) Effects of triethyl lead administration on the expression of glutathione S-transferase isoenzymes and quinone reductase in rat kidney and liver. Toxicology 117: 61-71

Derbel M, Igarashi T and Satoh T (1993) Differential induction of glutathione S-transferase subunits by phenobarbital, 3-methylcholanthrene and ethoxyquin in rat liver and kidney. Biochim Biophys Acta 1158: 175-180

DeLong E, DeLong D and Clarke-Pearson D (1988) Comparing the area under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 44: 837-845

Dvergsten, J., Carlos Manivel, J. Correa-Rotter, R. and Rosenberg, M. E. (1994). Expression of clusterin in human renal diseases. Kidney International, 45; 828-835.

Eger II EI, Koblin DD, Bowland T et al (1997) Nephrotoxicity of sevoflurane versus desflurane anesthesia in volunteers. Anesth Analg 84: 160-168

Eti S., Cheng S.Y., Marshall A. and Reidenberg M.M. (1993) Urinary clusterin in chronic nephrotoxicity in the rat. Proc. Soc. Exp. Biol. Med. 202, 487-490

Falkenberg FW, Hildebrand H, Lutte L et al (1996) Urinary antigens as markers of papillary toxicity. I Identification and characterization of rat papillary antigens with monoclonal antibodies. Arch Toxicol 71: 80-92

French L.E., Chonn A., Ducrest D., Baumann B., Belin D., Wohlwend A., Kiss J.Z. Sappino A.-P., Tschopp J. and Schifferli J.A. (1993) Murine clusterin: Molecular cloning and mRNA localization fo a gene associated with epithelial differentiation processes during embryogenesis. J. Cell. Biol. 122, 1119-1130.

Fritz I.B., Burdzy K., Setchell B. and Blashuck O. (1983) Ram rete testes fluid contains a protein (clusterin) which influences cell-cell interactions *in vitro*. Biol. Reprod. 28,1173-1188.

Giannini E, Risso D, Ceppa P, Botta F, Chiarbonello B, Fasoli A, Malfatti F, Romagnoli P, Lantieri PB, Testa R. 2000. Utility of alpha-glutathione S-transferase assessment in chronic hepatitis C patients with near normal alanine aminotransferase levels. Clin Biochem. 2000 Jun;33(4):297-301.

Girton RA, Sundin DP and Rosenberg ME (2002) Clusterin protects renal tubular epithelial cells from gentamicin-mediated cytotoxicity. Am. J. Physiol. Renal Physiol 282: F703–F709

Goodman and Gilman's The Pharmacological Basis of Therapeutics, ed by JG Hardman, LE Limbird and AG Gilman, 10th ed. McGraw-Hill Medical Publishing, NY, NY, 2001.

Griffen, P.S. et al. 2002, Alpha-Glutathione S-Transferase in assessment of hepatotoxicity – its diagnostic utility on comparison with other markers in the Wistar Han Rat. Toxicologic Pathol 30(3), 365-37

Harding M.A., Chadwick L.J., Gattone II V.H. and Calvet J.P. (1991) The SGP-2 gene is developmentally regulated in the mouse kidney and abnormally expressed in collecting duct cysts in polycystic kidney disease. Dev. Biol. 146, 483-490.

Hardy TL, Bach PH. (1984). The effect of N-phenylanthranilic acid-induced renal papillary necrosis on urinary acidification and renal electrolyte handling. Toxicol Appl Pharmacol. 75(2):265-77.

Harrison DJ, Kharbanda R, Scott Cunningham D et al (1989) Distribution of glutathione Stransferase isoenzymes in human kidney: basis for possible markers of renal injury. J Clin Pathol 42: 624-628

Helaly GF, Mahmoud MM. 2003 Diagnostic value of alpha-glutathione S-transferase as a sensitive marker of increased risk for hepatocellular damage in hepatitis C virus (HCV) infection: relation to HCV viraemia. J Egypt Public Health Assoc. 2003;78(3-4):209-23.

Hidaka S., Kranzlin B., Gretz N. and Witzgall R. (2002) Urinary clusterin levels in the rat correlate with the severity of tubular damage and may help to differentiate between glomerular and tubular injuries. Cell Tissue Res. 310, 289- 296.

Hildebrand H, Rinke M, Schluter G et al (1999) Urinary antigens as markers of papillary toxicity II Application of monoclonal antibodies for the determination of papillary antigens in rat urine. Arch Toxicol 73: 233-245

Ishii A, Sakai Y and Nakamura A (2007) Molecular pathological evaluation of clusterin in a rat model of unilateral ureteral obstruction as a possible biomarker of nephrotoxicity. Toxicol Pathol. 35: 376-382

Kharasch ED, Schroeder JL, Bammler T et al (2006) Gene Expression Profiling of Nephrotoxicity from the Sevoflurane Degradation Product Fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl Ether (‘‘Compound A’’) in Rats. Tox Sci 90: 419–431

Kharasch ED, Thorning D, Garton K et al (1997) Role of renal cysteine conjugate β-lyase in the mechanism of compound A nephrotoxicity in rats. Anesthesiology 86: 160-171

Kilty C, Doyle S, Hassett B, Manning F, (1998). Glutathione S-transferases as biomarkers of organ damage: applications of rodent and canine GST enzyme immunoassays. Chem Biol Interact. 111-112:123-35.

Kilty CG, Keenan J and Shaw M (2007) Histologically defined biomarkers in toxicology. Expert Opin Drug Saf 6: 207-215

Kirszbaum L., Bozas S.E. and Walker I.D. (1992) SP-40,40, a protein involved in the control of the complement pathway, possess a unique array of disulfide bridges. FEBS Lett. 297, 70-76.

Kirszbaum L., Sharpe J.A., Murphy B., d’Apice A.J.F., Classon B., Hudson P. and Walker I.D. (1989) Molecular cloning and characterization of the novel, human complement-associated protein, SP40,40: a link between the complement and reproductive systems. EMBO J. 8, 711-718.

Lee JW, Weiner RS, Sailstad JM, et al. (2005) Method Validation and Measurement of Biomarkers in Nonclinical and Clinical Samples in Drug Development: A Conference Report. Pharmaceutical Research, 22(4): 499-511.

Metz CE, 1978). Metz CE. (1978). Basic principles of ROC analysis. Semin Nucl Med. 8(4):283-98.

O’Bryan M.K., Cheema S. S., Bartlett P.F., Murphy B.F. and Pearse M.J. (1993) Clusterin levels increase during neuronal development. J. Neurobiol.I 24, 6617-6623.

Ozturk G, Ozturk N, Aksoy H, Akcay MN, Atamanalp SS, Acemoglu H. 2009. Hepatocellular damage following burn injury demonstrated by a more sensitive marker: alpha-glutathione S-transferase. J Burn Care Res. 2009 Jul-Aug;30(4):711-6.

Pearse M.J., O'Bryan M., Fisicaro N., Rogers L., Murphy B. and d'Apice A.J. (1992) Differential expression of clusterin in inducible models of apoptosis. Int. Immunol. 4, 1225-1231.

Rosenberg M.E. and Silkensen J. (1995) Clusterin and the kidney. Exp. Nephrol. 3, 9-14.

Rosenberg ME and Silkensen J (1995) Clusterin: physiologic and pathophysiologic considerations. Int J Biochem Cell Biol 27: 633-645

Rozell R, Hansson HA, Guthenberg M et al (1993) Glutathinone transferases of classes α, μ and π show selective expression in different regions of rat kidney. Xenobiotica 23: 835-849

Silkensen JR, Agarwal A, Nath KA et al (1997) Temporal induction of clusterin in cisplatin nephrotoxicity. J Am Soc Nephrol 8: 302-305

Sundberg AGM, Appelkvist E-L, Bäckman L et al (1994a) Urinary π-class glutathione transferase as an indicator of tubular damage in the human kidney. Nephron 67: 308-316

Sundberg AGM, Appelkvist E-L, Dallner G et al (1994b) Glutathione transferases in the urine: sensitive methods for detection of kidney damage induced by nephrotoxic agents in humans. Environ Health Perspect 102 (Suppl 3): 293-296

Sundberg AGM, Nilsson R, Appelkvist E-L et al (1993) Immunohistochemical localization of α and π class glutathione transferases in normal human tissues. Pharmacol Toxicol 72: 321-331

Tsuchiya Y, Tominaga Y, Matsubayashi K et al (2005) Investigation on urinary proteins and renal mRNA expression in canine renal papillary necrosis induced by nefiracetam. Arch Toxicol 79: 500-507

Witzgall R., Brown D., Schwarz C. and Bonventre J.V. (1994) Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the post-ischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. J. Clin. Invest. 93, 2175-2188.

Yamada, K, Hori, Y, Hanafusa, N, et al. (2003). Clusterin is up-regulated in glomerular mesangial cells in complement-mediated injury. Kid Int 59: 137-146.

Yoshida T, Kurella M, Beato F et al (2002) Monitoring changes in gene expression in renal ischemia-reperfusion in the rat. Kid Int 61: 1646-1654

Zhang F, Bartels MJ, Stott WT. 2004. Quantitation of human glutathione S-transferases in complex matrices by liquid chromatography/tandem mass spectrometry with signature peptides. Rapid Commun Mass Spectrom.;18(4):491-8.