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Division of Cardiovascular and Renal Products**

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Subject: Clinical Review of PSTC/FNIH Kidney Safety Biomarker Qualification Submission

Materials Reviewed:

- Briefing document: “Kidney Safety Project Qualification Submission”
- Briefing document appendices: “KSP clinical context of Use Briefing Book Appendices”
- Normal healthy volunteer study report: “Evaluation of normal reference ranges for novel urinary renal biomarkers in healthy volunteers”
- Merck mesothelioma protocol
- Minutes for EMA Scientific Advice Working Party Meeting held on January 13, 2016

Executive Summary:

On July 27, 2015, the Kidney Safety Project (KSP), supported jointly by the Foundation for the National Institute of Health Biomarkers Consortium and the Critical Path Institute’s Predictive Safety Testing Consortium (FNIH/PSTC), submitted data to the FDA and EMA to support the clinical qualification of a composite measure (CM) of 6 biomarkers [urine clusterin (CLU), cystatin-C (CysC), kidney injury molecule-1 (KIM-1), N-acetyl-beta-D-glucosaminidase (NAG),

neutrophil gelatinase-associated lipocalin (NGAL), and osteopontin (OPN)], hereafter referred to as the “PFC Index.” In brief, the PFC Index is a linear combination of the urine creatinine normalized fold-change from baseline for each of the six urine biomarkers and was derived using the fold-change from baseline in urine biomarker data from a study conducted in normal healthy volunteers (NHV). The proposed context of use is as follows: “A composite measure (CM) of urine CLU, CysC, KIM-1, NAG, NGAL, and OPN is a qualified safety biomarker of kidney tubular injury response for use in NHV trials supporting early drug development.” As discussed in the body of this review, the submission also includes a number of “Conditions of Qualified Use,” which address additional considerations related to the use of the PFC Index in NHV trials supporting early drug development. The submission does not propose the use of a set PFC Index threshold to define injury; rather the submission includes tables that provide information on the probability of obtaining a value greater than or equal to a particular value in a cohort of healthy volunteers of a particular sample size. Importantly, the submission does not assert that the PFC Index is the best way to utilize this group of biomarkers or that all of the biomarkers in the PFC Index are needed; rather, part of the motivation for requesting a limited qualification is to encourage further collection of data that can then be used to inform our understanding of whether and how best to use the biomarkers in the panel as tools in drug development. The PSTC/FNIH consortium is in the process of conducting clinical trials that should provide evidence to support qualification of the individual biomarkers for use as renal safety monitoring tools in clinical trials.

In support of the proposed context of use, the submitter has provided data from two observational studies, one conducted in normal healthy volunteers (“the PSTC Normal Healthy Volunteer Study”) and one conducted in patients with mesothelioma undergoing treatment with chemotherapy or surgery for their disease. The submission also references (1) biomarker data obtained in preclinical species showing a correlation between some of the biomarkers in the composite (i.e., CLU, CysC, Kim-1, NGAL and OPN) and histo-morphologic kidney damage;¹ and (2) information gleaned from the published literature on the sensitivity and specificity of each of the biomarkers that make up the PFC Index (see Appendix).

Overview of PSTC NHV Study

In brief, the PSTC NHV Study was a prospective observational biomarker study conducted in healthy volunteers. There was no prospective plan to use the data generated from the NHV study to develop the PFC Index. Rather, the stated primary objectives of the study were to characterize the mean values, normal range, and inter- and intra-subject variability of renal biomarkers (including, but not limited to, urine albumin, total protein, clusterin, cystatin C, beta₂-microglobulin, trefoil factor 3, and kidney injury molecule-1 [KIM-1]) in healthy subjects and to

¹ According to the submission, NAG appears to “have good performance in canines and nonhuman primates, but is not consistently predictive in rats.”

assess whether the mean values or variability are influenced by age, sex, fasting status or time of day. The secondary objectives were to evaluate correlations among biomarkers, establish assay performance criteria, collect blood for future exploratory studies correlating genomic patterns with biomarker expression and create a well-annotated sample set for evaluation of other biomarkers submitted by the Predictive Safety Testing Consortium in the future. The PSTC NHV Study was conducted at a single site and enrolled 89 subjects who were mostly Caucasian, nonhispanic and overweight. Of these, 76 subjects with biomarker samples at day 1 and day 21 were included in the analysis.

Overview of Mesothelioma Study

The mesothelioma study (MS) was a phase 1 single-center observational study that was not specifically designed to assess the performance of the PFC Index. Its main objective was to determine the maximum tolerated dose (MTD) of intracavitary heated chemotherapy using a lavage of cisplatin and gemcitabine after extrapleural pneumonectomy (EPP), after pleurectomy/decortication (P/DC), or after Tumor Debulking +/- Intrapleural Pneumonectomy (TD +/- IPP) with intravenous amifostine and sodium thiosulfate cytoprotection. Thirty-nine of the patients in the study who had no evidence of CKD at baseline and had evaluable specimens were used in the PSTC-FNIH analysis. Longitudinal sample collection occurred prior to surgery or cisplatin treatment, during surgery and after surgery and up to 6 post-op days but the collection timepoints for urine and serum samples were not consistent or precisely timed. The number of sCr measurements for each subject was also highly variable with a median of 19 (range = 9 to 35). Three subgroups were defined including Meso Surgery (N = 4 surgical control patients without exposure to cisplatin), Meso Controls (N = 22 patients exposed to cisplatin without clinical manifestation of treatment related renal injury), and Meso Cases (N = 13 patients exposed to cisplatin with clinical manifestation of treatment related renal injury). The PFC Index was calculated for each of the three mesothelioma subgroups and was used to assess whether it could distinguish the three subgroups from one another or if it could distinguish the mesothelioma cohort as a whole from the normal healthy volunteers.

Derivation of the PFC Index

Of the several biomarkers that were tested in the NHV study, 6 (CLU, CysC, KIM-1, NAG, NGAL, and OPN) were selected for purposes of deriving the PFC Index. First the fold changes were determined for each biomarker for each individual with evaluable data in the NHV study. Then, a principal component analysis (PCA) was performed to simplify and reduce the set of six individual biomarker measures identified above into a single index. Bootstrap resampling was performed to benchmark expectations in a future NHV population of various sample sizes, in order to assess the evidence that the PFC Index for a dose cohort will deviate from normal variability. Benchmark expectations in a future NHV population of various sample sizes were derived for a “weighted” and “equally-weighted” PFC Index. The “weighted” index described the contribution of the fold change from baseline in each biomarker to the index. The FNIH-

PSTC briefing book showed that the two indexes provided very similar results. The submission also explored the use of a longitudinal PFC Index. Because more than one post-baseline timepoint of biomarker concentrations will likely be available in future studies, and only two timepoints (one baseline and one post-baseline) were available from the NHV data, simulation was used to generate the multivariate posterior predictive distributions of longitudinal NHV biomarker concentration data across more than two timepoints (i.e., more than one post-baseline timepoint). These posterior predictive distributions were used to benchmark expectations in a future NHV population of various sample sizes when using the maximum fold change from baseline of each individual biomarker in an equally-weighted PFC Index. The submission proposes to apply one or all of these benchmark expectations that vary with various sample sizes to phase 1 studies in healthy volunteers to provide insight into the probability that a dose cohort deviates from expected normal variability.

To assess the performance of the PCA-weighted and equally-weighted indexes in the population of Mesothelioma patients with and without medically relevant increases in sCr, the PCA-weighted and equally-weighted PFC indexes were calculated for patients in the various subgroups of the Mesothelioma study at each timepoint, and compared to the derived thresholds based on the NHV subjects. Five thousand bootstrap samples of size $m = 6$ at timepoint $T = 12$ hours were generated to determine the likelihood that small samples from the Mesothelioma Control and Case patients would exceed the thresholds derived using the NHV subjects.

Statistical Assessment (as reported in Dr. Soukup's review)

The statistical review by Dr. Mat Soukup identified no consequential differences between the weighted and equally-weighted PFC Indexes. According to his review, there were insufficient data to validate a longitudinal PFC Index. Hence, Dr. Soukup identified the equally-weighted PFC Index as the most appropriate candidate for clinical qualification.

The statistical review by Dr. Mat Soukup also indicates that there were no substantial differences in the PFC Index among the mesothelioma subgroups. The mesothelioma dataset as a whole fell outside of the normal range of the PFC Index identified in the normal healthy volunteer study, even at baseline. This deviation from the PFC Index in a patient population supports limiting the qualification to use in phase 1 studies in healthy volunteers.

Clinical Assessment

The Drug Development Tools Qualification Program was created by the Center for Drug Evaluation and Research at FDA to provide a framework for the development and regulatory acceptance of scientific tools for use in drug development programs. Qualification is seen as a continuum, ranging from a limited context of use (COU) qualification to a more expanded COU qualification. A limited COU qualification, such as that proposed by the submitter, is intended to

provide a more circumscribed indication for the use of a biomarker and establish a platform for expanded qualification. With these objectives in mind, we note the following:

- There are important limitations to the submitted data.
 - The reliability of the derived thresholds and associated probabilities have not been validated using another dataset.
 - The assays have not been well-characterized and there are outstanding questions about the performance characteristics. In a recent communication from the PSTC/FNIH, it is now known that hematuria interferes with some of the biomarker assays; other potential sources of interference may also exist.
 - Finally, the submission does not address potential intra-subject variability due to factors such as diurnal variation and contains limited information on intrinsic or extrinsic factors that might affect variability.
- Other important gaps in our understanding of the PFC Index constrain its usefulness as a tool in drug development. One constraint is our limited understanding of the sensitivity and specificity of the index and its components for drug-induced renal toxicity. Another constraint is our limited understanding of the utility of such a measure given potential differences among biomarkers in terms of the rapidity of change in response to injury.

While we acknowledge these limitations and gaps in our understanding, we believe the PFC Index can be qualified for a constrained context of use assuming concerns related to analytical validation can be adequately addressed. Specifically, we believe the PFC Index can be qualified as a safety biomarker for the purpose of identifying a dose cohort that deviates from normal variability in a phase 1 study in normal healthy volunteers.²

Our rationale for the proposed limited qualification is as follows:

- Nonclinical and/or clinical data suggest that the component biomarkers have value for detecting acute kidney injury.
- Using the PFC Index in phase 1 NHV trials of drugs that are suspected to be nephrotoxicants could inform decision making.

² Our proposed context of use differs slightly from that proposed in the PSTC/FNIH submission. The proposed context of use by the PSTC/FNIH is to “identify kidney tubular injury response for use in NHV trials supporting early drug development”. Our suggested context of use does not imply that a deviation from the PFC Index indicates kidney injury; rather it indicates that there is a difference between what was seen in the dose cohort and what would be expected in a cohort of normal healthy volunteers, a finding that should prompt further investigation.

- The risks to study subjects associated with using the PFC Index is minimal because it will be used in conjunction with standard renal safety biomarkers.
- The risk of reaching a false conclusion can be minimized by qualifying the PFC Index for a limited context of use (COU) and specifying appropriate conditions of use (see Guidance for COU and conditions of use).
- The submitters are in the process of doing other studies to further the scientific understanding of these biomarkers which will, if their efforts are successful, expand the qualification and build confidence in their utility.

What information to include in the guidance document and how prescriptive the language should be regarding the use of these biomarkers has been a topic of internal discussion. In general, we believe that the goal of the biomarker qualification should be to describe the information content of a biomarker (or at least the Agency's interpretation of the information content). Given this goal, we believe that the guidance should highlight the information content of the PFC Index and reference the review for additional information on considerations related to use.

Below, we highlight what we believe are important considerations and sensible practices related to the use of the PFC Index; many of these practices were also suggested by the submitter.

- In general, the timing of biomarker measurements should be informed by the findings in animal studies and, if the concern for toxicity is based on the experience with other members of the pharmacologic class, an understanding of the time course of toxicity for these other members should be considered in determining the schedule of assessments.
- Because elevations in the PFC Index may reflect a non-renal etiology, elevations of the PFC Index should prompt further evaluation for renal as well as non-renal etiologies for the elevation.
- Placebo-treated subjects should be included in future studies to aid in the evaluation of the significance of any elevations in biomarkers or the PFC Index.
- Following the biomarker components of the PFC Index in real-time may maximize the utility of biomarker testing. Markedly high biomarker values in an individual subject should also prompt further investigation.

Disease Background and Unmet Need

Serum creatinine is widely used to monitor for drug-induced renal injury, however serum creatinine is a marker of renal function and is neither sensitive nor specific for identifying renal injury. When evaluating drugs with nonclinical signals of reversible kidney injury in early clinical studies, it is critical to ensure the safety of study subjects, particularly if the subjects are healthy volunteers, since healthy volunteers have no prospect of benefit from participation in the study. To mitigate risk to subjects, we often attempt to maintain a “sufficient” safety margin to the dose/exposure at which renal toxicity was seen in animals; however this may prevent development programs from evaluating doses/concentrations that are needed to achieve efficacy. Biomarkers that are more sensitive indicators of renal injury than current standard measures are needed for monitoring drug-induced renal injury in clinical trials so that renal injury can be detected at an early and reversible stage.

Background on the biomarkers that make up the PFC Index

The submission included a brief discussion of each biomarker. This summary is included in Appendix 1. The individual biomarker components of the PFC Index described in this submission are being actively studied in the nonclinical and clinical space and there are many publications describing conditions that appear to result in their increase. However, the sensitivity and specificity of the component urinary biomarkers for drug-induced renal tubular injury are not known.

Studies in rats with numerous compounds suggest that the induction of OPN, CLU, CysC, KIM-1, NAG, NGAL, microalbumin, and total protein occurs in response to nephrotoxic agents. These animal findings, coupled with findings in patients with kidney injury/diseases, suggest that these biomarkers may be able to detect renal injury in humans. However, a number of medical conditions in humans have also been found to confound or cause false elevations in urinary biomarker expression. For example, NGAL levels are elevated in response to inflammation, infection³, and gastrointestinal neoplasms⁴ and renal cell carcinoma⁵. CLU has been reported to be elevated in bladder cancer⁶, KIM-1 in renal cell carcinoma⁷, and NAG in laryngeal squamous cell carcinoma⁸. This speaks to the potential for low specificity and false positive results. Of note, the experience with some of these biomarkers as markers of kidney injury/disease in humans is also quite limited.

³ Liu KT, et al, BMC Infect Dis. 2016; 16: 441.

⁴ Michalak, L et al, Postepy Hig Med Dosw.2016: 1026-1031.

⁵ Shalabi, A et al, World J Urol. 2013; 31: 1541-5.

⁶ Soukup V, et al, Urol Int. 2015; 95: 56-64.

⁷ Mijugkovic M et al, Vojnosanit Preql. 2016; 73: 266-72.

⁸ Oktem F, et al, J Otolaryngol 2007; 36: 233-9.

Overview of PFC Index

The PFC Index is a group geometric mean (GM) of the fold changes from baseline of the six urine creatinine (uCr)-normalized urine biomarkers in subjects. A principal component analysis (PCA) was performed with the data to simplify and reduce the set of six individual biomarker measures identified above into a single CM. The PFC Index is first calculated for each individual subject and then the group PFC Index is calculated.

The PSTC/FNIH proposed three possible PFC Indices.

1. A principal component analysis (PCA) that used the NHV data to generate weights that described the contribution of the fold change from baseline in each biomarker to the PFC Index.
2. An equally-weighted individual subject PFC Index that provides equal weight to the fold change from baseline in each biomarker to the PFC Index.
3. A longitudinal individual subject PFC Index that considers the maximum values of the biomarkers over the course of a study period.

As discussed in the statistical review, there were inconsequential differences between the weighted and equally-weighted PFC Indexes and that there were insufficient data to validate a longitudinal PFC Index. For this reason, the equally weighted PFC Index was considered for qualification.

Context of Use Statement

The submission proposed the following context of use statement and conditions of qualified use:

Use Statement: The PFC Index, a composite measure of urine CLU, CysC, KIM-1, NAG, NGAL, and OPN, is a qualified safety biomarker of kidney tubular injury response for use in NHV trials supporting early drug development (Figure 1).

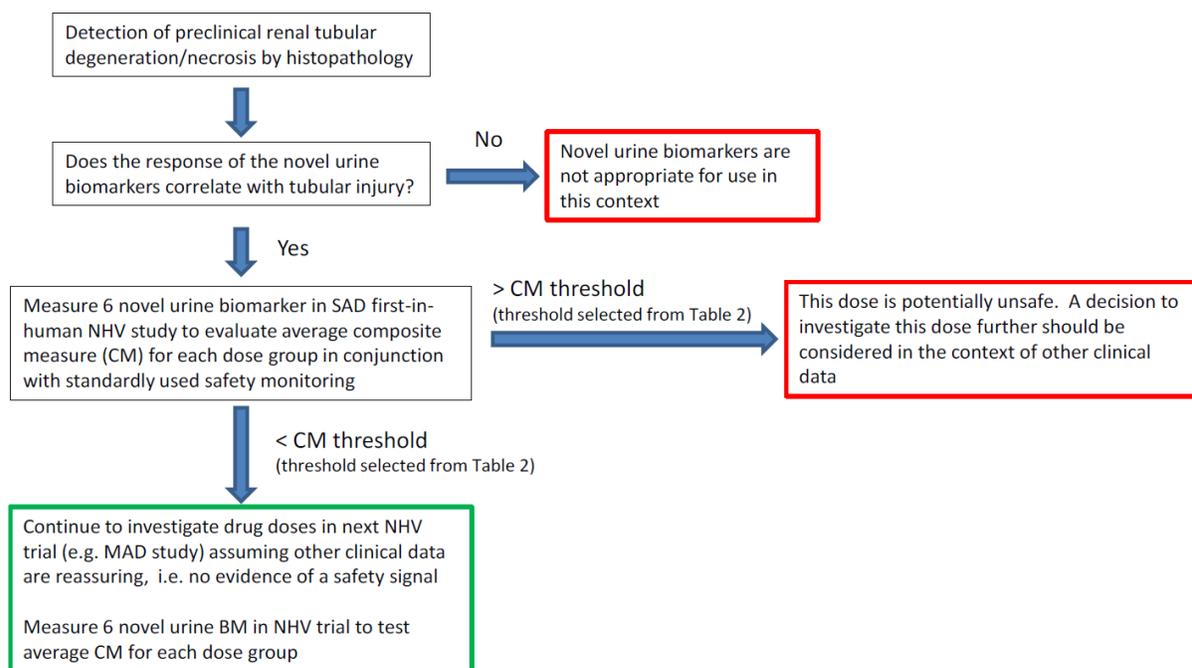
Conditions of Qualified Use:

1. The individual PFC Index is a measure of the fold change from baseline of urine CLU, CysC, KIM-1, NAG, NGAL, and OPN normalized to urine creatinine (uCr).
2. The group geometric mean, cohort PFC Index, is qualified for study Sponsors to determine if there is an increased likelihood of a renal injury response for a dose of an investigational drug in a dose cohort when benchmarked to results provided herein for NHVs. The CM is not currently qualified for individual patient safety monitoring.

3. The PFC Index is intended to complement the use of the standard biomarkers including serum creatinine (sCr), BUN, urine albumin and urine total protein for safety monitoring in a single or multiple dose escalation clinical trial with or without a comparator/placebo during drug development under an Investigational New Drug Application (IND)/Clinical Trial Application (CTA).
4. The PFC Index can be used for safety monitoring in clinical trials when nonclinical toxicology studies with a study drug demonstrate evidence of reversible histologic renal tubule damage that is associated with an elevation in any of the six urine biomarkers.
5. Urine for biomarker measurements should be collected at baseline and post-baseline to calculate the fold-change from baseline. Sample collection times should be informed by animal toxicology study data for the study drug.
6. There should be a plasma drug exposure margin relative to the anticipated clinically relevant dose range, such that the likelihood of kidney injury is considered low at the doses proposed for clinical investigation. Alternatively, data that support greater understanding of potential species-specific mechanisms of questionable human relevance can contribute to confidence that likelihood of kidney injury is low in the proposed clinical investigation. As always, risk-benefit considerations are expected to contribute to exposure-margin based dose selection decisions.
7. The PFC Index is qualified for use when standard biomarkers alone would be considered poor for initial detection of the renal tubule injury observed in animal toxicology studies. The PFC Index is not intended to replace standard measures of renal function including current biomarkers as described in #3 above.
8. The PFC Index is qualified for use in NHV studies.
9. The Sponsor's use of the PFC Index as a drug development tool in well-controlled clinical studies is encouraged.

The submitter provided an example decision tree for clinical use of the PFC Index in phase 1 NHV studies (see Figure 1).

Figure 1: Example decision tree for clinical use of qualified CM in Phase 1 NHV trials



Source: Kidney Safety Project Qualification Submission, p. 15

While not explicitly stated in the conditions of use, the PSTC/FNIH submission also proposes that the results of the PFC Index be interpreted within the context of the particular drug development program, i.e., the proposed indication (life-threatening, serious or mild condition) and whether there are other drugs that are approved for the indication and their toxicities. Certain scenarios (for instance, milder conditions or presence of other marketed drugs for the indication) might require stricter decisions in response to “abnormal” PFC Index results than other more challenging scenarios (for instance, life-threatening condition/ unmet medical need). Accordingly, the submission does not endorse the use of a particular PFC Index threshold; rather the submission includes tables that provide information on the probability of obtaining a value greater than or equal to a particular value in a cohort of healthy volunteers of a particular sample size.

Benchmark expectations in a future NHV population of various sample sizes are displayed in Table 1 and Table 2 for a “weighted” and “equally-weighted” PFC Index, respectively. The “weighted” index factors the contribution of the fold change from baseline in each biomarker into the threshold values. **Error! Reference source not found.** Table 1 shows the probability of obtaining a PCA-weighted PFC index threshold value greater than or equal to a particular value for a given sample size in a single-arm study in which all subjects are exposed to a non-nephrotoxic investigational product and in a two-arm study in which half of the subjects are exposed to a non-nephrotoxic investigational product and half are given placebo. When using the

PFC Index in a phase 1 trial in normal healthy volunteers who may be receiving a potentially nephrotoxic agent, these thresholds are intended to be applied when making decisions regarding dose-reduction, stopping or escalation on a dose cohort as a whole and not on a single individual (i.e. the thresholds are to be compared to the geometric mean/ PFC Index of the cohort).

Table 2 shows the probability of obtaining an equally-weighted PFC index threshold value greater than or equal to a particular value for a given sample size under the same circumstances as described above.

Table 1: Observed PFC Index (principal component analysis -weighted) thresholds based on alternative probabilities of consistency with a NHV population for various sample sizes

Sample Size (n/group)	Thresholds based on NHV population: observed measures greater than or equal to thresholds are expected to be observed with less than P% probability in a NHV population									
	Threshold when assessing a drug study group alone (PCA-weighted GM CM)					Threshold when assessing a drug study group relative to a comparator/placebo (ratio of PCA-weighted GM CMs)				
	P = 50%	P = 20%	P = 10%	P = 5%	P = 1%	P = 50%	P = 20%	P = 10%	P = 5%	P = 1%
6	1.07	1.18	1.24	1.28	1.38	1.00	1.15	1.24	1.33	1.51
8	1.07	1.16	1.21	1.25	1.33	1.00	1.13	1.21	1.28	1.41
10	1.07	1.15	1.19	1.23	1.31	1.00	1.11	1.18	1.24	1.36
12	1.07	1.14	1.18	1.22	1.28	1.00	1.10	1.16	1.22	1.32
14	1.07	1.14	1.18	1.20	1.26	1.00	1.10	1.15	1.20	1.29
16	1.07	1.13	1.17	1.20	1.25	1.00	1.09	1.14	1.18	1.27
18	1.07	1.13	1.16	1.19	1.24	1.00	1.08	1.13	1.17	1.26
20	1.07	1.13	1.16	1.18	1.23	1.00	1.08	1.12	1.16	1.24
22	1.07	1.12	1.15	1.18	1.23	1.00	1.08	1.12	1.15	1.23
24	1.07	1.12	1.15	1.17	1.22	1.00	1.07	1.11	1.15	1.22

GM = geometric mean

CM = composite measure of the fold change from baseline of urine CLU, CYsC, KIM-1, NAG, NGAL and OPN, normalized to urine creatinine using PCA-weights

Source: Kidney Safety Project Qualification Submission, p.47

Table 2: Observed cohort PFC Index thresholds using equal weights to calculate the individual PFC Indexes, and based on varying probabilities of consistency within an NHV population for sample sizes of n = 6 to 20 per group

Sample Size (m/group)	Thresholds based on NHV population: observed measures greater than or equal to thresholds are expected to be observed with less than P% probability in a NHV population									
	Threshold when assessing a drug study group alone (GM CM)					Threshold when assessing a drug study group relative to a comparator/placebo (ratio of GM CMs)				
	P = 50%	P = 20%	P = 10%	P = 5%	P = 1%	P = 50%	P = 20%	P = 10%	P = 5%	P = 1%
6	1.06	1.17	1.23	1.27	1.36	1.00	1.14	1.23	1.31	1.48
8	1.06	1.15	1.20	1.24	1.32	1.00	1.12	1.20	1.26	1.40
10	1.06	1.14	1.19	1.23	1.29	1.00	1.11	1.18	1.23	1.35
12	1.06	1.13	1.18	1.21	1.27	1.00	1.10	1.16	1.21	1.32
14	1.06	1.13	1.17	1.20	1.25	1.00	1.09	1.15	1.19	1.29
16	1.06	1.13	1.16	1.19	1.24	1.00	1.09	1.14	1.18	1.26
18	1.06	1.12	1.15	1.18	1.23	1.00	1.08	1.13	1.17	1.25
20	1.06	1.12	1.15	1.17	1.22	1.00	1.08	1.12	1.16	1.23

GM = geometric mean

CM = composite measure of the fold change from baseline of urine KIM-1, CLU, OPN, CysC, NAG, and NGAL normalized to uCr

Source: FNIH/PSTC Briefing Book, p.48

The statistical review aptly notes that because the PFC Index is a linear combination of all six biomarkers, the effective use of this method would require each subject in a dose cohort to have a recorded uCr-normalized fold-change from baseline for all six biomarkers. How missing values would be handled was not addressed in the submission and it is not clear what the most appropriate imputation approach should be. One approach that was proposed by the PSTC/FNIH was to include only those patients in the PFC Index who have results for all baseline and follow-up biomarkers.

Sources of data to support qualification:

To support the proposed context of use, the submission included analyses using data from one study in normal healthy volunteers and one study in patients being treated with chemotherapy or surgery for mesothelioma.

Normal Healthy Volunteer Study

Study Design

The normal healthy volunteer study was a single-center non-interventional study conducted at the Jasper Clinic, Kalamazoo, MI.

Primary Objectives:

- To characterize the mean values, normal range, and inter- and intra-subject variability of renal biomarkers (including, but not limited to, urine albumin, total protein, clusterin, cystatin C, beta2-microglobulin, trefoil factor 3, and kidney injury molecule-1 [KIM-1]) in healthy subjects. Healthy subjects were defined as those who have no documented disease that could affect renal function, and a calculated glomerular filtration rate (GFR) of at least 90 ml/min/1.73 m² for subjects 18 - 39 years of age and at least 75 ml/min/1.73 m² for subjects 40 - 70 years of age.
- To assess whether the mean values or variability are influenced by age, sex, fasting status or time of day.

Secondary objectives:

- To evaluate correlations between biomarkers, establish assay performance criteria, collect blood for future exploratory studies correlating genomic patterns with biomarker expression and create a well-annotated sample set for evaluation of other biomarkers submitted by the Predictive Safety Testing Consortium in the future.

Population: The aim was to recruit 60 subjects with equal numbers of male vs. female and younger (20-39 years) vs. older (40-70 years) subjects. The protocol stated that 40 additional subjects may complete the study at the discretion of the sponsor.

Inclusion/ Exclusion Criteria:

1. Between 18 and 70 years of age
2. No underlying diseases which require the use of chronic medications
3. No medications, vitamin/ mineral/ herbal/ creatine supplements
4. Sitting systolic/diastolic blood pressure < 140/90 mmHg and > 90/40 mmHg
5. eGFR (by the Cockcroft-Gault method) of ≥ 90 mL/min/1.73m² for subjects between 18-39 years of age and ≥ 75 mL/min/1.73m² for subjects 40-70 years of age
6. BMI < 35
7. Must be willing to refrain from illicit drug, alcohol, tobacco use or strenuous exercise during the study period.
8. Must not be pregnant if female by a urine pregnancy test
9. No history of urinary tract infection in the 6 months prior to enrolling

Schedule of Study Activities: The schedule of study activities is presented in Table 3.

Table 3: Originally Planned Schedule of Assessments

	Screening	Visit 1 (Day 1)	Visit 2 (Day 2)	Visit 3 (Days 5-7)	Visit 4 (Two wks after Visit 3)
Informed consent	X				
Inclusion/exclusion criteria	X				
Demographic data	X				
Height	X				
Weight	X	X	X	X	X
Vital signs	X	X	X	X	X
Medical history	X				
Physical examination	X				
Medication history	X	X	X	X	X
Blood/Urine Laboratory Panel ^a	X				
Blood collection for determination of serum creatinine, HIV, Hep B, and Hep C	X				
Blood collection of sample for possible genetic analysis		X			
Mental state, alcohol and tobacco use history	X	X	X	X	X
Standard of care assessment	X	X	X	X	X
Urine Drug Screen	X				X
Fasting Spot Urine for biomarkers and creatinine to be sent to analytical lab		X		X	X
Six hour timed urine collection in research unit		X		X ^b	X ^b
Blood collection for plasma for biomarkers to be sent to analytical laboratory		X		X	X
Blood collection for determination of serum creatinine and BUN at local lab		X		X	X
Blood collection for determination of serum creatinine, BUN and biomarkers to be sent to analytical laboratory		X		X	X
Remaining 3 x 6 hour urine for total of 24 hour collection for CrCl and urinary biomarkers, collected overnight out of the research unit		X ^c	X ^c		

^a Blood for CBC, SMA, thyroid function and urine for drug screen and routine urinalysis

^b Six hour timed urine collection in research unit. One of the two 6 hour timed urine collections scheduled for either Visit 3 or Visit 4 to be collected during a different 6 hour timed interval than the time of the first 6 hour

collection interval on Visit 1 (e.g., if Day 1 6 hour in unit interval is 9 AM – 3 PM; other intervals could either be 3 PM – 9 PM, 9 PM – 3 AM or 3 AM – 9 AM).

Urine collected in three 6 hour fractions to total a complete 24 hr collection beginning after completion of collection in unit of 6 hour timed collection on Day 1. The remaining urine will be collected out of the unit and returned on Day 2.

Source: Appendices of Kidney Safety Project Qualification Submission, p. 469-470/578.

Reviewer's comment: The protocol stated that subjects were supposed to complete a screening visit, and 4 additional clinic visits (original protocol) or 5 additional clinic visits (added in protocol addendum A) during an approximate 21-day period to provide urine and blood specimens for determination of biomarkers. However, according to the submitter, because of logistical and financial constraints, data from only 2 sample collection visits (the first and fourth study visits, which occurred ~21 days apart) were included in the analysis. Subject fasting status was not captured and samples at these 2 visits were collected at variable times during the first visit, and at time 0 (first morning void) during the fourth visit.

Sample collection: Urine samples were collected in preservative free urine collection cups and centrifuged at room temperature at 2000 X g for 10 minutes, aliquoted into cryotubes, and frozen at -70°C within 3 hours of collection. Serum was treated similarly. Samples were shipped on dry ice to centralized storage facility and analysis laboratories. At the laboratories, there were specific assay kits used for each biomarker. Urinary creatinine was measured using a rate-blanked modified Jaffe method (Roche).

According to the Kidney Safety Project Qualification Submission, urine samples were analyzed locally for creatinine, and total urine volume was measured and recorded. Urine samples were aliquoted and frozen and were analyzed later for biomarkers that included but were not limited to, albumin, total protein, clusterin, cystatin C, beta2-microglobulin, trefoil factor 3, and KIM-1 levels. Blood samples were analyzed for serum levels of BUN and creatinine and possibly other potential renal biomarkers. Biomarker values were normalized to urinary creatinine levels before analysis.

One tube of blood was sent to the local Jasper laboratory for analysis of sCr, sCysC, and BUN. The urine and additional blood were processed and sent to the biobank for storage. Urine samples were batch analyzed by PBI for renal biomarkers.

Urine samples from the PSTC NHV Study were analyzed by PBI and normalized to urine creatinine (uCr) for each of the six biomarkers. Not all timepoints collected during the PSTC NHV study were analyzed by PBI. For CLU and NAG three separate samples (Visits 1, 3 and 4) were analyzed from each subject. For the other biomarkers two samples (Visits 1 and 4) were analyzed. Table 5 shows the number of subjects who had each biomarker measured, the visit at which the biomarker was measured and the percentage of samples below the lower limit of quantification (LLOQ) by visit.

Analysis Plan: The analyses conducted to support the PFC were not prespecified in the statistical analysis plan for the NHV study.

RESULTS

Study Population

A total of 173 subjects were screened. Of these subjects, 89 were eligible for and enrolled in the study. Eight subjects prematurely discontinued voluntarily or due to protocol violations. A total of 81 volunteers completed the study. Of these, 76 subjects had samples collected on the first and fourth study visits and were included in the analysis.

Demographics

Demographic characteristics of the study population are presented in Table 4. The majority of participants were Caucasian and Non-Hispanic. There were approximately equal numbers of women and men and over two-thirds of the participants were overweight or obese. To what extent these demographic characteristics are similar to those seen in typical phase 1 studies in normal healthy volunteers is not clear. The statistical review examined the impact of race, age, gender, ethnicity and weight on the PFC index and found that there was no substantial difference in the individual PFC indexes among these demographic groups. However, minorities, including self-identified Blacks and Hispanics, were not well represented in the data set.

Table 4: Demographics at Study Entry for PSTC NHV Study (volunteers who completed study with biomarker data)

	Age Category		
	20 – 39 years N=41	40 – 70 years N=40	Total N=81
Age (years)			
Mean	29.4	50.8	40.0
Median	30.0	50.5	39.0
Min, Max	20.0, 39.0	40.0, 69.0	20.0, 69.0
Sex, n (%)			
Male	20 (48.4%)	20 (50%)	40 (49.4%)
Female	21 (51.2%)	20 (50%)	41 (50.6%)
Race, n (%)			
White	32 (78.0%)	36 (90.0%)	68 (84.0%)
Non-white	9 (22.0%)	4 (10.0%)	13 (16.0%)

Ethnicity, n (%)			
Hispanic	1 (2.4%)	1 (2.5%)	2 (2.5%)
Non-Hispanic	40 (97.6%)	39 (97.5%)	79 (97.5%)
BMI (lb/in2)*			
Mean	26.8	28.0	27.4
Median	27.8	27.9	27.8
Min, Max	19.1, 35.4	20.4, 37.6	19.1, 37.6
CDC BMI Category, n (%)			
Normal	18 (43.9%)	7 (17.5%)	25 (30.9%)
Overweight	10 (24.4%)	19 (47.5%)	29 (35.8%)
Obese	13 (31.7%)	14 (35.0%)	27 (33.3%)

Source: Kidney Safety Project Qualification Submission, p.33

Biomarker Results

A total of 76 subjects who had data on visits 1 and 4, approximately 21 days apart, contributed data to the analysis used to derive the PFC Index. Information on the percentage of samples that were below the LLOQ is provided in the table below.

Table 5: PSTC NHV study samples and percentage below LLOQ by Visit

Visit	N samples measured (% of Samples below LLOQ)					
	NAG	NGAL	CLU	CysC	KIM-1	OPN
1	79 (19.0%)	81 (1.2%)	80 (3.8%)	79 (8.9%)	79 (8.9%)	79 (0.0%)
3	80 (13.8%)	NA	80 (2.5%)	NA	NA	NA
4	78 (7.7%)	81 (0.0%)	78 (0.0%)	79 (2.5%)	79 (2.5%)	79 (1.3%)

LLOQ = Lower limit of quantification

Source: Kidney Safety Project Qualification Submission, p. 35/65

Standard biomarkers, including serum creatinine, cystatin C and BUN, and urine total protein and microalbumin were also measured. Results are shown in Table 6. Values were, as a whole, within the “normal” range; or at least suggestive of relatively well preserved renal function (bearing in mind that entry criteria excluded patients with clearance below some level).

Table 6: Standard renal biomarkers measured in serum and urine

Biomarker	Visit 1				Visit 3				Visit 4			
	N	GM	CV	(MIN, MAX)	N	GM	CV	(MIN, MAX)	N	GM	CV	(MIN, MAX)
sCr (mg/dL)	81	0.831	21.9	(0.5, 1.3)	81	0.860	21.1	(0.6, 1.3)	81	0.853	22.1	(0.6, 1.4)
sCysC (ng/mL)	81	620.7	22.8	(301, 919)	81	631.8	24.8	(254, 920)	79	626.5	26.9	(219, 986)
BUN (mg/dL)	81	12.63	29.5	(6, 25)	81	13.22	27.1	(5, 21)	81	13.04	27.6	(7, 23)
Urine Total Protein (mg/mg uCr)	79	0.050	83.7	(0.020, 0.385)					79	0.042	63.5	(0.017, 0.424)
Urine Microalbumin (µg/mg uCr)	79	3.76	131.3	(0.72, 58.50)					79	3.18	115.8	(0.45, 37.83)

GM = geometric mean; CV = coefficient of variation; max = maximum; min = minimum

Source: Kidney Safety Project Qualification Submission, p. 36/65

A geometric mean (GM) of the fold changes from baseline in each the six urine biomarkers (normalized to urine creatinine) was calculated. The standard deviation on log-transformed data was also calculated. The overall GM normalized concentration and the overall SD (log) normalized concentration combines the data from baseline and post-baseline. These calculations are displayed in Table 7.

Table 7: GM and SD of the 6 biomarkers at baseline and post-baseline

Biomarker	GM normalized concentration at baseline	GM normalized concentration post-baseline	Overall GM normalized concentration*	SD (log) normalized concentration at baseline	SD (log) normalized concentration post-baseline	Overall SD (log) normalized concentration
CLU	110.1	109.6	109.9	0.579	0.643	0.610
OPN	0.917	1.032	0.973	0.530	0.485	0.509
NAG	1.669	1.590	1.629	0.464	0.508	0.485
KIM-1	0.339	0.364	0.352	0.722	0.724	0.721
CysC	0.023	0.025	0.024	0.510	0.466	0.490
NGAL	10.72	11.88	11.28	1.043	1.075	1.057

GM = geometric mean

SD = standard deviation, calculated on log-transformed data

Note: The Overall GM normalized concentrations and SD (log) normalized concentrations were used in the simulation.

Units for CLU are ng/mg UCr, CysC: mcg/mg uCr, KIM-1: ng/mg uCr, NGAL: ng/mg uCr, OPN: mcg/mg uCr, NAG mU/mg uCr

Source: Kidney Safety Project Qualification Submission, p. 50/65

Reviewer’s Comment: Normalizing the biomarker values to urine creatinine provides a more accurate assessment when quantifying biomarker excretion (compared to not normalizing) because it adjusts for the inter- and intra-patient variability in urine flow.

Mesothelioma Study

The mesothelioma study (MS) was a phase 1 single-center observational study that was not specifically designed to assess the performance of the PFC Index. Its main objective was to determine the maximum tolerated dose (MTD) of intracavitary heated chemotherapy using a lavage of cisplatin and gemcitabine after extrapleural pneumonectomy (EPP), after pleurectomy/decortication (P/DC), or after Tumor Debulking +/- Intrapleural Pneumonectomy (TD +/- IPP) with intravenous amifostine and sodium thiosulfate cytoprotection. Thirty-nine of the patients in the study who had no evidence of CKD at baseline and who had evaluable specimens were used in the PSTC-FNIH analysis.

Procedures

Urine and serum were collected prior to surgery and cisplatin treatment, and over a period of up to 6 days following cisplatin treatment. Urine samples were centrifuged, aliquoted and frozen and stored at -80°C. Urine was collected the morning of surgery prior to the surgical procedure and post-operatively, centrifuged at 3200 x g for 5 minutes at 4°C. Urinary supernatants were then stored in 1.8 mL cryovials at -80°C. All samples were frozen within 6h of collection except for the 12h post-operative samples, which were stored at 4°C overnight prior to processing the following morning. Samples were frozen at -80°C until measurement. Blood samples were analyzed locally for levels of BUN and sCr. Several kidney urine biomarkers were measured by a central laboratory, Pacific Biomarkers, Inc. (PBI) in the aliquots of the urine samples. While longitudinal sample collection did occur, the collection timepoints for urine and serum samples were not consistent or precisely time stamped within or between patients.

Urine samples were collected prior to surgery or cisplatin treatment, during surgery and after surgery (up to 6 post-op days). The number of sCr measurements for each subject was highly variable with the median number of measurements equal to 19 (range = 9 to 35).

Exploratory Statistical Analysis:

Three subgroups were defined in the MS, including Meso Surgery (N = 4 surgical control patients without exposure to cisplatin), Meso Controls (N = 22 patients exposed to cisplatin without clinical manifestation of treatment related renal injury [i.e., patients could have maximum increases in sCr <50% and <0.3 mg/dL above baseline]), and Meso Cases (N = 13 patients exposed to cisplatin with clinical manifestation of treatment related renal injury [i.e., increases in sCr >50% and/or >0.3 mg/dL above baseline]). The CM calculated for the mesothelioma subgroups were used to assess the performance of the CM in patients with known exposure to the nephrotoxicant cisplatin in the three subgroups. Patients with history of chronic kidney disease were excluded from the analysis.

Individual timepoint concentrations for each biomarker were normalized to uCr by dividing the individual biomarker concentration by the concentration of uCr. The fold change from baseline for each individual timepoint concentration for each biomarker was calculated as the normalized concentration at a given timepoint divided by the normalized concentration at baseline.

To assess the performance of the PCA-weighted and equally-weighted CM in the population of mesothelioma patients with and without medically relevant increases in sCr, the PCA-weighted and equally-weighted CMs were calculated for patients in the various subgroups of the mesothelioma study at each timepoint, and compared to the derived thresholds based on the NHV subjects. Five thousand bootstrap samples of size $m = 6$ at timepoint $T = 12$ hours were generated to determine the likelihood that small samples from the mesothelioma control and case patients would exceed the thresholds derived using the NHV subjects.

To assess the performance of the equally-weighted longitudinal CM in the population of mesothelioma patients with and without medically relevant increases in sCr, the longitudinal equally-weighted CMs were calculated for patients in the various subgroups of the Mesothelioma study and compared to the derived thresholds.

Results

Demographics:

There was a paucity of information presented in the submission on the baseline characteristics of the mesothelioma study population. As shown in Table 8, the study population was mostly male and on average 20 years older than the normal healthy volunteer population used to derive the PFC Index. Of note, the table provides the demographics for the entire mesothelioma study population; however, the urine biomarkers were only analyzed in the subset of patients who had no evidence of CKD at baseline (n=39).

Table 8: Demographics of Mesothelioma Study

<i>Baseline Characteristics</i>		<i>N</i>
Gender	n (%) Male =48 (80%)	60
	n (%) Female =12 (20%)	
Age (years)	Mean = 63.9	60
	Median =65	
	Range= (33, 86)	
Baseline sCr (mg/dL)	Mean =0.91	59
	Median =0.90	
	Range = (0.51, 1.64)	
Cisplatin (0-250 mg/m2)	Mean = 371.5	55
	Median =366	
	Range = (254, 531)	

** 1 patient did not have a baseline sCr measure within 3.5 days of surgery and was excluded from the analysis*

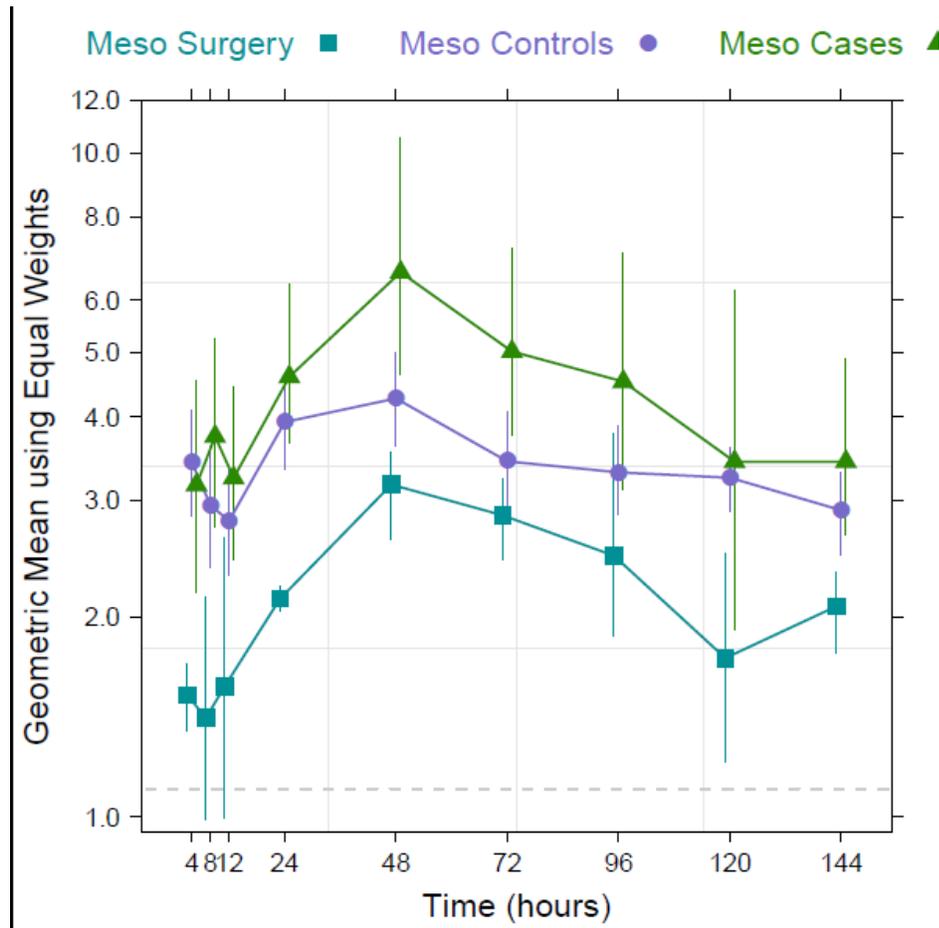
*** 5 patients did not receive Cisplatin, but surgical resection only and are used as Group 4 "controls" in the study*

Source: Kidney Safety Project Qualification Submission, p. 37/65

Biomarker Results

Figure 1 displays the GM of the equally- weighted CM for the 3 groups of subjects in the mesothelioma dataset over time, as well as the reference line associated with the GM CM observed in the 76 NHV subjects. The PCA-derived weighted CM graph is nearly identical to the equally-weighted analysis (not included in this review). The submitter determined the likelihood of exceeding the 99th percentile within-group threshold (1.38) and between-group threshold (1.49). GM CMs from the NHV subjects exceeded the within-group thresholds 1.04% of the time (as expected). GM CMs from the Meso Control and Meso Cases exceeded both the within and between-group thresholds > 99% of the time.

Figure 1: GM CM calculated using equal weights over time among categorized groups of the 39 patients in the mesothelioma dataset who had no evidence of CKD at baseline



Meso Surgery (N = 4 surgical control patients without exposure to cisplatin), Meso Controls (N = 22 patients exposed to cisplatin without clinical manifestation of treatment related renal injury [i.e., patients could have maximum increases in sCr <50% and <0.3 mg/dL above baseline]), and Meso Cases (N = 13 patients exposed to cisplatin with clinical manifestation of treatment related renal injury [i.e., increases in sCr >50% and/or >0.3 mg/dL above baseline])

Source: Mat Soukup, PhD, Statistical Review

Reviewer’s Comment: *The mesothelioma data are difficult to interpret and neither support nor refute the value of the PFC index in NHVs. Even at baseline, the PFC Index fell outside the range identified in the NHV study, indicating that the PFC index should not be qualified for use in patient populations.*

Appendix 1 (source, FNIH-PSTC Briefing Book, pp. 20-23, 59-64)

3.1.1 Urinary Clusterin (CLU)

CLU (product of the CLU gene) has a secreted and a nuclear isoform. Only the secreted isoform, a 76-80 kDa glycosylated protein with extensive post-translational modifications, is considered relevant in the context of kidney injury. CLU is constitutively expressed at high levels during early stages of renal development and later in response to kidney injury in the proximal and distal tubules, glomerulus, and collecting duct. Secreted CLU has been suggested to play an anti-apoptotic role and to be involved in cell protection, lipid recycling, cell aggregation and cell attachment (Rosenberg 1995). Expression of CLU mRNA is induced by different types of kidney injury in glomeruli, tubules and papilla of rats and dogs as a result of drug nephrotoxicity (Wadey 2014, Zhou 2014, Kharasch 2006, Rached 2008, Correa-Rotter 1998), surgery and ischemia (Nguan 2014, Tsuchiya 2005, Yoshida 2002, Ishii 2007) and in animal models of different renal diseases (Hidaka 2002). Changes in CLU protein levels have been measured in kidney and in the urine of many rat and dog studies (Vlasakova 2014, Hoffman 2010, Wadey 2014, Sasaki 2011, Betton 2012, Tsuchiya 2005, Correa-Rotter 1998, Ishii 2007, Hidaka 2002) as well as non-human primates treated with a triple reuptake inhibitor (Guha 2011). However, only recently has more clinical data appeared reporting the use of CLU as a biomarker for human kidney injury and disease (Saeidi 2015, Ariza, 2015, Cassidy 2015, Tsuchimoto 2014, Pianta 2015, Rosenberg 1995, Ghiggeri 2002). In the previous regulatory nonclinical qualification of biomarkers submitted by PSTC and Health and Environmental Sciences Institute (HESI), urinary CLU has proven to be a powerful diagnostic biomarker to monitor tubular injury and regeneration with a performance approximately equivalent to that seen with urinary KIM-1 in rat studies (Dieterle 2010, Harpur, 2011).

3.1.2 Urinary Cystatin-C (CysC)

CysC (product of the CST3 gene) is a protein marker that is freely filtered at the glomerulus and then reabsorbed by the renal tubular epithelium. In addition to its potential role as a biomarker of glomerular filtration, CysC can also be measured in the urine in the presence of tubular dysfunction. An impairment of re-absorption in proximal tubules can lead to a several hundred fold increase in urinary levels of CysC in humans and rats (Herget-Rosenthal 2007, Conti 2006). Reported reference ranges and average control values are very consistent in studies including nearly 2,000 healthy subjects in total and indicate a normal urinary CysC concentration below 0.3mg/L (Uchida 2002, Herget-Rosenthal 2004). In a study with 1670 healthy subjects the average urinary CysC concentration was 0.051mg/L \pm 0.0252 mg/L (Uchida 2002). Urinary CysC is becoming more commonly used, along with KIM-1, as a biomarker for both acute kidney injury (AKI) and chronic kidney disease (CKD). A recent study with 213 patients with AKI, of whom 59.6% had intrinsic AKI classified according to Acute Kidney Injury Network (AKIN) criteria, determined that CysC had both diagnostic and prognostic utility (Park 2013). CysC may also predict CKD progression in diabetic nephropathy (Kim 2013, Matys 2013). Urinary CysC has also been characterized in the context of different kidney diseases affecting glomerular integrity and proximal tubular re-absorption in humans (Herget-Rosenthal 2004, Tenstad 1996, Collé 1990). Urinary CysC levels were investigated in 50 patients with glomerular diseases and 22 patients with tubulointerstitial diseases, which were all proven by biopsy (Herget-Rosenthal 2007). Urinary CysC/uCr ratios > 11.3 mg/mmol were highly associated with tubular proteinuria, biopsy-proven tubulointerstitial disease and heavy proteinuria in this study. Both functional impairment due to protein overload in the case of heavy proteinuria (glomerular disease), as well as structural impairment due to tubulointerstitial disease were identified in this study as factors associated with increased urinary levels of CysC, with similar data also reported by others (Uchida 2002, Tkaczyk 2004, Herget-Rosenthal 2004).

3.1.3 Urinary Kidney Injury Molecule-1 (KIM-1)

Urinary KIM-1 (product of the TIM-1/HAVCR-1 gene) is a type I cell membrane glycoprotein containing a unique six-cysteine immunoglobulin-like domain and a mucin-rich extracellular region that is conserved across species in zebrafish, rodents, dogs, primates and humans (Ichimura 1998). KIM-1 mRNA levels are elevated after initiation of kidney injury more than any other known gene across these species (Ichimura 1998, Amin 2004). After injury, the ectodomain of KIM-1 is shed from proximal tubular kidney epithelial cells *in vitro* (Bailey 2002) and *in vivo* into urine in rodents (Ichimura 1998, Amin 2004, Prozialeck 2007, Nogueira 1998, Zhou 2008) and humans (Han 2002, Liangos 2007, Vaidya 2008, van Timmeren 2007). Following cisplatin treatment, KIM-1 protein levels were highly correlated in kidney tissue and in urine (Wadey 2014). Data from the PSTC across 16 rat studies using well established nephro- and hepatotoxicants conducted across multiple sites, showed that urinary KIM-1 significantly outperformed sCr and BUN, using area under the receiver operating characteristic (ROC) curve analyses (Vaidya, 2010). These results have been confirmed repeatedly in similarly sized rat datasets (Hoffman 2010, Rouse 2011, Vlasakova 2014, PSTC OPN NGAL submission, 2014).

The utility of KIM-1 as a biomarker to diagnose AKI and CKD in humans, and thus its utility as translational marker for drug-induced kidney injury (DIKI), has been shown in many different clinical contexts. In a study of 40 children undergoing cardiac surgery, for which NGAL levels were originally determined, urinary KIM-1 levels could diagnose AKI 12 hours after surgery with an area under the curve (AUC) value of 0.81 from ROC analyses, whereas increases in sCr were observed only after 24 to 72 hours (Han 2008). In a study in patients with non-diabetic renal disease, urinary KIM-1 levels were increased in patients with proteinuria and decreased in patients treated with a renin-angiotensin system inhibitor, sodium restriction or diuretic therapies. In those patients, KIM-1 correlated with proteinuria decrease, rendering it a potential alternative clinical endpoint (Waanders 2009).

In another study, urinary KIM-1, NGAL, NAG, CysC, IL-18 and α 1-Microglobulin were evaluated in 103 patients undergoing cardiac surgery, with 13% of the patients developing AKI. KIM-1 showed the highest diagnostic performance (AUC 0.78) and was the only marker independently associated with AKI after adjusting for pre-operative AKI score. The variance of reported results for the different markers in the context of cardiac surgery followed by AKI demonstrates that there is a pressing need to compile more evidence in different populations and assess all markers together in these cohorts to obtain consistent evidence of their utility in different clinical contexts.

In another cross-section study urinary KIM-1, MMP-9 and NAG levels were measured in 29 patients with AKI (due to sepsis and hypoperfusion, nephrotoxins and contrast-induced nephropathy) and compared to levels in 45 control patients (healthy volunteers, CKD patients and patients with urinary tract infection [UTI]) (Han 2008). The AUCs of the ROC analyses were 0.74 for MMP-9, 0.90 for KIM-1, 0.97 for NAG, and 1.0 for all three biomarkers combined. In a study with 201 patients with clinically established AKI, urinary KIM-1 levels and NAG levels correlated with the clinical composite endpoint of death or dialysis requirement, even after adjustment for disease severity and comorbidity (Liangos 2007).

KIM-1 has proven to be one of the most promising biomarkers to monitor AKI impacting proximal tubular epithelial cells due to rapidly increasing evidence of its pre-clinical and clinical utility in numerous contexts including its unique specificity, its sensitivity to detect various forms of tubular injury earlier than current diagnostic standards, its stability, and its translatability between different species.

3.1.4 Urinary N-Acetyl-beta-D-Glucosaminidase (NAG)

Urinary NAG (product of the nag1 gene) is a 140 kDa lysosomal brush-border enzyme with two isoforms (A and B) mainly expressed in proximal tubules where its function is the breakdown of

glycoproteins. Due to its size, plasma levels of NAG are normally not filtered by the glomeruli and its excretion into urine correlates with increased tubular lysosomal activity, tubular cell injury (leakage), and indirectly with increased proteinuria. NAG has been used for decades. In the context of renal diseases (diabetic and hypertensive nephropathy, focal segmental glomerulosclerosis), AKI, and treatment with nephrotoxic compounds, increased urinary NAG levels have typically been observed before increases in sCr and BUN (Sheira 2015, Westhuyzen 2003, Price 1992, Skálová 2005, Emeigh 2005, Ascione 1999). In hospitalized patients, increased NAG levels were associated with an adverse outcome (dialysis or death) (Liangos 2007).

As a translational biomarker for drug development, NAG differs from the other DIKI biomarkers evaluated in this project, as it is not as consistently reliable in rodents. Its responsiveness to drug-induced nephrotoxicity is well-established in dogs (Zhou 2014) and ongoing research indicates good responsiveness in non-human primates (PSTC unpublished data).

3.1.5 Urinary Neutrophil gelatinase-associated lipocalin (NGAL)

NGAL (product of the LCN2 gene) also known as human neutrophil lipocalin, lipocalin-2, siderocalin, or LCN2, is a 25-kDa protein initially identified in neutrophil specific granules. NGAL is expressed in various tissues at low levels, but induced in epithelial cells with inflammation or other types of injury including malignancy (Cowland 1997). NGAL functions in iron homeostasis through binding of siderophores, leading to iron chelation and inhibition of bacterial cell growth or inhibition of apoptosis or oxidative stress in mammalian cells (Schmidt-Ott 2007). With kidney injury, NGAL is upregulated in the thick ascending limb of the loop of Henle, distal tubule and collecting duct, and is secreted into the urine as well as plasma (Paragas 2011). In mouse models, strongly increased NGAL mRNA and protein in the kidney parenchyma and urine are observed shortly after cisplatin administration or renal ischemia and precede changes in sCr (Mishra 2003, Mishra 2004). Plasma levels of NGAL are normally low, and NGAL in the glomerular filtrate is nearly completely reabsorbed by the megalin-cubilin transporter complex in the proximal tubule. With increased urinary protein load (protein overload nephropathy), saturation of the re-absorption capacity of this complex can lead to increased urinary NGAL and tubular back-leak can result in increases in plasma NGAL. In addition, DIKI can cause increased expression and release of NGAL as a protective mechanism as shown for other “tubular stress” proteins such as KIM-1 (Bolignano 2008). As a consequence, conditions which lead either to saturation or impairment of the re-absorption complex or to increased *de novo* expression of NGAL in kidney are expected to demonstrate the utility of NGAL as a kidney biomarker in the context of drug development (Bolignano 2008, Cowland 1997, Devarajan 2010, Mishra 2003, Mishra 2004).

Urinary NGAL is actively being investigated, and in some cases utilized, for the prediction of AKI in a number of clinical settings including interventional trials for AKI, the diagnosis and management of cardiorenal syndrome and in patients undergoing cardiac surgery, in the emergency room, and in the intensive care unit (ICU). Recent reviews, from publications representing several thousand patients, summarize the promising clinical utility of NGAL for the prediction of AKI (Taub 2012, Devarajan 2014, Singer 2013, Tsigou 2013). sCr and urine output, current diagnostic measures of AKI, do not distinguish between hemodynamic changes due to reduced glomerular filtration rate (GFR) and structural kidney damage. Because NGAL is rapidly upregulated following kidney tissue injury, it is a highly attractive biomarker for the sensitive monitoring of DIKI in clinical trials.

3.1.6 Urinary Osteopontin (OPN)

OPN (product of the SPP1 gene) also known as secreted phosphoprotein I, sialoprotein I, uropontin, derives its name from its role in the regulation of osteoclast function during bone formation (Tanabe 2011). In the kidney, OPN has divergent roles. OPN is a protective agent against oxidative stress and ischemia (Fuchs 2011). OPN, also has pro-inflammatory and profibrotic activity. In normal mouse,

rat and human kidney, OPN is expressed at low levels in the distal nephron (thick ascending limb of the loop of Henle and distal convoluted tubules) (Hudkins 1999). With tissue injury, OPN expression has been demonstrated throughout the kidney, and OPN has proven to be a very sensitive and inducible indicator of different forms of AKI (Lyle 2012). Increased OPN mRNA and protein levels have been reported in the kidney in numerous animal models of renal disease and injury including after gentamicin administration (Xie 2001, Irita 2011, Lorenzen, 2008). OPN has gained recent attention as an accessible urinary protein biomarker resulting in a significant increase in activity to characterize its true value. Exploratory reagents have become commercially available to quantitatively measure OPN in the urine of rats, mice and humans. Compared to NGAL, the characterization of OPN in clinical kidney injury and disease settings is somewhat limited. Investigations in renal transplant and critically ill patients support its utility for predicting patient outcome (Jin 2013, Lorenzen 2011). However, potential confounding variables with respect to the use of OPN in clinical trials have not yet been identified or investigated.

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