White Paper

Detection of vancomycin-induced subacute nephrotoxicity using MILLIPLEX® MAP rat kidney toxicity multiplex panels

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About the Author

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Introduction

In the pharmaceutical and chemical industries, the kidney is routinely assessed during preclinical safety evaluations. The kidney is an important central detoxification organ, because there is an extraordinary exposure of renal tissue to drugs, reactive metabolites or environmental chemicals. This exposure can lead to cell damage, primarily due to high blood flow, xenobiotic metabolism or high clearance[1]. The frequency at which drug-induced nephrotoxicity occurs, relative to other drug-induced toxicities, is 2% to 20%[2]. The reason for this range may be due to the difficulty of assessing renal toxicity using traditional markers, which are well-known to be insensitive, as is the case with blood urea nitrogen (BUN) and serum creatinine (SerCrea). Although both are direct measurements of renal function, increases in serum concentrations of these biomarkers occur only after substantial renal injury, generally after loss of two-thirds of the nephrons' functional capacity[3]. In the case of acute kidney injury (AKI), the degeneration of renal tissue can occur after days or weeks of exposure[4,5].

For improved detection of acute nephrotoxicity, a set of novel urinary kidney biomarkers was recently approved by the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA) and Japan's Pharmaceuticals and Medical Devices Agency (PMDA). The Predictive Safety Testing Consortium (PSTC) — composed of these government entities and leading pharmaceutical companies, together with the nonprofit Critical Path Institute (C-Path) — led the discovery of a large number of transcriptional biomarkers and subsequent evaluation of 23 urinary proteins in the rat[6,7]. Only 8 of these markers have been accepted ("qualified") by the regulators for the detection of AKI in preclinical rodent studies up to 14 days. Table 1 gives an overview of the most relevant biomarkers measured in this study. Because the majority of the studies performed by the PSTC members included study time points of 1, 3, 7 and 14 days, for an improved risk assessment, we determined it was absolutely necessary to generate further information about the kinetics of the markers, as well as to test their utility in subacute (28 days) or subchronic (commonly 90 days) preclinical rodent studies. Therefore, this study focused on addressing how these urinary biomarkers perform after 28 days of treatment with a model nephrotoxic compound, vancomycin[8]. This compound is a glycopeptide antibiotic that has been previously shown to affect primarily the tubules in cases of vancomycin-induced kidney injury[7,8].

In this study, 120 Wistar rats were divided into groups of 10 animals (5 female, 5 male), and were treated with vancomycin at 2 doses (low dose: 50 mg/kg body weight [bw]; high dose: 300 mg/kg bw) for up to 28 days. The candidate proteins were measured in urine on days 4, 8, 15 and 29 using the MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Multiplex Panels to assess their ability to detect drug-induced renal damage. For interpretation of the data, standard clinicochemical and histopathological observations were performed. We have reported an earlier study with the same set of samples and similar methodology[8]. The data obtained from the two studies were consistent and reproducible. The ability to predict compound-dependent renal damage and distinguish it from acute renal failure, often associated with other risk factors, has the potential to positively affect drug development productivity.

Biomarker	Damage Location	Regulatory Status
AGP (α-1-acid-glycoprotein)	General	Exploratory
Albumin	Glomerulus	Accepted
β-2-Microglobulin	Proximal tubule	Accepted
Calbindin	Distal tubule and collecting duct[9]	Exploratory
Clusterin	General	Accepted
Cystatin C	Proximal tubule	Accepted (urine; serum is exploratory)
EGF	Distal tubule and collecting duct[9]	Exploratory
GSTα	Proximal tubule	Exploratory
IP-10	General	Exploratory
KIM-1	Proximal tubule	Accepted
Lipocalin-2/NGAL	General	Exploratory
Osteoactivin	Tubular epithelium	Exploratory
Osteopontin	General	Exploratory
RBP4	Proximal tubule	Exploratory
TFF-3	Tubular epithelium	Accepted
TIMP-1	Proximal tubule	Exploratory
VEGF	General (reno-protective responses)	Exploratory

Table 1.

Overview of the measured biomarkers, the prediction of the damage location and their acceptance status by the FDA, EMA and PDMA for nonclinical rat kidney studies. These, and additional biomarkers (not all are listed), are under clinical evaluation by the PSTC as well.

Materials and Methods

Animal Studies

The 28-day rat study was performed according to Good Laboratory Practice and in compliance with the German Law on the Protection of Animals (German Animal Welfare Act, Article 8a). Before the studies were conducted, a dose range finding study was carried out under the same conditions as planned for the main studies. Significant results of the dose range finding study have been reported[8]. Ten-week-old Wistar rats, purchased from Charles River Laboratories (Sulzbach, Germany), were randomly divided into 12 groups of 10 animals (5 females, 5 males). Animals were individually housed in type III isolated ventilated Makrolon® cages with a 12-hour light-dark cycle. Before treatment, male rats weighed 287 ± 9.0 g and female rats weighed 195 ± 5.8 g. Rats were treated with vancomycin (Ratiopharm, Ulm, Germany) at either a low dose of 50 mg/kg or a high dose of 300 mg/kg. Vancomycin was diluted in water and administered intraperitoneally (i.p.) daily for 7 days and thereafter only once per week. The control animals received an equal volume of 0.9% saline for 7 days by daily i.p. injection, followed by administration twice per week. On day 4, 8, 15 and 29, blood, urine and organ tissue samples were collected from all individual animals (n=10 for each group; 5 male, 5 female). The animals were housed in individual metabolism cages for 18 hours (fasted with free access to water). Urine was collected under cooled conditions and stored at -80°C until the urinary biomarkers were measured. Blood samples were taken by puncture of the sublingual vein under light isofluorane anesthesia. Blood samples and aliquots of urine were immediately used for routine clinical chemistry analyses. Frozen aliquots of urine for the determination of urinary protein biomarkers were all measured in parallel. The animals were sacrificed by CO_2 asphyxiation, and organs were collected and fixed in formalin for histopathological examination.

Clinical Pathology

Urine and plasma analyses were carried out on an ADVIA 1650 Autoanalyzer and Clinitek 100 Reflection Spectrophotometer (Siemens Medical Solution Diagnostics GmbH, Bad Nauheim, Germany) using standard protocols for the determination of multiple parameters, based on the recommendations from Weingand and colleagues[11,12] and according to the manufacturer's instructions.

Kidney Toxicity Biomarker Analysis

MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels using the Luminex® xMAP® platform were used for the safety biomarker analysis. This analysis requires a platform that will reliably quantify proteins from preclinical samples with high sensitivity and specificity. ELISAs are an alternate platform for accurate quantification; however, their low throughput is a major drawback. In addition, preclinical kidney toxicity studies typically use small rodents, resulting in limited available sample volumes.

To overcome these limitations, a number of emerging technology platforms, such as the Luminex® xMAP® platform and others, have been developed and compared[10], making it possible to measure several proteins in a single sample robustly and rapidly[13]. Multiplexed analysis offers significant advantages with respect to time, reagent cost, sample requirements and the amount of data that can be generated. Combined with high-quality antibody reagents and assay development expertise, multiplexed immunoassays on the Luminex® xMAP® platform can deliver the sensitivity, reproducibility, dynamic range (pg/mL to ng/mL), throughput and robustness demanded for quantitative biomarker analysis.

The MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 1 (Cat. No. RKTX1MAG-37K) includes multiplexed assays for Calbindin, Clusterin, GSTα, IP-10, KIM-1, Osteopontin, TIMP-1 and VEGF. This kit may be used for the simultaneous quantification of all or any combination of the analytes in urine, and requires only 25 µL of 1:2 diluted urine sample volume.

The MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 2 (Cat. No. RKTX2MAG-37K) includes multiplexed assays for AGP, Albumin, β-2-Microglobulin, Cystatin C, EGF and Lipocalin-2/NGAL. This kit may be used for the simultaneous quantification of all or any combination of the analytes in dilute urine samples, and requires only 25 µL of 1:500 diluted urine sample volume.

The results presented in this paper illustrate the application of MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels 1 and 2 in a preclinical study.

Statistical Analysis

Urinary multiplexing assay results were calculated from an 8-point standard curve and were normalized against the appropriate urinary creatinine value. Statistical analysis was performed by analysis of variance (ANOVA) and Tukey test. Values significantly different from control are indicated as:
*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

Renal histopathology of this cohort of rats was previously reported[8]. Macroscopic enlargement of kidneys in high dose-treated (HD) animals was observed. Histopathological observation of tissues prepared from formalin-fixed paraffin-embedded (FFPE) blocks (hematoxylin/eosin stained) showed that vancomycin treatment caused general tubular alterations in HD animals after 28 days of treatment. HD animals showed multifocal, massive to severe tubular degeneration in combination with a moderate to severe tubular dilation[8].

KIM-1 Immunohistochemistry

Gender-specific differences in the location of KIM-1 were identified at all time points in the HD groups (Figure 1A). An increase in protein level was observed up to Day 8. Cellular regeneration up to Day 15 led to a reduction in KIM-1. On Day 29, when massive tubular damage was again observed, KIM-1 was also up-regulated (Figure 1B).

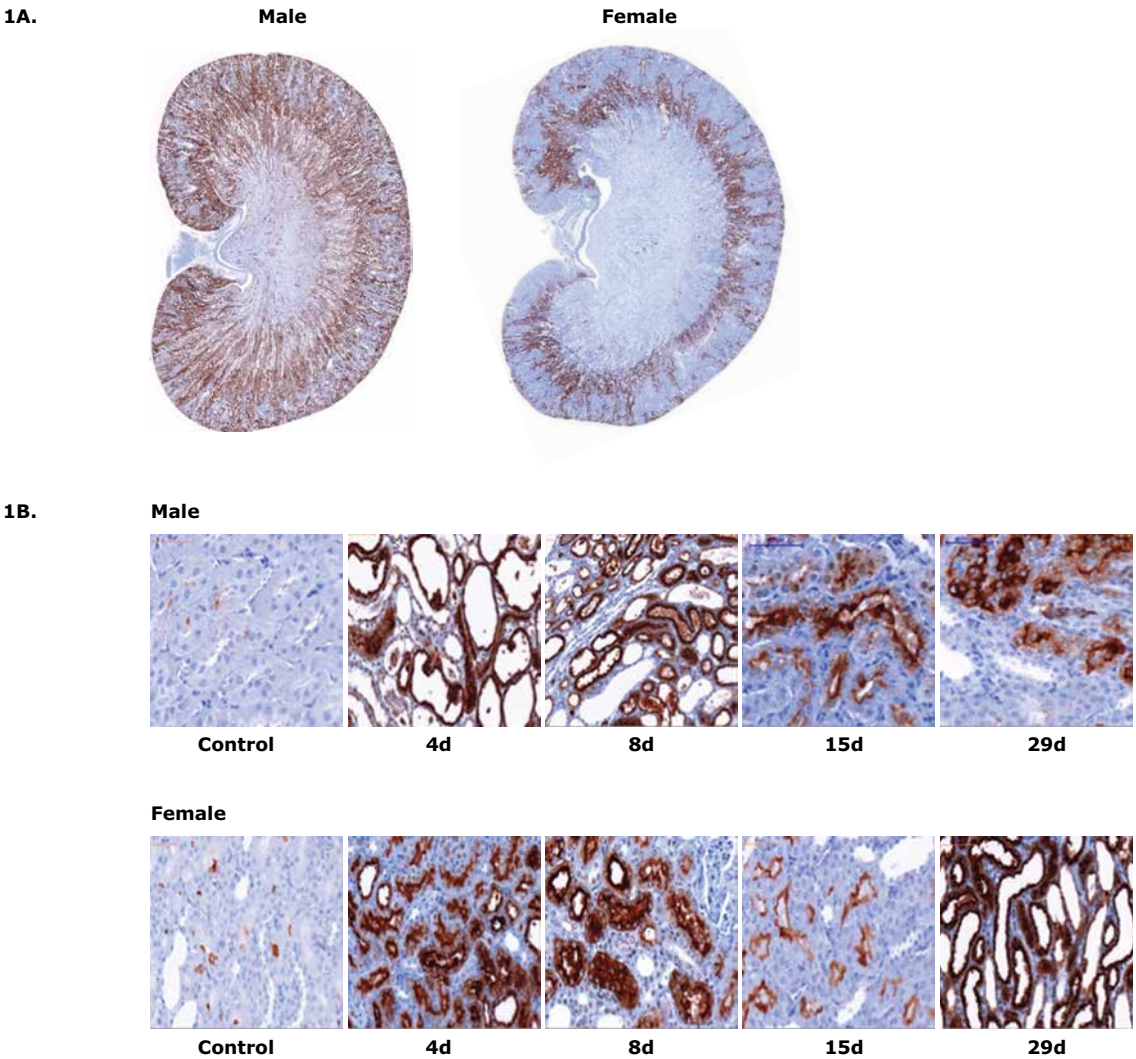

Figure 1.

(A) Gender-specific differences in the localization of KIM-1 at all time points in the HD animals (Day 8 shown).
(B) Time course of KIM-1 expression in male and female rats treated with high dose (300 mg/kg) vancomycin.

Serum Creatinine and Blood Urea Nitrogen

The classic serum markers for detecting the loss of renal function, SerCrea and BUN, showed strong effects after 7 and 28 days of 300 mg/kg (HD) vancomycin treatment (Figure 2).

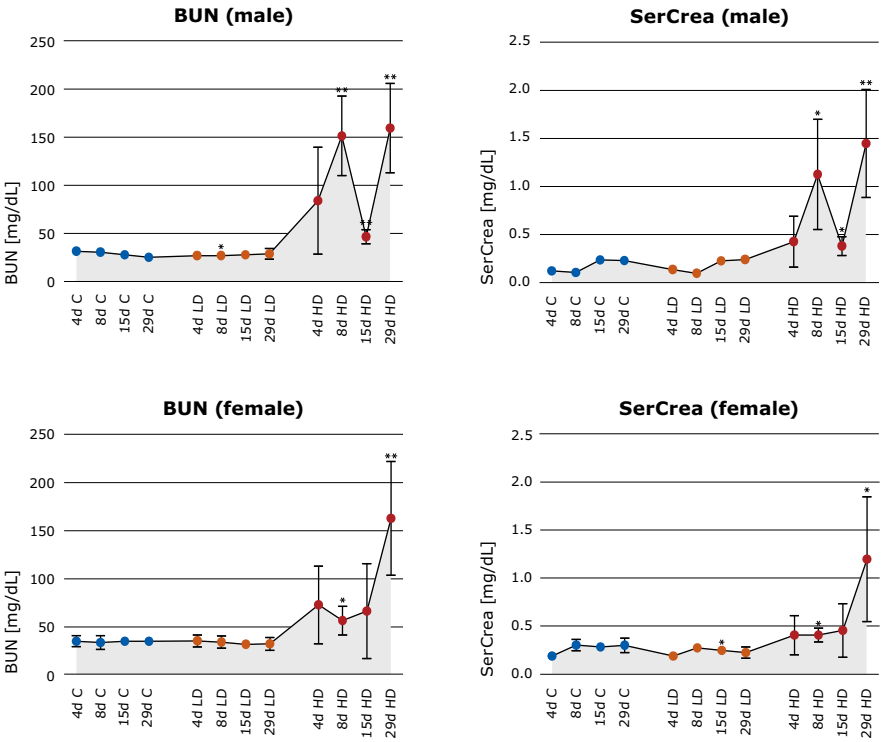

Figure 2.

Significant increases were observed at several time points in the high dose-treated group of both genders in **BUN** and **SerCrea**. No changes in low dose-treated animals could be detected. ANOVA + Dunnett p-values: * <0.05, ** <0.01, *** <0.001.

Analysis of Urinary Kidney Biomarkers

Fourteen urinary protein biomarkers were measured using the MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels 1 and 2: AGP, Albumin, β2M, Calbindin, Clusterin, Cystatin C, EGF, GSTα, IP-10, KIM-1, NGAL, OPN, TIMP-1 and VEGF.

No urinary markers showed significant differences in the low dose-treated (LD) groups throughout the 28-day time course. In the HD groups, increases in urinary Clusterin, KIM-1, NGAL, Osteopontin (OPN) and TIMP-1 (Figure 3A) reflected the renal regeneration and strength of vancomycin-induced response, correlating with histopathological findings (data not shown[8]). Interpretation of AGP, Albumin, Cystatin C and EGF levels was limited by gender differences, illustrating significant and related alterations to vancomycin treatments in the male groups but not in the female groups (Figure 3B). β2M, Calbindin, GSTα, IP-10 and VEGF demonstrated minimal changes in this study, and therefore were not predictive of vancomycin-induced renal damage (Figure 3C).

3A.

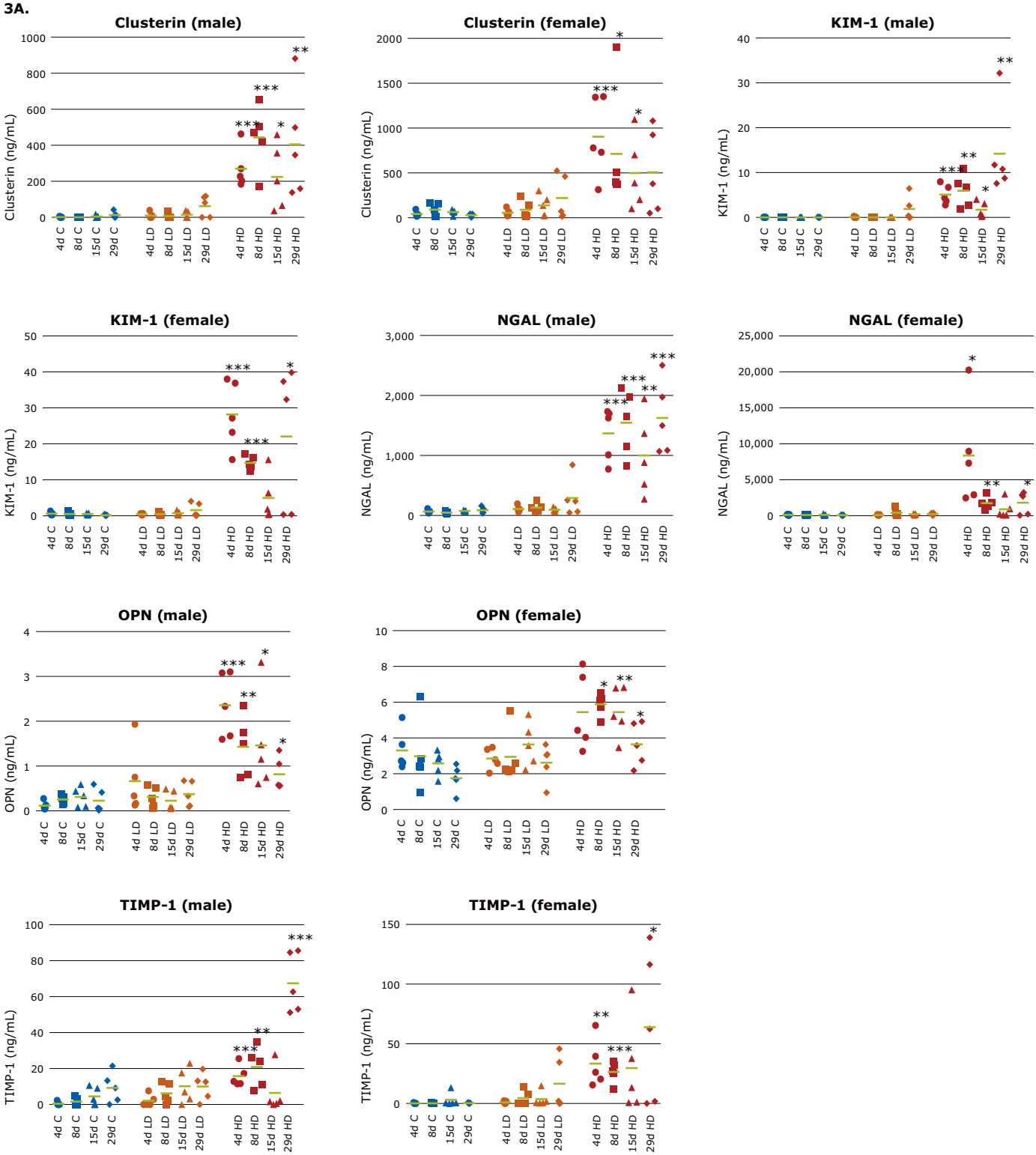

Figure 3A.

Urinary kidney biomarker analysis by MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Multiplex Panels to predict vancomycin-induced kidney damage: Clusterin, KIM-1, NGAL, Osteopontin (OPN) and TIMP-1. Data represent individual animals, and mean values are represented by a line. Statistical significance was determined by ANOVA + Dunnett-Test: *p<0.05, **p<0.01, ***p<0.001.

3B.

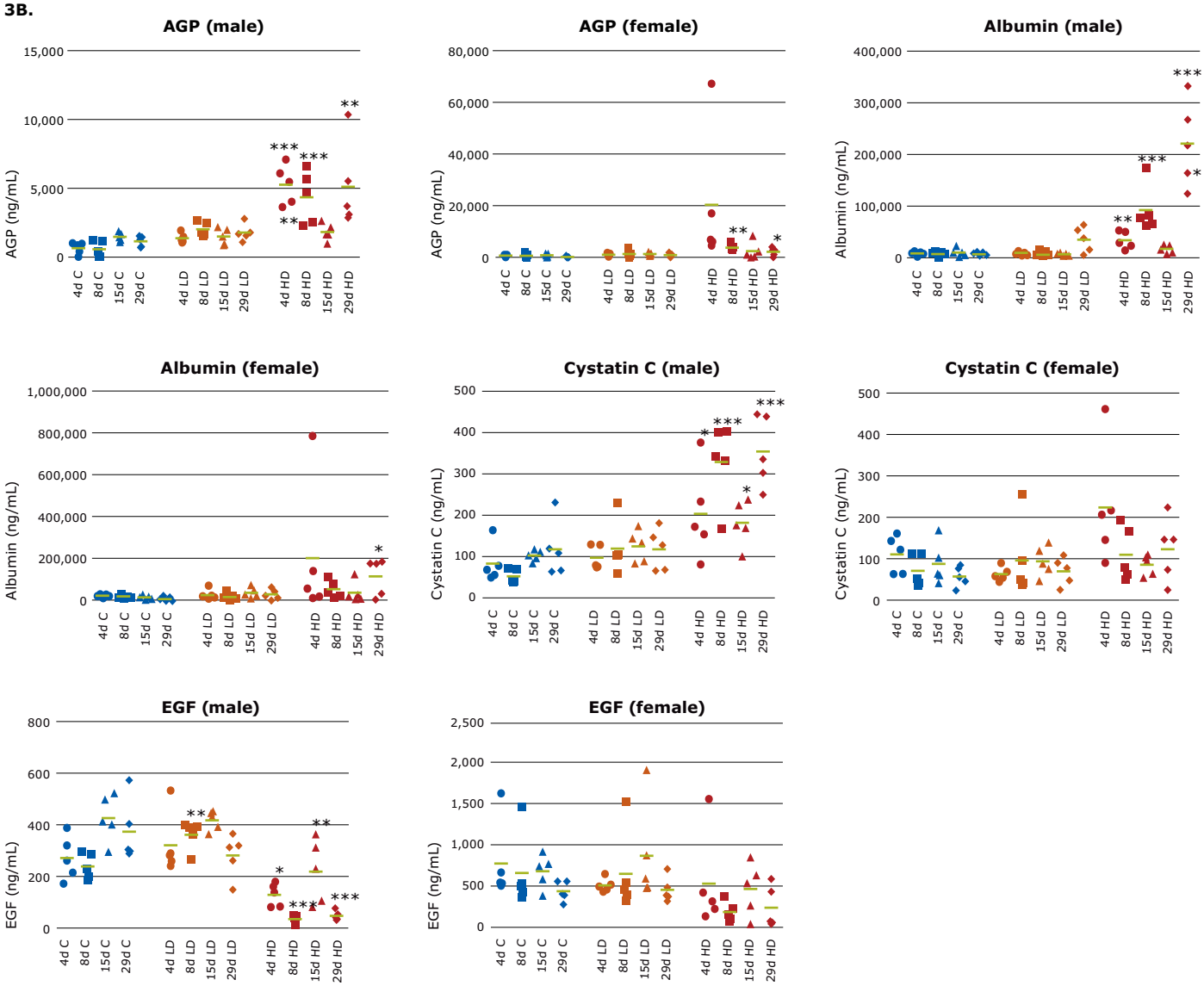

Figure 3B.

Urinary kidney biomarker analysis by MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Multiplex Panels to predict vancomycin-induced kidney damage: AGP, Albumin, Cystatin C and EGF. Data represent individual animals, and mean values are represented by a line. Statistical significance was determined by ANOVA + Dunnett-Test: *p<0.05, **p<0.01, ***p<0.001.

3C.

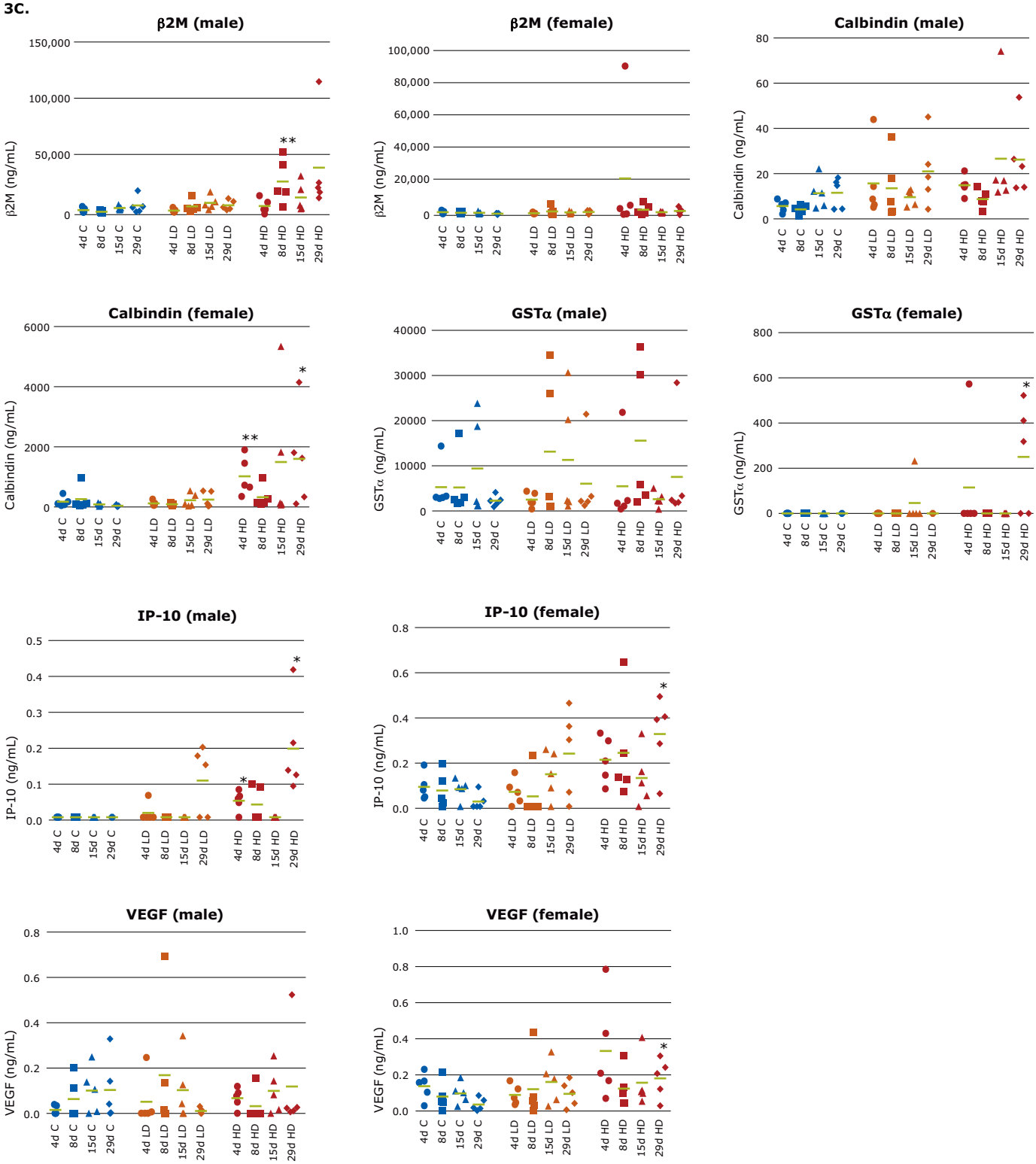

Figure 3C.

Urinary kidney biomarker analysis by MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Multiplex Panels to predict vancomycin-induced kidney damage: β2M, Calbindin, GST, IP-10 and VEGF. Data represent individual animals, and mean values are represented by a line. Statistical significance was determined by ANOVA + Dunnett-Test: *p<0.05, **p<0.01, ***p<0.001.

Conclusion

In this study, acute nephrotoxicity biomarkers were investigated for their ability to detect renal damage in a vancomycin-induced toxicity study in rats over a period of 28 days at 4 time points. MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels, based on Luminex® xMAP® multiplexing technology, were employed and compared to histopathology and immunohistochemistry data.

Our data demonstrate the high accuracy and predictivity of several of these new markers, even after 28-day subacute treatment with one well-described nephrotoxin, vancomycin, which caused a very distinct kidney tubular damage in rats. The data were consistent with previous studies of subacute vancomycin treatment[8]. The classic markers, BUN and SerCrea, showed significant increases after 7 days of treatment (Day 8 groups, Figure 2). No significant changes were observed on Day 4. In contrast, significant changes in urinary Clusterin, KIM-1, NGAL, Osteopontin (OPN) and TIMP-1 levels could be detected on Day 4, demonstrating a higher sensitivity and specificity of these novel urinary protein biomarkers (Figure 3A). Furthermore, the strong increase of KIM-1 after vancomycin treatment reflected a severe insult on the renal proximal tubular cells and delivered additional information on the location of damage, compared with the traditional parameters alone. Interestingly, a group of urinary markers (AGP, Albumin, Cystatin C and EGF) showed a gender-specific detection of vancomycin-induced kidney toxicity only in the male groups and not in the female groups (Figure 3B). Based on the known toxicity of vancomycin, these biomarker results, predicting primarily tubular damage, are consistent with the literature on kidney injury[7,8].

Many questions remain with regards to the utility of these novel nephrotoxicity biomarkers. It is of major interest to discover the optimum timeline of biomarker excretion, especially against the background of the high regenerative properties of renal tissue. It is also of interest to identify any potential recovery from renal injury after cessation of drug exposure, and this can be assessed based on excretion of the urinary biomarkers.

In addition, besides age dependency in biomarker excretion, which has recently been shown[14], gender differences in urinary biomarker excretion have been illustrated in this study. These differences could influence the sensitivity and/or specificity of some of the markers. Therefore, before implementing these assays into preclinical studies, further investigations are required to differentiate between treatment-induced effects and general variations in the biomarker expression pattern across a population. Further investigations should also address excretion and the ability to discriminate gender-specific toxic insults induced by xenobiotics.

The Luminex® xMAP® based MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels are able to accommodate a broad context of study designs. The complexity of accumulating standardized preclinical data, interpreting the data and ranking the new markers is a compelling reason to use these biomarker panels, which are comprised of several markers, as opposed to inferring kidney injury based on a single biomarker or a small number of biomarkers. Practically, these panels enable researchers to measure multiple nephrotoxicity biomarkers in a very small sample volume, enhancing the predictive power of preclinical models while minimizing time, animal and reagent costs.

Ordering Information

MILLIPLEX® MAP Toxicity Biomarker Multiplex Assays

Bead-based multiplex immunoassays enable precise, multiparametric analysis of the concurrent processes that underlie toxicity. No single biomarker has the power to tell you what you need to know to profile the effect on your model, so we provide you a growing portfolio of protein biomarkers in multiplex format, using the Luminex® xMAP® bead-based technology.

Our MILLIPLEX® MAP multiplex toxicity biomarker panels make us the leading partner for toxicity testing research.

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	Cat. No. RKTX1 MAG-37K	Cat. No. RKTX2 MAG-37K	Cat. No. CKT1 MAG-97K	Cat. No. CKT2 MAG-97K	Cat. No. MKI1 MAG-94K	Cat. No. MKI2 MAG-94K	Cat. No. HKI1 MAG-99K	Cat. No. HKI2 MAG-99K	Cat. No. HKI3 MAG-99K	Cat. No. HKI4 MAG-99K	Cat. No. HKI5 MAG-99K	Cat. No. HKI6 MAG-99K
Analyte												
α-1-Microglobulin								•			•	
AGP		•										
Albumin		•		•				•				
β-2-Microglobulin		•		•	•				•			•
Calbindin	•						•					
Clusterin	•		•			••		•				•
Collagen IV							•				•	
Cystatin C		•	•			••		•				•
EGF		•				•		•		•		
FABP1							•			•		
GSTα	•						•					
IL-8			•									
IP-10	•				••		•			•		
KIM-1	•		•		••		•			•		
Lipocalin-2/NGAL		•	•			••		•			•	
MCP-1			•									
Osteoactivin							•				•	
Osteopontin	•		•			••		•		•		
PTH										•		
RBP4				•					•			•
Renin					••		•			•		
TFF-3				•			•					
TIMP-1	•				••		•				•	
Uromodulin									•		•	
VEGF	•				•							

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