University of Cincinnati

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I, Andrew Bukowinski , hereby submit this original work as part of the requirements for the degree of Master of Science in Clinical and Translational Research .

It is entitled:

Novel Urinary Biomarkers of Acute Kidney Injury to Detect Toxicity and Predict Clearance in Pediatric Oncology Patients Treated with High Dose Methotrexate

Student's name: **Andrew Bukowinski**

This work and its defense approved by:

Committee chair: Erin Nicole Haynes, Dr.P.H.

Committee member: Adam C. Lane, Ph.D.

Committee member: Maureen O'Brien, M.D., M.S.

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Novel Urinary Biomarkers of Acute Kidney Injury to Detect Toxicity and Predict Clearance in Pediatric Oncology Patients Treated with High Dose Methotrexate

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by

Andrew Bukowinski

M. D., University at Buffalo School of Medicine and Biomedical Sciences, June 2009

B.S., Western New England College, May 2001

Committee Chair: Erin Haynes, DrPH

Abstract:

High-dose methotrexate (HD-MTX) is a critical component of therapy for pediatric malignancies. The ability to identify patients at risk for delayed MTX clearance and acute kidney injury (AKI) is limited. Kidney Injury molecule-1 (KIM-1) is a urinary biomarker which is an early indicator of tubular injury. The current study evaluates associations between urinary KIM-1, delayed MTX clearance, and MTX exposure. **Methods:** 47 patients (31 male, 16 female; median age 9.5 years, range 3-31) received 1-12 g/m^2 HD-MTX over 4 or 24 hours for a total of 96 courses of MTX. Diagnoses included: leukemia/lymphoma (42 pts), osteosarcoma (5 pts). Data was collected on up to 4 courses of HD-MTX. Serum creatinine (SCr) and MTX were measured per standard clinical practice. Urine samples were obtained prior to the infusion and at 12, 24, and 36-48 hours after start. KIM-1 was measured by enzyme linked immunosorbent assay and normalized to urine creatinine. Delayed methotrexate clearance (DC) was defined by plasma MTX levels exceeding "high risk" concentrations in standard oncology treatment regimens, failure to clear MTX within 72 hours, or a 50% rise in SCr. Toxicity data was collected by chart review. Univariate analyses were conducted using Wilcoxon Rank sum testing. MTX area under the curve (AUC) was calculated with MW/Pharm software using Bayesian estimation. **Results:** Patients with DC had higher AUC levels ($p < 0.05$). Kim-1 at 12 hours correlated with AUC ($p < 0.04$) in leukemia patients receiving 1st course of HD-MTX. KIM-1 is associated with DC at 12 and 24 hours for 1st and 2nd courses of HD-MTX ($p < 0.05$). Prior to infusion KIM-1 is associated with previous DC ($p < 0.05$). **Conclusions:** Increased KIM-1 is associated with both MTX AUC and DC and is indicative of renal tubular injury. This association is most pronounced in course 1 and may be due to attrition of patients with severe toxicity, or dose reduction and enhanced supportive care in subsequent courses. KIM-1 provides an early indication of kidney injury from HD-MTX and with additional evaluation may be a target for enhanced supportive care strategies.

Acknowledgments:

All or part of this study will be presented at the American Society of Clinical Oncology in May, 2015.

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Background:

Methotrexate is a widely used chemotherapeutic agent which when given in high doses has improved outcomes of pediatric oncology patients with leukemia, lymphoma, and osteosarcoma. [1-4] High-dose methotrexate, doses between 1-12 grams/m2, is delivered intravenously over a prolonged infusion period of 4-24 hours depending on the regimen in order to achieve adequate intracellular concentrations of drug which is polyglutamated and retained.[5, 6] These polyglutamated metabolites then block nucleotide synthesis primarily by inhibition of dihydrofolate reductase.[5, 6] The toxic effects of methotrexate on both malignant cells and normal tissues depend on concentration and time of exposure, with renal, hepatic, and CNS injury occurring with exposure to high doses and gastrointestinal and bone marrow toxicity with prolonged exposure to low concentrations.[5, 6]

Methotrexate is predominantly (~90%) cleared by the kidneys and is poorly soluble at acidic pH; optimal solubility is achieved at pH 7-8.[6] Methotrexate renal dysfunction is mediated by precipitation of methotrexate and its metabolites in the renal tubules or by a direct toxic effect.[5] As a result, standard management of HD-MTX infusion requires aggressive hydration and alkalization with close monitoring of urinary specific gravity and pH. Serum methotrexate levels are monitored per oncology treatment protocol at periodic intervals until serum methotrexate levels have decreased to a safe level (typically less than 0.1 micromolar). Patients also receive folate rescue with leucovorin at specified time points after completion of the infusion to mitigate toxic effects on normal tissues.[7, 8] Despite appropriate fluid and pH management, some patients sustain renal injury which results in elevated plasma methotrexate levels and severe toxicity or even death in extreme cases. Most patients with renal dysfunction are asymptomatic and do not have oliguria, so their acute kidney injury (AKI) goes undetected until the completion of the infusion and the discovery of rising creatinine or elevated methotrexate levels.[9] Once elevated MTX levels are noted, leucovorin rescue is

pharmacokinetically adjusted and intravenous (IV) fluid rate increased to mitigate toxicity per established nomograms. [10] With modern fluid and leucovorin management, 1.8% of osteosarcoma patients developed grade 2 or greater (WHO criteria) nephrotoxicity, although this number does not capture those patients who sustain recurrent subclinical AKI with courses of methotrexate.[11] Large cohort studies have shown that rates of hypertension in survivors of a pediatric cancer diagnosis may be as high as 15% which may be in part due to chronic kidney disease.[12-14] While methotrexate has not been implicated as a medication which has been associated with long term morbidity from a renal standpoint, this is possibly due to the imperfect measurement of kidney dysfunction. [3, 13-17] Studies utilizing more sensitive methods for detection of renal injury (such as radioisotope testing) have noted that methotrexate may be responsible for more renal damage than previously described. [16, 18]

Serum creatinine is the most commonly employed biomarker to detect acute kidney injury, but is an imperfect measure since it is affected by gender, ethnicity, muscular composition, and nutritional status. [19, 20] Furthermore, approximately half of the kidney nephron mass needs to be injured in order for an elevation to occur in serum creatinine and even at this point it may take days for the serum concentration of creatinine to reach steady state therefore it does not offer the opportunity for an early intervention. [19, 20] Research in the field of nephrology is moving towards the establishment of other biomarkers which are more sensitive and specific for the diagnosis of AKI. Two of the biomarkers which have shown promise are Neutrophil Gelatinase Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (KIM-1). NGAL is a 25 kDA protein which is markedly induced in sites of epithelial injury, including the kidney. [20] KIM-1 is expressed in proliferating and regenerating proximal tubules, and serves as a mediator of T cell recruitment to the affected area. [21] Both NGAL and KIM-1 levels are not affected by the same variables that alter creatinine and have shown promise in the early detection of AKI in the setting of nephrotoxic (kidney injuring) medications,

cardiopulmonary bypass, and severe infection requiring ICU intervention (sepsis). [12, 20-27] KIM-1 has also been studied as a predictor of nephrotoxicity from another nephrotoxic chemotherapy agent, cisplatin. [27] Given that the urinary proteins NGAL and KIM-1 can be used to predict renal injury in a variety of settings and that methotrexate is a nephrotoxic chemotherapeutic agent, we hypothesize that NGAL and KIM-1 levels will correlate with AKI from MTX and delayed methotrexate clearance.

Method:

Our hypothesis was evaluated using an IRB-approved, prospective cohort study of patients receiving High-Dose Methotrexate (HD-MTX) at Cincinnati Children's Hospital Medical Center (CCHMC). HD-MTX is defined as a dose between 1g/m2-12g/m2 and is administered in an inpatient hospitalization. Patients were identified as eligible to participate in the study based upon hospital schedules for planned methotrexate admissions which was generated on a monthly basis. Patients were approached for enrollment prior to HD-MTX administration. Since a majority of the patients in this study were below the age of consent parents/legal guardians consented to participate in this study. Patients were determined meet eligibility for this study if they were receiving a dose of HD-MTX between 1g/m2-12g/m2 as part of a Children's Oncology Group (COG) treatment protocol, received their treatment at CCHMC, and were able to understand the English consent document. Patients receiving HD-MTX as parts of their oncology treatment protocol have monitoring of serum methotrexate levels as well as serum creatinine levels at various time points. (Please refer to table 1, 2 for additional information) The first 18 patients enrolled on study served as a pilot group and urine samples for biomarker analysis were collected in the following fashion: Patients with leukemia had urine samples collected prior to infusion, at 12 and 24 hours after the infusion start, and every morning thereafter. Patients with lymphoma or osteosarcoma had urine samples collected prior to infusion, at , 12, and 24 hour after the infusion start, and every morning thereafter. Based upon

the results of the pilot the IRB was amended to allow for the collection of multiple courses of HD-MTX. Patients not receiving steroids as a part of their anti-emetic regimen (leukemia only) had serum cystatin-C obtained prior to first infusion and on day 3 of each high dose methotrexate infusion. Urine samples for all patients were collected as mentioned above. Each patient could be collected on a total of 4 courses of HD-MTX. Urine samples were processed within 72 hours of collection and frozen and stored in Cincinnati Children's Center for Acute Care Nephrology (CACN) Core Laboratory and thawed and analyzed over a three day time period. NGAL levels and KIM-1 levels were measured by commercially available Enzyme Linked Immunosorbent Assays (ELISA) kits. Urine creatinine was measured for each of the urine samples. NGAL and KIM-1 levels were normalized to urine creatinine levels prior to statistical evaluation. NGAL/Cr levels were expressed as nanograms/milliliter/milligram/deciliter, and KIM-1/Cr levels were expressed as pictograms/milliliter/milligram/deciliter.

Table 1: Experimental Design for Leukemia patients

Prior to Infusion	Urine Biomarker 24 hours		Urine Biomarkers daily until cleared (<0.1microM)			
Urine Biomarker Level	Urine Biomarker at 12 hours			Serum Cystatin-C		
Serum Cystatin C (prior to course one only)						
	Methotrexate Infusion	Day 1	Day 2	Day 3	Day 4	Day…
24 hour Methotrexate Administration High Risk Leukemia: Dose of 5g/m2 **Children's Oncology Group Protocols: AALL0232, AALL0232, AALL0331 AALL0434, AALL0622 AALL1132** Average Risk Leukemia: Dose of 1g/m2 **Children's Oncology Group Protocol: AALL0932**		Methotrexate Serum Level at 24 hours per oncology Protocol* Serum Creatinine at 24 hours* *if 24 hour levels are elevated draw 36 hour levels	Methotrexate Serum Level at 42 and 48 hours per oncology Protocol Serum Creatinine at 48 and 48 hours	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol

Table 2: Experimental Design for Lymphoma/Osteosarcoma patients

Prior to Infusion	Urine Biomarker 24 hours		Urine Biomarkers daily until cleared (<0.1microM)			
Urine Biomarker Level	Urine Biomarker at 12 hours				Serum Cystatin-C		
Serum Cystatin C (prior to course one only)	Urine Biomarker 4 hours into infusion						
	Methotrexate Infusion		Day 1	Day 2	Day 3	Day 4	Day…
3-4 hour Methotrexate Administration Lymphoma: Dose 3g/m2 (3 hours)or 8g/m2 (4 hours) **Children's Oncology Group Protocol: ANHL12P1, ANHL1131** Osteosarcoma: Dose of 12g/m2 (4 hours) **Children's Oncology Group Protocol: AOST0331**			Methotrexate Serum Level at 24 hours per oncology Protocol Serum Creatinine at 24 hours *if 24 hour levels are elevated draw 36 hour levels	Methotrexate Serum Level at 42 and 48 hours per oncology Protocol Serum Creatinine at 42 and 48 hours	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol	Methotrexate Serum Level daily until <0.1mM until cleared per oncology protocol

Patient clinical data was collected by chart review from CCHMC electronic health record (EPIC) including, age, gender, diagnosis, MTX dosing, and variables which could affect MTX clearance including urine pH, intravenous hydration rate, administration of diuretics, administration of bicarbonate boluses, presence of fever, and presences of known medications which has been associated with delayed methotrexate clearance. The following clinical endpoints were evaluated:

- Development of AKI as defined by the KDIGO criteria (50% increase in serum creatinine) [19]
- Delayed Methotrexate Clearance defined as a patient failing to clear methotrexate prior to 72 hours (i.e. failure to obtain a serum methotrexate level <0.1 micromolar) or a serum MTX level >150 micromolar at 24 hours, >3 micromolar at 36 hours, or >1 micromolar at 42 hours.

Pharmacokinetic data including MTX serum levels, creatinine levels, and body surface area was collected from EPIC and analyzed using MWPharm (ver. 3.82, Mediware, The Netherlands) software to generate Area Under the Curve (AUC) for approximation for methotrexate exposure and clearance. Study data were collected and managed using REDCap electronic data capture tools hosted at CCHMC. [28] REDCap (Research Electronic Data Capture) is a secure, web-

based application designed to support data capture for research studies, providing 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

Urine NGAL and KIM-1 were not normally distributed so the data was analyzed using non parametric testing to evaluate the differences in levels between the two groups (Wilcoxon Rank Sum). A two tailed p value of <0.05 was considered statistically significant. Toxicity information based on demographics were analyzed using t test or Fischer exact test as appropriate. A two tailed p value of <0.05 was considered statistically significant. To determine sensitivity and specificity the optimal cut point was determined using Youden's statistic to predict toxicity where the most significant difference was seen in the analysis. (12 hours into the infusion for leukemia patients) All statistical analyses were completed using SAS statistical software (version 9.4)

Results:

Demographic Results:

Forty-seven patients enrolled to participate in the study between 2009-2014 (31 males and 16 females). The median age of the cohort was 9.5 years of age with the age range between 3 and 31 years of age. Forty-two patients were being treated for leukemia or lymphoma on the study. 5 patients were being treated for osteosarcoma. To analyze the predictive value of KIM-1 and NGAL patients were grouped according to methotrexate course. 37 patients were captured as receiving first course of HD-MTX, 27 of which had leukemia. A total of 25 patients were captured for course 2, 17 of which had leukemia. 19 patients were receiving course number 3 of HD-MTX, 14 of which were leukemia patients. 19 patients received course number 4 of HD-MTX for this study, 15 of which were leukemia patients. A total of 10 patients were captured for all four courses of HD-MTX, all of which were leukemia patients. A total of 96 courses of

methotrexate were captured for analyses of urinary biomarkers. Table 4 illustrates the finding that there is no difference between the delayed clearance group and those who cleared the drug normally other than a trend toward greater drug exposure.

Table 4: Demographic and Toxicity Information

Course 1	Delayed Clearance Group	Unaffected Group	p value
Diagnosis			
Leukemia	10 (83%)	17 (70%)	0.83
Lymphoma	1 (8.3%)	5 (20%)	0.83
Osteosarcoma	1 (8.3%)	2 (8%)	0.83
Gender and Age			
Number of Males	8	17	1
Number of Females	4	7	1
Mean Age	11.75	9.88	0.36
Prior Kidney Injury			
prior AKI	4 (36%)	7 (31%)	1
Supportive Care Measures			
Fever	2	1	0.17
Bicarbonate Bolus >1	1	3	0.82
Diuretic >1	6	4	0.06
Change in Leucovorin	1	0	0.33
MTX Clearance Hour (MEAN)	92	66.7	0.007*
Total Area Under the Curve (MEAN)	3028	1767.3	0.06
Course 2	Delayed Clearance Group	Unaffected Group	p value
Diagnosis			
Leukemia	6	12	0.11
Lymphoma	4	1	0.11
Osteosarcoma	0	2	0.11
Gender and Age			
Number of Males	8	10	0.65
Number of Females	2	5	0.65
Mean Age	9.9	11	0.55
Prior Kidney Injury			
Prior AKI	4 (40%)	2 (13.3%)	0.17
Prior Delayed MTX Clearance	4	2	0.17
Supportive Care Measures			
Dose Reduction	0	1	1
Fever	1	0	0.37
Bicarbonate Bolus >1	2	1	0.38
Diuretic >1	4	4	0.29
Change in Leucovorin	2	0	0.16
MTX Clearance Hour (MEAN)	98.2	63	0.005*
Course 3	Delayed Clearance Group	Unaffected Group	p value
Diagnosis			
Leukemia	3	10	1
Lymphoma	0	2	1
Osteosaroma	0	3	1
Gender and Age			
Number of Males	1	12	0.17
Number of Females	2	3	0.17
Mean Age	7.3	11.3	0.31
Prior Kidney Injury			
Prior AKI	2 (66%)	3 (23%)	0.21
Prior Delayed MTX Clearance	3	3	0.04*
Supportive Care Measures			
Dose Reduction	0	1	1
Fever	3	1	1
Bicarbonate Bolus >1	0	3	0.08
Diruretic >1	0	7	0.39
Change in Leucovorin	0	1	0.33
MTX Clearance Hour (MEAN)	80.6	76.7	0.77
Total Area Under Curve (MEAN)	2496.1	1583.3	0.03*

Course 4	Delayed Clearance Group	Unaffected Group	p value
Diagnosis			
Leukemia	3	11	1
Lymphoma	0	0	1
Osteosarcoma	1	3	1
Gender and Age			
Number of Males	2	9	1
Number of Females	2	5	1
Mean Age	9.75	11.1	0.7
Prior Kidney Injury			
Prior AKI	2 (50%)	3 (25%)	0.55
Prior Delayed MTX Clearance	4	3	0.04*
Supportive Care Measures			
Dose Reduction	0	1	1
Fever	0	1	1
Bicarbonate Bolus >1	1	3	0.9
Diuretic >1	2	6	0.48
Change in Leucovorin	0	1	0.33
MTX Clearance Hour (MEAN)	126.8	64.78	0.008*
Total Area Under Curve (MEAN)	1776.3	1812.7	0.92

Analysis of NGAL

Urine samples were collected at set intervals and frozen for processing at a later date, as outlined in the methods section. The data was analyzed first for the cohort of patients for each

Figure 1: Corrected NGAL levels for Cohort Courses 1-4

MTX Course	Prior To Infusion NGAL Level (median)		p value
	Delayed Clearance	Unaffected	
1	0.024	0.032	0.92
2	0.007	0.013	0.78
3	0.004	0.013	0.9
4	0.001	0.024	0.01*
	12 hrs into Infusion NGAL Level (median)		
	Delayed Clearance	Unaffected	
1	0.068	0.016	0.23
2	0.006	0.015	0.46
3	0.004	0.013	0.2
4	0.005	0.02	0.54
	24 hrs into Infusion NGAL Level (median)		
	Delayed Clearance	Unaffected	
1	0.104	0.012	0.06
2	0.02	0.009	1
3	0.006	0.0625	0.14
4	0.003	0.025	0.26
	36-48 hrs into Infusion NGAL level (median)		
	Delayed Clearance	Unaffected	
1	0.008	0.007	0.1
2	0.01	0.005	0.26
3	0.003	0.038	0.11
4	0.006	0.028	0.32

treatment course and then analyzed after stratification by diagnosis given differences in MTX dose and administration protocol for different diagnoses. Figure 1 illustrates the NGAL levels in patients who had delayed clearance of HD-MTX and those patients that did not experience this toxicity. At baseline, prior to infusion urinary NGAL level, was not different in patients experiencing AKI from any cause (p=0.42). A difference in NGAL level was not seen between patients with a history of delayed methotrexate (patients receiving greater than their first course of HD-MTX) (p=0.13). Only one statistical difference was seen with prior to infusion NGAL levels being lower in the group with delayed clearance in course 4. Furthermore when analyzed by diagnosis there were no differences appreciated in patients with leukemia with delayed clearance from HD-MTX. (data not shown)

Analysis of KIM-1

Urine samples were collected at set intervals and frozen for processing at a later date, as outlined in the methods section. The data was analyzed first for the cohort of patients for each treatment course and then stratified by diagnosis. Figure 2 illustrates the KIM-1 levels in patients who had delayed clearance of HD-MTX and those patients that did not experience this toxicity. KIM-1 levels at baseline were not elevated in patients with a prior history of AKI.

Figure 2: Corrected KIM-1 levels for Cohort Courses 1-4

MTX Course	Prior To Infusion KIM-1 Level (median)		p value
	Delayed Clearance	Unaffected	
1	8.05	3.88	0.1
2	8.06	4.04	0.27
3	7.71	4.06	0.11
4	5.3	4.97	0.71
	12 hrs into Infusion KIM-1 Level (median)		
	Delayed Clearance	Unaffected	
1	11.09	5.09	0.054
2	10.95	2.67	0.03*
3	12.47	6.78	0.41
4	3.94	4.97	0.61
	24 hrs into Infusion KIM-1 Level (median)		
	Delayed Clearance	Unaffected	
1	16.79	5.686	0.04*
2	13.34	4.28	0.32
3	14.03	5.86	0.34
4	11.49	2.76	0.12
	36-48 hrs into Infusion KIM-1 level (median)		
	Delayed Clearance	Unaffected	
1	9.54	6.47	0.02*
2	13.33	6.03	0.09
3	17.54	7.81	0.19
4	11.12	5.61	0.43

(p=0.41) However, there was a difference seen between patients experiencing delayed clearance from HD-MTX. During course 1 there was difference in the 12 hour urinary KIM-1 level which approached significance between the delayed clearance group and the unaffected group (p=0.054). Twenty four hours into first course of HD-MTX there was a significant difference between the two groups ($p<0.05$) which is illustrated in Figure 2. This effect persisted until 36-48 hours into the infusion.

A significant difference was also seen at 12 hours into the infusion for course 2 and persisted until 24 hours post infusion ($p<0.05$) but the effect was not appreciated at 36-48 hours into the infusion. Given these findings the results were analyzed for the leukemia patients only as they comprise largest oncology diagnosis in the cohort. Figure 3 illustrates the differences in KIM-1 levels for leukemia patients with and without delayed clearance. It should be noted that leukemia patients that developed delayed clearance had a higher urinary KIM-1 level prior to MTX infusion compared to the group that did not experience delayed clearance but this effect did not reach statistical significance. ($p=0.06$) Subsequently patients had a higher 12 and 24 hours into infusion urinary KIM-1 level when the patient experienced delayed clearance of HD-MTX ($p<0.05$). The change in KIM-1 level over course 1 of HD-MTX for patients with leukemia is shown graphically in figure 4.

Figure 3: Corrected KIM-1 levels for Leukemia Patients Courses 1-4

MTX Course	Prior To Infusion KIM-1 Level (median)		p value
	Delayed Clearance	Unaffected	
1	12.19	3.19	0.06
2	4.89	4.03	1
3	7.72	4.7	0.2
4	5.15	5.88	0.87
	12 hrs into Infusion KIM-1 Level (median)		
	Delayed Clearance	Unaffected	
1	11.09	3.46	0.02*
2	7.07	2.66	0.3
3	12.47	5.8	0.32
4	6.29	1.46	0.43
	24 hrs into Infusion KIM-1 Level (median)		
	Delayed Clearance	Unaffected	
1	16.79	4.52	0.02*
2	3.24	4.08	0.82
3	14.02	4.75	0.35
4	14.07	3.23	0.2
	36-48 hrs into Infusion KIM-1 level (median)		
	Delayed Clearance	Unaffected	
1	9.32	5.23	0.1
2	10.9	4.74	0.36
3	17.54	4.48	0.11
4	16.21	5.67	0.53

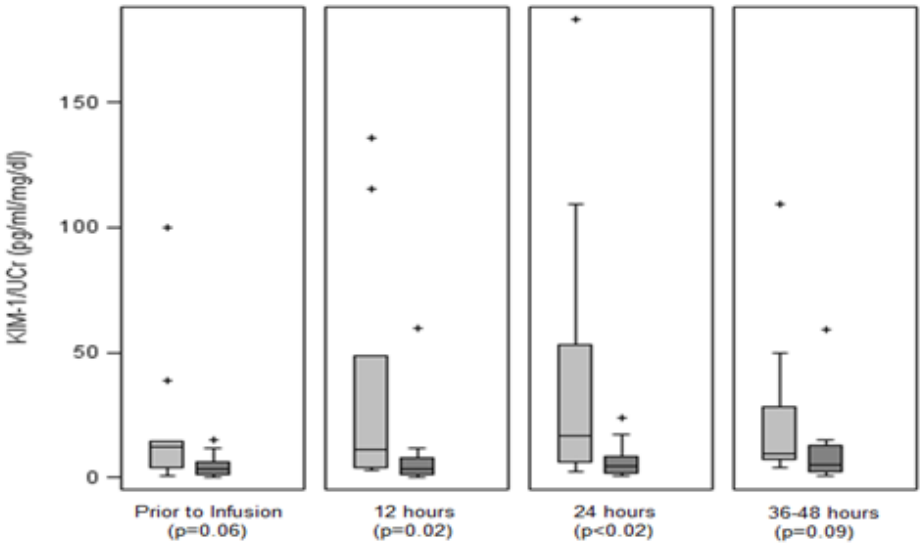

Figure 4: Graph Representing Leukemia Group (28 patients) receiving HD-MTX. Delayed Clearance Group is shown with light grey bar; unaffected group is represented as dark grey bar. Line in bar plot represents the median for the group. It should be noted that outliers were consistently elevated throughout HD-MTX course. (Outliers prior to infusion were outliers at 12 hours, 24 hours, and 36 hours)

Given the strong association of elevated urinary KIM-1 level at 12 hours into the infusion for leukemia patients a receiver operator curve was developed to determine the sensitivity and specificity for KIM-1 at 12 hours to predict which patients are at risk for delayed clearance.

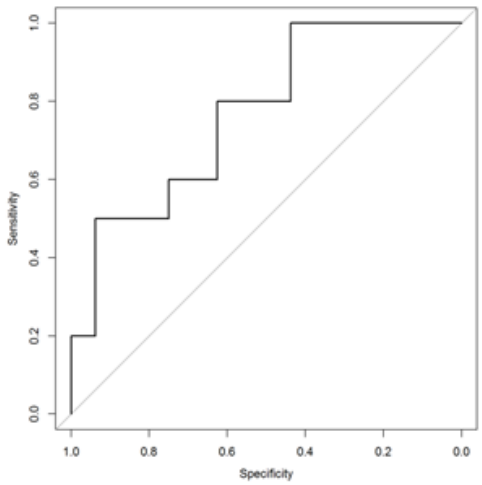

Figure 5: ROC curve for Corrected Urinary KIM-1 marker at 12 hours into infusion for leukemia patients. A KIM-1 level of 12 pg/ml/mg/dl had a sensitivity of 50% and a specificity of 93.75% to predict delayed clearance from HD-MTX

(Figure 5) At 12 hours a KIM-1 level corrected for urine creatinine greater than 12 pg/ml/mg/dl had a sensitivity of 50% and a specificity of 93.75% to predict delayed clearance from HD-MTX infusion.

Discussion:

This is the first study which attempted to use urinary biomarkers of Acute Kidney Injury with administration of HD-MTX. The biomarker NGAL was not associated with delayed clearance in this cohort and this is not entirely unexpected. NGAL appears to be a more robust predictor of AKI in patients who have nephrotoxic injury due to hypoxia. [12, 20, 22-25] KIM-1 rises secondary to tubular injury which is the proposed mechanism of kidney injury from HD-MTX [5-8, 11, 21, 26, 27, 29, 30] In our cohort KIM-1 was associated with delayed clearance from high dose methotrexate in courses 1 and 2 from 12 hours into the infusion and continuing through the end of infusion at 24 hours. The effect was strongest in the leukemia cohort. It is important to note that the leukemia patients who had delayed MTX clearance had higher pre infusion KIM-1 levels. (See Figure 3) This is likely reflective of previous acute kidney injury from a variety of other causes. Approximately one third of patients in the cohort had experienced AKI prior to their first course of HD-MTX likely as a consequence of treatment related complications including other nephrotoxic chemotherapies, nephrotoxic supportive care medications (antibiotics and antifungal agents), and contrast nephropathy. In patients that were not methotrexate naïve (greater than course 1 of HD-MTX) pre infusion KIM-1 was associated with previous course of delayed methotrexate clearance. ($p<0.05$). Given the frequency at which HD-MTX infusions are given (every 2 weeks for leukemia patients, weekly for osteosarcoma patients) it is reasonable to conclude that any kidney injury which has recently occurred is likely secondary to HD-MTX. Although the effect was not statistically significant there was a trend towards an increase in prior to infusion KIM-1 levels in the 10 patients were all 4 courses of HD-MTX were captured.

The effect seen with KIM-1 elevation decreases with subsequent courses and the reason why this occurs is not completely clear. Part of this may be related to attrition rates with patients with severe toxicity. It is unlikely to be the only explanation since many of the course 2

patients were not captured and new patients were added during this course thereby increasing variability. Of the patients who received first course of HD-MTX only 2 had chemotherapy course changed due to severe toxicity and did not receive further courses. Thirteen patients received course 1 of therapy but course 2 was not collected because patient methotrexate courses were collected as a part of the pilot study, or that courses were unable to be captured due to extenuating circumstances. It is also possible that a majority of kidney injury from HD-MTX occurs in the early courses of HD-MTX and it is therefore more difficult to detect a difference in patients who have delayed clearance and those who do not. This is supported by a higher prior to infusion KIM-1 level in the patients that had data captured for all 4 courses of HD-MTX but this did not reach statistical significance. Host factors may also affect HD-MTX methotrexate clearance as the drug is polyglutamated in the tissues which is not reflected in serum concentrations. This is illustrated by the fact that patients with delayed MTX clearance do not always have a larger AUC than those that do not experience this toxicity. (Table 4). A number of genetic polymorphisms have also been identified and it is possible that patients experiencing delayed methotrexate clearance may have one of these common genetic mutations which affect methotrexate clearance.[31-33]

The study is not without the following limitations. The small sample size and multiple courses per patient can bias our data. It is possible that we have a cohort of patients that tolerate methotrexate poorly, or that patients who do tolerate HD-MTX infusions well are biasing our results since they have received more courses without issues. Our findings of delayed clearance are compatible with national norms, so it is unlikely that we have a population of patients that are more likely to experience toxicity. [7, 8, 11, 29, 32] A variety of diagnoses are represented here but definitive conclusions about osteosarcoma and lymphoma patients are not able to be made from this data set given their small numbers in our cohort. Another shortfall of

this study is that we were unable to capture 4 courses of HD-MTX on all patients making it difficult to make definitive conclusions about changes which happen over the course of therapy.

Conclusions

KIM-1 shows an acute rise in the early courses of patients receiving HD-MTX and may serve as a harbinger of delayed clearance and acute kidney injury at 12 hours into the MTX infusion. In contrast, for leukemia patients, serum methotrexate and creatinine levels are currently obtained at the end of a high dose methotrexate infusion at 24 hour with current end of infusion MTX level greater than 150 micromolar has a sensitivity of 0.27 and specificity of 0.94 to predict delayed clearance of HD-MTX and serum creatinine has a sensitivity of 0.32 and 0.97 to predict delayed clearance of HD-MTX. [30] The elevation in urinary KIM-1 shows an early elevation twelve hours into infusion with a higher sensitivity than the current standard of care and may allow for additional therapeutic intervention prior to twenty four hours. For the patients with leukemia only half of the dose has finished at this time, therefore additional supportive care measures may be utilized (such as increased hydration) to decrease the risk of nephrotoxicity from HD-MTX. Future studies focusing on the change in urinary KIM-1 at 12 hours into infusion and interventions in supportive care measures will help to verify the current findings and determine if these changes can enhance excretion of high dose methotrexate.

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