

# Highlighting Levels of Indoxyl Sulphate among Critically Ill Patients with Acute Nephrotoxicity; Correlations Between Indoxyl Sulphate Levels and Patients' Characteristics

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## Abstract

**Background:** Many animal studies suggested that the uremic toxin indoxyl sulphate can add to renal damage following induced nephrotoxicity and this effect has not been proved in patients with such complication.

**Methods:** This is a prospective, case-control, and an observational study conducted on 74 critically ill patients with acute nephrotoxicity. It was designed to measure serum levels of indoxyl sulphate on the day of enrollment and over the course of their illness using high performance liquid chromatography (HPLC-UV) and to test the correlation between these levels and patient's demographics, clinical characteristics, physiological variables, and their outcomes.

**Results:** Critically ill patients with acute nephrotoxicity had significantly higher total (tIS) and free (fIS) indoxyl sulphate than healthy controls and significantly lower than patients with end-stage renal disease (ESRD). Although, no correlation was found between tIS or fIS and mortality, among survivors, tIS, fIS, creatinine and eGFR were independently associated with no renal recovery.

**Conclusions:** Serum indoxyl sulphate levels were elevated in critically ill patients with acute nephrotoxicity. There is an association between high levels of indoxyl sulphate and no renal-recovery outcome among survivors of acute nephrotoxicity. Early removal of indoxyl sulphate from patients' blood may improve their outcomes.

**Keywords:** HPLC, Indoxyl sulphate, Mortality, Prognosis, Toxic acute kidney injury.

## Introduction

Acute kidney injury (AKI) is a common disorder in critical care medicine (CCM), characterized by an abrupt decrease in renal function with retention of body's waste products and electrolyte disturbance (1-2). Toxins affecting the kidney are called nephrotoxins; are responsible for about 20% of hospital acquired AKIs hence the name toxic AKI. Nephrotoxins may be exogenous or endogenous; drugs, radiocontrast materials. and

environmental pollutants are the most common nephrotoxins encountered (3,4). AKI mechanisms differ according to the type of nephrotoxin. Nephrotoxins cause renal parenchymal damage, acute tubular necrosis, acute interstitial nephritis, or renal arterioles vasoconstriction and pre-renal ischemia (2,4). After AKI, renal function can be completely or partially recovered, or may be chronically lost, according to severity and time of

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injury and generation capacity of the kidneys (5-7). AKI is a major health problem accompanied by high resources utilization, with a growing interest in providing tools for early diagnosis and prognosis (8) and for achieving such goals, kidney structure and function should be linked by the gold standard; renal biopsy which is impractical in acutely ill patients, so novel renal imaging techniques and panels of biomarkers might help (9-13). AKI can be detected in line with RIFLE, or AKIN definitions (9,13,14).

When kidney function decreases, serum levels of some endogenous solutes were found to be elevated (5,7). Those interacting with biological functions were called uremic toxins and were classified into small water soluble, middle, and protein-bound toxins (15,16). Indoxyl sulphate (IS) is a small protein-bound uremic toxin synthesized in liver from indole, a sulphate conjugate of tryptophan produced by action of intestinal flora on dietary-derived proteins. Circulating IS is 90% bound to plasma proteins with a strong albumin binding at Sudlow II site and it is cleared from blood through renal proximal tubular secretion (17). Normal concentrations reported for free indoxyl sulphate (*fIS*) was 0.0- 0.2  $\mu\text{mol/l}$  and for total indoxyl sulphate (*tIS*) was 0.7- 6.3  $\mu\text{mol/l}$  (18). High concentrations of IS were identified among elders, men and those with lower renal functions (19).

Survivors from AKI may suffer from adverse short-term renal outcomes, in the form of delayed renal recovery, dependence on renal replacement therapy, or progression to RIFLE stages L (Loss of kidney function) or E (End-stage renal disease). The increased levels of cytokines, uremic toxins and other circulating mediators may explain the adverse effects following AKI (7, 20-22).

In uremia, the role of the uremic toxin indoxyl sulphate (IS) was extensively studied and high levels were found to promote the progression to renal failure (23-25). Elevated IS levels were reported in animal models of AKI (26-27). Nevertheless, high IS levels were shown to add to tubular injury and delayed renal recovery through induction of tubulo-interstitial fibrosis and glomerular sclerosis, production of intra-renal free radicals, induction of oxidative stress in endothelial cells after injury (28, 29). Otherwise,

the role of such toxin in patients with AKI is not yet understood.

The current study aimed at identifying serum levels and short-term predictive role of indoxyl sulphate in critically ill patients with toxic AKI.

## Materials and Methods

### *Study cohort*

This study was a prospective, case-control observational study conducted at Alexandria University hospital, Egypt, (March 2017 to January 2019) after approval of the Ethics Committee and obtaining written consents.

### *Study protocol*

#### *Selection of the studied population*

This study was conducted on seventy-four patients in the critical care medicine (CCM) department diagnosed as toxic AKI (group I). Patients were excluded from the study if; below 20 or above 60 years, pregnant, unable to give a written consent, with AKI due to other causes or combined causes, received renal replacement therapy (RRT) due to non-renal causes, or have a history of chronic kidney disease (CKD), liver disease or renal transplant.

Forty patients with end stage renal disease (ESRD), on maintenance hemodialysis for at least three months, were selected as controls (group II), in addition to forty healthy individuals as healthy control subjects (group III).

Controls were excluded if; not age matched (20-60 years), pregnant, had recent infection or recent intake of antibiotics or immunosuppressive agents.

#### *Definition and staging of AKI*

AKI was defined and staged according to abrupt and sustained (>24 hours) rise in serum creatinine level by 1.5-fold or more the baseline value, which is known in the last 6 months, and by a decrease in glomerular filtration rate (GFR) by 25% or more, as defined by RIFLE criteria (Table 1) (14).

Staging of patients was based on the rise in serum creatinine and decrease in GFR and not on the urine output (12). A threshold value of serum creatinine was set at 150  $\mu\text{mol/L}$  for men and 120  $\mu\text{mol/L}$  for women beyond which it was considered as raised.

**Table 1.** RIFLE criteria for classification and prognostication of AKI.

Stage	Serum Creatinine or Gfr	Urine Output
Risk (R)	Increase in serum creatinine 1.5 x of baseline or GFR decrease > 25%	< 0.5 ml/kg/h for > 6 consecutive hours
Injury (I)	Increase in serum creatinine 2 x of baseline or GFR decrease > 50%	< 0.5 ml/kg/h for > 12 consecutive hours
Failure (F)	Increase in serum creatinine 3 x of baseline or increase in serum creatinine to $\geq 354$ $\mu\text{mol/L}$ with an acute rise > 44 $\mu\text{mol/L}$ or GFR decreased > 75%	< 0.3 ml/kg/h for > 24 consecutive hours or anuria for 12 hours
Loss (L)	Persistent AKI= complete loss of kidney function > 4 weeks	
End-Stage Renal Disease (E)	ESRD > 3 months	

GFR, glomerular filtration rate.

### **Defining the patients' outcomes**

Primary outcome will be mortality and secondary outcome will be no-renal recovery (in the form of partial renal recovery or on maintenance RRT).

Complete renal recovery was defined by fall of serum creatinine below threshold.

Partial renal recovery was defined by serum creatinine above threshold but not on maintenance RRT.

Estimated GFR (eGFR) was calculated for each patient using Modification of Diet in Renal Disease 4-variable (MDRD) formula.

$$\text{eGFR (ml/min/1.73m}^2\text{)} = 186 \times (\text{Creatinine } (\mu\text{mol/l}) / 88.4)^{-1.154} \times (\text{age})^{-0.203} \times 0.742 \text{ [if female]} \times 1.212 \text{ [if black]}$$

### **Measurement of indoxyl sulphate (29-31)**

#### **Sample collection and storage**

Morning blood samples were withdrawn on serum separator tubes from group I patients within 48 hours after defining toxic AKI then followed by two serial measurements at week 1 and week 2 (or as ended earlier), from group II controls once just before dialysis, and from group III controls once at their routine laboratory visit, then serum was aliquoted and stored at  $-80^\circ\text{C}$  till analysis time.

2-naphthalenesulphonic acid (NSA) (0.2 mmol/L) was used as the internal standard, prepared in ultrapure water, and was stable for at least four weeks at  $4^\circ\text{C}$ .

### **Sample preparation**

For total indoxyl sulphate (tIS): serum was deproteinized by adding a double volume (1:2) of acetonitrile, vortex mixing for 1 minute, centrifuged at 14,049 G for 15 minutes, then supernatant was filtered by 0.22 mm filters to remove large particles, then 200  $\mu\text{l}$  of NSA was added to each 600  $\mu\text{l}$  of the prepared serum in a 2 ml auto-sampler tube vials.

For free indoxyl sulphate (fIS): ultrafiltrates were obtained from ultrafiltration of 500  $\mu\text{l}$  of serum using Amicon ultra 0.5 centrifugal filter devices, 30 kDa (Milipore sigma, Taunton, USA), and this was facilitated by centrifugation at 14,049 G for 10 minutes at room temperature, then serum was deproteinized as was done for tIS.

2-naphthalenesulphonic acid (NSA) was used as the internal standard and was added after deproteinization because it was only water soluble.

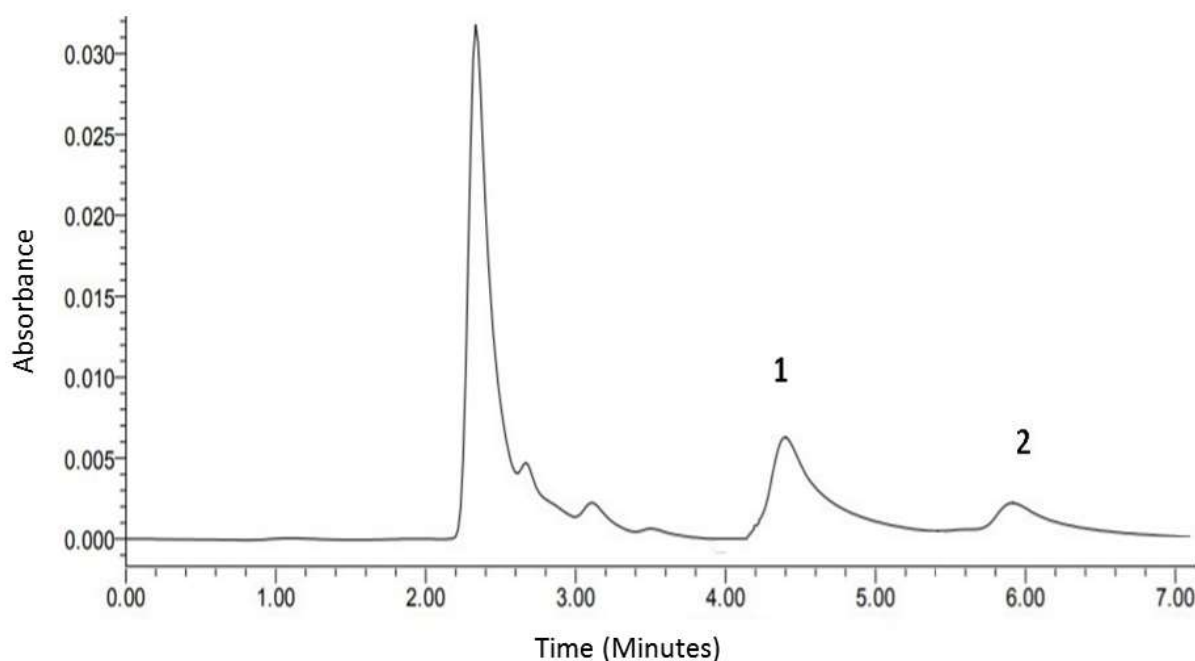
### **High performance liquid chromatography (HPLC)**

Chromatography was performed using a Waters separation module series 2690 with an autosampler, empower 3 software data station, and an UV/Visible Waters detector series 2489.

HPLC conditions were: Zorbax C18, 5  $\mu\text{m}$  (4.6 x 250 mm) column (Agilent instruments, Wilmington, DE, USA), Mobile phase: an isocratic elution system was used as; 80% buffer A (Formic acid adjusted to pH 4 with ammonium

hydroxide) and 20% acetonitrile. Run time was 8 minutes for *tIS* and 6 minutes for *fIS*. Injection volume was 10  $\mu$ l for both *tIS* and *fIS* with flow rate maintained at 1 mL/min. Column temperature was 35 °C and the UV absorbance was monitored at 280 nm. Indoxyl sulphate peak

was identified by superimposing the sulphate potassium salt in methanol. Peak heights of *tIS* were expressed as relative to the peak height of internal standard in order to obtain results independent of variations in sample size and detector sensitivity (Fig. 1).



**Fig. 1.** HPLC chromatogram of pooled normal serum spiked with 200  $\mu$ mol/l 3-indoxyl sulphate potassium salt in methanol buffer (peak 1) and internal standard (peak 2). Under these conditions, *tIS* was eluted at ~4.45 minutes, *fIS* at ~4.5 minutes and the internal standard at ~6 minutes. (N.B. These are mean elution times for total and free IS, as not all samples were analyzed in the same day).

### Statistical analysis

All tests of significance were two-sided, and differences were considered significant when  $p$  value < 0.05.

Data were statistically analyzed and graphed using Graph Pad Prism 7 for windows.

Serum levels of *tIS* and *fIS* were highly skewed and then non-parametric statistics were used or they were log-transformed before inference testing.

Linear regression model analysis generalized linear model analysis, multivariable analysis, mixed effect models and binary logistic regression of models were analyzed using R version 3.5.2 (2018-12-20) -- "Eggshell Igloo".

The trends of *tIS*, *fIS* and creatinine over time were estimated using mixed-effect model which was selected based on Akaike's information criterion.

### Results

#### Patients' characteristics

The demographics and clinical characteristics of patients with toxic AKI according to prognosis at hospital discharge are summarized in Table 2.

#### Defining outcomes

The patients' outcome at discharge and after 3 months follow up is outlined in Figure 2.

**Table 2.** Demographics and clinical characteristics of patients with toxic AKI according to prognosis at hospital discharge.

Demographics and clinical characteristics	Total N= 74	Survivors N= 57		p <sub>1</sub> value	Non-survivors N= 17	p <sub>2</sub> value
		Recovery N= 36	No-recovery N= 21			
<b>Age (years), median (range)</b>	59(20-60)	59(21-60)	60(22-60)	0.443 <sup>a</sup>	60(35-60)	0.863 <sup>a</sup>
<b>Male, n (%)</b>	44(59.5)	20(55.6)	15(71.4)	0.235 <sup>b</sup>	9(52.9)	0.532 <sup>b</sup>
<b>Smokers, n (%)</b>	17(22.9)	6(18.8)	5(23.8)	0.656 <sup>b</sup>	6(35.3)	0.128 <sup>b</sup>
<b>Alcohol drinkers, n (%)</b>	4(5.4)	1(3.1)	2(9.5)	0.324 <sup>b</sup>	1(5.8)	0.001 <sup>b</sup>
<b>Hospital stays (days), median (range)</b>	11(2-70)	14(3-70)	10(3-30)	0.111 <sup>a</sup>	12(2-30)	0.096 <sup>a</sup>
<b>RIFLE criteria, n (%)</b>						
RIFLE class R	15(20.3)	13(36.1)	1(4.8)	0.025 <sup>b</sup>	1(5.9)	0.020 <sup>b</sup>
RIFLE class I	28(37.8)	12(33.3)	12(57.2)		4(23.5)	
RIFLE class F	31(41.9)	11(30.6)	8(38)		12(70.6)	
<b>APACHE II score, mean (SD)</b>	17.8(7.8)	16.6(6.2)	16.6(5.8)	0.890 <sup>c</sup>	24(9.2)	< 0.001 <sup>c</sup>
<b>Renal replacement therapy (RRT), n (%)</b>	14(18.9)	2(6.2)	5(23.8)	0.064 <sup>b</sup>	7(41.3)	0.012 <sup>b</sup>
<b>Co-morbid conditions, n (%)</b>						
Diabetes mellitus	25(33.8)	11(30.6)	7(33.3)	0.937 <sup>b</sup>	8(47.1)	0.330 <sup>b</sup>
Cardiac disease	46(62.1)	24(66.7)	11(52.4)	0.398 <sup>b</sup>	12(70.6)	0.489 <sup>b</sup>
Pulmonary disease	17(23)	10(27.8)	3(14.3)	0.160 <sup>b</sup>	5(29.4)	0.685 <sup>b</sup>
Malignancy	8(10.8)	2(5.6)	4(19)	0.150 <sup>b</sup>	2(11.8)	0.960 <sup>b</sup>
<b>End organ failure, n (%)</b>						
Sepsis	5(7.1)	3(9.4)	0	–	3(17.6)	0.124 <sup>b</sup>
DIC	1(1.4)	0	0	–	1(5.9)	–
Thrombosis	2(2.8)	2(6.3)	0	–	0	–
<b>Mortality day, median (range)</b>					12(2-30)	
<b>CCM mortality, n (%)</b>					14(82.4)	
<b>30-day mortality, n (%)</b>					17(100)	

RIFLE criteria, (risk of renal dysfunction (R), injury to kidney (I), failure of kidney function (F), loss of kidney function (L), end-stage kidney disease (E); APACHE II score, Acute Physiology and Chronic Health Evaluation score; DIC, disseminated intravascular coagulation; CCM, critical care medicine; RIFLE class L, loss of kidney function> 4 weeks; RIFLE class E, End-stage renal disease (ESRD)>3 months; N; number of patients in each group.

**p<sub>1</sub> value:** between recovery and no-recovery groups. **p<sub>2</sub> value:** between survivors and non-survivors. <sup>a</sup> Denotes p value derived from Mann-Whitney, (significant< 0.05). <sup>b</sup> Denotes p value derived from Chi-square test, (significant< 0.05). <sup>c</sup> Denotes p value derived from unpaired t-test, (significant< 0.05).

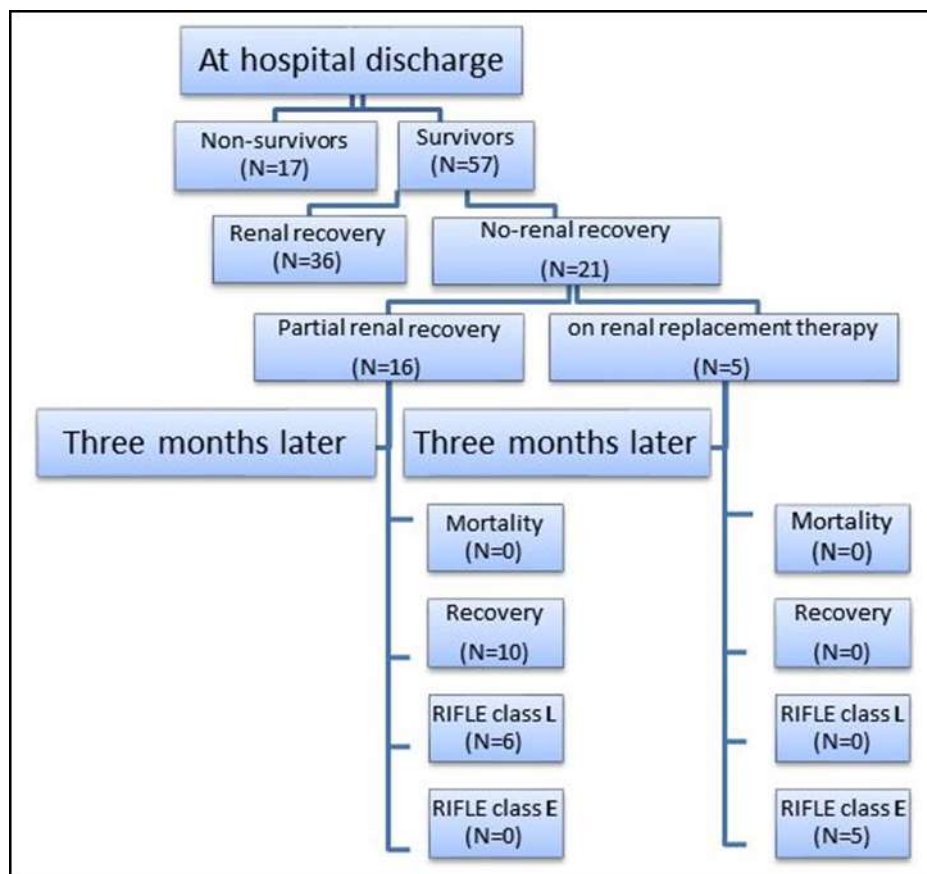


Fig. 2. Flow chart of outcomes at hospital discharge and three months of follow up.

### Basal serum indoxyl sulphate levels

The basal *tIS* and *fIS* levels measured in serum of critically ill patients with toxic AKI (group I) were significantly lower than those with ESRD group II)

( $p < 0.0001$ ) and significantly higher than those levels measured in serum of healthy controls (group III) ( $p < 0.0001$ ) (Fig. 3).

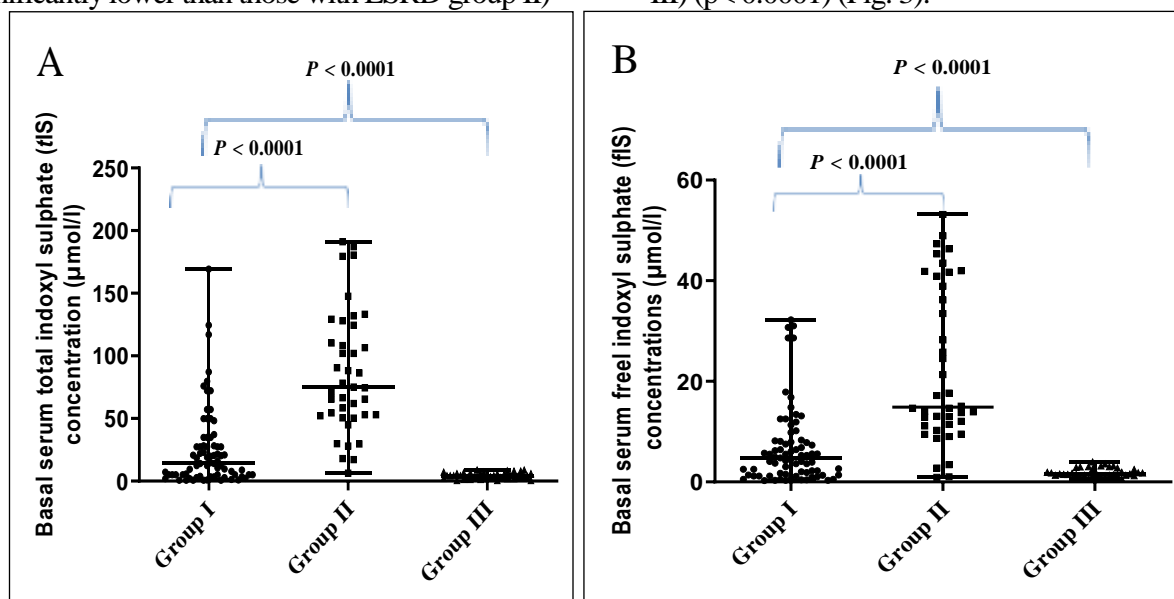


Fig. 3. Comparison of basal serum total indoxyl sulphate (*tIS*) concentrations (A) and basal serum free indoxyl sulphate (*fIS*) concentrations (B) among the three studied groups. Data were analyzed using Kruskal-Wallis test between the all groups:  $p$  value is significant at  $< 0.05$ , and Klotmogorov-Simimov test between groups (I versus II) and (I versus III):  $p$  value is significant at  $< 0.05$ .

As summarized in Table 3; among the studied patients with toxic AKI, basal serum levels of both *tIS* and *fIS* showed significant positive correlations with each other ( $p < 0.0001$ ), with

serum creatinine ( $p < 0.0001$  for both) and with BUN ( $p = 0.042$  for *tIS* and  $p = 0.028$  for *fIS*), and a significant negative correlation with eGFR ( $p < 0.0001$  for both).

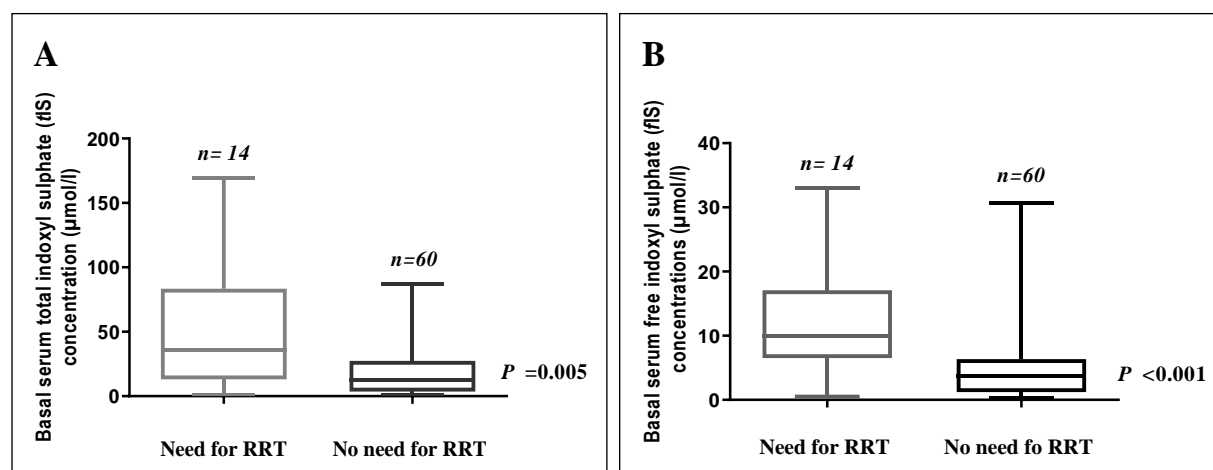
**Table 3.** Correlations between both of basal serum levels of *tIS* and *fIS* and patients' demographics and physiological variables among studied toxic AKI patients.

Demographics and clinical characteristics	<i>tIS</i> ( $\mu\text{mol/l}$ )	<i>fIS</i> ( $\mu\text{mol/l}$ )
Age (years)	$r = 0.044$ $p = 0.708$	$r = 0.037$ $p = 0.753$
APACHE II score	$r = 0.123$ $p = 0.293$	$r = 0.153$ $p = 0.191$
Length of hospital stay (days)	$r = 0.027$ $p = 0.819$	$r = -0.001$ $p = 0.991$
<b>Laboratory data:</b>		
Serum creatinine ( $\mu\text{mol/l}$ )	$r = 0.490$ $p < 0.0001^*$	$r = 0.503$ $p < 0.0001^*$
Blood urea nitrogen (mmol/l)	$r = 0.236$	$r = 0.254$
eGFR (ml/min/1.73m <sup>2</sup> )	$p = 0.042^*$ $r = -0.534$ $p < 0.0001^*$	$p = 0.028^*$ $r = -0.526$ $p < 0.0001^*$
<i>tIS</i> ( $\mu\text{mol/l}$ )	-----	$r = 0.920$ $p < 0.0001^*$

*tIS*, total indoxyl sulphate; *fIS*, free indoxyl sulphate; eGFR, estimated glomerular filtration rate. Data were presented as spearman correlation coefficient  $r$  above and  $p$  value below,  $p$  significant at  $< 0.05$ .

As depicted in Figure 4; among patients with toxic AKI, basal serum levels of both *tIS* ( $p = 0.005$ ) and

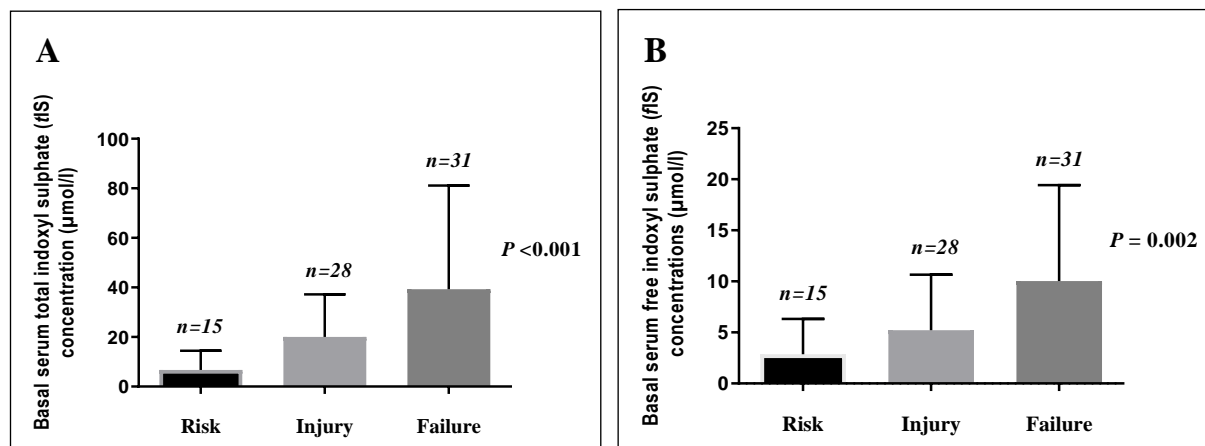
*fIS* ( $p < 0.001$ ) were significantly higher among patients who received RRT than among who did not.



**Fig. 4.** Basal serum total indoxyl sulphate (*tIS*) (A) and basal serum free indoxyl sulphate (*fIS*) (B) concentrations among patients with toxic AKI according to need for renal replacement therapy (RRT). Bars and error bars represent median and range for each group. Data were analyzed using Mann-Whitney test;  $p$  value is significant at  $< 0.05$ .

Basal serum levels of *tIS* and *fIS* showed statistically significant differences when compared among the

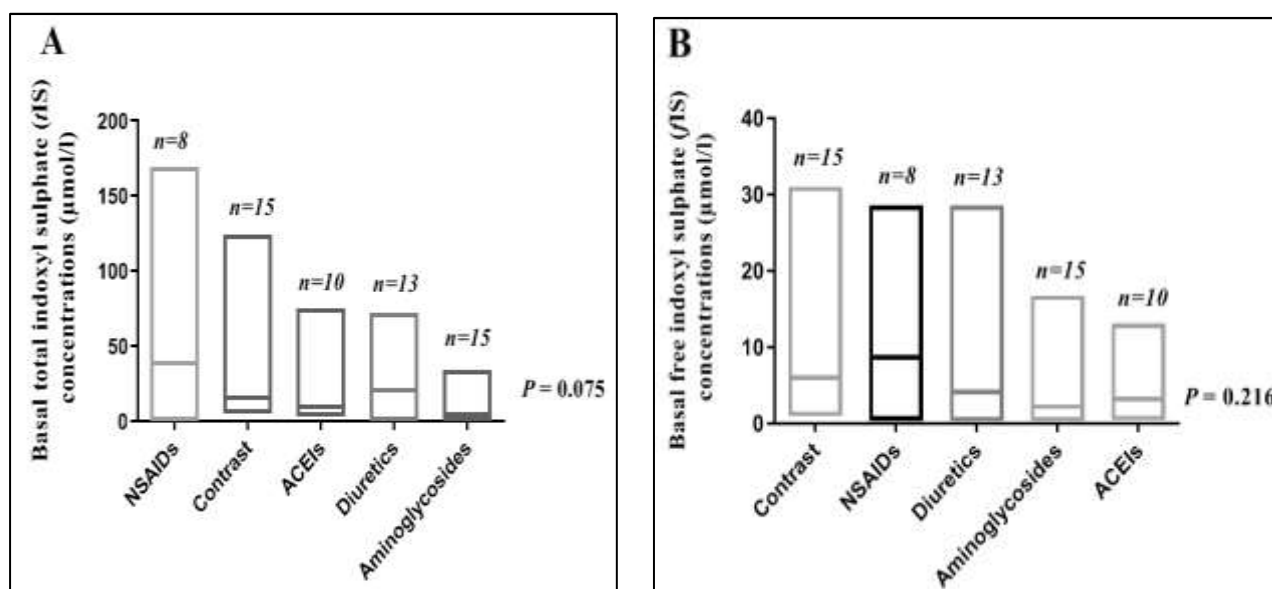
studied patients with toxic AKI according to the RIFLE classes ( $p < 0.001$  and  $p = 0.002$ , respectively) (Fig. 5).



**Fig. 5.** Basal serum total indoxyl sulphate (*tIS*) (A) and basal serum free indoxyl sulphate (*fIS*) concentrations (B) among patients with toxic AKI according to RIFLE classes. Bars and error bars represent median and range for each stage. Data were analyzed using Kruskal-Wallis test;  $P$  value is significant at  $< 0.05$ .

In this study, AKI was due to exogenous nephrotoxins in sixty-eight (91.9%) patients, while six patients (8.1%) had AKI due to endogenous nephrotoxins; light chains, myoglobin and high uric acid. Figure 6 shows the major causes of AKI

among the studied groups and shows that basal serum levels of *tIS* and *fIS* did not differ significantly when compared among the studied patients with toxic AKI according to the most frequent nephrotoxins.



**Fig. 6.** Basal serum total indoxyl sulphate (*tIS*) (A) and basal serum free indoxyl sulphate (*fIS*) (B) concentrations among patients with toxic AKI according to the nephrotoxin type. Bars represent range with line at median for each nephrotoxin. Data were analyzed using Kruskal-Wallis test;  $P$  value is significant at  $< 0.05$ .



**What can indoxyl sulphate levels predict, mortality (primary outcome) or no recovery (secondary outcome) among patients of toxic AKI?**

Patients' basal laboratory data according to prognosis at hospital discharge are summarized in Table 4, where basal serum levels of creatinine were significantly higher and eGFR were significantly lower among no-recovery than among recovery

groups ( $p=0.003$  and  $p=0.001$  respectively), and among non-survivors than among survivors ( $p=0.047$ ,  $p=0.001$  respectively). Moreover, basal serum levels of both *tIS* and *fIS* were significantly higher among no-recovery than among recovery groups ( $p<0.001$ ,  $p=0.005$  respectively), while there was no statistically significant difference in these levels among survivors and non-survivors.

**Table 4.** Patients' basal laboratory data according to prognosis at hospital discharge.

Basal laboratory data	Survivors N= 57		$p_1$ value	Non-survivors N=17	$p_2$ value
	Recovery N= 36	No-recovery N=21			
Serum creatinine ( $\mu\text{mol/l}$ ), mean (SD)	249.1(141.2)	462(83.7)	0.003 <sup>a</sup>	294.7(293.6)	0.047 <sup>a</sup>
BUN ( $\text{mmol/l}$ ), mean (SD)	39.4(50.5)	24.6(10.5)	0.096 <sup>a</sup>	25.0(13.9)	0.119 <sup>a</sup>
eGFR ( $\text{ml/min/1.73m}^2$ ), mean (SD)	40.4(14.9)	24.8(12.4)	0.001 <sup>a</sup>	21.3(9.7)	0.001 <sup>a</sup>
Serum <i>tIS</i> ( $\mu\text{mol/l}$ ), median (range)	5.4(0.7-79.6)	27.4(0.8-124)	<0.001 <sup>b</sup>	14(0.8-169)	0.826 <sup>b</sup>
Serum <i>fIS</i> ( $\mu\text{mol/l}$ ), median (range)	2.5(0.3-30.7)	6.4(0.4-33)	0.005 <sup>b</sup>	5.5(0.4-23)	0.876 <sup>b</sup>

BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; *tIS*, total indoxyl sulphate; *fIS*, free indoxyl sulphate.

$p_1$  value: between recovery and no-recovery groups.  $p_2$  value: between survivors and non-survivors. <sup>a</sup> Denotes  $p$  value derived from unpaired  $t$ -test, (significant< 0.05). <sup>b</sup> Denotes  $p$  value derived from Mann-Whitney, (significant< 0.05)

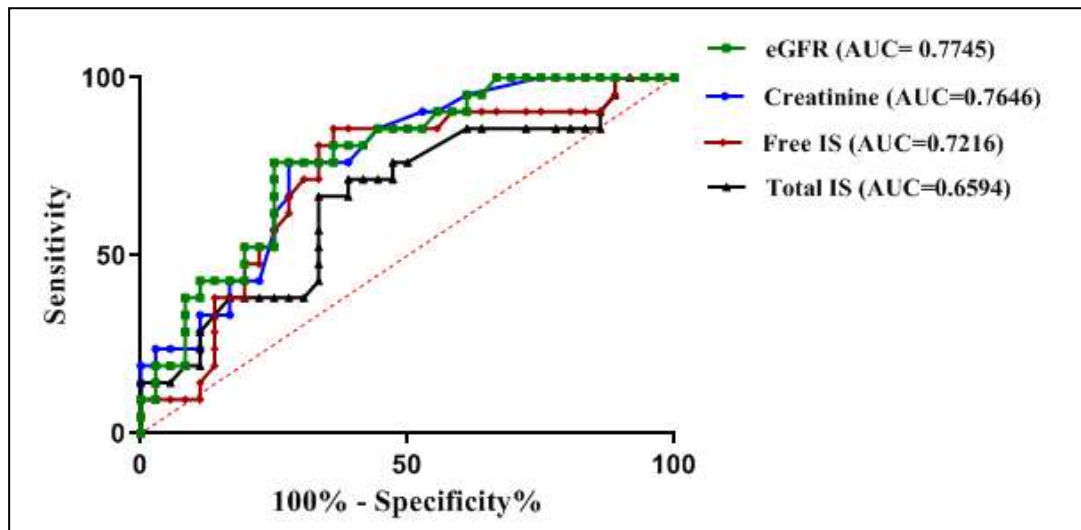
**Role of indoxyl sulphate as a predictor of no-recovery among survivors of toxic AKI**

Using generalized linear models (GLM), neither age, sex, smoking, alcohol drinking, co-morbid conditions, BUN nor APACHE II score had significant association with no-recovery, while *tIS*, *fIS*, creatinine and eGFR were independently associated with no-recovery ( $p=0.04$ ,  $p=0.04$ ,  $p=0.04$ ,  $p=0.01$  respectively).

Using multiple variable analysis, both of *tIS* and *fIS* had no significant association with either of age,

sex, smoking, alcohol drinking, co-morbid conditions, APACHE II score, creatinine, BUN or eGFR, while both of *tIS* and *fIS* have significant positive association with each other ( $p<0.001$ ), and significant negative association with the associated liver disease ( $p=0.044$  for *tIS* and  $p=0.049$  *fIS*).

ROC curve analysis denoted cut-off values; for estimated glomerular filtration rate= 29.4  $\text{ml/min/1.73m}^2$ , for creatinine= 245  $\mu\text{mol/l}$ , for *tIS* =13.25  $\mu\text{mol/l}$  and for *fIS*= 4.0  $\mu\text{mol/l}$  (Fig. 7).

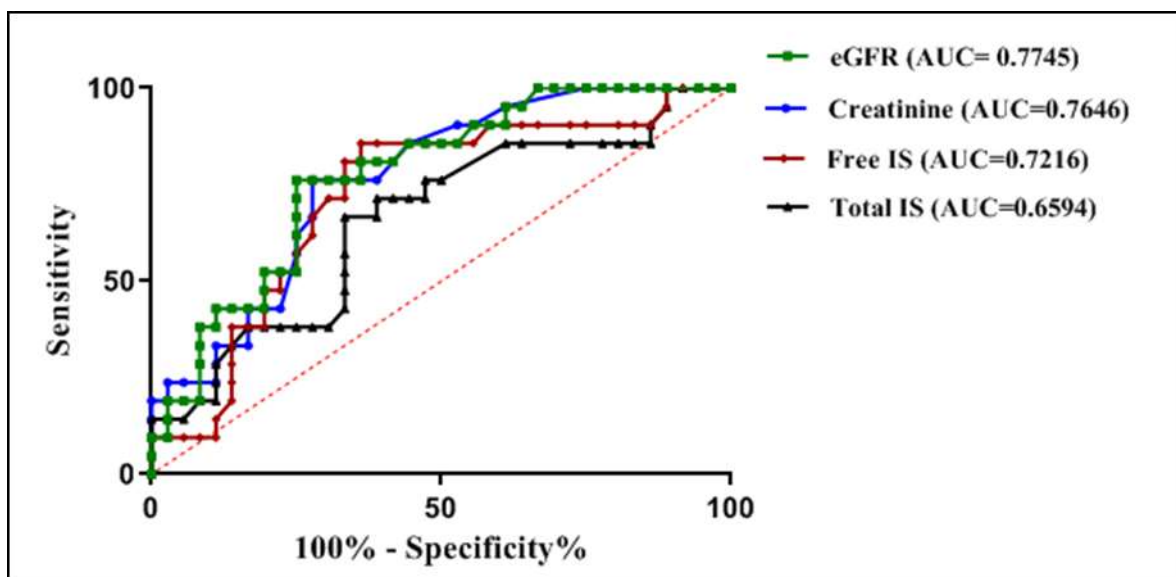


**Fig. 7.** Receiver-operating characteristics (ROC) curve of weight for comparison of the performance of eGFR, creatinine, free IS and total IS in prediction of no-recovery among survivors of toxic AKI patients. The cut-off values were defined by calculating Youden index scores and were chosen as the closest scores to 1.

Based on the previous cut-off values, the positive predictive value for prediction of no-recovery among survivors of toxic AKI patients of creatinine, eGFR, *f*IS, and *t*IS were respectively 63%, 59%, 58% and 52%.

Using binary logistic regression of models

as depicted in Figure 8, in model 2; *t*IS added a value of 0.0152 to the original model (model 1), while in model 3; *f*IS did not add any value to the original model, otherwise, in model 4; adding both *t*IS and *f*IS to the original model added a value of 0.0614.



**Fig. 8.** Receiver-operating characteristics (ROC) curve of weight for comparison of models in prediction of no-recovery among survivors of patients with toxic AKI.

Model 1= creatinine + eGFR, AUC= 0.7785

Model 2= creatinine + eGFR + *t*IS, AUC= 0.7937

Model 3= creatinine + eGFR + *f*IS, AUC= 0.7778

Model 4= creatinine + eGFR + *t*IS + *f*IS, AUC= 0.8399

### Changes of total and free indoxyl sulphate among survivors of toxic AKI patients over time

As presented in Table 5, serum levels of *tIS* and *fIS* over two weeks were significantly higher among no-recovery than among recovery groups ( $p < 0.001$

and  $p < 0.004$  respectively). Moreover, changes in serum levels of *tIS* and *fIS* over two weeks were significantly different when compared among recovery and no-recovery groups ( $p = 0.007$  and  $p = 0.041$  respectively).

**Table 5.** Relationship of changes in serum levels of total and free indoxyl sulphate over two weeks to renal status at hospital discharge.

	Total N	Recovery		No-recovery		p value
	N			N		
<b><i>tIS</i> (<math>\mu\text{mol/l}</math>) median (range)</b>						$<0.001^a$
Basal	57	36	5.4(0.7-79.6)	21	27.4(0.8-124)	0.006 <sup>b</sup>
First week	15	9	14.1(0.8-58.8)	6	53.4(7.6-153)	
Second week	7	3	30.8(0.8-35)	4	51.4(19.9-61.2)	
<b><i>fIS</i> (<math>\mu\text{mol/l}</math>) median (range)</b>						0.004 <sup>a</sup>
Basal	57	36	2.5(0.3-30.7)	21	6.4(0.4-33.0)	
First week	15	9	6.8(0.6-19.4)	6	7.1(0.45-19.6)	0.041 <sup>b</sup>
Second week	7	3	7.6(1.4-7.8)	4	7.3(5.6-17.3)	

*tIS*, total indoxyl sulphate; *fIS*, free indoxyl sulphate; N, number of patients at each point of time. Both of *tIS* and *fIS* had non-parametric distribution and were log transformed before analysis.

<sup>a</sup> Denotes p value derived from linear regression model for comparison of each of *tIS* and *fIS* over two weeks among recovery and no-recovery groups, (significant  $< 0.05$ ). <sup>b</sup> Denotes p value derived from mixed-effect model for changes in indoxyl sulphate concentrations over two weeks, (significant  $< 0.05$ ).

### Three months of follow up of the no-recovery group (N= 21)

After three months of follow up following hospital discharge, ten (47.6%) patients had renal recovery (recovery) and eleven (52.4%) patients did not have and were classified according to RIFLE classes into class L or class E (no-recovery).

As presented in Table 6; basal serum levels of creatinine, *tIS* and *fIS* were significantly higher and eGFR were significantly lower among the group with no-renal recovery than the group who had renal recovery ( $p = 0.043$ ,  $p = 0.013$ ,  $p = 0.019$  and  $p < 0.0001$  respectively).

**Table 6.** Relationships between basal laboratory data and renal outcome (Secondary outcome) of no-recovery group after three months of follow up following hospital discharge.

Basal laboratory data	Recovery N= 10	No-recovery N= 11	p value
<b>Serum creatinine (<math>\mu\text{mol/l}</math>), mean (SD)</b>	425 (85)	711 (410)	0.043 <sup>a</sup>
<b>BUN (mmol/l), median (range)</b>	18.2 (12.1-40.3)	27.8 (8.9-44.3)	0.081 <sup>b</sup>
<b>eGFR (ml/min/1.73m<sup>2</sup>), mean (SD)</b>	33.4 (10.1)	17.1 (8.64)	$<0.001^a$
<b>Serum <i>tIS</i> (<math>\mu\text{mol/l}</math>), median (range)</b>	20.45 (0.8-49.8)	48.2 (3.1-6.2)	0.013 <sup>b</sup>
<b>Serum <i>fIS</i> (<math>\mu\text{mol/l}</math>), median (range)</b>	5.1 (0.4-13.1)	9.8 (4.1-32)	0.019 <sup>b</sup>

BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; *tIS*, total indoxyl sulphate; *fIS*, free indoxyl sulphate.

<sup>a</sup> Denotes p value derived from unpaired t-test (significant  $< 0.05$ ). <sup>b</sup> Denotes p value derived from Mann-Whitney test (significant  $< 0.05$ ).

## Discussion

Despite of the reported significant role of high levels of indoxyl sulphate in *in-vitro* models of AKI, its *in-vivo* role is still obscure. In this study we observed that the IS levels were significantly higher in patients with toxic AKI than those measured in age and sex matched healthy subjects and were significantly lower than those measured in age and sex matched patients with ESRD. Those results matched with those reported by Veldman et al (2019) in a recent *in-vivo* study, on 194 patients admitted to intensive care unit with sepsis, as they found a significant increase in levels of IS in patients with sepsis who developed AKI than in those who did not, moreover, these levels were significantly lower than those measured in serum creatinine matched CKD patients (32).

Indoxyl sulphate levels were observed to correlate significantly with renal function in general population and in uremia (19,24,25). In accordance, among the studied toxic AKI patients, we proved that basal serum levels of *tIS* and *fIS* correlated significantly to the basal renal function.

An increased incidence of mortality was observed following AKI in critically ill patients (8,20,21). Similarly, the in-hospital mortalities were 31.4% and 70.6% in RIFLE class F which matched with the rates previously reported (14,33,34,35). In the current study, the non-survivors had significantly higher basal creatinine and significantly lower glomerular filtration rates, and 70.6% of them were classified as RIFLE class F with significantly different distribution of classes when compared to the survivors, besides, 41.3% of them needed RRT that differed significantly than the survivors. However, no significant difference was noticed in basal levels of either *tIS* or *fIS* among the two groups. Subsequently, severe AKI can be deduced to be a leading cause of death in the studied patients and indoxyl sulphate could not be considered as a predictor of mortality among such patients.

In uremia, indoxyl sulphate was linked to the morbidity following CKD (36-38). Despite of high rate of reversibility of AKI, increased morbidity was also observed, and it is a great health and money costing problem. Deterioration of renal functions and other end-organ failures may follow

AKI and are still unexplainable. In the current study, we tried to raise an answer to the question; is the short-term exposure of the renal tissues to accumulating IS after AKI will affect the progression of renal disease and cause adverse renal outcomes? We observed among the survivors of toxic AKI that basal levels of creatinine, *tIS* and *fIS* were significantly higher and eGFR were significantly lower among no-recovery than among recovery groups at hospital discharge and changes in serum levels of *tIS* and *fIS* over two weeks were significantly different among such groups. Nevertheless, basal serum levels of *tIS*, *fIS* creatinine and eGFR were independent predictors of no-recovery. Moreover, basal serum levels of *tIS* and *fIS* were significantly lower among patients with associated liver disease than among those who had not. Besides, measurement of *tIS*, *fIS*, creatinine and eGFR at time of enrollment was more accurate in prediction of no-recovery than measuring creatinine and eGFR only. Moreover, after three months of follow up of the no-recovery group following hospital discharge, basal serum levels of creatinine, *tIS* and *fIS* were still significantly higher among no-recovery (RIFLE classes L and E) than among recovery outcomes. The results recently reported by Veldman et al after studying patients with sepsis who developed AKI showed harmony with our observations whereas it was reported that basal levels of creatinine and IS measured in patients with sepsis who developed AKI were significantly higher among the group with unfavorable evolution than that with favorable one and changes in serum levels of creatinine and IS correlated significantly to the evolution in renal function (32).

Limitations to this study are First: the small sample size and so the numbers of followed up patients were progressively decreasing. Second, all patients were enrolled from one medical center. Third; causes of nephrotoxicity were heterogeneous and therefore, we could not assess the relation between indoxyl sulphate levels and the pathology of AKI. Fourth; We did not compare indoxyl sulphate levels in patients who were exposed to nephrotoxins and did not develop AKI to those who developed. Finally, in order to establish IS as a

prognostic marker, its clinical significance must be validated further by researchers. We recommend future studies to investigate the role of IS in other AKI populations besides, the role of reduction of serum concentration of IS in improving clinical outcomes of AKI.

In conclusion, serum indoxyl sulphate levels were elevated in critically ill patients with toxic AKI and at time of diagnosis, and measurement of

creatinine, eGFR, *tIS* and *fIS* together can be used as a model for early prediction of no-renal recovery outcome in patients with toxic AKI.

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